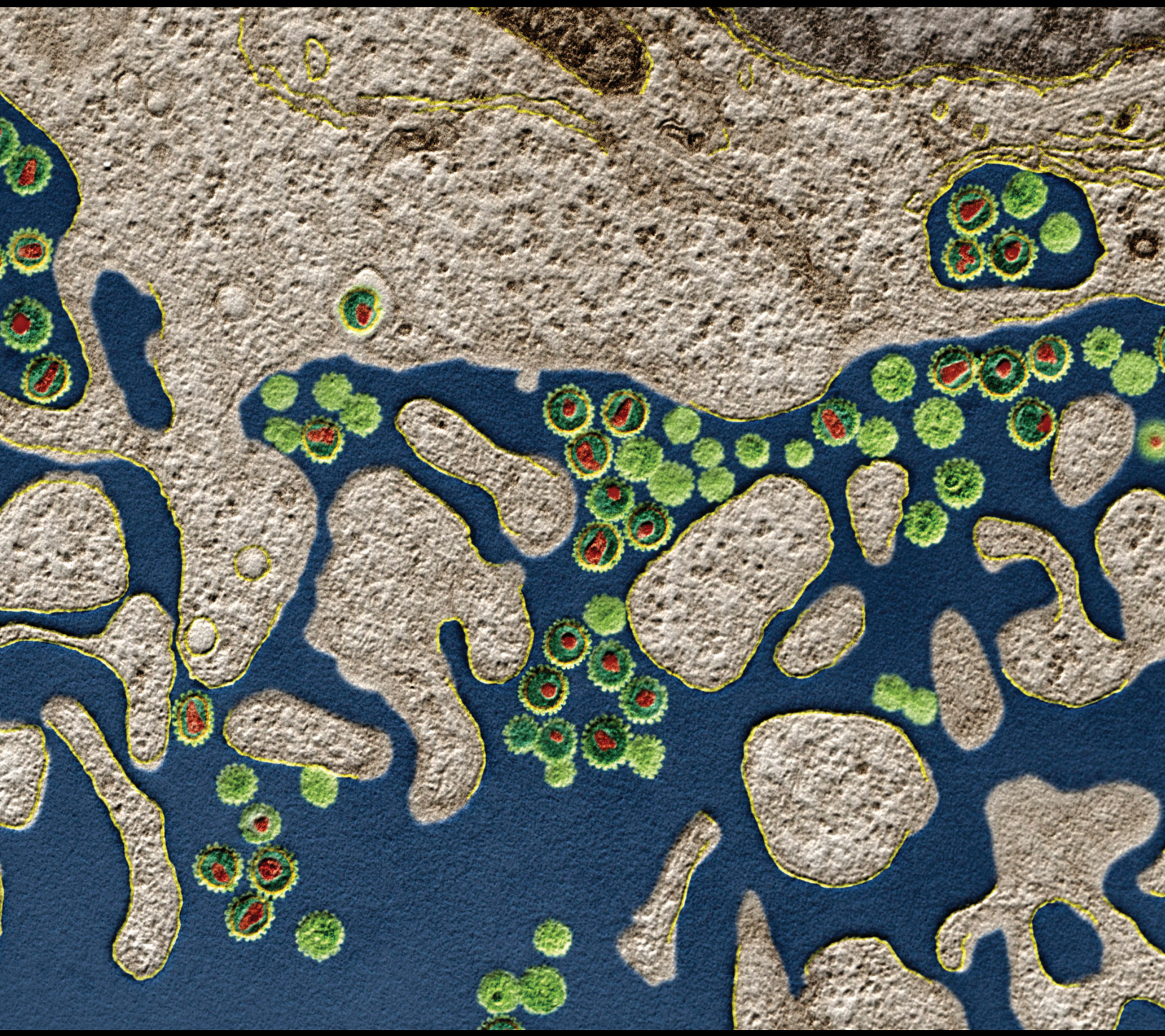
Journal of Immunology Research

Cancer Immunology and Cancer Immunodiagnosis

Guest Editors: Jianying Zhang, Suxia Han, Bin Zhang, and Yi Zhang



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Editorial Board

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Editorial

Cancer Immunology and Cancer Immunodiagnosis

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Cancer immunology is the study of interactions between the immune system and cancer cells, which is a rapid growing field of research that aims to identify biomarkers in cancer immunodiagnosis and to develop innovative cancer immunotherapeutic strategies. The immune response, including the recognition of cancer-specific antigens, is of particular interest in cancer immunology field, which can further drive the development of new vaccines and antibody therapies. It is also well demonstrated that the immune system can recognize the antigenic changes in cancer cell and further develop antibody against these cellular antigens that have been generally called tumor-associated antigens (TAAs) [1–4]. These cancer-associated anti-TAAs autoantibodies might be considered as "reporters" from the immune system, to identify the antigenic changes in cellular proteins involved in the transformation process [5, 6]. There has been a growing interest in using serum autoantibodies against TAAs as biomarkers in cancer immunodiagnosis. The persistence and stability of these antibodies in the serum samples of cancer patients is an advantage over other potential markers, including the TAAs themselves, some of which are released by tumors but are rapidly degraded or cleared after circulating in the serum for a limited time [7]. In recent years, the potential utility of TAA-autoantibody systems as early cancer biomarker tools to monitor therapeutic outcomes or as indicators of disease prognosis has been explored.

Activation of the immune system for therapeutic benefit in cancer has long been a goal in immunology and oncology. The passive cancer immunotherapy has been well established for several decades, and continued advances in antibody and T-cell engineering should further enhance their clinical impact in the years to come. In contrast to these passive immunotherapy strategies, the active cancer immunotherapy has been proved elusive. In the context of advances in the understanding of how tolerance, immunity, and immunosuppression regulate antitumour immune responses together with the advent of targeted therapies, these successes suggest that active immunotherapy represents a path to obtain a durable and long-lasting response in cancer patients [8]. The key to cancer immunodiagnosis and immunotherapy is an improved understanding of the immune response during malignant transformation.

According to this background, we have invited investigators to contribute original research articles as well as review articles describing cancer immunodiagnosis and cancer immunotherapy and assembled this special issue for updating the recent advances in this field. In this special issue, we have included a total of 18 papers including 12 original research papers and 6 review papers, in which 7 research papers deal with cancer immunotherapy and 5 research papers deal with cancer immunodiagnosis. For example, a paper of Z. B. Wu et al. has demonstrated that glioma-associated antigen HEATR1 can induce functional cytotoxic T lymphocytes in patients with glioma; a paper of S. I. Kim et al. has discussed the impact of underweight after treatment on prognosis of advanced-stage ovarian cancer; a paper of J. Ma et al. has indicated that the intensity of radiotherapy-elicited immune response is associated with esophageal cancer clearance; a paper of J. Li et al. has discussed the selective depletion of regulatory T cell subsets by docetaxel treatment in patients

with non-small cell lung cancer. Papers from P. Wang et al., L. Wang et al., L. Chen et al., L. Borska et al., and J. Gu et al. have, respectively, discussed different cancer-associated protein biomarkers in cancer immunodiagnosis and cancer prognosis. In addition, review papers cover many aspects relating to cancer immunotherapy and cancer immunodiagnosis. For example, a review paper of Palacios-Arreola MI et al. has discussed the role of chemokines in breast cancer pathology and its possible use as therapeutic targets; a paper of J. Lacombe et al. has discussed the use of autoantibodies in detection of breast cancer; a paper of D.-S. Chung et al. has discussed a new hope of immunotherapy for malignant gliomas; a paper of D. A. Erkes and S. R. Selvan has extensively reviewed the hapten-induced contact hypersensitivity, autoimmune reactions, and tumor regression.

In summary, this special issue covers many important aspects in cancer immunology, including recent advances in the identification and evaluation of TAA and anti-TAA biomarkers in cancer immunodiagnosis, as well as the basic and clinical studies relating to cancer immunotherapy. We hope that this special issue can provide some useful information to investigators in the field of cancer immunodiagnosis and cancer immunotherapy and also give the readers a sense of some of the advancements made in this field.

Jianying Zhang
Suxia Han
Bin Zhang
Yi Zhang

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Review Article

The Role of Chemokines in Breast Cancer Pathology and Its Possible Use as Therapeutic Targets

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Chemokines are small proteins that primarily regulate the traffic of leukocytes under homeostatic conditions and during specific immune responses. The chemokine-chemokine receptor system comprises almost 50 chemokines and approximately 20 chemokine receptors; thus, there is no unique ligand for each receptor and the binding of different chemokines to the same receptor might have disparate effects. Complicating the system further, these effects depend on the cellular milieu. In cancer, although chemokines are associated primarily with the generation of a protumoral microenvironment and organ-directed metastasis, they also mediate other phenomena related to disease progression, such as angiogenesis and even chemoresistance. Therefore, the chemokine system is becoming a target in cancer therapeutics. We review the emerging data and correlations between chemokines/chemokine receptors and breast cancer, their implications in cancer progression, and possible therapeutic strategies that exploit the chemokine system.

1. Introduction

Chemokines are small proteins that primarily regulate the trafficking of leukocytes under homeostatic conditions and during specific immune responses. They share a secondary structure, and based on their amino acid composition—specifically, the presence of a conserved tetra-cysteine motif—they are grouped into 4 families: C, CC, CXC, and CX3C [1, 2]. Chemokines guide the migration and adhesion of leukocytes and influence other cellular functions, such as proliferation, maturation, angiogenesis, and malignant transformation [1, 2]. These effects are mediated by binding to G-protein-coupled receptors (GPCRs) with 7 transmembrane domains [2].

The chemokine system comprises almost 50 chemokines and approximately 20 chemokine receptors [2, 3]. Upon the binding of different chemokines to the same receptor, there can be a variety of biological effects. Complicating the system further, the effects also depend on the cellular microenvironment.

Breast cancer has gained particular relevance in recent years due to the high incidence in both developed and less developed regions [4]. Breast cancer is basically defined by the presence of a malignant tumor that originates from breast tissue, either from lobes, ducts, or stroma. The tumor cells proliferate and are able to invade surrounding tissues, lymph nodes and distant organs. According to the size of the primary tumor, the involvement of lymph nodes, and the presence of distant metastasis, the stage of breast cancer can be determined, ranging from stage 0 to stage IV [5].

The intervention of the immune system in cancer does not begin with the fighting and effort to restrain an established tumoral mass but with the detection of transformed cells since they began proliferating. In the late 50's, Burnet proposed the *immunosurveillance* theory [6], which proposes that the immune system cells are capable of detecting

transformed cells, attacking them and eliciting an specific (adaptive) response to eventually succeed and eliminate them all or fail, leading then to the formation of a tumoral mass and cancer onset.

Nowadays, it is known that this process is more complex than previously thought and consists not only of that simple event series, but can comprise alternative processes, such as *immunoediting* and even *immunosubversion* [7, 8]. The previous could be depicted by the paradoxical fact that a greater infiltration of immune cells in breast cancer neoplasia has been correlated with a worse disease prognostic, and how this can also be explained by the polarization phenomena that immune cells experience in the tumoral microenvironment, which induces the acquisition of a protumoral phenotype.

Chemokines and chemokine receptors play a key role along these processes, since they not only comprise the main regulatory system leading leukocyte infiltration in primary tumors, but also intervene in cancer cells proliferation and in metastasis guidance.

Nearly every tissue expresses chemokines and chemokine receptors. Normal breast expresses a set of chemokines at generally low levels [9]. We review the emerging data and correlations between chemokines and breast cancer, from their implications in cancer progression to therapeutic strategies that exploit the chemokine system.

2. Breast Cancer Cells Proliferation and Tumor Growth: Is There a Function for Chemokines?

Chemokines not only are associated with the establishment of a protumoral microenvironment and organ-directed metastasis, but also mediate disease progression, favoring the growth and proliferation of tumor cells. Several chemokines have been described as participating in these processes.

One of those chemokines implicated in breast cancer progression is CCL2 (formerly known as MCP-1), which is a potent chemotactic factor that regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells, signaling through CCR2 and CCR4 [10]. CCL2 is expressed at high levels in both tumor and tumor microenvironment cells, exerting its protumoral effects indirectly by promoting angiogenesis and enriching leukocyte infiltration [11, 12], primarily with tumor-associated macrophages (TAMs), which produce immunomodulatory factors that promote angiogenesis and tumor growth.

Moreover, CCL2 mediates development of the cancer stem cell (CSC) phenotype. Cancer-associated fibroblasts (CAFs) and fibroblasts that are activated by coculture with cancer cells secrete high levels of CCL2, which affects the sphere-forming phenotype (stem cell-specific) of breast cancer cells and CSC self-renewal [13]. The promotion or CSC phenotype is of great relevance in cancer biology, given that this population of self-renewing, chemo- and radioresistant cells is thought to maintain the tumor heterogeneity, as well as giving rise to metastasis.

Another member of the CC family of chemokines that has been related to breast cancer progression is CCL20. This chemokine primarily targets lymphocytes and dendritic cells, although it also attracts neutrophils weakly, upon binding to receptor CCR6. Recently, Marsigliante and colleagues [14] correlated high CCL20 concentrations with extensive cellular proliferation, mediated by increased cyclin E (which is required for the transition from G1 to S phase) and decreased p27 (an inhibitor of cyclin D).

Cell cycle regulation is clearly a key element in cancer progression and recently CXCL8, a member of the CXC family, has been identified as a promoter of cell cycle progression. Shao and colleagues demonstrated that silencing CXCL8 using siRNA in the MDA-MB-231 breast cancer cell line resulted in the upregulation of p27, downregulation of cyclin D1, and thus a delay in the progression from G1 to S phase [15]. Besides cell cycle regulation, CXCL8 has been implicated in CSC phenotype. It has been reported that breast cancer stem cells express CXCR1, which upon binding of CXCL8 increase their activity (measured as sphere-formation) and self-renewal [16].

Although traditionally implicated in organ-directed metastasis, CXCR4 is a chemokine receptor that has been linked to cancer progression. Its constitutive activation in MCF-7 breast cancer cells enhances tumor growth and metastasis, which can be reversed by its inhibition [17].

In contrast, the chemokine CXCL14, which is abundantly expressed in normal tissue but downregulated in breast cancer tissue and cell lines, negatively regulates the growth and metastasis of breast cancer as its expression is positively associated with patient survival and a lower incidence of metastasis. Overexpression of CXCL14 was recently reported to inhibit cell proliferation *in vitro* and decrease xenograft tumor growth *in vivo* [18].

3. Chemokines and Tumor Microenvironment

Cancer cells are not the only decisive factor in the course of the disease—there are other factors, both systemic and in the tumor microenvironment, that can limit or promote the growth of cancer cells and their mobility and dissemination to other organs.

When talking about the progression of cancer, one of the key elements of the tumor microenvironment is the myeloid cell population, particularly macrophages. On recruitment to the tumor microenvironment, macrophages are influenced by the cytokine milieu and local growth factors, resulting in the acquisition of a protumoral phenotype. The resulting TAMs produce angiogenic and immunomodulatory factors (e.g., IL-10 and TGF-β) which induce regulatory T lymphocytes (T_{reg}) and facilitate remodeling of the extracellular matrix, promoting cancer cell motility. Because a primary function of chemokines is to attract and direct leukocytes (Figure 1), their significance is evident, regarding leukocyte infiltration into the tumor microenvironment.

CCL2 and CCL5 (RANTES) chemokines have been extensively studied in breast cancer. As mentioned before, CCL2 is a potent chemoattractant of monocytes (Figure 1); in addition to monocytes, CCL5 recruits T helper type 2 lymphocytes (Th2) and eosinophils, signaling through

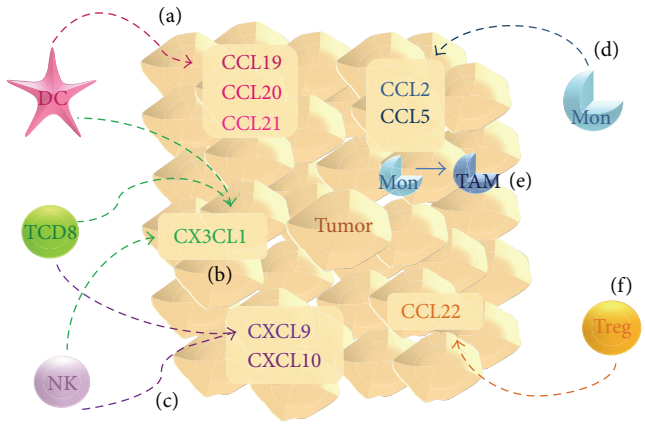

Figure 1: Chemokines influx the tumor microenvironment. (a) CCL19, CCL20, and CCL21 act as chemoattractants for dendritic cells (DC); (b) CX3CL1 has been related to infiltration of DCs as well as activated (cytotoxic) CD8+ T lymphocytes and NK cells. These last two populations are also chemoattracted by (c) CXCL9 and CXCL10. DCs, T CD8+ lymphocytes, and NK cells are thought to contribute to antitumoral immune response. (d) CCL2 and CCL5 are both chemoattractants for monocytes (Mon), which (e) within tumor microenvironment acquire a TAM phenotype. (f) CCL22 expression correlates with T_{reg} infiltration, which together with TAMs promotes tumor survival and progression.

the receptors CCR1, CCR3, and CCR5 [2, 11]. These chemokines have similar expression patterns—they are detected in cancer cells in primary tumors, tumor-infiltrated lymph nodes, distant metastases, and cells that are adjacent to the tumor (e.g., TAMs and fibroblasts) but are expressed at low levels in healthy breast tissue [11, 12].

An increase in the CCL2 expression levels correlate with the extent of TAM infiltration in primary tumors and in animal xenograft models, a causal relationship based on findings where blocking CCL2 with neutralizing antibodies decreases macrophage infiltration, tumor growth, and angiogenesis (associated to some extent with the presence of TAMs) in a mouse model of breast cancer [19, 20]. Although CCL5 is also linked to macrophage infiltration in animal models of breast cancer [21, 22], in human breast xenografts, CCL5 expression correlates negatively with macrophage recruitment [19], implying the participation of other chemokines.

In addition to macrophages, the leukocyte infiltrate in the tumoral niche includes T lymphocytes, dendritic cells (DCs), NK cells, and other granulocytes, which also influence the fate of cancer cells at the cellular level and by modifying the tumoral microenvironment with cytokines and chemokines. The chemokines CCL19, CCL20, and CCL21 regulate the traffic of DCs (Figure 1), and their overexpression in experimental tumor systems has antitumoral effects [12]. Although the function of DCs in cancer immunology is still under investigation, the effects of the overexpression of these chemokines correlate with increased DC infiltration.

T lymphocytes and NK cells are important populations in tumor immunology—activated lymphocytes orchestrate immune response against cancer cells, and NK cells are central innate effectors that recognize and have cytotoxic effects on stressed and transformed cells. In this context, CXC chemokines CXCL9 (also known as Mig) and CXCL10 (IP-10) control the migration of activated T cells and NK cells [23, 24], which can enhance the antitumoral response (Figure 1). Moreover, these chemokines belong to the antiangiogenic ELR(−) CXC subfamily (further discussed later) [25, 26]. In addition, CXCL12 (SDF-1) was recently shown to promote $CD8^+$ cytotoxic T lymphocyte activity when overexpressed in a syngeneic model of breast carcinoma [27].

CX3CL1 (also known as Fractalkine) is the only known member of the CX3C chemokine family and signals through CX3CR1. This receptor is expressed on monocytes, NK cells, and T lymphocytes, to mediate several functions, including migration, adhesion, and proliferation [28]. As discussed, T lymphocytes and NK cells are key populations in antitumoral immunity—a response in which CX3CL1 is thus expected to be involved. Park and colleagues [29] described a positive correlation between CX3CL1 expression in breast carcinoma specimens and the number of stromal T $CD8^+$ lymphocytes, intratumoral DCs, and stromal NK cells (Figure 1). Consistent with these results and the antitumoral properties of these subpopulations, elevated CX3CL1 expression may be associated with significantly better disease-free survival.

Conversely, T_{reg} infiltration is associated with a poor prognosis. It is now clear that T_{regs} induce an immunomodulatory state by producing IL-10 and TGF-β, which inhibit APC maturation and the expression of costimulatory molecules as well as decreasing the cytotoxic potential of NK cells and cytotoxic T lymphocytes. CCL22 is a chemokine that signals through CCR4 and is a chemoattractant for monocytes, dendritic cells, NK cells, and chronically activated T lymphocytes. Recently, CCL22 was linked to T_{reg} infiltration (Figure 1) in gastric, esophageal, and ovarian carcinomas [30–32]. Consistent with its significance in T_{reg} infiltration, breast tumors that lack CCL22 are not infiltrated by the T_{reg} subpopulation [33]. Moreover, in human breast carcinoma cell lines, CCL22 was secreted at low basal levels and upregulated in response to inflammatory signals.

4. The Function of Chemokines in Angiogenesis

Angiogenesis is a hallmark of cancer. The resulting tumor-associated neovasculature that is generated addresses the tumor's growing demands for nutrients and oxygen [34] and enables the tumor to grow and avoid excessive necrosis.

The CXC chemokine family comprises angiogenic and antiangiogenic chemokines (Table 1). Angiogenic chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8, are generally distinguished by an ELR motif. These chemokines mediate their angiogenicity through CXCR2 and interact alone or with other angiogenic factors (e.g., VEGF) to effect angiogenesis (Figure 2) [26]. CXCL8 is considered one of the most potent inducers of angiogenic processes [12] such as rapid stress fiber assembly, chemotaxis, enhanced proliferation of and tube formation by endothelial cells [26]. Noteworthy, Haim and coworkers [35] reported that estrogen upregulates the transcription and secretion of CXCL8 in

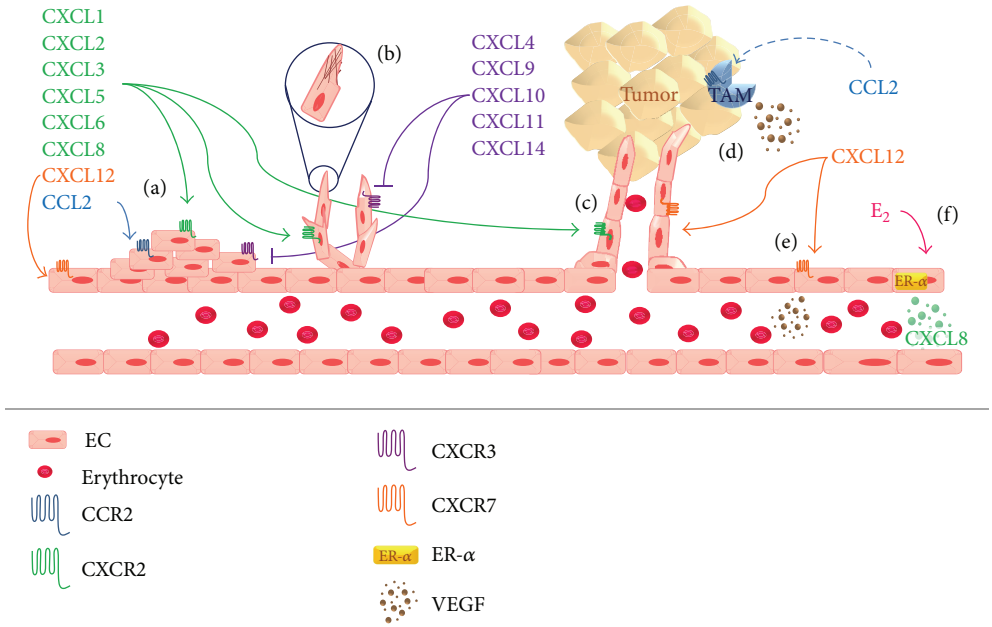

Figure 2: Chemokines involvement in angiogenesis. Angiogenic ELR$^+$ chemokines act through CXCR2 receptor to promote (a) proliferation of endothelial cells (EC), (b) stress fibre assembly, and (c) tube formation. On its behalf, antiangiogenic non-ELR$^+$ chemokines, via CXCR3, inhibit these processes. CCL2 also promotes angiogenesis via CCR2, (a) stimulating EC proliferation and (d) in an indirect manner by increasing TAM infiltration, which secrete angiogenic factors like VEGF. CXCL12 acts through CXCR7 to promote (a) EC proliferation and (e) VEGF production by these cells. It has been reported that (f) estrogen (E_2) stimulates EC secretion of CXCL8.

Table 1: Angiogenic and antiangiogenic members of CXC chemokine family.

Angiogenic ELR$^+$ chemokines	Antiangiogenic non-ELR$^+$ chemokines
CXCL1	CXCL4
CXCL2	CXCL9
CXCL3	CXCL10
CXCL5	CXCL11
CXCL6	CXCL14
CXCL7	
CXCL8	

breast tumor cells additively through estrogen receptor α (ERα), adding a novel role of estrogen in promoting tumor growth (Figure 2).

Although CXCL12 is a non-ELR-CXC chemokine, it has been implicated as an angiogenic chemokine based on evidence of its involvement in blood vessel formation, inducing endothelial cell migration and proliferation, stimulating tube formation, and enhancing VEGF release (Figure 2) [12].

Angiostatic CXC chemokine family members include CXCL4, CXCL9, CXCL10, CXCL11, and CXCL14. CXCL4, CXCL9, and CXCL10 signal through CXCR3, which, on ligand engagement, blocks microvascular endothelial cell migration and proliferation (Figure 2) in response to various angiogenic factors [26].

CCL2 and CCL5 have been also suggested to shift the balance in the tumor microenvironment towards increased vascularity. CCL2 acts directly on endothelial cells to promote angiogenesis and correlates closely with positive endothelial growth regulators, such as vascular endothelial growth factor (VEGF), thymidine phosphorylase (TP), and CXCL8 [20]. In an indirect manner, CCL2 increases the presence of TAMs, which produce other angiogenic factors [11] (Figure 2).

In addition to the development of new blood vessels, tumors also undergo a process denominated Lymphangiogenesis—the growth of lymphatic vessels—which contributes to lymphatic metastasis [36] and is thus a major event in the development and spread of cancer. Peritumoral lymphangiogenesis involves the secretion of VEGF-C and VEGF-D, which act on the lymphatic endothelium and are upregulated in the MCF10 breast cancer cell line [37]. Further, CXCL12 is a chemoattractant for lymphangiogenic endothelial cells (LECs), inducing the migration and tubule formation of LECs *in vitro* and lymphangiogenesis *in vivo* and correlating with lymphatic vessel density in cancer tissues [36].

5. The Function of Chemokines in Metastasis

Metastasis is the dissemination of cancer cells to distant organs and tissues, such as the liver, lung, brain, and bone. This process is the most devastating attribute of cancer and significantly influences its morbidity and mortality [38]. Cancer metastasis is not a fortuitous or randomly driven process but is governed by many factors that, for example, allow cancer cells to move, detach from the ECM (which is achieved by the expression of matrix metalloproteases

and heparanase), intravasate, migrate to distant organs and be able to fluorish in a different niche from the one in which they developed. How other cellular populations in the tumor microenvironment contribute to ECM remodeling is beyond the scope of this section, but notably cancer-associated fibroblasts (CAFs) have a significant function in this process.

The CXCL12-CXCR4 axis is one of the most extensively studied pairs in metastasis, primarily with regard to its involvement in organ-directed metastasis. Its function in metastasis begins with cancer cell mobility—the binding of CXCL12 to CXCR4 activates various intracellular signal transduction pathways and effector molecules that regulate chemotaxis, migration, and adhesion. Low-CXCR4-expressing MCF-7 cells fail to metastasize when injected into mice, whereas CXCR4-high MDA-231 cells are efficient in forming distant organ metastases [39]. Similarly, CCL21, through its receptor CCR7, triggers actin polymerization, pseudopodia formation, and the directional migration and invasion of breast cancer cells, particularly to lymph nodes, where CCL21 is highly expressed [40].

CXCR4 expression is higher in malignant breast tumor compared with its normal counterpart [40]. It controls chemotaxis toward its ligand, CXCL12, which is highly expressed in the lung, bone, liver, and lymph nodes, organs to which breast cancer cells preferentially metastasizes [41, 42]. With regard to brain metastasis, it remains unknown how cancer cells breach the brain-blood barrier (BBB) and invade this tissue, but it is possible that CXCL12-expressing CNS cells are chemoattractants for metastatic breast cancer cells [43].

Triple-negative breast cancer (TNBC) is a very aggressive subtype with few therapeutic alternatives and a poor prognosis. CXCR4 expression was recently reported to be more frequent in TNBC versus other subtypes, and CXCR4-positive patients had a significantly higher rate of metastasis, larger primary tumors, and shorter overall- and disease-free survival [44].

Chemokines that are expressed by osteoblasts and bone marrow endothelial cells have been implicated in driving bone metastasis. During their differentiation into osteoblasts, mesenchymal stem cells secrete CCL2, which is believed to mediate the migration of cancer cells, a process that is partially inhibited by anti-CCL2 [45]. CX3CL1 is expressed in a membrane-bound form and is exposed to the luminal side of human bone marrow endothelial cells, whereas its receptor, CX3CR1, is expressed in normal and malignant mammary glands [46]. Breast cancer cells that express high levels of CX3CR1 have a greater propensity toward bone metastasis; consistent with these data, studies in CX3CL1-null transgenic mice suggest that the absence of this chemokine impairs the dissemination of cancer cells to bone [46].

Notably, Hernandez et al. [47] reported that the binding of CXCL12 to CXCR4 and CXCR7 elicits disparate cellular responses. CXCR4 controls chemotactic and invasive behavior (*in vivo* motility and intravasation) in response to CXCL12, whereas CXCR7 enhances primary tumor growth and angiogenesis but decreases *in vivo* invasion, intravasation, and metastasis formation.

As discussed, CCL2 mediates TAM infiltration and generates an amplification loop, upregulating CCL2 in TAMs, which is associated with the expression of membrane type 1-matrix metalloprotease (MT1-MMP) [11]. Similarly, other chemokines and receptors, such as CCL5, CCL20 (via CCR6), CXCL12, and CXCR7, induce or increase the expression of MMPs [12, 14, 47]. CXCL7 has been reported to be linked to greater heparanase activity in MCF-7 breast cancer cells [37].

Recently, Chen and colleagues [48] linked TAM-produced CCL18 with cancer cell invasiveness and identified PITPNM3 (a membrane-associated phosphatidylinositol transfer protein) as its receptor. In this report, CCL18 colocalized with CD68 (a TAM marker) in most invasive breast carcinoma samples. In *in vitro* experiments, the group showed that the invasiveness of MDA-MB-231 cells and primary breast cancer cells was enhanced equally by the addition of recombinant CCL18 and coculture with TAMs, whereas the addition of anti-CCL18 and CCL18-siRNAs reduced the number of invasive cancer cells. Based on these findings, it is concluded that TAMs are a source of CCL18 and there may be a close relationship between CCL18 and invasiveness.

6. Therapeutic Targeting of Chemokines in Breast Cancer

Chemoresistance is a significant obstacle in cancer treatment, because cancer cell subpopulations that survive chemotherapy can proliferate and reemerge as a more aggressive variant, limiting subsequent therapeutic options. CCL25, via CCR9, confers a survival advantage to breast cancer cells by inhibiting cisplatin-induced apoptosis in a PI3K-dependent manner, in addition to activating cell survival signals through Akt [49]. Acharyya et al. reported a notable feedback mechanism between chemotherapy treatment and chemokine-induced chemoresistance [50], in which CXCL1 and CXCL2 attract $CD11b^{+}GR1^{+}$ myeloid cells, which produce other chemokines, including S100A8/9, that enhance cancer cell survival. Although chemotherapeutic agents kill cancer cells, they induce TNF-α production by endothelial and stromal cells, which upregulates CXCL1 and CXCL2 in cancer cells, amplifying the CXCL1/2-S100A8/9 loop and affecting chemoresistance.

Chemokines and chemokine receptors are appealing targets for cancer treatment, based on the wide range of processes that they influence. For instance, chemokine receptors mediate critical steps in the development and spread of cancer, for which antagonists have been designed to inhibit signal transduction and impede the undesired effects of chemokines. In CXCL1- and CXCL2-induced chemoresistance, CXCR2 blockers have been shown to break the chemokine-chemoresistance cycle described above, augmenting the efficacy of chemotherapy [50].

As described, CCL5 mediates cancer cell invasiveness and signals through CCR5. CCR5 antagonists slow the invasion of basal breast carcinoma cells *in vitro* and decrease pulmonary metastasis in a preclinical mouse model of breast cancer,

suggesting that CCR5 antagonists can be used as adjuvant therapy in patients with this breast cancer subtype [51].

Concerning the chemoresistant CSC population, it is known that chemotherapy induces the expression of CXCL8 in injured cells, which increases the activity and self-renewal of the former. Thus, the blockade of CXCL8 receptors CXCR1 and CXCR2 arises as a promising side-therapy attempting to avoid tumor recurrence [16, 52]. Furthermore, not only the number of CSCs but also a reduction in general tumor cell viability is achieved by the use of CXCR1 inhibitors [52].

Because the CXCL12-CXCR4 axis has significant function in breast cancer metastasis, it has also been targeted using CXCR4 antagonists. Williams and colleagues [27] reported that a CXCL12 analog with antagonist activity (CXCL12(P2G)) significantly inhibited metastasis in a syngeneic mouse model of breast carcinoma. Conversely, T140 analogs are peptidic CXCR4 antagonists, originally developed as anti-HIV agents that inhibit CXCL12-induced migration of MDA-MB-231 breast cancer cells *in vitro* and mitigate pulmonary metastasis *in vivo* [53].

Chemokines that promote and enhance the activity and interaction of immune cells have been exploited as a prophylactic approach. CCL19 and CCL21 regulate the encounter between DCs and T lymphocytes in lymph nodes, for which they can be considered as important natural adjuvants for immune response [54, 55]. These chemokines have been used in DNA vaccines, amplifying their immunogenicity, inducing a Th1-polarized immune response, and substantially improving their protective effects in BALB/c mice [55]. Oncolytic viruses are also promising cancer treatments; this virotherapy, in combination with a CXCR4 antagonist, has increased efficacy over oncolysis alone in a triple-negative breast carcinoma syngeneic mouse model; systemic delivery of the armed virus after resection of the primary tumor inhibits the development of metastasis and increases overall tumor-free survival [56].

7. Concluding Remarks

Rapidly accumulating data in breast cancer immunology from recent years suggest that many established and widely accepted paradigms should be revised. In breast cancer, whereas chemokines are primarily associated with the generation of a protumoral microenvironment and organ-directed metastasis, they also appear to mediate disease progression, favoring the growth and proliferation of tumor cells.

Recent studies suggest that inhibiting local chemokines signaling in the tumor by blocking particular receptors or using analogs with antagonist activity could be a new rationale promising strategy for controlling tumor development and progression. Thus, the development of drugs that specifically target the chemokine axis will be invaluable in the treatment of breast cancer, in which inflammation has a major role.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Use of Autoantibodies to Detect the Onset of Breast Cancer

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The widespread use of screening mammography has resulted in increased detection of early-stage breast disease, particularly for *in situ* carcinoma and early-stage breast cancer. However, the majority of women with abnormalities noted on screening mammograms are not diagnosed with cancer because of several factors, including radiologist assessment, patient age, breast density, malpractice concerns, and quality control procedures. Although magnetic resonance imaging is a highly sensitive detection tool that has become standard for women at very high risk of developing breast cancer, it lacks sufficient specificity and costeffectiveness for use as a general screening tool. Therefore, there is an important need to improve screening and diagnosis of early-invasive and noninvasive tumors, that is, *in situ* carcinoma. The great potential for molecular tools to improve breast cancer outcomes based on early diagnosis has driven the search for diagnostic biomarkers. Identification of tumor-specific markers capable of eliciting an immune response in the early stages of tumor development seems to provide an effective approach for early diagnosis. The aim of this review is to describe several autoantibodies identified during breast cancer diagnosis. We will focus on these molecules highlighted in the past two years and discuss the potential future use of autoantibodies as biomarkers of early-stage breast cancer.

1. Introduction

Breast cancer is the most common malignancy and the second most common cause of cancer-related mortality in women [1]. Successful strategies for screening, early diagnosis, prognosis, and risk stratification are needed to decrease mortality and increase the probability of curing the disease. Currently, mammography is the gold standard of breast cancer screening and remains the only screening test proven to reduce mortality. However, not all cancers can be visualized on screening mammograms. Indeed, mammographic sensitivity decreases significantly as breast density increases, with sensitivity reported to be as low as 45% in women with extremely dense breasts [2]. Conversely, mammography can also lead to overdiagnosis (i.e., detection of tumors that might not need intervention) and can lead to unnecessary treatment of some patients [3]. Therefore, considerable efforts have been undertaken to produce an effective screening method for early-stage breast cancer. Both full-field digital mammography and computer-aided detection programs have been proposed, but results from these methods remain controversial [4]. The ability of magnetic resonance imaging (MRI) to detect the presence and extent of small tumors seems to exceed that of both mammography and ultrasound, with low specificity. However, additional investigations are still required to confirm this finding [5]. Finally, improving early-stage breast cancer screening is needed, particularly for women with high breast density [6].

2. Current Biomarkers and Clinical Utility of Autoantibodies

For early detection to be an effective and practical approach, screening tests must satisfy four basic requirements. First, screening tests should be able to distinguish healthy individuals from cancer cases with a high degree of accuracy, sensitivity, and specificity (namely, low false-negative and false-positive rates). Second, detection should be possible before the disease progresses to an advanced stage, or even

prior to the first manifestation of clinical signs, when it is still curable. Third, the test should ideally allow discrimination between lesions that are aggressive and require treatment and those that ultimately will do no harm, thus reducing the problem of overdiagnosis. Fourth, tests should be inexpensive and well accepted by the target population [7]. Breast cancer markers currently in use do not satisfy all these requirements. Therefore, FDA-approved protein tumor markers currently used in clinical practice, such as circulating tumor cell (CTC) proteins, estrogen receptor (ER), progesterone receptor (PR), Her-2/neu, CA 15-3, and CA27.29, are not approved for screening or early diagnosis [8]. Rather, ER and PR assays are recommended for prognosis and determining response to therapy, while Her-2/neu is used to assess appropriate therapeutic options. Moreover, ER, PR, and Her-2/neu measurements are based on immunohistochemistry, a method that requires invasive intervention such as biopsy to obtain samples. The level of CTCs is reported to be associated with cancer progression and survival. Finally, CA15-3 and CA27.29 serum biomarkers are only recommended for monitoring disease state and response to therapy.

Development of a sensitive, specific, and reproducible assay to identify biomarkers that can accurately determine the onset of breast cancer, particularly noninvasive tumors, is an attractive goal. This assay should be applicable for routine clinical use, require minimal time, and present little risk for the patient (e.g., venipuncture). Based on these criteria, autoantibodies have great potential as breast cancer biomarkers. Indeed, autoantibodies are secreted and are therefore easily accessible. Autoantibodies are present in sera before tumor-associated antigens (TAAs) can be detected. Autoantibodies also correspond to efficient biological amplification of TAAs and are secreted into serum prior to first clinical signs. Moreover, antibodies are highly stable in serum samples and, unlike other polypeptides, are not subject to proteolysis, simplifying sample handling. They have a long lifetime in blood ($T_{1/2}$ between 7 to 30 days, depending on the subclass of immunoglobulin) and may persist as long as a corresponding autoantigen that elicited the original specific humoral response. Finally, antibodies are biochemically well-characterized and many reagents and techniques are available for their detection, simplifying assay development [9].

3. First Steps toward the Identification of Autoantibodies in Cancer Patients

In 1955, Baldwin was the first to demonstrate that the immune system could react to a developing tumor [10]. He showed that tumor extracts could cause considerable tissue destruction when injected into growing tumors in rats. Tumors in these rats regressed, and the animals remained free of recurrent tumor growth. Furthermore, injected rats were found to be immune to subsequent implants of the same tumor. These results suggested that the development of tumor immunity depends upon the presence of immunogenetic differences in the tumor-host relationship. Also in 1955, Graham J. B. and R. M. Graham screened autoantibodies in the sera of 48 patients with gynecological cancers including cervical, ovarian, and uterine lesions using the complement-fixation technique [11]. Twelve of these samples demonstrated significant autoantibody titers, suggesting for the first time that autoantibodies could be used as a diagnostic tool for cancer. In 1970, Taylor and Odili identified the first neoantigen, which was highly similar to the T antigen of oncogenic DNA virus, eliciting a specific humoral response in breast cancer [12]. In the following years, using immunofluorescence approaches Priori et al. confirmed the presence of autoantibodies in random sera from breast cancer patients [13]. In 1975, Wasserman et al. demonstrated that the incidence of autoantibodies at diagnosis of breast carcinoma was higher in patients who developed local recurrences or distal metastases within 2 years than in patients free from recurrence [14]. Although these results have not been be confirmed, this study was the first to use autoantibodies as prognostic biomarkers.

4. Autoantibodies to Individual TAAs

Considerable efforts have been made to identify autoantibodies and their antigen counterparts to detect and/or monitor cancer progression. Over the past 10 years, several technical approaches have been developed (Figure 1), and many studies have demonstrated the potential use of autoantibodies for early breast cancer detection. These molecules included p53, MUC-1, heat shock proteins (HSP-27, HSP-60, and HSP-90), HER2/neu/c-erg B2, GIPC-1, c-myc, c-myb, cancer-testis antigens (NY-ESO-1), BRCA1, BRCA2, endostatin, lipophilin B, cyclin B1, cyclin D1, fibulin, insulin-like growth factor binding protein 2 (IGFBP-2), topoisomerase II alpha (TOPO2α), and cathepsin D (for review see [9, 15]). Recently, an original study aimed to address the temporal relationship between breast cancer development and serum antibody responses against two previously identified TAAs (p53 and HER-2/neu) with sera collected prior to diagnosis, at diagnosis, and during treatment [16]. At the time of treatment, p53 and HER-2/neu autoantibodies were significantly increased in the sera collected from patients with breast cancer. Interestingly, comparison of antibody responses in prediagnostic samples and controls demonstrated that HER2/neu and p53 antibodies can be detected in sera collected, on average, more than 150 days prior to diagnosis. Although sample sizes were relatively small (33 cases and 45 controls), and although the percentage of patients producing autoantibodies against HER2 and p53 in prediagnostic samples was also low (15% and 6%, resp.), these results confirm the potential usefulness of these markers as indicators of the early stages of carcinogenesis.

In the past two years, new autoantibodies have been identified. Sun et al. provided the first evidence for the presence of circulating SOX2 antibodies in breast cancer [17]. The authors determined the expression levels of SOX2 antibodies in sera from 282 breast cancer patients, 78 benign breast disease patients, and 194 healthy women, using indirect enzyme-linked immunosorbent assay (ELISA). The results showed that SOX2 antibodies were more prevalent in patients with breast cancer (18.4%) than in healthy women (2.6%, $P < 0.0001$) and patients with benign breast disease (6.4%,

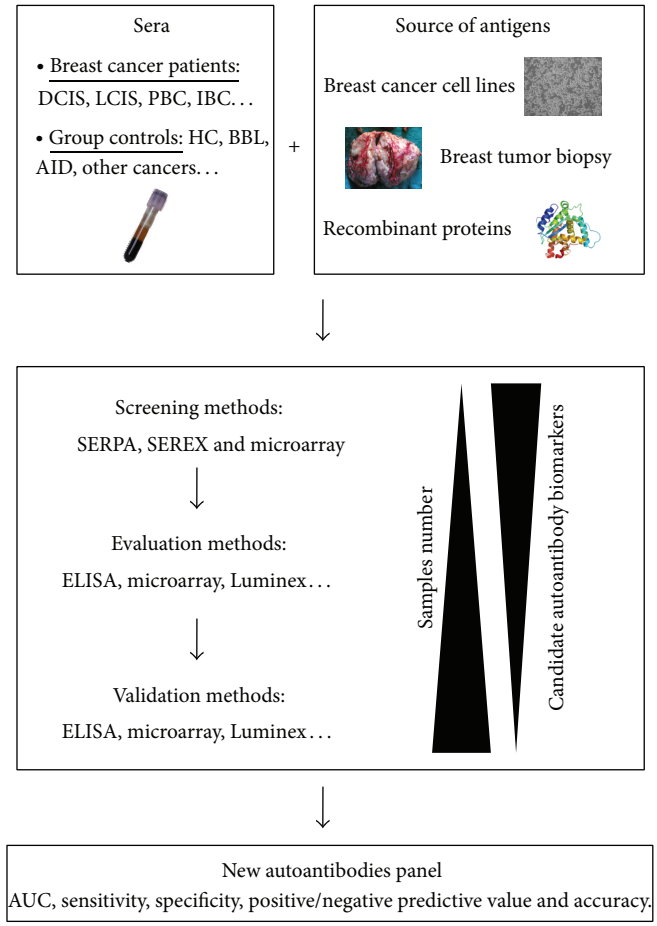

Figure 1: Breast cancer autoantibody research pipeline. Methodologies for identification of TAAs consist to locate specific immunogenic proteins specific from a source of antigens (recombinant protein or extracted from cell cultures or tumors). Different screening methods have been developed in order to identify TAAs such as SEREX (serological identification of antigens by recombinant expression cloning), SERPA (serological proteome analysis), or more recently microarray. TAAs were subsequently evaluated and validated using ELISA on multiplex methodologies such as Luminex or microarray. DCIS (ductal carcinoma *in situ*); LCIS (lobular carcinoma *in situ*); PBC (primary breast cancer); IBC (invasive breast cancer); HC (healthy control); BBL (benign breast lesions); AID (autoimmune disease); AUC (area under curve).

$P = 0.011$). Based on the concentration of circulating SOX2 antibodies, the investigators were able to discriminate between breast cancer patients and healthy controls ($P < 0.001$) and between breast cancer patients and those with benign breast disease ($P < 0.001$). Furthermore, in breast cancer patients the prevalence of SOX2 antibodies was associated with higher tumor grade and positive nodal status. Liu et al. also identified a specific humoral response against the p90/CIP2A antigen [18]. In 256 sera samples (168 from breast cancer patients and 88 from normal individuals), the authors showed that p90/CIP2A elicited higher autoantibody production in breast cancer (19.1%) than in normal volunteers (2.3%). These results were supported by the higher frequency of p90/CIP2A expression in breast cancer tissues than in adjacent normal tissues. Ye et al. have assessed the levels of CD25 and FOXP3 autoantibodies levels, previously identified in lung [19] and esophageal cancer [20, 21], in 152 breast cancer patients and 112 healthy individuals [22]. No significant differences were observed between breast cancer patients and controls. However, patients with stage I primary breast cancer exhibited higher expression of CD25 autoantibodies than healthy controls. In addition, Heo et al. observed by ELISA that a mimotope for circulating anti-cytokeratin 8/18 autoantibody discriminated breast cancer patients from normal subjects with a sensitivity and a specificity of 50% and 82.61%, respectively [23].

5. Tailor-Made Autoantibody Panels in Breast Cancer

Usually, only 10–30% of cancer patients elicited a specific humoral response against a single TAA [24]. The reason for this low sensitivity could lie in the heterogeneous nature of breast cancer, whereby different proteins are aberrantly processed or regulated in patients with the same type of cancer [25]. Therefore, several studies have evaluated the usefulness of detecting various autoantibodies as a panel to increase the accuracy of a potential diagnostic test (Table 1). Chapman et al. were the first to assess the frequency of

Table 1: Comparison between individual autoantibodies and autoantibody signatures in breast cancer.

TAA composition	Serum samples	Methods	Individual autoantibodies			Panel			Ref.
			AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	
p53, c-myc, HER2, NY-ESO-1, BRCA1, BRCA2, and MUC1	40 DCIS versus 97 PBC versus 90 HC	ELISA	—	24; 13; 18; 26; 8; 34; 20% (PBC versus HC) 15; 8; 13; 8; 3; 23; 13% (DCIS versus HC)	96; 97; 94; 94; 91; 92; 98%	—	64% (PBC versus HC) 45% (DCIS versus HC)	85%	[26]
p53, p62, c-myc, cyclin B1, survivin, and IMP1	64 breast cancers versus 346 HC	ELISA	Frequency of AABs to seven cancer-associated antigens: 7.8; 7.8; 18.8; 4.7; 7.8; 7.8% (breast cancer) and 1.4; 2; 1.2; 1.7; 2; 2% (HC)			Frequency of AABs to any seven antigens: 43.8% (breast cancer) and 10.1% (HC)			[31, 32]
						—	67–92%	85–95%	
MUC1, cyclin D1, cathepsin D, p53, HER2, IGFBP-2, and TOPO2α	184 late-stage IBC versus 134 HC	ELISA	Frequency of AABs to seven cancer-associated antigens: 20; 8; 5; 10; 13; 14; 7% (breast cancer) and 3; 5; 3; 1; 5; 1; 3% (HC)			Frequency of AABs to any p53. HER2. MUC1 and TOPO2α antigens: 31% (breast cancer)			[33]
			0.48 (p53)	—	—	0.61 (p53 + HER2) 0.63 (p53 + HER2 + IGFBP-2 + TOPO2α)	—	—	
ASB-9, SERAC1, and RELT	*Training set*: 5 breast cancers versus 5 HC *Validation set*: 87 breast cancers versus 87 HC	SEREX ELISA	0.593; 0.642; 0.727	41; 47; 53% (training set) 59; 53; 65% (validation set)	100; 100; 100% (training set) 65; 71; 77% (validation set)	0.861	80% (training set) 77% (validation set)	100% (training set) 82.8% (validation set)	[34]
HSP60, MUC1, FKBP52, PPIA, PRDX2, HSP60, and MUC1	*Training set*: 20 breast cancers versus 20 other cancers versus 20 AID versus 20 HC *Validation set*: 82 DCIS versus 60 early-stage PBC versus 93 HC	SERPA ELISA	0.69; 0.69; 0.66; 0.57; 0.59 (HC versus cancer) 0.63; 0.68; 0.66; 0.64; 0.63 (HC versus early-stage PBC) 0.73; 0.70; 0.65; 0.51; 0.56 (HC versus DCIS)	35; 37; 50; 50; 45% (HC versus cancer) 44; 43; 43; 48; 45% (HC versus early-stage PBC) 27; 32; 55; 51; 45% (HC versus DCIS)	87; 88; 87; 87; 86% (HC versus cancer) 86; 87; 90; 89; 87% (HC versus early-stage PBC) 90; 89; 87; 86; 84% (HC versus DCIS)	0.74 (HC versus cancer) 0.73 (HC versus early-stage PBC) 0.80 (HC versus DCIS)	60.5% (HC versus cancer) 55.2% (HC versus early-stage PBC) 72.2% (HC versus DCIS)	77.2% (HC versus cancer) 87.9% (HC versus early-stage PBC) 72.6% (HC versus DCIS)	[27]

Table 1: Continued.

TAA composition	Serum samples	Methods	Individual autoantibodies AUC	Sensitivity	Specificity	Panel AUC	Sensitivity	Specificity	Ref.
GAL3, PAK2, PHB2, RACK1, and RUVBL1	*Training set*: 20 cancers (10 DCIS and 10 early-stage PBC) versus 20 BBL versus 20 AID versus 20 HC *Validation set*: 55 DCIS versus 59 early-stage PBC versus 68 HC	SERPA ELISA	0.61; 0.56; 0.55; 0.59; 0.52 (HC versus cancer) 0.67; 0.59; 0.62; 0.61; 0.59 (HC versus early-stage PBC) 0.56; 0.52; 0.50; 0.57; 0.50 (HC versus DCIS)	32; 25; 24; 31; 24% (HC versus cancer) 47; 31; 32; 31; 31% (HC versus early-stage PBC) 24; 27; 18; 29; 18% (HC versus DCIS)	94; 96; 97; 94; 94% (HC versus cancer) 88; 94; 94; 97; 93% (HC versus early-stage PBC) 97; 91; 97; 94; 94% (HC versus DCIS)	0.81 (HC versus cancer) 0.81 (HC versus early-stage PBC) 0.85 (HC versus DCIS)	62–66% (HC versus cancer) 63–71% (HC versus early-stage PBC) 73–82% (HC versus DCIS)	83–87% (HC versus cancer) 81–84% (HC versus early-stage PBC) 74–82% (HC versus DCIS)	[28]
p62, p53, c-myc, survivin, p16, cyclin B1, cyclin D1, and CDK2	*Training set*: 1 cancer versus 4 HC *Validation set*: 41 cancers versus 82 HC	Miniarray ELISA	Frequency of AABs to eight cancer-associated antigens: 12.2; 12.2; 22; 22; 12.2; 17.1; 17.1; 9.8% (breast cancer) and 1.2; 2.4; 0; 1.2; 2.4; 1.2; 2.4; 1.2% (HC) —	22% (c-myc)	100% (c-myc)	Frequency of AABs to any eight antigens: 61% (breast cancer) and 11% (HC) —	61%	89%	[29]
hnRNPF and FTH1	*Training set*: 5 cancers versus 5 HC *Validation set*: 155 breast cancers versus 155 HC versus 40 others cancers	SEREX ELISA	0.725; 0.686	84.2; 81.2%	60.8; 56.1%	0.816	91.1% 89.3% (when combined with CA15-3)	72% 93.8% (when combined with CA15-3)	[30]
RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1	*Training set*: 20 DCIS versus 20 early-stage PBC *Validation set*: 61 DCIS versus 59 early-stage PBC	Microarray ELISA	0.57; 0.58; 0.51; 0.51; 0.54	62.7; 59.3; 16.9; 80.6; 59.3%	57.4; 54.1; 93.4; 31.8; 60.7%	0.794	83.3–86.1%	72.7–75%	[35]

TAA: tumor-associated antigens; AABs: autoantibodies; AUC: area under curve; DCIS: ductal carcinoma *in situ*; PBC: primary breast cancer; HC: healthy control; AID: autoimmune disease; BBL: benign breast lesions; Ref: references.

seven autoantibodies (p53, c-Myc, HER2, NY-ESO-1, BRCA1, BRCA2, and MUC1) in a ductal carcinoma *in situ* (DCIS) population of 40 patients [26]. Interestingly, reproducibly elevated serum levels of autoantibodies were seen in at least one of the six antigens in 64% of primary breast cancer patients and 45% of patients with DCIS, at a specificity of 85%. Desmetz et al. reported a multimarker signature combining HSP60, MUC1, FKBP52, PPIA, and PRDX2 that reached sensitivity, specificity, and accuracy of 72.2, 72.6, and 72.4%, respectively, in DCIS compared with healthy individuals [27]. Recently, the same group identified a panel of five new autoantibodies from 80 subjects (20 patients with early-stage DCIS or primary breast cancer, 20 women with benign breast lesions, 20 healthy controls, and 20 women with autoimmune disease) [28]. This panel consisted of GAL3, PAK2, PHB2, RACK1, and RUVBL1. The expression levels of these five markers were validated by ELISA on a second set of sera (182 patients: 59 patients with primary breast cancer, 55 patients with DCIS, and 68 healthy controls). The signature significantly discriminated early-stage cancer from healthy individuals (AUC = 0.81; 95% CI: 0.74–0.86). Interestingly, this value was high in both node-negative early-stage primary breast cancer patients (AUC = 0.81; 95% CI: 0.72–0.88) as well as in DCIS patients (AUC = 0.85; 95% CI: 0.76–0.95). Using microarray, Ye et al. assessed Imp1, p62, Koc, p53, cmyc, survivin, p16, cyclin B1, cyclin D1, and CDK2 autoantibody levels in 122 patients [29]. The antibody frequency to the individual TAAs in breast cancer was variable and ranged between 7.3% and 22.0%. However, with the successive addition of TAAs to a total of eight antigens, there was a stepwise increase in positive antibody reactions, reaching a sensitivity of 61.0% and a specificity of 89.0% in breast cancer. The positive and negative likelihood ratios were 5.545 and 0.438, respectively, which showed that the clinical diagnostic value of a parallel assay of eight TAAs was high. Moreover, the positive predictive value (PPV) was 73.5% and the negative predictive value (NPV) was 82.0%. Using a T7 breast cancer complementary deoxyribonucleic acid phage library for tumor-associated antigens, Dong et al. identified hnRNPF and FTH1 autoantibodies in breast cancer [30]. Autoantibodies have been evaluated by ELISA in 150 breast cancer, 150 normal, and 40 non-breast-cancer serum samples. Autoantibodies were significantly higher in breast cancer patients relative to controls ($P < 0.01$), with an AUC of 0.73 and 0.69 for hnRNPF and FTH1 autoantibodies, respectively. Specificities remained relatively low (56.1% for FTH1 and 60.8% for hnRNPF autoantibodies). Even when the two biomarkers were combined, the specificity remained low (72.0%), while the sensitivity increased to 91.0%. However, when both of these autoantibody biomarkers combined with serum CA 15-3 values, the AUC increased to 0.93, with 89.3% sensitivity and 93.8% specificity.

Autoantibody assessment as a prognostic biomarker has been poorly investigated. Using protein microarrays, Mangé et al. described significant and consistent differences in the level of autoantibodies targeting specific antigens in a population of 20 patients with DCIS and 20 with early-stage breast cancer [35]. In this protein microarray experimental study, a set of five autoantibody targets (RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1) with the highest differential signal intensities was used to establish an autoantibody signature of the transition from DCIS to early-stage cancer. The performance of this humoral signature was then assessed in an independent set of 120 newly diagnosed patients using ELISA. The results showed that this signature could significantly discriminate DCIS from invasive breast cancer (AUC = 0.794; 95% CI: 0.674–0.877). Moreover, this panel could clearly distinguish low-grade DCIS from high-grade DCIS (AUC = 0.749; 95% CI: 0.581–0.866). Interestingly, the autoantibody signature could significantly divide the DCIS patients into groups with either poor prognosis or good prognosis ($P = 0.01$). Taken together, these results suggested that examining the humoral response to preinvasive lesions could identify potential markers that accurately detect DCIS patients at high risk for subsequent local recurrence.

6. Clinical Implications

Until now, a wide range of autoantibodies has been identified. Although several studies present hopeful preliminary results, there is a need to validate autoantibody signatures on a large prospective population. Indeed, for biomarkers reach to the clinic, their original performance must be independently reproduced in subsequent validation studies [36]. However, most of the studies cited above are limited by the size of the validation sample set. As an example in a breast cancer population, when MUC1 autoantibody was assessed in prediagnostic sera from over 2000 women, distributed across one discovery set (273 cases versus 273 controls) and two validation sets (426 cases versus 426 controls and 303 cases versus 606 controls), no differences could be observed between cases and controls. This result demonstrates the need to validate results in several independent cohorts. Validation is usually performed by ELISA, an assay that is rapid and simple to carry out and can handle a large number of samples in parallel. However, multiplex analysis remains difficult because ELISA processing is usually time consuming and expensive. Currently, two types of techniques allow for multiplex analysis. The Lumina immunobead platform (LabMAP, FlowMetrix) uses digital signal processing capable of classifying polystyrene beads (microspheres) dyed with distinct proportions of red and near-infrared fluorophores. These proportions define a "spectral addresses" for each bead population. As a result, up to one hundred different detection reactions can be carried out simultaneously on the various bead populations in very small sample volumes [37]. This technology has already been utilized in non-small-cell lung cancer autoantibodies detection [38, 39]. In 2009, Kim et al. used the bead array platform for discovering signatures specific to primary nonmetastatic breast cancer and differentiating these patients from normal subjects using sensitive combinatorial classifiers [40]. In his work, an antibody bead array of 35 markers was constructed, and an initial study population consisting of 98 breast cancer patients and 96 normal subjects was analyzed. Multivariate classification algorithms were then used to find discriminating biomarkers, which were validated with another independent population

of 90 breast cancer subjects and 79 healthy controls. Serum concentrations of three autoantibodies (against epidermal growth factor, soluble CD40-ligand, and proapolipoprotein A1) were increased in breast cancer patients, whereas five autoantibodies (against high-molecular-weight-kininogen, apolipoprotein A1, soluble vascular cell adhesion molecule-1, plasminogen activator inhibitor-1, vitamin-D binding protein, and vitronectin) were decreased. The classifier was able to discriminate breast cancer patients from the normal population with high accuracy (87.6% to 91.8% according to classification method). The second multiplex approach consists of protein microarray technology. This technique was developed for high throughput and multiparametric assays that allow for the identification of multiple tumor markers. Combining various TAAs onto microstructured microarray under optimized conditions (spotting pH buffer, surface chemistry, and blocking procedure) could improve sensitivity and specificity of anti-TAA autoantibody detection. A recent paper showed the utility of protein microarray for autoantibody detection in sera of breast cancer patients [41]. The authors investigated both surface chemistry and protein immobilization conditions to improve sensitivity of the detection of tumor autoantibodies on these microarrays. Ten proteins (CEA, p53, HER2, NY-ESO-1, Hsp60, Hsp70, MYCL1, CHEK2, HNRNPK, and NME1) were immobilized onto microstructured glass slides functionalized with six different surface chemistries to detect autoantibodies in sera of breast cancer patients. The authors demonstrated that there is not a unique surface chemistry suitable for all proteins and that immobilization parameters must be optimized for each protein. Thus, to validate the best surfaces for protein immobilization and biological activity, sera from 29 breast cancer patients and 28 healthy donors were tested on TAA microarrays. Through a combination of five TAAs (Hsp60, p53, Her2-Fc, NY-ESO-1, and Hsp70) immobilized on an optimized surface chemistry, 82.7% of breast cancer patients were specifically detected. The potential cost and time savings that could be realized by using these technologies relative to other methods provide a strong impetus for their routine use in both research and clinical settings. Nevertheless, as with all clinical laboratory tests, questions of reproducibility, precision, and accuracy must be addressed to validate these assays [37].

With the inherent heterogeneity of breast tumors and our limited understanding of the humoral immune response to cancer, there are some obstacles to autoantibody identification and their routine clinical use for early breast cancer detection. However, this promising type of diagnostic strategy should continue to be developed. Recent published reports indicate an encouraging future for the implementation of sensitive and specific tests. It is conceivable that a humoral signature based on the detection of specific autoantibodies can be applied to the detection of cancer as well as to the tracking of disease progression and response to therapy. The most significant hope may be the use of such a signature for detection of cancers to which patients are predisposed by monitoring autoantibody profiles before the first clinical manifestation of symptoms. Finally, investigators should pursue a transition from the current system of retrospective studies to prospective analyses of patients' autoantibody responses and an assessment of this method's efficacy in clinical settings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Glioma-Associated Antigen HEATR1 Induces Functional Cytotoxic T Lymphocytes in Patients with Glioma

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A2B5+ glioblastoma (GBM) cells have glioma stem-like cell (GSC) properties that are crucial to chemotherapy resistance and GBM relapse. T-cell-based antigens derived from A2B5+ GBM cells provide important information for immunotherapy. Here, we show that HEAT repeat containing 1 (HEATR1) expression in GBM tissues was significantly higher than that in control brain tissues. Furthermore, HEATR1 expression in A2B5+ U87 cells was higher than that in A2B5−U87 cells ($P = 0.016$). Six peptides of HEATR1 presented by HLA-A∗02 were selected for testing of their ability to induce T-cell responses in patients with GBM. When peripheral blood mononuclear cells from healthy donors ($n = 6$) and patients with glioma ($n = 33$) were stimulated with the peptide mixture, eight patients with malignant gliomas had positive reactivity with a significantly increased number of responding T-cells. The peptides $HEATR1_{682–690}$, $HEATR1_{1126–1134}$, and $HEATR1_{757–765}$ had high affinity for binding to HLA-A∗02:01 and a strong capacity to induce CTL response. CTLs against HEATR1 peptides were capable of recognizing and lysing GBM cells and GSCs. These data are the first to demonstrate that HEATR1 could induce specific CTL responses targeting both GBM cells and GSCs, implicating that HEATR1 peptide-based immunotherapy could be a novel promising strategy for treating patients with GBM.

1. Introduction

Human glioblastoma (GBM) accounts for approximately 60–70% of malignant gliomas, the most common and deadly brain tumors [1]. Despite improvements in standard therapies including surgery, radiation, and chemotherapy, the poor prognosis of patients with GBM has not been obviously improved. Immunotherapy represents a promising treatment designed to reshape the immune system to specifically eradicate malignant cells. The effort of T-cell-mediated immunotherapy to selectively kill remnant glioma cells that could not be completely removed using microsurgery has been highlighted [2–4].

Glioma stem-like cells (GSCs) may be capable of initiating tumor growth [5, 6] and are likely to be responsible for the malignant behavior of tumors because of their acquired resistance to chemotherapy, radiotherapy, and immunotherapy induced by glioma-associated antigens, which results in the ineffectiveness of existing conventional therapies [7–9]. Thus, GSCs could be a novel target for cancer therapy, including immunotherapy. Our recent study findings indicated that glioma stem-like cell-associated antigens (SAAs) from CD133+ GSCs bear highly immunogenic antigens and induce significant responses from cytotoxic T lymphocytes (CTLs) [10]. Several other studies have tried immunotherapy targeting GSCs [11–16].

A2B5 is considered a marker for immature glial-committed progenitors that are permanently generated in the subventricular zone. Glial progenitor cells are defined as cells that give rise to glial cell types such as astrocytes and oligodendrocytes. In GBM tissues, A2B5+ cells include A2B5+/CD133+ and A2B5+/CD133− cells. Furthermore, A2B5+ cells from human GBM have cancer stem-like cell properties that are crucial for the initiation and maintenance of GBM [17, 18]. Thus, A2B5+ GBM cells could be an ideal target for GBM immunotherapy. Our recent study found that vaccination with A2B5+ GL261 cell lysate-pulsed dendritic cells had a preventive effect for mouse glioma [19]. However, T-cell epitopes derived from A2B5+ GBM progenitor cells for immunotherapy have not been reported.

To identify novel genes selectively overexpressed in A2B5+ GBM as the target for T-cell mediated immunotherapy, we sequenced the mRNA profile of A2B5+ GBM cells from U87 cell lines using fluorescence-activated cell sorting (FACS) by Solexa sequencing (data not shown) and identified that the HEAT repeat containing 1 (HEATR1) gene (gene ID: 55127) was overexpressed in A2B5+ GBM cells. Recently, Bleakley et al. reported that HEATR1 was highly expressed in testis and ovary than in other tissues including liver, colon, small intestine, lung, brain, and heart [20]. Identification of epitope derived from HEATR1 is likely to provide alternative candidates for the design of antitumor vaccine with high efficacy in the future.

In the present study, we confirm the selective HEATR1 overexpression in A2B5+ GBM cells and in the vast majority of GBM. In addition, we identify several HEATR1-derived T-cell epitopes in tumor carrier patients. Our results emphasize the suitability of this protein for T-cell-based immunotherapy in patients with GBM.

2. Materials and Methods

2.1. Ethics Statement. The study protocol was approved by the Local Independent Ethics Committee at Huashan Hospital, Fudan University. Some samples in this study were used in our previous reports [21, 22]. Written informed consent was obtained from each donor of the samples used in our research.

2.2. Cell Lines. Human GBM cell lines U87, A172, and SHG66 were used in this study. SHG66 came from a 47-year-old man with a right parietal glioblastoma (World Health Organization grade IV) [10]. U87 and A172 cells were purchased from the cell bank of the Shanghai Branch of Chinese Academy of Sciences. A172 cells did not express HLA-A∗02:01 [23, 24], while the other two GBM cell lines expressed HLA-A∗02:01 according to flow cytometry [25, 26]. The HLA-A∗02:01, expressing human tumor cells T2 (deficient in TAP1 and TAP2 transporters), and BB7.2 hybridoma, producing anti-HLA-A∗02 monoclonal antibody (mAb), were purchased from American Type Culture Collection (USA). All cell lines were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 100 U/mL penicillin/streptomycin (Gibco) and maintained in a humidified atmosphere with 5% CO_2 at 37°C.

The GSC lines (U87, A172, and SHG66) were established and characterized as described previously [10, 13]. Short-term tumor spheres of the GBM cell lines were cultured in serum-free medium (SFM) consisting of DMEM/F12 (Invitrogen) supplemented with 20 ng/mL recombinant human basic fibroblast growth factor (bFGF; Chemicon), 20 ng/mL recombinant human epidermal growth factor (EGF; Chemicon), and B27 (Invitrogen). The GSC tumor spheres exhibited stem cell-like characteristics [10, 15].

2.3. Patients. A total of 22 frozen GBM tumor tissues were obtained from the Department of Neurosurgery, Huashan Hospital, to analyze the expression level of HEATR1 mRNA. Additionally, eight control brain tissue samples were obtained from adjacent brain tissues of patients with traumatic brain injury who suffered contusion and laceration. In addition, 10 GBM formalin-fixed, paraffin-embedded (FFPE) tissue sections and 10 normal brain tissues were analyzed by IHC.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque (Biochrom, Berlin, Germany) density gradient centrifugation of heparinized blood obtained from healthy donors ($n = 6$) and patients (benign tumors, 5; grade 2 astrocytoma, 7; grade 3 anaplastic glioma, 10; glioblastoma, 16). The patients' clinical characteristics are listed in Table 1.

2.4. FACS with A2B5. The U87 cells were resuspended at a density of 1×10^5 cells/mL in SFM consisting of DMEM/F12 (Invitrogen) supplemented with 20 ng/mL recombinant human bFGF, 20 ng/mL recombinant human EGF, and B27. U87 cells were cultured for 2 weeks. A2B5-PE antibody (Miltenyi Biotec) was used in this study for FACS. Cell sorting was performed on a BD FACSVantage Cell Sorter (BD Biosciences) according to the manufacturer's instructions.

2.5. Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of HEATR1 Expression. Total RNA was extracted from GBM and control brain tissues or from the GBM cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were synthesized using a High-Capacity cDNA Archive Kit. Each cDNA (2 μL) was amplified in a SYBR Green Real-time PCR Master Mix (final volume, 20 μL) and loaded on an Applied Biosystems 7900 Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions for quantitative RT-PCR (qRT-PCR) were as follows: the first step, 95°C for 10 min and the ensuing 40 cycles, 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s. The qRT-PCR primers used were as follows: HEATR1 (forward) 5′-TCCTTTTTGATACCCAGCATTTTAT-3′ and HEATR1 (reverse) 5′-TGATCCACCAGAGGCATCATC-3′; actin (forward) 5′-CCCTGGCACCCAGCAC-3′ and actin (reverse) 5′-GCCGATCCACACGGAGTAC-3′. All samples were analyzed in triplicate. To validate that the efficiencies of the target gene amplification and *β-ACTIN* amplification were approximately equal, we plotted standard curves of log input amount versus ΔCT (CT_{target}–$CT_{control}$) for every

Table 1: Clinical characteristics of healthy donors and patients.

Number	Gender	Years	Tumor location	Pathology	Grade	ELISpot response	HLA-A2
198	M	54		Healthy		Negative	Yes
203	M	55		Healthy		Negative	Yes
205	F	56		Healthy		Negative	Yes
209	F	45		Healthy		Negative	Yes
219	M	22		Healthy		Negative	No
306	M	31		Healthy		Negative	No
215	F	48	Right frontal	Meningioma	0	Negative	Yes
216	F	62	Cerebellum	Hemangioblastoma	0	Negative	No
255	F	59	Left frontal	Meningioma	0	Negative	Yes
226	F	49	Sellar region	Pituitary adenoma	0	Negative	Yes
261	M	21	Sellar region	Pituitary adenoma	0	Negative	No
122	M	45	Right temporal¶	Astrocytoma	2	Negative	No
135	F	37	Right temporal¶	Astrocytoma	2	Negative	Yes
217	M	48	Right frontal¶	Astrocytoma	2	Negative	No
246	F	30	Left parietal	Astrocytoma	2	Negative	Yes
252	M	50	Right temporal	Oligodendroglioma	2	Negative	No
264	M	45	Right frontal-callosum	Astrocytoma	2	Negative	Yes
262	M	40	Left temporal	Oligodendroglioma	2	Negative	Yes
218	M	58	Right temporal	AA	3	Negative	Yes
238	F	56	Right callosal convolution¶	AO	3	Negative	No
254	F	33	Right frontal	AOA	3	Negative	Yes
256	F	46	Right temporal-basal ganglia	AA	3	Negative	No
259	F	58	Right parietal	AO	3	Negative	No
265	M	56	Right frontal-parietal¶	AOA	3	Negative	No
127	F	42	Left frontal	AA	3	Positive	No
140	M	45	Left temporal	AA	3	Positive	A0201/A1101
156	M	48	Left occipital	AE	3	Positive	A0203/A3001
239	M	16	Left temporal	AA	3	Positive	A0207/A1102
129	F	38	Left occipital	GBM	4	Negative	Yes
133	F	48	Left temporal	GBM	4	Negative	No
147	F	61	Left temporal	GBM	4	Negative	Yes
150	M	48	Right frontal-temporal	GBM	4	Negative	Yes
151	M	30	Right temporal	GBM	4	Negative	Yes
214	M	66	Right parietal-occipital	GBM	4	Negative	No
223	M	37	Left frontal-temporal¶	GBM	4	Negative	Yes
224	M	59	Left temporal-occipital	GBM	4	Negative	Yes
225	M	31	Left frontal¶	GBM	4	Negative	No
231	M	36	Left frontal	GBM	4	Negative	No
241	M	58	Right frontal¶	GBM	4	Negative	Yes
253	M	54	Left frontal	GBM	4	Negative	No
132	M	68	Right temporal	GBM	4	Positive	A0201/A0203
141	F	12	Right parietal-occipital¶	GBM	4	Positive	No
220	M	47	Left temporal	GBM	4	Positive	A0201/A3303
221	F	39	Right temporal	GBM	4	Positive	No

¶: recurrence; F: female; M: male; GBM: glioblastoma multiforme; AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma; AOA: anaplastic oligoastrocytoma; AE: anaplastic ependymoma.

gene and all the slopes of the plot <0.1. The ΔΔCT method recommended by the manufacturer was used to compare the relative expression levels between samples.

2.6. IHC Analysis. Human GBM FFPE tissue sections were provided and IHC stained with HEATR1-specific antibody made against COOH-terminal peptide of human HEATR1 (Sigma-Aldrich) using a DakoCytomation EnVision+ System-HRP (DAB) detection kit. Briefly, 5 μm tissue sections were dehydrated and subjected to peroxidase blocking. HEATR1 antibody was added at a dilution of 1 : 20 and incubated at room temperature for 30 min on the Dako Autostainer using the DakoCytomation EnVision+ System-HRP (DAB) detection kit. The slides were counterstained with hematoxylin. The stained slides were observed under a microscope and images were acquired. Cytoplasm staining was considered positive. To evaluate HEATR1 expression, 10 high-power fields (400x) within the tumor showing cytoplasm staining were selected. IHC signals were visually quantified by L.F. Sempere using a quick score system combining staining intensity and positive cell percentage (staining intensity: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong; percentage: 0 = 0%, 1 = <25%, 2 = ≥25%, and 3 = ≥50%). All of the IHC stained sections were evaluated by two senior neuropathologists blinded to the clinical parameters.

2.7. Peptide HLA-A∗02:01 Binding Affinity. The binding activity of selected peptides to the HLA-A∗02 molecule was determined semiquantitatively by measuring peptide-induced expression of HLA-A∗02:01 on T2 cells using flow cytometry. The T2 cells were incubated for 4 h with the candidate peptides, respectively, at a concentration of 20 μg/mL in SFM. After being washed with phosphate buffered saline-fetal calf serum (PBS-FCS), the T2 cells were incubated with supernatant containing murine mAb against HLA-A∗02:01 derived from BB7.2 cells for 30 min at 4°C. The T2 cells were washed twice with PBS-FCS and stained with 5 μg/mL diluted fluorescein isothiocyanate-conjugated immunoglobulin G which reacts to mouse immunoglobulin for 30 min. The cells were then rinsed three times with PBS-FCS and analyzed using a FACSAria flow cytometer. The percent mean fluorescence index (% MFI) increase of HLA-A∗02:01 molecules was calculated as follows: % MFI increase = [(MFI with peptide − MFI without peptide)]/(MFI without peptide) × 100 [27].

2.8. Interferon-γ- (IFN-γ-) Based Enzyme-Linked Immunosorbent Spot (ELISpot) Assay. A human IFN-γ ELISpot kit (552138; BD Pharmingen, CA) was used to quantify the CTL response in PBMCs. Several 96-well plates were coated with purified anti-human IFN-γ monoclonal antibodies at the concentration of 5 μg/mL at 100 μL/well and incubated at 4°C overnight and then washed once with 200 μL/well of RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin-L-glutamine (R10) and blocked with 200 μL/well R10 for 2 h at room temperature. PBMCs were then washed twice with R10 and resuspended in R10 complete culture medium. After being counted, the cells were then adjusted to the concentration of 1×10^6 cells/mL and plated onto a 96-well ELISpot plate at 50 μL/well (5×10^4 cells/well) with the addition of 50 μL of the peptide. The final concentration of each peptide was 5 μg/mL. The 96-well ELISpot plates were incubated for about 20 h at 37°C in 5% CO_2. After incubation, the ELISpot plates were developed according to the kit instructions. Finally, the plates were air-dried and the resulting spots were counted with ChampSpot IV Bioreader (Beijing SAGE Creation Science, Beijing, China). Peptide-specific IFN-γ ELISpot responses were considered positive only when the number of spots was twofold greater than the control peptide stimulation and there were >50 spots per 1×10^6 PBMCs [28, 29].

2.9. Cytotoxicity Assay by Measuring Lactate Dehydrogenase (LDH) Activity. CytoTox 96 Nonradioactive Cytotoxicity Assay (Cat. number G1780, Promega) was used to determine the cell-mediated cytotoxicity [27, 30]. U87, SHG66, and A172 cells serving as target cells (1×10^5) were loaded with 4 μg/mL peptide for 2 h at 37°C and 5% CO_2. Effector PBMCs (1×10^6) were added to peptide-loaded or blank target cells and cultured for additional 4 h at 37°C and 5% CO_2. To measure the LDH activity, 50 μL of the reconstituted substrate mix was added to 50 μL of the culture supernatant and incubated at room temperature protected from light for 30 min. A total of 50 μL of the stop solution was added to each well of the plate. The concentrations of the colorimetric product were recorded as absorbance at 490 nm by a spectrometer [27].

2.10. Statistical Analysis. All statistical analyses were carried out using the SPSS 16.0 statistical software package. Continuous variables are expressed as mean ± SEM. Statistical differences between the two groups were evaluated using the unpaired Student's t-test. The correlation between ELISpot response and glioma grades was evaluated using the χ^2 test. P values < 0.05 were considered statistically significant (two-tailed test).

3. Results

3.1. HEATR1 Overexpression in GBM and A2B5+ GBM Cells. First, we investigated whether HEATR1 was overexpressed in GBM cells. We investigated the expression profile of HEATR1 mRNA in 22 primary GBM tissues and eight control brain tissues using quantitative RT-PCR. As shown in Figure 1(a), the expression of HEATR1 mRNA in GBM tissues was higher than that in control brain tissues ($P < 0.01$). In addition, IHC was initially performed in FFPE tissue sections of primary GBM ($n = 10$) and normal brain tissues ($n = 10$). As shown in Figure 1(b), HEATR1 protein was mainly localized in the tumor cell cytoplasm and nuclei. The average IHC score of HEATR1 expression in GBM and normal brain tissues was 4.4 ± 0.7 and 2.1 ± 0.4, respectively. GBM tissues had higher expressions of HEATR1 protein than normal brain tissues (Figure 1(c), $P = 0.015$). However, the expression level of HEATR1 proteins did not appear to be correlated with glioma grade (data not shown).

Next, we investigated whether HEATR1 expression in A2B5+GBM cells was higher than that in A2B5−GBM cells.

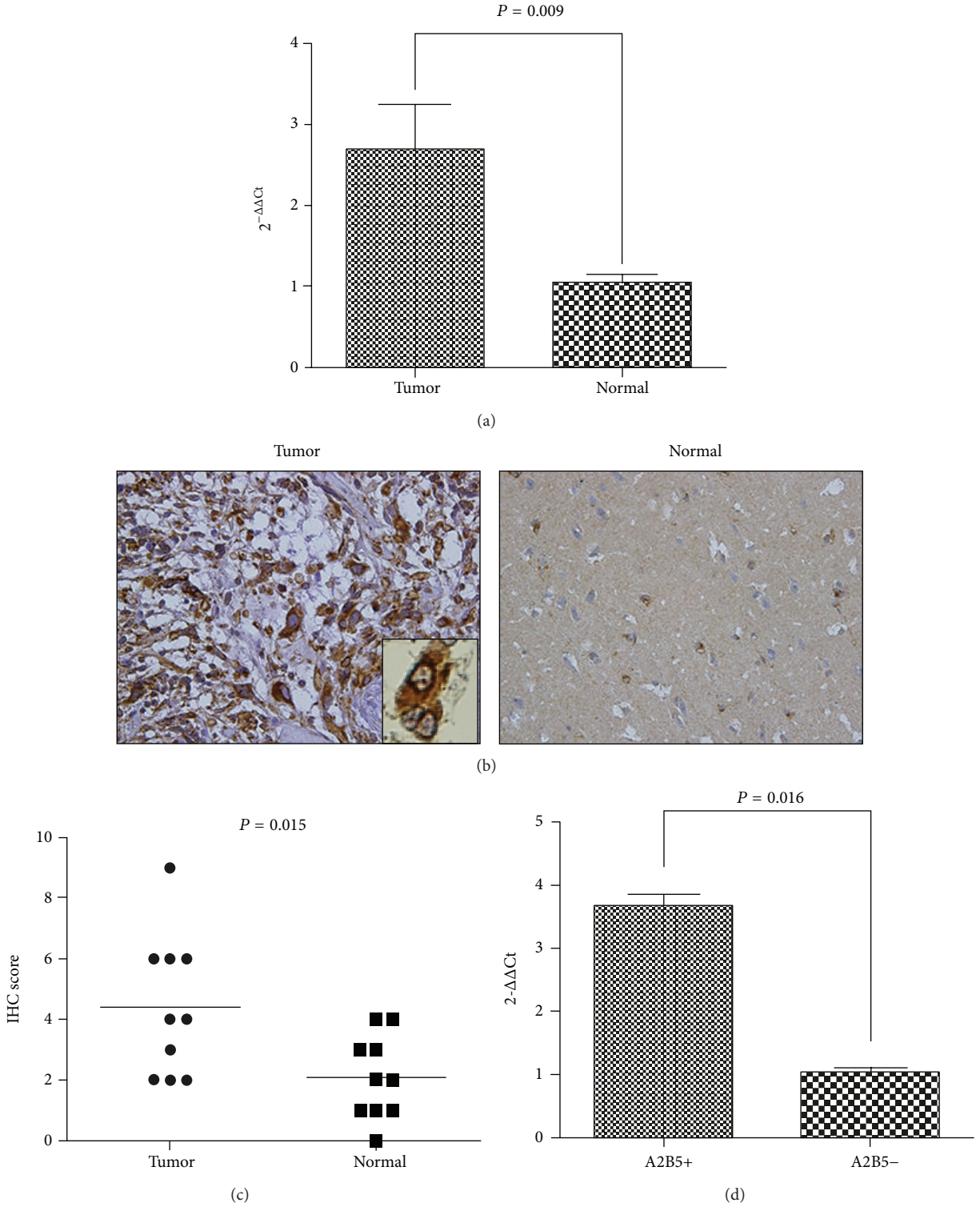

Figure 1: HEATR1 was overexpressed in GBM and in A2B5+GSCs. (a) qRT-PCR was performed to analyze the differential expression between GBM tissues ($n = 22$) and controlled brain tissues ($n = 8$). (b)-(c) IHC was performed in FFPE tissue sections of 10 primary GBM tissues (left, ×400) and 10 normal brain tissues (right, ×400). GBM tissues had higher staining score of HEATR1 protein than normal brain tissues ($P = 0.015$). (d) qRT-PCR was performed to analyze the differential expression between A2B5+U87 cells and A2B5−U87 cells ($P = 0.0016$).

Our previous study showed that U87 cells cultured in SFM for 2 weeks had stem-like features [10]. Furthermore, those A2B5+ U87 cells were double-positive for CD133 and nestin or vimentin (Supplementary Figures 1, 2, and 3, resp., in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/131494). Prior to sorting, the percentage of A2B5+ cells accounted for 6.5%. HEATR1 mRNA in sorted A2B5+ U87 cells was significantly higher than that in A2B5−U87 cells quantified by qRT-PCR (P = 0.016, Figure 1(d)).

3.2. Prediction of Candidate HLA-A∗02-Binding Peptides Derived from HEATR1. Due to HEATR1 overexpression in GBM, we sought to determine whether HEATR1-derived epitopes that could be presented by antigen process machinery and induce the CTL response in patients with GBM. Since HLA-A∗02:01 is expressed by 30–40% of Asians as the most common subtype of HLA-I class [31, 32], epitopes potentially binding to HLA-A∗02:01 were generated using the HLA Peptide Binding Predictions Program (http://www-bimas.cit.nih.gov/molbio/hla_bind/) of the Bioinformatics and Molecular Analysis Section [12]. Six peptides with binding scores >1000 were selected as the candidate epitope peptides (Table 2). Peptides including $HEATR1_{2003–2011}$ (2003–2011, FLFDTQHFI), $HEATR1_{1126–1134}$ (1126–1134, KLLRMLFDL), $HEATR1_{2102–2110}$ (2102–2110, LLPESIPFL), $HEATR1_{1411–1419}$ (1411–1419, FLWILLILL), $HEATR1_{682–690}$ (682–670, KMVEDLISV), and $HEATR1_{757–765}$ (757–765, LMLDRGIPV) were synthesized by GL Biochem (Shanghai) Ltd. with >95% purity as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis. The negative control peptides (CFLPVFLAQPPSGQR) were also synthesized.

3.3. Affinity of Candidate Epitope Peptides for HLA-A∗02 Molecule. The T2-cell-peptide binding test was used to evaluate the binding affinity of these candidate epitope peptides for HLA-A∗02 with flow cytometry *in vitro* (Figure 2(a)). As shown in Figure 2(b), $HEATR1_{682–690}$ had the highest affinity for HLA-A∗02:01 and the percentage of MFI increase was 308.5 ± 4.8%. The percentages of MFI increase of $HEATR1_{2102–2110}$, $HEATR1_{1126–1134}$, and $HEATR1_{757–765}$ were 285.2 ± 49.2%, 287.2 ± 7.7%, and 228.7 ± 5.4%, respectively. $HEATR1_{2003–2011}$ was a lower affinity peptide, while $HEATR1_{1411–1419}$ had the lowest affinity for binding to HLA-A∗02.

3.4. HEATR1-Derived Peptides Induced CTL Responses. In the next set of experiments, we tested whether those candidate peptides are epitopes that can be recognized by the host immune system *in vivo*. PBMCs from glioma carriers were incubated with those six mixed peptides and the IFN-γ secretion was tested by the ELISpot. As shown in Table 1, we found that eight patients (anaplastic astrocytomas/ependymoma in four and glioblastoma in four) had positive reactivity with a significant increase of ELISpot-detected spots (Figure 3(a)). The frequency of positive reactivity in malignant gliomas

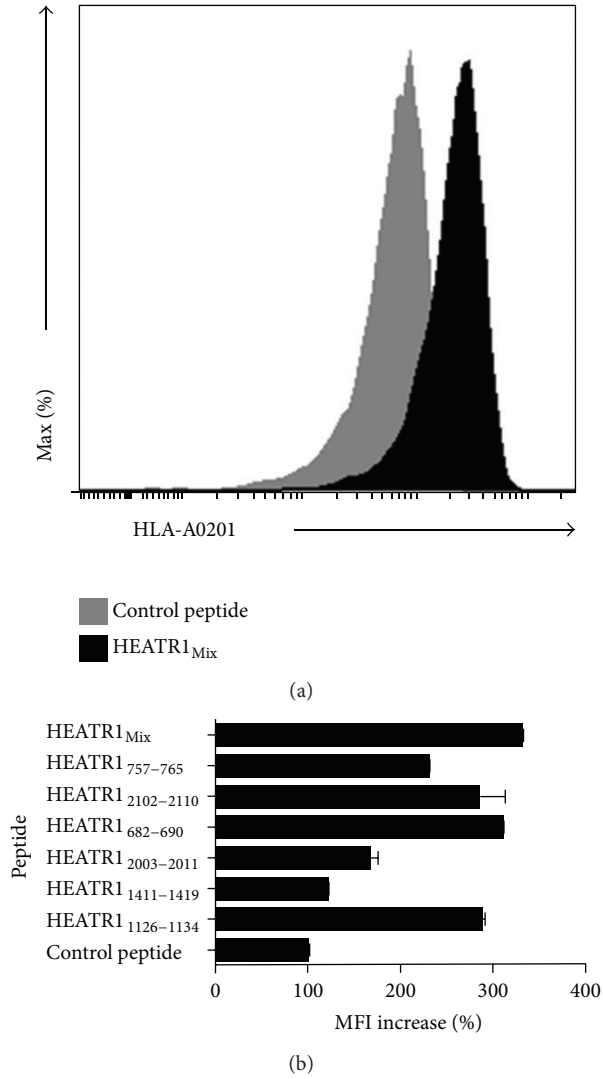

Figure 2: HLA-A02 binding affinity of six candidate peptides. (a) Flow cytometry results of $HEATR1_{mix}$. (b) The binding activity of selected peptides to HLA-A∗02 molecule was determined semi-quantitatively by measuring peptide-induced expression of HLA-A∗02 on T2 cells with flow cytometry. Data from three independent experiments were expressed as the mean ± SE. Unrelated 15-mer peptides were considered as control peptide.

accounts for about 31%. In this study, those positive responses were only observed in the malignant glioma, indicating that those epitopes could be considered specific for malignant gliomas and significantly higher than healthy donors and low-grade glioma carriers (Figure 3(b), P = 0.022). In addition, three of eight patients with positive reactivity were non-HLA-A∗02 (Supplementary Table 1), indicating that these peptides might not be exclusively presented by HLA-A∗02.

Furthermore, we investigated which individual HEATR1-derived peptide could induce the CTL responses. PBMCs from HLA-A∗02+ patients, five patients with GBM and one control patient with a benign tumor, were stimulated with

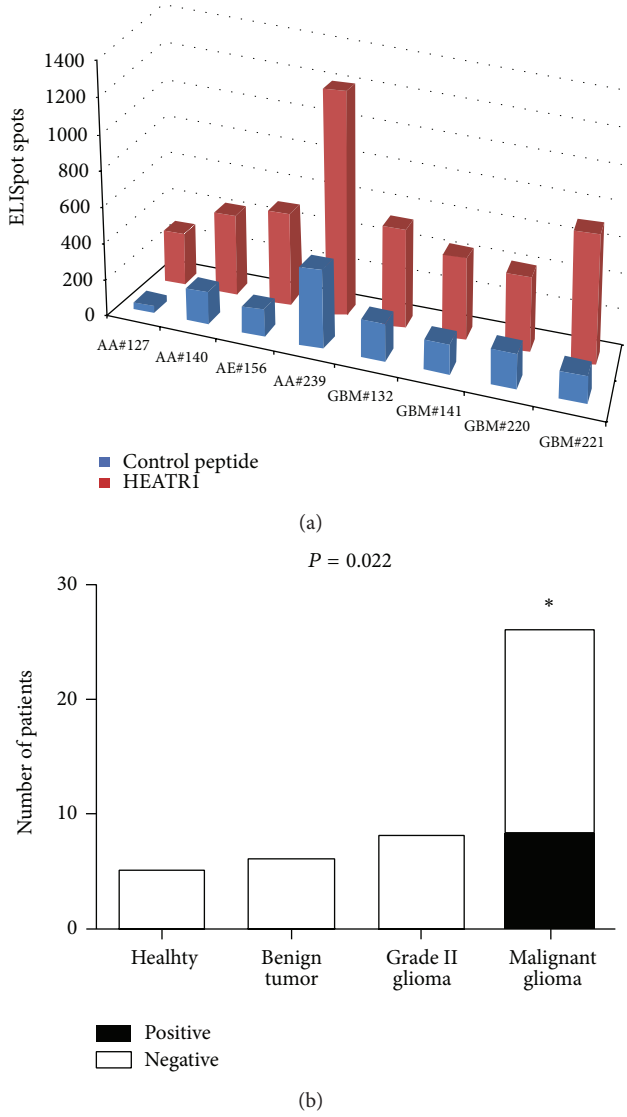

Figure 3: Six epitope peptides derived from the HEATR1 induce the IFN-γ response. (a) ELISpot result of 8 malignant gliomas with positive reactivity. The number of IFN-γ formingspots was calculated per 1×10^6 PBMCs. (b) The positive reactivity among 6 healthy donors and 38 patients only occurred in 8 malignant gliomas ($P = 0.022$). GBM: glioblastoma multiforme; AA: anaplastic astrocytoma; AE: anaplastic ependymoma. This is a representative experiment from two independent experiments. No peptide stimulation was negative control. Correlation between ELISpot response and glioma grades was evaluated using a χ^2 test.

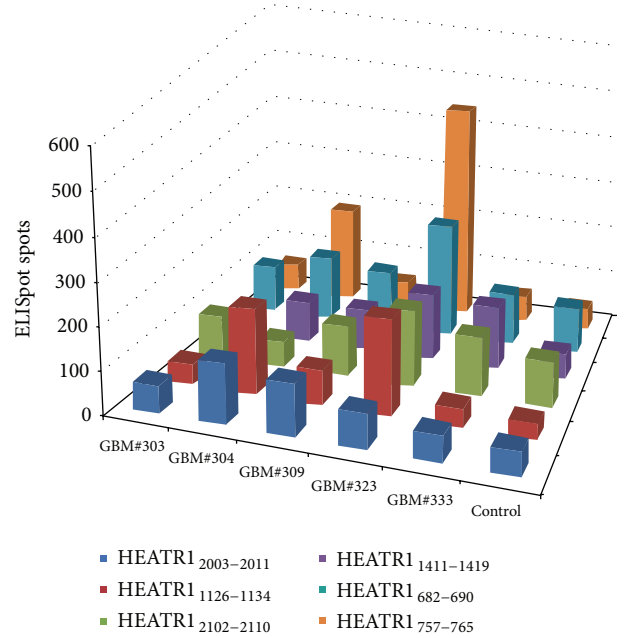

Figure 4: Single epitope peptide derived from the HEATR1 induces the IFN-γ response using ELISpot assay. PBMCs were extracted from 5 patients with HLA-A2+ GBM and 1 controlled patient with HLA-A$*$02+ benign tumor. IFN-γ formingspots were calculated per 1×10^6 PBMC.

individual peptide. As shown in Figure 4, $HEATR1_{757–765}$ had the highest ELISpot response, indicating that it is the most immunogenic *in vivo*. In addition, the ELISpot responses induced by $HEATR1_{682–690}$ and $HEATR1_{1126–1134}$ were higher than the others (Figure 5). These data indicate that these three peptides possess the ability to induce CTLs *in vivo*.

3.5. HEATR1-Specific CTLs Lyse GBM Cells and GSCs. Finally, we evaluated the ability of HEATR1-specific CTLs to lyse GBM cell lines endogenously expressing HEATR1 *in vitro*; all three GBM cell lines (U87, SHG66, and A172) are capable of expressing endogenous HEATR1 with the highest expression in U87 cell lines (Figure 5(a)). The cytotoxic activity of patients' PBMCs (effector cells) was evaluated using an LDH-release assay. PBMCs of patient 323 (positive ELISpot response with HLA-A$*$02+; Table 1) were incubated with three GBM cell lines (U87, SHG66, and A172) as target cells, respectively. The results showed that peptide-stimulated PBMCs could lyse 37.4% of U87 and 23.1% of SHG66 target cells expressing both HEATR1 and HLA-A$*$02 at an E : T ratio of 10 : 1 but not A172 cells that are HLA-A$*$02-negative (Figure 5(b)). We further evaluated whether CTLs recognizing the HEATR1 peptides could kill A2B5+ GSCs. PBMCs from patient 323 demonstrated the ability to kill 76.8% of A2B5+ U87 GSCs and 20.4% of A2B5+ SHG66 GSCs at an E : T ratio of 10 : 1 (Figure 5(c)). These data suggest that HEATR1-specific CTLs are effective to lyse target cells endogenously expressing HEATR1; the cytotoxicity is associated with the expression level of endogenous HEATR1.

4. Discussion

To our knowledge, we reported first here that HEATR1 was especially overexpressed in GBM cells and A2B5+GBM cells. T-cell epitopes derived from HEATR1 could significantly induce the CTL response *in vivo* and these CTLs were able to lyse both GBM cells and GSCs. These results indicate that HEATR1 has great potential for the development of glioma immunotherapy.

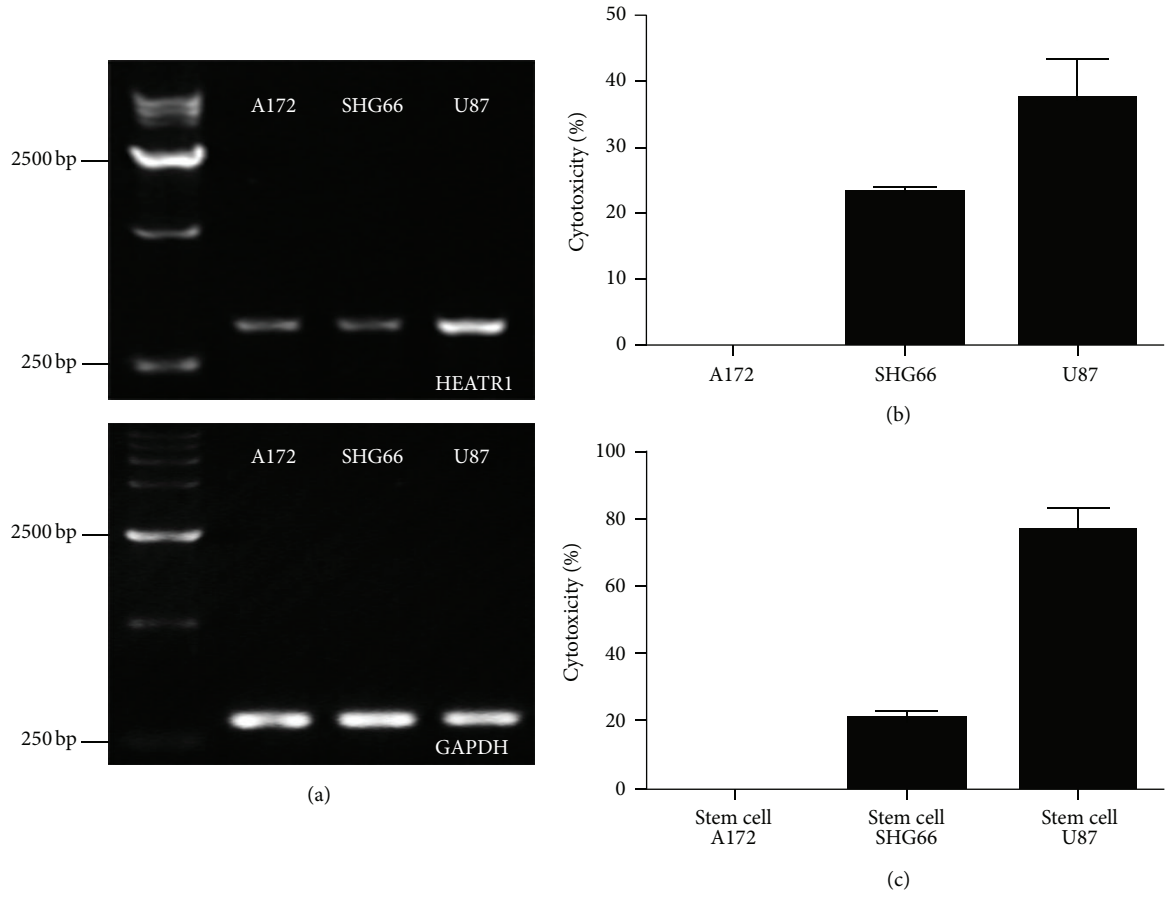

Figure 5: HEATR1-specific-peptide CTLs kill HLA-A∗02+ gliomas that express HEATR1. (a) RNA was isolated from three GBM cell lines and mRNA expression of HEATR1 was investigated by RT-PCR. (b) The values shown represent the mean ± SD of triplicate assays from PBMCs of patient number 323. U87, SHG66, and A172 were loaded with or without peptides and used as target cells in a LDH-release assay. The results showed that 6-peptides-stimulated PBMCs significantly lysed U87 and SHG66 target cells expressing both HEATR1 and HLA-A∗02 but not A172 cells that do not express HLA-A∗02 at an E/T ratio of 10 : 1. (c) Six-peptides-stimulated PBMCs from patient number 323 also significantly lysed the U87 and SHG66 GSCs at an E/T ratio of 10 : 1. Statistical differences between two groups were evaluated by the unpaired Student's *t*-test.

The HEATR1 gene is a multiple spliced 7-kb gene that encodes bap28, a protein involved in nucleolar processing of pre-18S ribosomal RNA and ribosome biosynthesis. In the zebrafish central nervous system, bap28 is required for cell survival through its role in rRNA synthesis and processing, and its mutation leads to abnormalities in the brain starting at mid-somitogenesis stages [33]. A recent study indicated that HEATR1 is an ideal minor histocompatibility antigen that is expressed by leukemia stem cells [20, 34]. Moreover, HEATR1 expression detected using TaqMan PCR was higher in testicular and ovarian tissues than in liver, colon, small intestine, lung, brain, and heart tissues [20]. In the meantime, the novel polymorphic minor histocompatibility antigen encoded by the HEATR1 gene could be recognized by one of the CTL clones. In GBM, we first confirmed that HEATR1 expression was significantly higher in most of the GBM samples than in control brain tissues. Although HEATR1 overexpression was not detected in a few cases of GBM, it might contribute to the vast genetic aberrations and their heterogeneity of GBM or GBM samples from the tumor-surrounding tissues. Furthermore, HEATR1 was overexpressed in A2B5+ GSC cells compared to A2B5−tumor cells.

To date, T-cell epitopes derived from several glioma-associated antigens have been shown to elicit T-cell responses against gliomas of several genes, including SART-1 and -3, interleukin-13 receptor a2 chain, ARF4L, GALT3, AIM-2, EphA2, EGFRvIII, HER-2, gp100, MAGE-1, glioma big potassium (gBK), *TRP-2*, *SOX2*, *SOX11*, *SOX6*, and 3′ β-hydroxysteroid dehydrogenase type 7 gene [12, 24, 35–50]. Dutoit et al. recently reported that the peptidomes from *ex vivo* GBM samples, which consisted of 10 glioblastoma-associated antigen epitopes, induced specific tumor cell lysis by patients' CD8+ T-cells *in vitro* and *in vivo* [51]. Geet al. confirmed that gBK channel-specific peptides could induce HLA-A∗02-restricted human CD8+ CTLs that killed gBK+ tumor cells [50]. In our study, we confirmed that peptide

Table 2: Binding score of HEATR1-derived peptides to HLA-A02 molecules.

Peptide	HLA molecule	Amino acid position	Subsequence residue listing	Score (estimate of half time of disassociation of a molecule containing this subsequence)
$HEATR1_{2003–2011}$	A_0201	2003–2011	F**L**FDTQHF**I**	4004.119
$HEATR1_{1126–1134}$ [&]	A_0201	1126–1134	K**L**LRMLFD**L**	3690.419
$HEATR1_{2102–2110}$	A_0201	2102–2110	L**L**PESIPF**L**	1883.533
$HEATR1_{1411–1419}$ [¶]	A_0201	1411–1419	F**L**WILLIL**L**	1875.918
$HEATR1_{682–670}$	A_0201	682–670	K**M**VEDLIS**V**	1657.907
$HEATR1_{757–765}$	A_0201	757–765	L**M**LDRGIP**V**	1295.433

[&] $HEATR1_{1126–1134}$ was also predicted to bind to HLA-03 and HLA-B08.
[¶] $HEATR1_{1411–1419}$ was also predicted to bind to HLA-B08, HLA-B40, and HLA-B3801.

epitopes derived from HEATR1 could significantly induce the CTL response of killing both GBM cells and A2B5+ GBM progenitor cells.

The CTL response in this study occurred in a non-HLA-A∗02-dependent manner. We found that $HEATR1_{1126–1134}$ and $HEATR1_{1411–1419}$ were also predicted to bind in the HLA-A∗03, HLA-B∗08, HLA-B∗38:01, and HLA-B∗40 regions using the epitope prediction system of SYFPEITHI analysis database (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm). Furthermore, positions 2 and 9 anchor peptides in the HLA-A∗02-peptide-binding groove are critical for optimal binding to HLA-A∗02. Positions 2 and 9 anchor peptides of those six peptides derived from HEATR1 were LL, LI, and MV, respectively (Table 2). More than 120 predicted peptides in non-HLA-A∗02 MHC class I (especially in HLA-B∗08) were found, where the 2nd and 9th positions were LL, LI, and MV. In addition, the HEATR1 region also was predicted to bind at least 1000 different 15-mers to the HLA-DR regions in the SYFPEITHI analysis database that could stimulate various CD4+ T-cells. Thus, six HEATR1 peptides in this study could cross-bind to the MHC class I or MHC class II region and potentially can be used to treat patients with GBM.

Several studies have used brain tumor stem-like initiating cells or cancer stem-like cells as sources of antigens for DC vaccination against human GBM with the achievement of CSC targeting and enhancing antitumor immunity [11–14]. GBM-associated tumor antigens including EGFR, HER2, TRP2, MRP3, AIM2, and SOX2 were twofold to >200-fold higher in CSCs than those in adherent cells [11]. Brown et al. reported that IL13-zetakine$^+$ CTLs were capable of efficient recognition and killing of both IL13Rα2^{pos} GSCs and IL13Rα2^{pos} differentiated cells *in vitro* and *in vivo* [15]. Sampson et al. reported that EGFRvIII is expressed in GSC lines and EGFRvIII chimeric antigen receptors-engineered T-cells effectively target these lines [52]. However, the number of GSC-associated proteins' peptide epitopes known to elicit T-cell responses is rather limited, and sox6 is the first protein expressed in glioma stem cells whose peptides are potentially immunogenic in patients with HLA-A∗24 or -A∗02 positive glioma [12]. A2B5 is considered a marker for glioma progenitor cells and A2B5+ cells from human GBM have cancer stem cell properties that are crucial to GBM initiation and maintenance [17, 18]. In our study, we confirmed that HEATR1-derived peptide epitopes could significantly induce the CTL response and then lyse cells from the GBMs and the GSCs, which should be considered a promising strategy for effective T-cell-based immunotherapy for patients with GBM.

HEATR1 expression in normal brain tissues was very low, unlike *ARF4L* and *GALT3*, which were markedly expressed in various normal tissues [43, 44]. Interestingly, HEATR1-specific CTLs are only detectable in PBMC derived from patients with malignant gliomas but not in PBMC from healthy donors. Two reasons might account for this discrepancy. First, the induction of HEATR1-specific CTLs may require higher level of HEATR1 expression. As shown in Figure 1, HEATR1 expressions are significantly higher in tumors than in normal tissues. In other words, it is possible that the epitopes expressed in normal tissues are below the threshold level to stimulate T-cell responses [53]. Second, tumor induced microinflammation may result in the increase of permeability of blood-brain barrier and thereby help CTL to access and recognize the presented HEATR1-derived peptide on tumor cells. Furthermore, although the MAGE-1, MAGE-3, Melan-A, gp100, tyrosinase, HER-2, and NY-ESO-1 are expressed in normal testicular, retinal, and/or brain tissues, no autoimmune responses have been elicited in the clinical trials or animal experiments of cancer vaccines [54–58]. Of course, our results require further *in vivo* experiments to confirm the safety and effectiveness of those HEATR1-derived epitope peptides as future immunotherapy for patients with GBM.

5. Conclusion

In this study, we demonstrated the selective overexpression of HEATR1 in A2B5+ GBM cells, whose epitopes could induce specific CTL responses targeting GBM cells and GSCs, suggesting that immunotherapy selectively targeting GSCs could be a novel effective strategy to treat patients with malignant glioma. Combined with other therapeutic avenues, epitope-based GSC-targeting immunotherapy may represent a new promising paradigm for the treatment of patients with GBM [59, 60]. Moreover, those novel CTL epitopes may serve

as an attractive component of personalized peptide-based vaccines in the treatment of GBM.

Abbreviations

GBM:	Glioblastoma
GSCs:	Gliomas stem-like cells
CSCs:	Cancer stem-like cells
FACS:	Fluorescence-activated cell sorting
DC:	Dendritic cell
HEATR1:	HEAT repeat containing 1
PBMCs:	Peripheral blood mononuclear cells
CTL:	Cytotoxic T lymphocyte
Q-RT-PCR:	Quantitative reverse transcription-polymerase chain reaction
HLA:	Human leukocyte antigen
IFN:	Interferon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zhe Bao Wu, Chao Qiu, and An Li Zhang contributed equally to this paper.

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Research Article

Impact of Underweight after Treatment on Prognosis of Advanced-Stage Ovarian Cancer

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This study aimed to investigate the impact of underweight status on the prognosis of advanced-stage ovarian cancer. A total of 360 patients with stage III-IV epithelial ovarian cancer were enrolled and divided into three groups by body mass indexes (BMIs): underweight (BMI < 18.5 kg/m^2); normal weight to overweight (18.5 kg/m^2 BMI < 27.5 kg/m^2); obesity (BMI ≥ 27.5 kg/m^2). Progression-free survival (PFS), overall survival (OS), CA-125, and neutrophil to lymphocyte ratio (NLR) as a marker reflecting host inflammation and immunity were compared among the three groups according to the three treatment times: at diagnosis; after surgery; and after treatment. Only underweight status after treatment was associated with poor OS in comparison with normal weight to overweight or obesity (mean value, 44.9 versus 78.8 or 67.4 months; $P = 0.05$); it was also an unfavorable factor for OS (adjusted HR, 2.29; 95% CI, 1.08–4.85). Furthermore, NLR was higher in patients with underweight than in those with obesity after treatment (median value, 2.15 versus 1.47; $P = 0.03$), in spite of no difference in CA-125 among the three groups at the three treatment times. In conclusion, underweight status after treatment may be a poor prognostic factor in patients with advanced-stage ovarian cancer, which accompanies increased host inflammation and decreased immunity.

1. Introduction

Excessive bodyweight is an established risk factor for several types of cancer. In particular, epidemiologic data show that obesity defined as body mass index (BMI) ≥ 30 kg/m^2 increases cancer risk and cancer-specific mortality [1, 2]. Although the precise mechanism is not clear, some obesity-related changes are expected to contribute to an increased risk of cancer. Insulin resistance and hyperinsulinemia are commonly observed in obesity. In this condition, secretion of insulin-like growth factor-1 (IGF-1) and various cytokines, such as adipokines, are stimulated. These factors promote cell proliferation, cell survival, and angiogenesis [3, 4]. Moreover, reactive oxygen radicals, increased by obesity, lead to systemic inflammation contributing to cancer development [5]. Recent epidemiologic studies supported these mechanisms, suggesting that obesity may affect poor prognosis in some cancers [6, 7].

However, the impact of underweight status on prognosis has not been adequately addressed. Although underweight status has been reported to be a high-risk factor for recurrence and death in patient with breast cancer [8], its role has not been evaluated in ovarian cancer. Furthermore, even in a recent meta-analysis, which showed slightly worse survival in obesity patients with ovarian cancer, the impact of BMI including underweight status, as well as obesity, was unclear because of a large amount of interstudy variation [9, 10].

Therefore, we investigated the impact of underweight status on prognosis in patients with advanced-stage ovarian cancer, depending on the time of measurement of BMI in

relation to the treatment. Thus, we evaluated the relationship between underweight status and cancer progression, with related changes of systemic inflammation and immunity.

2. Materials and Methods

2.1. Study Population. Clinicopathologic data for the current study were retrieved from a database of 360 patients registered in two tertiary medical centers (Seoul National University Hospital and Seoul National University Bundang Hospital) between 2000 and 2011. The current study was approved by the Institutional Review Board of Seoul National University Hospital. The patients' medical records were reviewed retrospectively. Informed consent was not required since the current study was conducted by a retrospective review of medical records.

2.2. Inclusion or Exclusion Criteria. We included patients with the following inclusion criteria: those with epithelial ovarian cancer; those with advanced-stage disease, in particular, the International Federation of Gynecology and Obstetrics (FIGO) stage III-IV disease; those who underwent staging operation and taxane- and platinum-based chemotherapy; those with BMIs measured at three treatment points including "at diagnosis," "after surgery," and "after treatment." We excluded patients with nonepithelial ovarian cancer, synchronous or metachronous cancer, and insufficient data for investigating the impact of BMI on survival.

2.3. Data Collection. BMIs at diagnosis, after surgery, and after treatment were defined as those measured at diagnosis, before the first administration of adjuvant chemotherapy, and after the last administration of adjuvant chemotherapy. Furthermore, all patients were classified into four groups based on the following BMI criteria suggested by the World Health Organization for the Asian population: underweight (BMI $< 18.5\,kg/m^2$); normal ($18.5\,kg/m^2 \leq$ BMI $< 23.0\,kg/m^2$); overweight ($23.0\,kg/m^2 \leq$ BMI $< 27.5\,kg/m^2$); and obesity (BMI $\geq 27.5\,kg/m^2$) [11].

To evaluate the potential of cancer progression and related changes of systemic inflammation and immunity, serum CA-125 level and neutrophil to lymphocyte ratio (NLR) were investigated. NLR is known as a prognostic factor for recurrence and death in patients with ovarian cancer [12, 13]. Since increased inflammation and decreased immunity by cancer contribute to secondary hematological changes, including relative neutrophilia and lymphocytopenia, NLR tends to increase in several types of malignancy [14, 15]. Thus, we measured CA-125 as a tumor marker and NLR as a marker of systemic inflammation and immunity, using a radioimmunoassay kit (Fujirebio Diagnostics, Malvern, PA, USA) and SYSMEX XE-2100 (TOA Medical Electronics, Kobe, Japan) at diagnosis, after surgery, and after treatment, respectively.

Clinicopathologic characteristics including age, grade, FIGO stage, histology, neoadjuvant chemotherapy, cycles of adjuvant chemotherapy, optimal debulking surgery, progression-free survival (PFS), and overall survival (OS) were collected. Patients treated with neoadjuvant chemotherapy received three cycles of taxane- and platinum-based chemotherapy before surgery, and optimal debulking surgery was considered when the size of residual tumor was less than 1 cm in the longest diameter. PFS was defined as the time that elapsed from the date after completion of the primary treatment to the date of clinically proven recurrence. OS was defined as the time that elapsed from the date of diagnosis to the date of cancer-related death or end of the study.

2.4. Statistical Methods. Kruskal-Wallis, Mann-Whitney U, and Chi-square tests were used to determine differences in clinicopathologic characteristics among underweight, normal to overweight, and obesity patients. Furthermore, univariate and multivariate analyses for investigating factors affecting survival were performed using the Kaplan-Meier method with log-rank test and Cox's proportional hazard regression model with hazard ratio (HR) and 95% confidence interval (CI). We conducted these statistical analyses using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patients' Characteristics. Clinicopathologic characteristics of all patients are depicted in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/349546). The mean age was 53.9 years (range, 18–80 years) and 5 (1.4%), 23 (6.4%), 256 (71.1%), and 76 (21.1%) patients had stage IIIA, IIIB, IIIC, and IV diseases, respectively. Furthermore, serous carcinoma was identified in 276 (76.7%) patients while endometrioid, clear cell, mucinous, undifferentiated, and mixed carcinomas were observed in 29 (8.1%), 20 (5.6%), 13 (3.6%), 7 (1.9%), and 15 (4.2%), respectively. Three cycles of neoadjuvant chemotherapy using taxane and platinum were administered in 57 patients (15.8%), and the mean value of cycles of adjuvant chemotherapy using the same regimen was 6 (range, 3–12).

Among 360 patients, the following conditions were identified: underweight, normal, overweight, and obesity in 12 (3.3%), 162 (45.0%), 150 (41.7%), and 36 (10.0%) patients, respectively, *at diagnosis*; 32 (8.9%), 183 (50.8%), 118 (32.8%), and 27 (7.5%) patients, respectively, *after surgery*; 29 (8.1%), 146 (40.6%), 157 (43.6%), and 28 (7.8%) patients, respectively, *after treatment*. In particular, 7 patients (58.3%) who showed underweight status at diagnosis were underweight even at the after treatment time point (Figure 1).

After treatment, patients with hypertension were observed in 2 out of 29 underweight (6.9%), 20 out of 146 normal (13.7%), 19 out of 157 overweight (12.1%), and 13 out of 28 obesity (46.4%). The prevalence of hypertension significantly increased as the patient's BMI after treatment increased toward obesity ($P = 0.003$). After treatment, patients with diabetes were observed in 9 out of 146 normal (6.2%), 10 out of 157 overweight (6.4%), and 2 out of 28 obesity (7.1%). The prevalence of diabetes had the same trends, but without statistical significance ($P = 0.372$).

Table 1: Clinicopathologic factors affecting progression-free and overall survivals in all 360 patients with advanced-stage ovarian cancer.

Characteristics	Univariate			Multivariate		
	HR	95% CI	*P* value	Adjusted HR	95% CI	*P* value
Progression-free survival						
≥53 years	1.04	0.82–1.32	0.74	—	—	—
Stage IV disease	1.27	0.95–1.69	0.11	—	—	—
Grade 3 disease	1.11	0.80–1.53	0.53	—	—	—
Nonserous histology	1.26	0.95–1.67	0.11	—	—	—
No neoadjuvant chemotherapy	1.62	1.19–2.20	<0.01	1.84	1.18–2.87	<0.01
≤6 cycles of adjuvant chemotherapy	1.03	0.81–1.32	0.81	—	—	—
Suboptimal debulking surgery	1.54	1.21–1.96	<0.01	1.71	1.22–2.39	<0.01
Underweight after treatment	1.25	0.80–1.93	0.33	—	—	—
Overall survival						
≥53 years	1.12	0.79–1.58	0.52	—	—	—
Stage IV disease	1.21	0.80–1.84	0.36	—	—	—
Grade 3 disease	1.21	0.75–1.93	0.44	—	—	—
Nonserous histology	1.58	1.07–2.33	0.02	—	—	—
No neoadjuvant chemotherapy	1.65	1.08–2.54	0.02	1.88	1.28–2.77	<0.01
≤6 cycles of adjuvant chemotherapy	1.16	0.82–1.64	0.42	—	—	—
Suboptimal debulking surgery	1.49	1.05–2.11	0.03	1.67	1.23–2.28	<0.01
Underweight after treatment	2.01	1.13–3.58	0.02	2.29	1.08–4.85	0.03

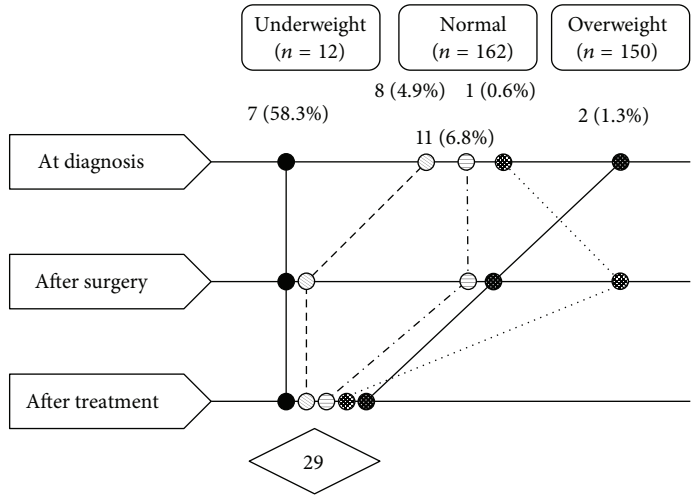

Figure 1: Underweight patients with advanced-stage ovarian cancer according to the treatment time.

3.2. Underweight Status Effects on Prognosis or Systemic Inflammation and Immunity. We compared PFS and OS among underweight, normal to overweight, and obesity patients, according to the treatment time. As a result, only patients with underweight status *after treatment* showed poor OS in comparison with those with normal to overweight or obesity (mean value, 44.9 versus 78.8 or 67.4 months; $P = 0.05$; Figure 2). When we adjusted the result with clinicopathologic characteristics, underweight status after treatment was an unfavorable factor for OS (adjusted HR, 2.29; 95% CI, 1.08–4.85; Table 1).

Next, we compared CA-125 and NLR among underweight, normal to overweight, and obesity patients according to the treatment time (Table 2). As a result, CA-125 *at diagnosis* was higher in patients with normal to overweight status or obesity than in those with underweight status (median value, 865 or 912.5 versus 185.5 U/mL; $P = 0.04$). Since underweight patients *at diagnosis* achieved more frequent optimal debulking surgery than those with normal weight to overweight or obesity, we did subgroup analyses based on whether optimal debulking surgery was performed. As a result, there were no differences in CA-125 and NLR among underweight, normal to overweight, and obesity patients who underwent optimal debulking surgery (median value of CA-125, 161.5 versus 555 versus 490 U/mL; $P = 0.37$: median value of NLR, 2.93 versus 2.51 versus 2.54;

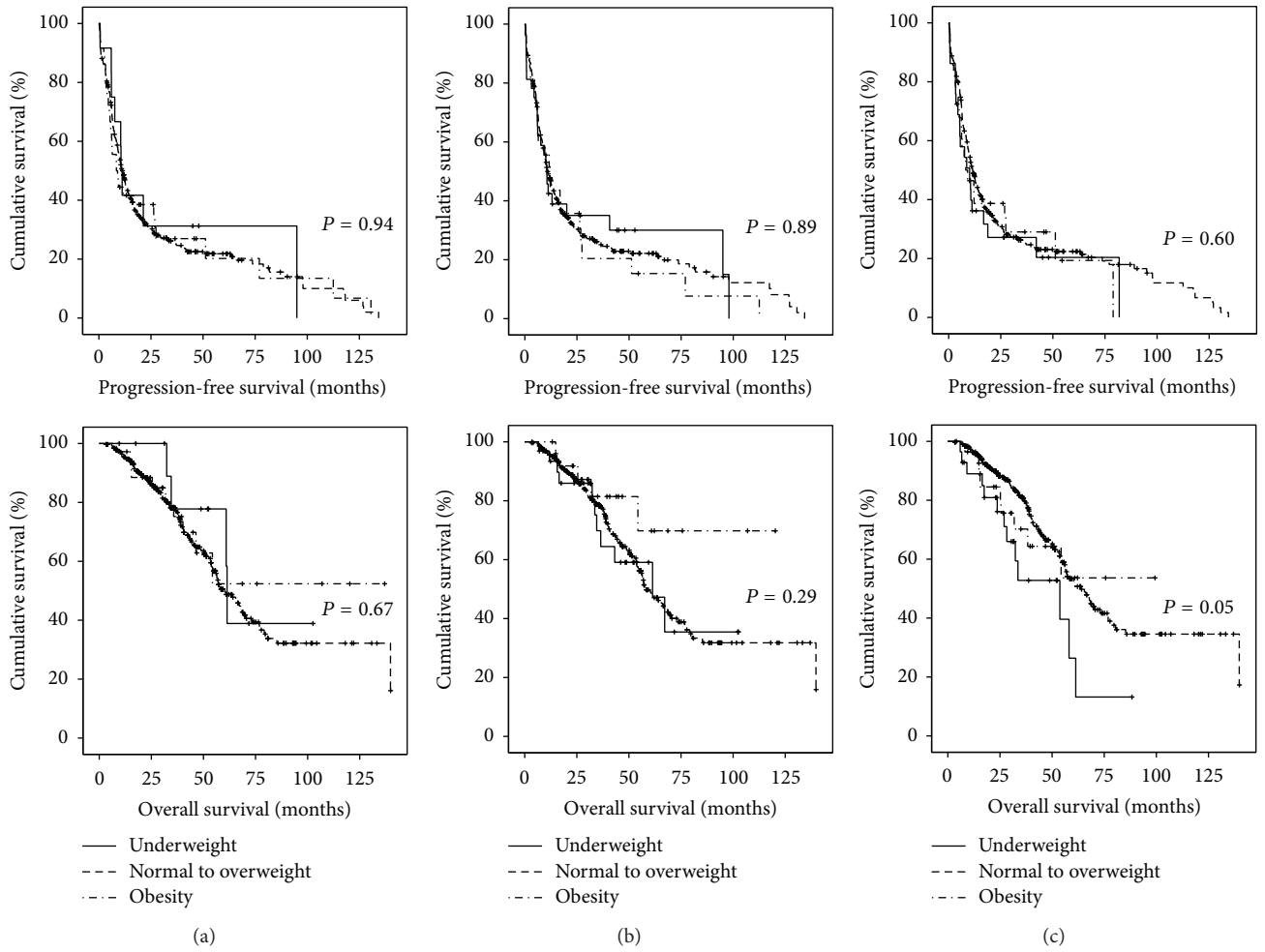

Figure 2: Kaplan-Meier analyses with the log-rank test for comparing progression-free survival and overall survival among patients with underweight, normal to overweight, and obesity with advanced-stage ovarian cancer: (a) at diagnosis; (b) after surgery; (c) after treatment.

$P = 0.86$) and suboptimal debulking surgery (median value of CA-125, 956 versus 1,043 versus 930 U/mL; $P = 0.68$: median value of NLR, 3.29 versus 3.49 versus 3.43; $P = 0.55$).

Furthermore, the rate of optimal debulking surgery was also different between underweight and obesity patient groups *after surgery*, in spite of no differences of CA-125 and NLR. Thus, we also did subgroup analyses according to whether optimal debulking surgery was performed and observed that CA-125 and NLR were not different among underweight, normal to overweight, and obesity patients who underwent optimal debulking surgery (median value of CA-125, 71 versus 76.5 versus 65 U/mL; $P = 0.39$: median value of NLR, 2.58 versus 2.37 versus 2.68; $P = 0.43$) and suboptimal debulking surgery (median value of CA-125, 216.8 versus 202 versus 105 U/mL; $P = 0.52$: median value of NLR, 2.53 versus 3.05 versus 3.71; $P = 0.15$).

On the other hand, underweight patients *after treatment* showed higher NLR than those with obesity, in spite of no differences of confounding factors between two groups (median value, 2.15 versus 1.47; $P = 0.03$).

3.3. Degree of Weight Loss Effects on Prognosis in Underweight Patients after Treatment. Next, we compared clinicopathologic characteristics and prognosis according to the degree of weight loss, only in patients with underweight status *after treatment*. All 29 patients with underweight status *after treatment* were divided into two subgroups according to the following criteria: weight loss ≥10% versus <10% from the body weight at diagnosis. Clinicopathologic characteristics based on the degree of weight loss are summarized in Supplementary Table 2. Although there were no differences in age, FIGO stage, histology, grade, neoadjuvant chemotherapy, cycles of adjuvant chemotherapy, CA-125, and NLR between two subgroups, the success rate of optimal debulking surgery was higher in underweight patients with weight loss <10% than in those with weight loss ≥10% (83.3% versus 36.4%; $P = 0.02$).

Furthermore, underweight patients with weight loss ≥10% showed poor PFS and OS in comparison with those with weight loss <10% (PFS, median value, 3.5 versus 16.8 months; OS, median value, 23.7 versus 58.1 months; Figure 3). Weight loss ≥10% was also a poor prognostic factor for PFS

Table 2: Comparison of CA-125 and neutrophil to lymphocyte ratio (NLR) among underweight, normal to overweight, and obesity patients according to the treatment time.

BMI	CA-125 (median, U/mL)	NLR (median)	Confounding factors			
			Stage IV disease	Grade 3 disease	Nonserous histology	Suboptimal debulking
At diagnosis						
Underweight	185.5	8*,†	1 (8.3)*,†	3 (25.0)*,†	4 (33.3)*,†	2 (16.7)*
Normal to overweight	865*	9*,‡	69 (22.3)*,‡	156 (50.3)*,‡	69 (22.3)*,‡	149 (48.1)*,†
Obesity	912.5*	6.75†,‡	5 (13.9)†,‡	21 (58.3)†,‡	5 (13.9)†,‡	21 (58.3)†
P value	0.04	0.47	0.28	0.14	0.33	0.04
After surgery						
Underweight	95.6*,†	2.58*,†	6 (18.8)*,†	15 (46.9)*,†	3 (9.4)*,†	10 (32.2)*,†
Normal to overweight	190.0*,‡	2.67*,‡	65 (22.3)*,‡	146 (50)*,‡	67 (22.9)*,‡	143 (49)*,‡
Obesity	87.5†,‡	3.14†,‡	5 (18.5)†,‡	16 (59.3)†,‡	5 (17.9)†,‡	16 (59.3)†,‡
P value	0.78	0.23	0.83	0.60	0.29	0.08
After treatment						
Underweight	8*,†	2.15*	5 (17.2)*,†	13 (44.8)*,†	6 (20.7)*,†	10 (34.5)*,†
Normal to overweight	9*,‡	1.56*,†	62 (20.7)*,‡	148 (50.7)*,‡	67 (22.4)*,‡	146 (48.8)*,‡
Obesity	6.8†,‡	1.47†	8 (28.6)†,‡	18 (66.7)†,‡	4 (14.3)†,‡	13 (46.4)†,‡
P value	0.21	0.09	0.54	0.27	0.60	0.33

BMI: body mass index; *,†,‡no significant difference between two groups with the same symbol.

Table 3: Clinicopathologic factors affecting progression-free and overall survivals in 29 patients who showed underweight after treatment.

Characteristics	Univariate			Multivariate		
	HR	95% CI	*P* value	Adjusted HR	95% CI	*P* value
Progression-free survival						
≥53 years	1.91	0.79–4.63	0.15	—	—	—
Stage IV disease	1.88	0.68–5.18	0.23	4.89	1.14–20.94	0.03
Grade 3 disease	0.94	0.33–2.70	0.91	—	—	—
Nonserous histology	2.99	1.13–7.90	0.03	—	—	—
No neoadjuvant chemotherapy	1.54	0.51–4.64	0.45	—	—	—
≤6 cycles of adjuvant chemotherapy	1.10	0.40–3.01	0.86	—	—	—
Suboptimal debulking surgery	3.89	1.55–9.74	<0.01	10.04	1.48–68.13	0.02
Weight loss ≥10%	4.07	1.55–10.64	<0.01	6.90	1.51–31.54	0.01
Overall survival						
≥53 years	2.07	0.62–6.92	0.24	—	—	—
Stage IV disease	1.92	0.40–9.25	0.42	11.9	1.00–141.1	0.05
Grade 3 disease	0.77	0.17–3.47	0.73	—	—	—
Nonserous histology	2.58	0.73–9.15	0.14	—	—	—
No neoadjuvant chemotherapy	1.27	0.27–6.02	0.77	—	—	—
≤6 cycles of adjuvant chemotherapy	3.84	0.78–18.91	0.10	—	—	—
Suboptimal debulking surgery	2.64	0.88–7.91	0.08	—	—	—
Weight loss ≥10%	12.81	2.54–64.65	<0.01	15.27	1.42–164.5	0.02

and OS when adjusted with other clinicopathologic factors (adjusted HRs, 6.90 and 15.27; 95% CIs, 1.51–31.54 and 1.42–164.5; Table 3).

4. Discussion

In terms of the association between BMI and cancer risk and prognosis, most of the studies have focused mainly on the impact of obesity, because deleterious mechanisms related to obesity are expected to be unfavorable to cancer patients [16]. The metabolic syndrome, a cluster of risk factors for cardiovascular disease and type 2 diabetes, is considered to play a central role in this relationship [17–19].

However, excessive weight loss can also be associated with poor prognosis, because it has similar features to cancer cachexia, a complex metabolic condition characterized by

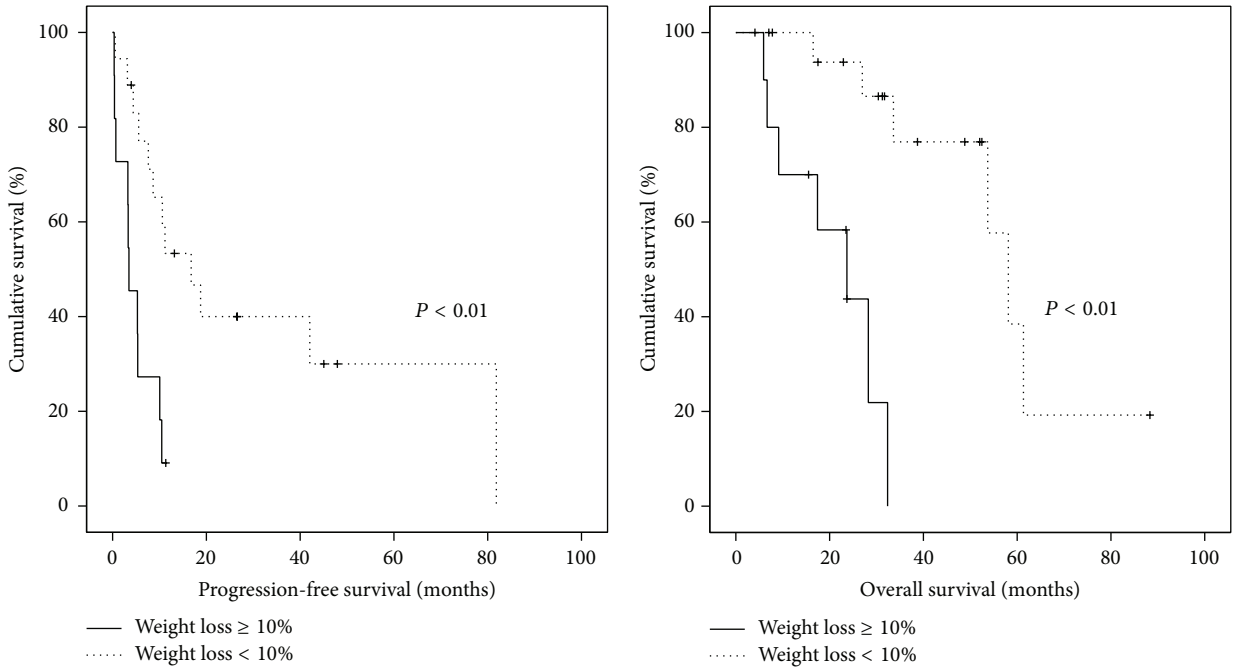

Figure 3: Kaplan-Meier analyses with the log-rank test for comparing progression-free survival and overall survival between weight loss ≥10% and <10% in underweight patients after treatment.

loss of skeletal muscle and body weight, developed in progressive disease [20, 21].

Epidemiologically, the relationship between the risk of mortality and BMI is known to be U-shaped with the increased risk related to either cachexia showing very low BMI or obesity demonstrating very high BMI, whereas emerging data indicate that obesity is associated paradoxically with better prognosis in cancer patients [22]. In the current study, we also found that underweight status after treatment was an unfavorable factor for OS in patients with advanced-stage ovarian cancer (adjusted HR, 2.29; 95% CI, 1.08–4.85), whereas obesity was not associated with prognosis, regardless of the treatment time. The lack of an association between obesity and prognosis can be explained by the following reasons. The cut-off value defining obesity is relatively lower in the Asian population than in the Western population (27.5 kg/m^2 versus 30 kg/m^2), and it may result in different effects of obesity based on race. Moreover, obesity can help patients endure the increased resting energy expenditure (REE) which occurs in cancer [20]. This endurance can help patients with advanced-stage ovarian cancer, with a 5-year survival rate of approximately 30% [23], to maintain their general condition thereby improving survival. These hypotheses were supported by recent epidemiologic data showing no association between obesity and poor prognosis in Asian patients with ovarian cancer [24].

However, underweight status can act as a poor prognostic factor in these patients. Theoretically, most patients with advanced-stage ovarian cancer should recover from their underweight status after treatment because the Warburg effect, that is, increased glucose uptake by tumors for glycolysis to generate ATP, is expected to reduce with the decrease of tumor burden after treatment [25]. Inversely, failure to regain weight after treatment indicates that the cancer has potentially progressed, and it is easily identified in patients with cancer cachexia. In cancer cachexia, systemic inflammation is induced and persists due to increased tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1, and interferon-gamma (IFN-γ). This results in decrease of protein anabolism and caloric intake, while promoting increase of protein catabolism, insulin resistance, lipolysis, and REE. Eventually, loss of muscle mass and strength, loss of whole body fat, ineffective host's antitumor response, and impaired immunity occur and lead patients to physical disability, diminished quality of life, and reduced survival [20, 26–29].

To prove this hypothesis clinically, we investigated CA-125 as a tumor marker and NLR as a marker reflecting inflammation and immunity among underweight, normal weight to overweight, and obesity patients according to the treatment time. After treatment, although there were no differences of CA-125 among the three groups, underweight patients showed the highest NLR compared with normal weight to overweight and obesity patients, suggesting increased systemic inflammation (neutrophilia) and decreased immunity (lymphocytopenia) in these patients. This means that underweight status after treatment is a condition which increases the likelihood of cancer progression, and it can be considered as an early marker for poor prognosis in patients with advanced-stage ovarian cancer.

Chronic systemic inflammation is also known to be related to metabolic syndrome, which is in state of central obesity or excessive adiposity [30]. However, in the current study, underweight patients after treatment showed relatively higher increase in systemic inflammation compared

to obesity patients. This can be explained as follows. First, inflammatory state of underweight patients may reflect ethnic variation. The Asian subjects are known to develop metabolic syndrome at a relatively low level of BMI compared to the Western populations [31], which can be explained by ethnic variations in body fat distribution [32]. Second, BMI alone does not exactly predict fat distribution and adiposity in individuals. Third, although the prevalence of metabolic syndrome in the enrolled patients was not known due to the lack of data, including waist circumference and serum levels of triglyceride or high density lipoprotein, patients with hypertension and/or diabetes were more common in obesity patients and all of them were under adequate and specific medications. The antihypertensive and/or antidiabetic drugs could counterbalance the underlying proinflammatory state which was generated from metabolic syndrome [33]. Lastly, underweight patients after treatment in advanced-stage ovarian cancer include patients with cancer cachexia, which is well known to have chronic systemic inflammation that can result in poor prognosis [26–29].

In terms of cancer cachexia, it was hard to assess exactly how many underweight patients after treatment were in cachectic state according to the retrospective analysis of medical records. To consider cancer patients to be in cachectic state, at least all three key features of cachexia should be presented as follows: weight loss >10%; systemic inflammation (C-reactive protein (CRP) > 10 mg/L); and reduced food intake (<1,500 kcal/day) [20]. However, serum CRP levels and food intake and/or nutritional status of patients have not been routinely observed in our institute. Only the change in each patient's weight from the diagnosis was able to be retrieved.

Thus, we divided all underweight patients after treatment into two subgroups on the basis of weight loss by 10% from the bodyweight at diagnosis, considering the features of cancer cachexia. As a result, the risk of suboptimal surgery increased in underweight patients with weight loss ≥10% (63.6% versus 16.7%; $P = 0.02$), and weight loss ≥10% was an independent poor prognostic factor for PFS and OS (adjusted HRs, 6.90 and 15.27; 95% CIs, 1.51–31.54 and 1.42–164.5). These data indicated that severe weight loss (≥10%) after treatment was associated with more unresectable tumors and an increased risk of cancer progression. However, there was no difference in NLR between the two subgroups in spite of the tendency that it was higher in underweight patients with weight loss ≥10% than in those with weight loss <10% (median value, 2.15 versus 2.04). The small number of underweight patients enrolled in the study likely led to no statistical difference; a large-scale cohort is needed in future studies.

The current study is the first report demonstrating the impact of underweight status after treatment on prognosis of gynecologic cancer. However, there were some limitations. Firstly, we could not evaluate the impact of underweight status on prognosis of patients with early-stage ovarian cancer, because they showed good prognosis. Secondly, we measured only NLR as an indicator of host inflammation and immunity because other proinflammatory markers or cytokines were not included in the clinical setting. Thirdly, all patients in the current study were ethnically homogenous Asians, so the results may not be applicable to other ethnic groups.

In conclusion, we found that underweight status after treatment may be a poor prognostic factor in patients with advanced-stage ovarian cancer, and it is accompanied by increased tumor-induced inflammation and decreased immunity. Underweight status can act as an early marker to predict poor prognosis. In particular, paying attention to weight change is required during the treatment period, because more than half of underweight patients at diagnosis failed to gain weight and a weight loss ≥10% after treatment was associated with an increased risk of disease recurrence and mortality.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Hsp74, a Potential Bladder Cancer Marker, Has Direct Interaction with Keratin 1

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Early diagnosis and prognosis monitoring are very important for the survival of patients with bladder cancer. To identify candidate biomarkers of bladder cancer, we used a combination of techniques including 2-DE, co-IP, western blot, LC-MS/MS, and immunohistochemistry. Hsp74 was identified with high expression in bladder cancer. The cellular location of expression products of gene Hsp74 showed that they were distributed into cytoplasm and keratin 1 was found to be associated with Hsp74. The results provide a new idea to understand the molecular basis of bladder cancer progression and pinpoint new potential molecular target for early diagnosis and therapeutic monitoring of bladder cancer.

1. Introduction

Bladder cancer is the 7th most common cancer in men and the 17th most common cancer in women in the world [1]. Occupational risks, environmental risks, dietary habits, and cigarette smoking are lifestyle factors influencing the development of bladder cancer. It is a common malignancy requiring a high degree of surveillance because of the frequent recurrences and the poor clinical outcome of invasive disease. Hematuria is often the onset symptom of bladder cancer; therefore, cytologic analysis of urine becomes the initial evaluation method, followed by cystoscopy with or without biopsy [2]. However, cytologic analysis has a limited value because it is operator-dependent and has low sensitivity. Cystoscopy still has some limitations [3, 4]; for example, it is invasive, time-consuming, and expensive, requires sedation or anesthesia, and sometimes leads to iatrogenic injury. Also, evaluation of lesions located in the base or neck of the bladder or in the diverticulum is difficult because of the limited perspective of the cystoscope [5, 6]. To date, serum biomarkers for bladder cancer have some practical value, but they lack optimal sensitivity and specificity in diagnosis and disease categorization. Recently, many radiologic imaging techniques have been used to detect and evaluate bladder tumors, but none is reliable in detection of bladder cancer. Therefore, it is important to establish early detection methods with high sensitivity and specificity for bladder cancer.

In this study, Hsp74 as a potential tumor antigen was identified by 2-DE and western blot using bladder cancer cell line BLZ211. Moreover, keratin 1 as an associated protein with Hsp74 was found by coimmunoprecipitation. These two molecules, in conjunction, might play a certain role in the progression of bladder cancer and might be seen as potential therapeutic target, which is more inspiring, though further investigations are needed. In addition, these data represent the first report, to our knowledge, of a functional link between Hsp74 and keratin 1 in bladder cancer cells.

2. Materials and Methods

2.1. Cell Line and Preparation of Monoclonal Antibody (McAb). Established bladder cancer cell line BLZ-211 was described previously [7–9]. Cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified

atmosphere of 5% CO_2. BLZ211 cells were harvested from monolayer cultures by trypsinization and the cell pellet was washed with sterile PBS and suspended in the same medium. Approximately 6×10^6 cells were injected i.p. into 6-week-old female BALB/c mice. The animals were given 3 i.p. booster injections a week apart and final injection 3 days prior to fusion experiment. Spleen cells from these mice were fused with Sp2/0 myeloma cells (spleen cells versus myeloma cell = 5 : 1). 2-3 weeks after fusion, culture supernatants were analyzed using ELISA method for antibody production. Positive clones were selected and subcloned twice by semisolid cloning.

2.2. Two-Dimensional Electrophoresis. 1×10^7 BLZ211 cells were suspended in 200 μL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 0.5% IPG buffer, 2 mM TBP, and 50 mM DTT) supplemented with protease inhibitor cocktail and vortexed at 4°C for 1 h, and then the cell lysate was clarified by centrifugation at 40,000 ×g for 1 h. The supernatant was collected and protein concentration was determined by Bradford Assay using BSA as a standard. Two gels were prepared at the same time. An IPGPhor apparatus (Amersham-Pharmacia Biotech, Uppsala Sweden) was used for IEF with 13 cm pH 3–10 no-linear or pH 4–7 immobilized pH gradient (IPG) strips (GE Healthcare Bio-Science AB) at 20°C.The strips were rehydrated overnight with 250 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 0.5% IPG buffer, 2 mM TBP, and 30 mM DTT) containing 50 μg proteins. Isoelectric focusing protocol was followed as (1) 60 V, 12 h, Step and Hold mode; (2) 200 V, 2 h; Step and Hold mode; (3) 500 V, 1 h, Gradient mode; (4) 1000 V, 1 h, Gradient mode; (5) 8000 V, 1 h, Gradient mode; (6) 8000 V, Step and Hold mode until 30 kVhT was reached. Strips were then equilibrated in 10 mL equilibration buffer (6 M urea, 30% w/v glycerin, 4% w/v SDS, 50 mM Tris-HCl pH 8.8) with 1% w/v DTT for 15 min at room temperature. Strips were removed and incubated in equilibration buffer with 2.5% w/v iodoacetamide for another 15 min. After equilibration, the strips were embedded onto an 11%, 1 mm SDS/PAGE gel and were fixed in place with a 0.5% w/v agarose overlay. Gels were run in a SE600 (Amersham-Pharmacia Biotech, Uppsala Sweden) at 10 mA/gel for 20 min then 20 mA/gel until the bromophenol blue dye reached the bottom of the gel.

2.3. Western Blot. One of 2-DE gels with separated proteins were immunoblotted onto nitrocellulose membrane (0.45 μm pore size; Bio-Rad) and blocked overnight at 4°C in SuperBlock Blocking Buffer in Tris-bufferedsaline (Pierce) to block the nonspecific bindingsite. As first antibodies, the MAb diluted 1 : 10 in blocking buffer and the membranes were incubated for 1 h at room temperature. After one hour incubation at room temperaturewith the second antibodies, the immunoproducts were visualizedwith ECL western blotting detection reagents (AmershamBiosciences) and exposure to X-ray films. Each wash was performedwith Tris-buffered saline-Tween (10 mmol/L Tris-HCl (pH 7.6), 100 mmol/L NaCl, and 1 mL/L Tween 20).

2.4. Silver Staining and Image Analysis. One of 2-DE gels was fixed in 30% alcohol containing 1% acetic acid for 2 h and was sensitized in 30% alcohol containing 0.2% $Na_2S_2O_3$ and 6.8% sodium acetate for 30 min. The grids were removed with plastic forceps and washed by 3 immersions of 5 min each in double-distilled water. The gel was stained in 0.25% silver nitrate for 30 min. The grids were washed by 3 immersions of 1 min each in double-distilled water. 2-DE gel was developed in 0.74% formaldehyde containing 2.5% Na_2CO_3 for 4–8 min. At clear spot times, add 5% acetic acid for 10 min. The grids were washed by 3 immersions of 5 min each in double-distilled water. Gels were scanned with a ScanMaker 8700 scanister (MICROTEK, China). The images were saved in TIFF format and then exported to the ImageMaster 2D platinum Software 5.0 (Amersham-Pharmacia, Sweden). Spot detection parameters were best adjusted using, first, the Smooth parameter which was set to a value of 1.5 allowing the detection of all real spots and split as many as possible overlapping spots. Then, the minimum area was set to eliminate spots that have an area smaller than 15 pixels. Finally, the saliency parameter was experimentally adjusted to 1 to filter out artifacts.

2.5. Coimmunoprecipitation. Total protein of BLZ211 cells was used to immunoprecipitate and coimmunoprecipitate interacting proteins (prey proteins) according to the protocol of ProFound Co-Immunoprecipitation (Co-IP) Kit (PIERCE). The purified antibody was put into the spin cup containing the gel for antibody immobilization. The BLZ211 cells were washed once with PBS (Product number 28372; 0.1 M phosphate, 0.15 M NaCl, and pH 7.2). The 200 μL M-PER reagent was added to the plate. Lysate was collected and transferred to a microcentrifuge tube for centrifugation of samples at ~13,000 ×g for 5–10 minutes to pellet the cell debris. The prey complex and controls were put to the appropriate gel in the spin cups, and 0.4 mL of Co-IP Buffer was added. The tubes were inverted 10 times and centrifuged. 100 μL elution buffer was put to the gel in the spin cup and the tube was centrifuged. 5 μL of the 5X sample buffer was added to the sample and applied to the gel for electrophoresis.

2.6. LC-MS/MS. The mobile phase buffer A contained H_2O and 0.1% methanoic acid. The mobile phase buffer B contained acetonitrile and 0.1% methanoic acid, 120 min linear gradient elution, and flowed at 1 μL/min. This consisted of a full mass scan (m/z 400–2000), zoom scan on the most abundant ion to determine charge state, and a tandem mass spectrometry (MS/MS) scan to collect collision-induced dissociation (CID) spectra on peptides. Automated analysis of CID spectra to determine the amino acid sequence of peptides was performed on computer (SEQUEST software; ThermoFinnigan) as described by Yates III et al. [10].

2.7. Immunofluorescence. BLZ211 cells were cultured in 6-well plate with cover slips for 24 hr. The cells on cover slip were fixed for 10 min by immersion in −20°C precooling methanol and washed in phosphate-buffered saline (PBS) three times. Cells were incubated in 0.5% TritonX-100/PBS

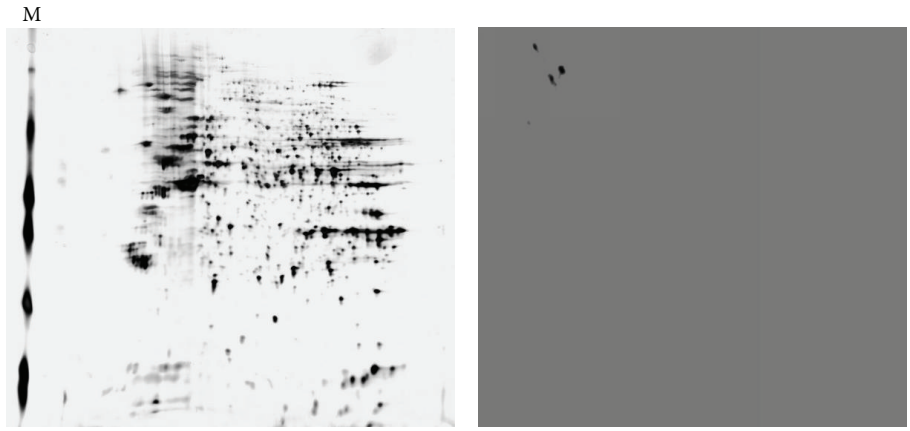


Figure 1: (a) 2-DE gel showing the proteome map of total soluble protein from BLZ211 cells. M: mw markers. (b) Western blot showing 3 spots.

for 5 min at room temperature and washed in PBS three times and incubated with goat serum for 1 h at room temperature. Cells were incubated for 4 h at 4°C on a rocker platform in 1:10 dilutions of experimental McAb in PBS. The control group uses PBS instead of McAb as primary antibody. Then cells were washed in PBS three times, incubated with secondary antibody for 40 min at 4°C in 1 : 40 dilutions of fluorescein-conjugated goat anti-mouse IgG in a PBS media, followed by incubation with DAPI for 5 min at room temperature, and washed in PBS three times. The cover slips were subjected to another washing cycle before being monitored for specific fluorescence under an immunofluorescence microscope.

2.8. Immunohistochemistry Procedures. 35 bladder cancer tissues and adjacent normal tissues were used to analyze the expression of Hsp74 with McAb. Paraffin sections of bladder cancer tissues were dewaxed and dehydrated by graded ethanol. Endogenous peroxidase activity was quenched and antigen retrieval was done by 5 min heating in citric acid. After blocking, sections were incubated with Hsp74 McAb at 4°C overnight. Immunoreactivity was detected using horseradish peroxide conjugated second antibody and DAB. Tissue structures were visualized by counterstaining with hematoxylin. Comparisons between groups for bladder cancer tissues and adjacent normal tissues were performed by applying chi-square test as indicated. A P value of ≤ 0.05 was considered significant.

3. Results

3.1. Monoclonal Antibody. Fusion cells were inoculated in a 96-well plate, in which clone growth appeared in 62 wells. Using immunohistochemistry method, 29% of the 62 clones were found secreting specific antibody. The subcloning with limited dilution assay did not stop until the preliminary screening positive hybridoma cells were 100%.

3.2. 2-DE and Western Blot. To analyze the proteome of BLZ211 cells, soluble proteins from the BLZ211 cells were separated by 2-DE (Figure 1(a)). Western blot showed 3 protein spots were more intense in BLZ211 cells (Figure 1(b)). One of the protein spots was detected by LC-MS/MS with an apparent mass of 94299.96 Da, score 70.4, accession 6226869, peptides (Hits)7(70000), and pI of 5.18, which was identified as Hsp74 (Figure 2).

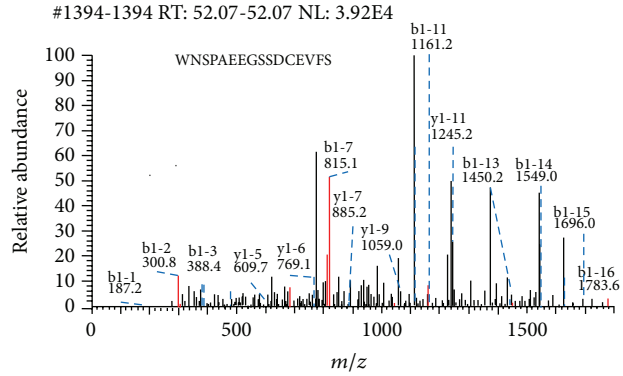


Figure 2: Representative MS/MS spectrum of the peptide of HSP74.

3.3. Identified Keratin 1. Keratin 1 (Figure 4) was identified associated with Hsp74 by coimmunoprecipitation (Figure 3), LC-MS/MS. Compared with NCBInr database showed score 198.2, accession 11935049.0, peptides (Hits)20(191000), theory PI 8.16, and MW 66066.74 Da.

3.4. Cellular Location. The cellular location of expression products of gene Hsp74 was analyzed using immunofluorescence. The results showed that they are all distributed in cellular membrane, cytoplasm and nucleus (Figure 5). The cellular membrane and cytoplasm show all red, and the cellular nucleus shows some red and some blue.

3.5. Hsp74 Expression in Tissue. Among the 35 bladder cancer cases, the positive expression rate of Hsp74 in bladder cancer

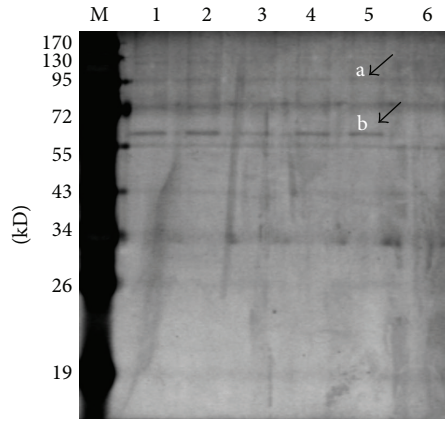


Figure 3: Demonstration of MAb with BLZ211 cells protein reaction by coimmunoprecipitation. M: marker; (a) Hsp74 (b) keratin 1. Lanes 1–5: BLZ211 cells protein; lane 6: negative control; lanes identified by LC-MS/MS.

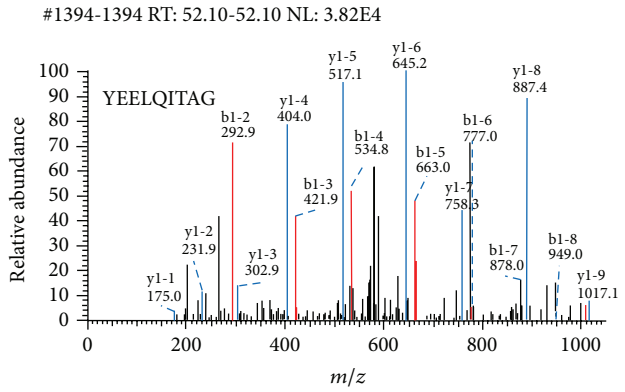


Figure 4: Representative MS/MS spectrum of the peptide of keratin 1.

tissues was 74.2%, which was similar to that in adjacent normal tissues ($X^2 = 0.063$, $0.75 < P < 0.9$). But there was a significant difference in the expression intensity distribution between the two groups ($X^2 = 21.86$, $P < 0.005$). In most cases, the expression intensity of Hsp74 in bladder cancer tissues was higher than in normal bladder tissue (Table 1, Figure 6).

4. Discussion

Bladder cancer is a prevalent disease that causes substantial morbidity and mortality. Despite the continued refinement of surgical techniques, namely, radical cystectomy, the prognosis of muscle-invasive bladder cancer has remained unchanged for the past 30 years with five-year survival rate remaining disappointingly low at 40%. Understanding the biology mechanism underlying tumorigenesis and tumor progression of bladder cancer is essential for improving the capacity to diagnose and treat the disease. Unraveling the biological complexity underlying these processes is expected

Table 1: Hsp74 expression in bladder cancer tissue and adjacent normal tissues.

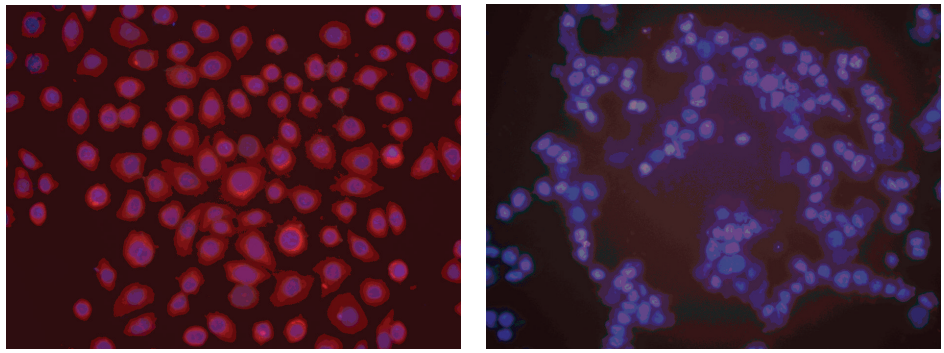
Pathology	Number	Hsp74 (n)				Positive rate (%)
		−	+	++	+++	
Bladder cancer tissue	35	9	4	19	3	74.2
Adjacent normal tissues	35	10	20	5	0	71.4

to provide novel tools of predictive nature and to enable identification of therapeutic targets by selecting those molecular targets significantly and differentially expressed in bladder tumors. Numerous markers that correlate to some extent with bladder cancer stage and prognosis have been identified. However, the ability of most markers in predicting the clinical outcome of individual tumors is limited, and alternative markers are still needed for detection of the disease and for predictive purposes.

We did a related research of bladder cancer using immunoscreening technology previously [11]. Recent advances in expression profiling of cancer cells by proteomic technologies, high-resolution 2-dimensional electrophoresis (2-DE), and mass spectrometry have made it possible to identify candidate proteins as tumor markers in various cancers. In bladder cancer, Celis et al. [12] performed proteome analysis by extensive 2-DE and developed a comprehensive 2-DE database for bladder cancer that includes profiles of both transitional and squamous cell carcinoma. Therefore we used 2-dimensional electrophoresis and mass spectrometry to carry out exploratory research in bladder cancer and identified Hsp74 as potential target. Immunofluorescence analysis showed that Hsp74 protein was distributed in cytoplasm. This result gives us the bladder cancer intracellular localization of Hsp74 in the preliminary concept.

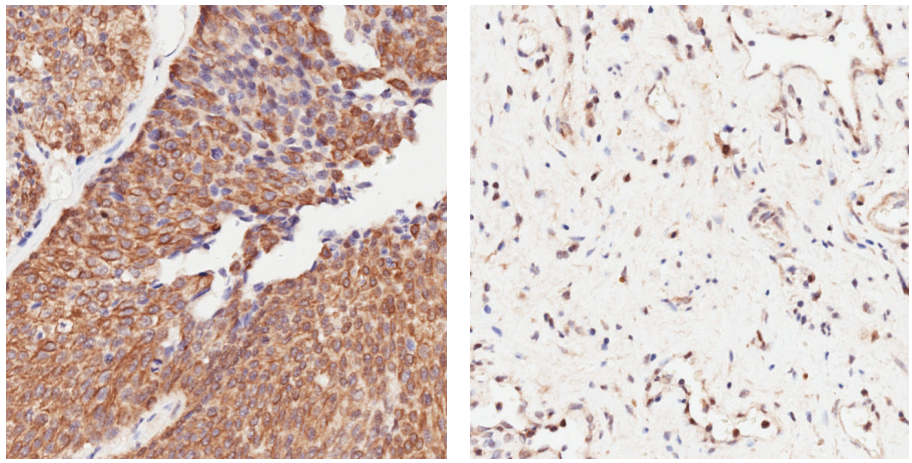
Hsp74 belongs to a member of the family heat-shock (stress) proteins (HSPs). In the face of thermal, chemical, or physiological stresses that cause unfolding of proteins, cells preferentially express HSPs, a process called the heat-shock (or stress) response. Related researches on Hsp can draw the following conclusions: (1) Hsp has relevance with cancer diagnosis; (2) detection of Hsp can help identifying abnormalities in carcinogenesis; (3) Hsp is associated with some tumor differentiation degree; (4) Hsp has significant correlation with some specific molecule. HSP expression is associated with carcinogenesis, cell proliferation, differentiation, and apoptosis. So the detection of Hsp has important significance in the program of individualized cancer therapy, regular followup, and avoidance of excessive use of cytotoxic cancer drug. Research on Hsp provides a new encouraged approach and a new target for immunotherapy [13].

It is common for a constitutively expressed Hsp70 cognate (hsc70) to be present in cells under all conditions, where it functions as a chaperone during normal protein synthesis. As one of Hsp70 isoforms, Hsp74 has been demonstrated to regulate cytoskeletal stability [14]. Mucous membrane of urinary bladder is exposed for longtime to the urine which contains various kinds of mutagenic activity materials and toxic metabolites. Elevated Hsp74 expression might be the



(a) (b)

Figure 5: Immunofluorescence of the cellular location analysis of expression products of gene HSP74 showed that they are all distributed in cellular membrane, cytoplasm and nucleus (a), negative control (b) ×200.



(a) (b)

Figure 6: Immunohistochemistry of the tissue location analysis of expression products of gene HSP74 showed that they are distributed positively in bladder cancer tissues (a) and negatively in bladder cancer adjacent normal tissues (b) ×20.

result of mucous membrane of urinary bladder chronic stress reaction.

Hsp74 was associated with keratin 1 as determined by coimmunoprecipitation from bladder cancer cell line BLZ211. Keratin 1 belongs to keratin family and is a specific marker of mammal epithelium terminal differentiation since keratin 1 protein mainly locates in epithelial prickle cell layer and epithelial granular cell layer. Monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) provide an important means of examining keratin expression in epidermal tumors and keratinizing disorders [15]. Cytokeratins 1, 7, and 14 immunoexpression is helpful in the diagnosis of basaloid squamous carcinoma [16]. It remains to be determined whether the binding of Hsp74 to keratin 1 is dependent on a linear sequence of keratin 1 or a conformation of keratin 1 tetramers or polymers. Furthermore, although our data suggest a direct interaction of Hsp74 with keratin 1, we cannot exclude the possibility that this interaction may be mediated by one or more additional proteins that form a large complex.

To detect tumor mark by monoclonal antibodies is a kind of classical method. Using a combination of techniques including 2-DE, co-IP, western blot, LC-MS/MS, immunofluorescence, and immunohistochemistry, we find the expression intensity of Hsp74 in bladder cancer tissues is higher than that in normal bladder tissue. The results of this research suggest that Hsp74 might be a potential marker of bladder cancer, although further validation is still needed. To use this antigen to detect tumor has more problems which need to be resolved. The relevance of Hsp74 expression with clinical pathological characteristics and survival time is worth to follow-up study. Specifically, in tumor formation the interaction mechanism of keratin 1 and Hsp74 need to be clarified further.

Conflict of Interests

The authors declared that there are no potential conflict of interests, neither financial nor of other nature, to disclose.

Acknowledgments

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Review Article

A New Hope in Immunotherapy for Malignant Gliomas: Adoptive T Cell Transfer Therapy

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Immunotherapy emerged as a promising therapeutic approach to highly incurable malignant gliomas due to tumor-specific cytotoxicity, minimal side effect, and a durable antitumor effect by memory T cells. But, antitumor activities of endogenously activated T cells induced by immunotherapy such as vaccination are not sufficient to control tumors because tumor-specific antigens may be self-antigens and tumors have immune evasion mechanisms to avoid immune surveillance system of host. Although recent clinical results from vaccine strategy for malignant gliomas are encouraging, these trials have some limitations, particularly their failure to expand tumor antigen-specific T cells reproducibly and effectively. An alternative strategy to overcome these limitations is adoptive T cell transfer therapy, in which tumor-specific T cells are expanded *ex vivo* rapidly and then transferred to patients. Moreover, enhanced biologic functions of T cells generated by genetic engineering and modified immunosuppressive microenvironment of host by homeostatic T cell expansion and/or elimination of immunosuppressive cells and molecules can induce more potent antitumor T cell responses and make this strategy hold promise in promoting a patient response for malignant glioma treatment. Here we will review the past and current progresses and discuss a new hope in adoptive T cell therapy for malignant gliomas.

1. Introduction

The prognosis of malignant glioma patients is grim despite the advanced multimodality therapies including surgery, radiotherapy, and chemotherapy. Immunotherapy emerged as a potential therapeutic approach to the highly incurable malignant gliomas, for which, however, either encouraging results or disappointing limitations were revealed as an alternative strategy [1, 2].

Tumor-specific $CD8^+$cytotoxic T lymphocytes (CTLs) are generated by repetitive stimulation of peripheral blood mononuclear cells (PBMCs) with tumor-associated antigen (TAA) expressing antigen-presenting cells (APC) such as dendritic cells (DCs) and certain cytokines including interleukin- (IL-) 2, IL-7, IL-12, IL-15, and IL-21 [3, 4]. These cells can be expanded rapidly *ex vivo* to use them for adoptive cell therapy (ACT). Antigen sources for this procedure include major histocompatibility complex- (MHC-) restricted peptides, recombinant proteins, tumor lysates, and genetically introduced tumor antigen genes. $CD4^+$ T cells may also exert antitumor effector functions mainly through the secretion of interferon- (IFN-) γ [5].

Theoretically, tumor-specific CTLs can move to TAA-overexpressed tumor cells specifically and kill them without adverse effects on normal cells. But, immune system may recognize these TAAs as self-antigens, leading to decreased T cell response to tumor cells because TAAs are also somewhat expressed in normal tissues [6, 7]. T cells with high affinity to self-antigen may be physiologically removed through the mechanisms of immune tolerance, so the endogenously activated tumor-specific T cells have low affinity to self-antigen, inducing limited T cell response [8]. Furthermore, tumors

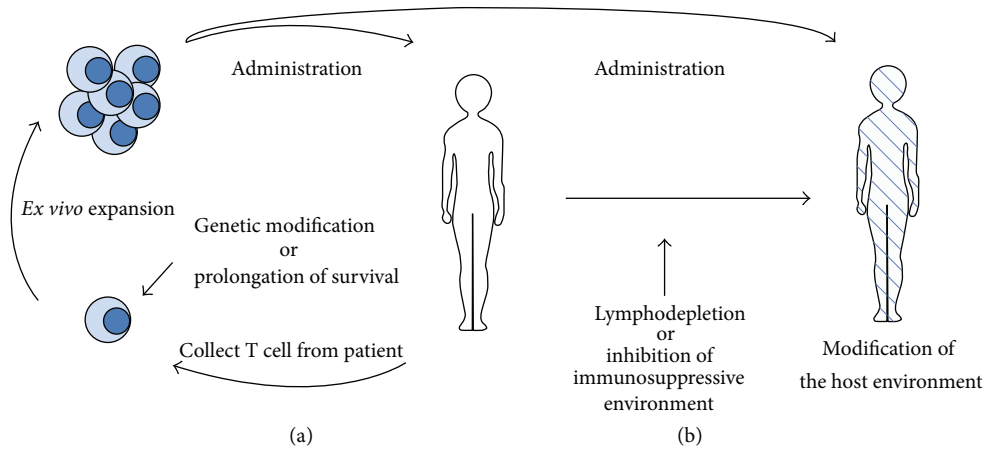

Figure 1: Adoptive T cell transfer therapy. (a) Enhancement of tumor-specific T cell function. (b) Modification of the host environment.

have evolved numerous mechanisms to evade both innate and adaptive immunity. These include modulation of MHC antigens and costimulatory molecules, expression of Fas ligand and other apoptotic molecules on the cell surface, production of inhibitory molecules such as transforming growth factor- (TGF-) β and IL-10, constitutive expression of the tryptophan-depleting enzyme, indoleamine 2,3-dioxygenase (IDO), and recruitment of regulatory T cells (Tregs) [9].

Results from recent immunotherapeutic clinical trials with tumor cell or DC vaccines for malignant glioma patients were encouraging [10–13]. These trials, however, have shown some limitations, particularly their failure to expand tumor antigen-specific T cells reproducibly and effectively, suggesting that endogenous activation of T cells is insufficient to control tumors. A strategy to overcome these limitations is adoptive T cell transfer, in which tumor-specific T cells are expanded *ex vivo* rapidly and then transferred to patients. Moreover, a recent advance in delivering therapeutic genes into somatic cells has been applicable to T cell therapy for tumors. T cells used in ACT can be modified to increase their specificity and survival for the tumor or to make them resistant to immune evasion mechanisms [14–25] (Figure 1). T cell response for malignant gliomas also can be improved by combination with other therapeutic modalities [26, 27].

Here we will review past experiences and discuss current promising strategies of adoptive T cell therapy for malignant gliomas.

2. Immune Environment of Malignant Glioma

The brain has long been considered to be immunologically privileged due to immediate inability to reject intracranial xenograft in early report [28], physical isolation from the systemic immune system by the blood-brain-barrier (BBB), and lack of connections to the lymphatic system. Subsequent studies, however, have described the efficient rejection of intracranial xenografts and allografts in immunocompetent hosts abundantly [29], capability of activated T cells to cross the BBB [30, 31], and the drainage of cerebrospinal fluid into systemic lymphatics [32]. In addition, no specific CNS-associated antigens have been known that are systematically immunogenic but evade immune surveillance within the brain unlike testes, other immunologically privileged site [33]. Microglia, resident APCs in the brain, play a crucial role in the CNS immune response [34]. Collectively, these results clearly indicate that the brain is not an immunologically privileged site, but may be an organ that has immunologically particular environment although not fully understood.

Table 1: Glioma-associated antigens.

Classification	Antigens [references]
Mutated antigens	EGFRvIII [35]
Cancer-testis antigens	MAGE [36], GAGE [37], and SOX6 [38]
Tissue-specific antigens	Gp100 [39], TRP-2 [40]
Others	IL-13Rα2 [41], EphA2 [42], EphB6 [43], HER-2[39], AIM-2 [44], SOX11 [45], surviving [46], telomerase [47], Mart-1 [48], and KIF3C [49]

A critical step for an efficient stimulation of adaptive immune response even in the brain is the identification of suitable tumor-specific or tumor-associated antigens that can be recognized and eliminated by the immune system. Malignant glioma is known to be genetically heterogenous with a variety of antigen profile [48], so glioma cells are inefficient for antigen processing. Difficulty in identification of ideal tumor antigens for immunotherapy as well as the above-mentioned immune evasion mechanisms and the presence of immune inhibitory cells may render malignant glioma resistant to T cell responses. The source of antigen used in initial immunotherapeutic approaches to the malignant glioma was tumor lysates derived from autologous irradiated glioma cells [50]. Numerous glioma-associated antigens have been identified over the past decades and the antigens most suitable for activating the host-specific T cell response are still under investigation (Table 1). The glioma-specific antigens used in recent preclinical or clinical studies showing potent

Table 2: Comparison of the effector cells used in adoptive T cell therapy for malignant glioma.

Effector cells	Advantages	Disadvantages
Lymphokine-activated killer (LAK) cells	MHC-independent cytotoxicity Easy preparation of cells	Nonspecific killing IL-2 related toxicities
Natural killer (NK) cells	MHC-independent cytotoxicity Immediate response Can be modified to target tumor antigens genetically	Nonspecific killing
$\gamma\delta$ T cells	MHC-independent cytotoxicity Immediate response	Nonspecific killing
Tumor infiltrating lymphocytes (TILs)	Presumably tumor-specific killing	Need T cells from tumor tissue Technical difficulty to expand *ex vivo*
$CD4^+$ cytotoxic T lymphocytes	Tumor-specific killing	MHC class II-dependent cytotoxicity
$CD8^+$ cytotoxic T lymphocytes	Tumor-specific killing Can be modified to target tumor antigens genetically	MHC class I-dependent cytotoxicity
Genetically modified cytotoxic T lymphocytes	MHC-independent cytotoxicity Rapid and elaborate tumor-specific killing	Induction of antigen loss variants at tumor recurrence Possible overreactivity on same target antigens expressed in normal tissue

antiglioma effect include IL-13Rα2, human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor variant III (EGFRvIII), and erythropoietin-producing hepatocellular carcinoma A2 (EphA2) [19–22, 51].

Recruitment of lymphocytes is a key of immune response. Immune cells can infiltrate to malignant glioma at later stage of tumor growth with destruction of the BBB [52] and peripherally infused CTLs can enter the CNS in patients with malignant glioma [53]. Glioma-derived chemokines such as CCL2, CCL7, or CCL20 can mediate the recruitment of immune cells [54, 55].

3. Antitumor Immune Responses of Effector Cells

Effector cells used in ACT for the malignant glioma have developed from lymphokine-activated killer (LAK) cells with nonspecific cytotoxicity to more tumor-specific genetically engineered CTLs over time. The advantages and the disadvantages of the effector cells used in ACT for malignant glioma are summarized in Table 2.

3.1. LAK Cells. Autologous LAK cells are a mixture of IL-2 activated T cells and natural killer (NK) cells and are generally obtained by culture of PBMCs in the presence of IL-2. Major therapeutic limitation of these cells against tumors is that their lytic properties are not specifically directed against tumor cells. Autologous tumor cells were usually used as antigen source in ACT using LAK cells for malignant gliomas [56–59].

Although several clinical trials by intratumoral injection of LAK cells combined with IL-2 for the glioblastoma patients have been carried out, most of their therapeutic effects have not shown a significant survival benefit [60–70]. In addition, the use of LAK cells in combination with IL-2 was not superior to the use of IL-2 alone in the phase III trial for other tumors [71]. Moreover, IL-2 related toxicities that emerged in some studies such as brain edema and aseptic meningitis have disturbed widespread use of this strategy for malignant gliomas [63, 65, 70].

3.2. NK Cells. In contrast to adaptive immune responses, innate lymphocytes such as NK cells and $\gamma\delta$ T cells broadly recognize and immediately respond to a certain range of antigens in a MHC-independent fashion [72]. NK cells, $CD3^-CD56^+$ lymphocytes, play potential role in cancer immunosurveillance as innate immune cells. They initially recognize the tumor cells via cellular stress or danger signals. Activated NK cells can directly kill tumor cells without MHC restriction, interact with DCs to facilitate the generation of antigen-specific CTL response by enhancing their antigen uptake and presentation, and induce $CD8^+$ T cells to become CTLs by producing cytokines such as IFN-γ. Cytokines produced by NK cells can also regulate antitumor antibodies produced by B cells [73–75]. Both allogeneic and autologous IL-2 activated NK cells, furthermore, recognize and kill human glioblastoma cells with stem cell-like properties [76].

Although clinical trials with ACT using LAK cells did not show a significant clinical benefit for malignant gliomas as discussed above, recent advances in NK cell immunobiology and results in animal studies showing favorable antitumor effect in glioma-bearing mice treated with activated NK cells take a growing interest in ACT using activated NK cells again. NK cells can do traffic to the brain directly [77], so both peripheral and intratumoral route of administration are available in the treatment of malignant gliomas. In a rat glioma model, no therapeutic effect was observed in animals treated with intradermally injected paraformaldehyde-fixed tumor vaccine alone, but intratumoral injection of IL-2-activated rat NK cells strongly enhanced antitumor effect

of the vaccine [78]. Also, intracranial injection of cytokine-induced killer cells markedly inhibited intracranial xeno-transplanted glioma growth in mice [79].

Safe antitumor response was shown in a clinical trial that exclusively used *ex vivo* expanded autologous NK cells to treat recurrent malignant glioma patients [80]. In this study, two (22%) of the nine patients injected focally and intravenously showed partial response. Additionally, prolonged survival of the patients with malignant glioma treated by tumor-loaded DCs vaccine may be associated with NK cell response such as high level of circulating IFN-γ and increased NK cell vaccine/baseline (V/B) ratio that was inversely correlated with TGF-β2 V/B ratio [81]. These results suggest that a strategy of ACT using *ex vivo* activated NK cells following tumor-loaded vaccine can have a potent antiglioma effect as in animal studies.

Tumor cells, however, have various mechanisms to avoid NK cell recognition including the expression of MHC class I and ligands for inhibitory receptors on NK cells [82, 83]. In order to overcome this resistance of tumor cells to NK-mediated cytotoxicity and enhance tumor recognition of NK cells, gene modification can be utilized. Antitumor activity of NK cells can be enhanced by genetic modification to highly express cytokines, Fc receptors, and/or chimeric antigen receptors (CARs) [84–86]. CAR directly recognizes tumor cell surface antigens and provides specificity of engineered cells regardless of antigen processing or MHC-restricted presentation. Cytokine gene transfer such as IL-2 [87–89], IL-12 [88, 90], IL-15 [91–93], and stem cell factor (SCF) [94] induces NK cell proliferation and survival, and gene transfer of CARs against HERs/neu [95], carcinoembryonic antigen (CEA) [96], and CD33 [97] shows increased specificity [85] *in vitro* and *in vivo* studies. These results suggest ACT using genetically modified NK cells can be a challenge to patients with cancer including malignant gliomas.

NK cell-based immunotherapy has several potential limitations including the immunosuppressive microenvironment of the tumors. Activation of myeloid derived suppressor cells (MDSCs) and Tregs, especially, are known to be major barriers. MDSCs, a heterogeneous population of $CD11b^+$, $Gr\text{-}1^+$ cells of immature myeloid origin, consist of myeloid progenitors and precursors of macrophages, granulocytes, and DCs and have a strong ability to suppress a variety of T cell and NK cell functions [98–100]. MDSCs can also modulate the induction of Tregs [101, 102]. MDSCs increase in malignant glioma-bearing mice [77] and effectively inhibit NK cell-mediated tumor suppression. Circulating number of these tumor suppressor cells also increases in the patients with malignant gliomas [103, 104]. Although there have been no published studies on human glioma-infiltrating MDSCs to date, many preclinical studies to improve antitumor effect by reducing MDSCs in tumor-bearing animal models have been carried out [105, 106].

Tregs are potential inhibitors of NK cell activity in malignant gliomas [107]. Tregs directly inhibit NKG2D-mediated NK cell cytotoxicity, effectively suppressing NK cell-mediated tumor rejection by a TGF-β dependent mechanism and independent of IL-10 and depletion of Tregs via NKG2D before NK cell activation markedly enhances NK cell-mediated suppression of tumor growth and metastases in animal studies [108]. Tregs also decrease NK cell cytotoxicity and downregulate the IFN-γ secretion of NK cell responding to IL-12 activation in a TGF-β dependent manner [109]. Elimination or inhibition of these immunosuppressive cells, therefore, can improve the antitumor effect of ACT using NK cells.

3.3. $\gamma\delta$ T Cells. $\gamma\delta$ T cells are a subpopulation of T lymphocytes, which express T-cell receptors (TCRs) consisting of one γ chain and one δ chain. Unlike the conventional $\alpha\beta$ T cells that recognize only MHC-related antigens, $\gamma\delta$ T cells can broadly recognize and immediately respond to a range of antigens in a MHC-independent manner.

$\gamma\delta$ T cells have potent cytotoxic activity against malignant glioma cells [110, 111]. Antiglioma effect of human $\gamma\delta$ T cells can be increased by the addition of IL-12 [112, 113]. Intracranial infusion of expanded and activated $\gamma\delta$ T cells can mediate killing of new or established glioblastoma xenografts and reduce tumor progression [114]. *Ex vivo* expanded and activated $\gamma\delta$ T cells from both patients and healthy volunteers can recognize and kill glioblastoma cell lines and primary glioblastoma culture cells, but $\gamma\delta$ T cell counts and mitogen-stimulated proliferative response of $\gamma\delta$ T cells are markedly decreased in glioblastoma patients prior to treatment, suggesting that allogeneic therapy could be a reasonable option in adoptive $\gamma\delta$ T cell immunotherapy [115].

Despite of the theoretical basis of $\gamma\delta$ T cell-based immunotherapy, there have been no clinical studies designed to assess the immunotherapeutic potential of $\gamma\delta$ cell therapy against malignant gliomas to date. A recent report that gene modified $\gamma\delta$ T cells have greater cytotoxicity to temozolomide (TMZ) resistant glioblastoma cell lines in the presence of TMZ than unmodified cells [116] suggests combined TMZ resistant $\gamma\delta$ T cell immunotherapy and high dose TMZ chemotherapy could be a new therapeutic challenge to the glioblastoma patients.

3.4. TILs. Tumor infiltrating lymphocytes (TILs) are effector cells presumably thought to be able to recognize and respond to the specific tumor antigens because they are already present in the tumor. Although antitumor activity of endogenous TILs may not be sufficient to conquer tumor-induced immunosuppressive environment, *ex vivo* expansion of these cells may overcome this immunologic barrier and be a tool of ACT for tumors. *Ex vivo* expanded TILs have the properties to proliferate *in vivo* and display functional activity and trafficking to tumor [117]. Significantly increased antitumor activities of *ex vivo* expanded TILs therapy have been shown in clinical trials for melanoma especially in combination of lymphodepletion with intensive chemoradiation [118, 119].

It is difficult, however, to expand TILs from tumor tissues in most cancers including malignant glioma except melanomas [120]. In a pilot study exclusively performed to date against patients with recurrent malignant gliomas that were treated with intratumoral infusion of *ex vivo* expanded autologous TILs with IL-2, one of six patients showed complete remission, two had partial responses, and three died of

tumor progression [56]. The cytotoxic activity of TILs against autologous tumors *in vitro* was variously dependent on the patients and was not correlated with the clinical outcome in this study. These results suggest that clinical benefit from ACT for malignant gliomas using *ex vivo* expanded TILs may be limited.

3.5. Antigen-Specific CTLs. Antigen-specific CTLs commonly generated by *ex vivo* antigen stimulation of PBMCs with autologous inactivated tumor cells have potent antitumor immune response compared with T cell response to endogenous stimulation. These CTLs are also able to migrate to antigen-expressed tumor cells following administration and have durable antitumor effect by memory T cells. *Ex vivo* expansion of CTLs for strong priming of T cells with antigens and for rapid increase of effector T cell numbers makes these cells feasible to be used in ACT for cancers.

To date, 4 phase I trials to evaluate CTLs generated from PBMCs [57–59, 121] and 3 phase I and 2 pilot studies examining CTLs obtained by lymphocytes from tumor draining lymph nodes or PBMCs after vaccination with irradiated autologous tumor cells [53, 122–125] against malignant gliomas have been described. Total 9 clinical trials of ACT using antigen-specific CTLs showed 2 complete response (CR), 26 partial response (PR), and 16 stable disease (SD) in 87 patients with malignant gliomas (65 glioblastoma). Data from 49 patients with glioblastoma exclusively in 8 trials except a study that did not describe the results from the distinguished tumor grade [125] demonstrated a result of no CR, 11 PR, and 6 SD. A pilot study for 19 patients with recurrent malignant gliomas (16 glioblastoma) that did not distinguish tumor grade in treatment outcome displayed a favorable result of 1 CR, 7 PR, and 9 SD [125]. More improved median survival of 12 months after tumor recurrence compared with 6 months for controls and a positive correlation between increased survival and delayed-type hypersensitivity response were described in this study [125]. Similarly, a positive correlation between CD4/CD8 composition of infused cells and clinical response was reported [124]. Most other trials, however, did not show survival benefit and a clear association between the concentration of injected T cells and clinical outcome.

3.6. $CD4^+$ T Cells. $CD4^+$ T cells contribute to the immunologic antitumor activity through their ability to mediate tumor cell destruction independent of $CD8^+$ T cells as well as help activate $CD8^+$ T cells classically [126–128]. Identification of MHC class II-restricted isotopes derived from several TAAs including melanoma differentiation antigens and several cancer-testis antigens becomes feasible to generate antigen-specific $CD4^+$ T cells which can be used in ACT [129–131]. Several preclinical studies have described antitumor effect of ACT using $CD4^+$ T cell population, and CD4+ T cells have cytolytic activity dependent on class II-restricted recognition of tumors [132–134]. In a recent early-phase dose escalation study of ACT for patient with metastatic melanoma using $CD4^+$ T cell clones, the patients experienced partial responses including a case of a complete durable response [128, 135].

Table 3: Genetic modification of T cells to improve the efficacy of ACT for cancers.

	References
Enhanced specificity	
Expression of $\alpha\beta$ TCR	[16, 17, 136]
Expression of CARs	[18–22]
Coexpression of costimulatory molecules	[23–25, 137]
Increased survival and proliferation	
Expression of proliferative cytokines	[138–141]
Expression of antiapoptotic genes	[142–144]
Ectopic expression of gene for telomere elongation (hTERT)	[145–148]
Enhanced trafficking	
Expression of chemokine receptors	[149–152]
Enhanced trafficking	
Expression of negative TGF-β receptor	[153–156]
Downregulation of Fas	[157]
Integration with conventional therapy	
Expression of chemoresistant genes	[116]

4. Enhancement of Tumor-Specific T Cell Function

4.1. Genetically Modified T Cells. Recently, gene modification of T cells has been developed for enhancing the efficacy of ACT. Gene engineering of T cells by a variety of gene transfer techniques is able to allow T cells to make them more resistant to immune evasion mechanisms of tumor cells or modify the tumor environment to make it less inhibitory to T cell activation and effector function [9] (Table 3). Retroviral or lentiviral vectors are usually used for gene delivery [14, 15].

Two most common approaches can be used for enhancement of T cell specificity: (a) gene modification with TCR variable α and β chains cloned from high affinity TAA-specific T cells and (b) insertion of chimeric antigen receptors (CARs) that recognize tumors through single-chain variable fragment (scFv) isolated from TAA-specific Abs.

Genes encoding TCRs of T cells isolated from patients showing an excellent response to ACT can be cloned into viral vectors and then be used to alter T cells from other patients with matching HLA restriction elements to be treated [17]. These genes can also be isolated from humanized mice that have been primed to recognize TAAs. Humanized mice that have been cloned human MHC class I or class II molecules can express human MHC molecules and can be immunized with human TAAs of interest. Mouse T cells specific for certain MHC-restricted epitope can then be isolated, and their TCR genes are cloned into viral vectors that can be used to genetically modify T cells from the patient [17, 158].

Some clinical studies for patients with metastatic melanoma using T cells genetically modified with tumor antigen-specific T cell receptors for patient with melanoma have been conducted [16, 17]. In a recent clinical study assessing ACT using a high-avidity TCR recognized MART-1 and gp 100 for patients with metastatic melanoma, cancer

regression was seen in 30% and 19% of patients who received the human or mouse TCR, but patients exhibited destruction of normal melanocytes throughout the body including skin, eye, and ear as the result of CTL responses to cognate antigen-containing cells [17]. No clinical study for malignant gliomas, however, has been performed to date. This procedure allows the rapid production of TAA-specific T cells but has a basic limitation that T cells engineered by this procedure can mainly recognize antigens that have processed and presented in MHC-restricted patterns.

An alternative approach to overcome this limitation is the use of CARs, genes encoding monoclonal antibody chains specific for TAAs [18]. T cells modified with CARs can be directed toward any antigen expressed on the cell surface because CARs provide T cell activation regardless of MHC-restricted presentation. CARs are synthetic molecules that consist of an extracellular antigen binding domain that usually contains the heavy and light chain variable regions of a monoclonal antibody, referred to as a single chain Fc (scFv) molecule, joined to transmembrane and cytoplasmic signaling domains derived from CD3-ζ chain or Fc receptor γ chains (FcRγ) and from costimulatory molecules. Engineered T cells activated by both tumor-specific TCR and costimulatory molecules such as CD28, 4-1BB, OX40, and inducible costimulator (ICOS) have enhanced antitumor activity to tumors [23–25, 159, 160].

T cells and expressing CARs for the glioma-specific antigens including IL-13Rα2, HER2, EGFRvIII, and EphA2 show potent antiglioma activity in preclinical animal studies [19–22, 51]. In a study, T cells from glioblastoma patients could be modified with HER2-specific chimeric antigen receptors to produce effector cells and killed autologous HER2-postive glioblastoma cells including CD133-positive glioblastoma stem cells. These HER2-specific T cells also had a potent antitumor activity against autologous tumors in an orthotopic xenogeneic SCID mouse model [20]. Recently, cytomegalovirus has emerged as a target for the treatment of malignant gliomas. Expression of genes unique to cytomegalovirus (CMV) in malignant gliomas has raised the possibility of CMV-specific T cells as a therapeutic tool [161–164]. Data from a recent clinical study to evaluate antiglioma response of ACT using CMV-specific T cells in combination with TMZ into a patient with recurrent glioblastoma showing a long-term disease free survival [164] suggest CMV can be a challenging target of ACT for malignant gliomas and provide an important clue for further evaluation of combined ACT and TMZ chemotherapy.

Although clinical experience of ACT using T cells expressing TAA-specific CARs is limited, therapeutic limitations of these cells have emerged. In a clinical study targeting three glioblastoma patients treated by intracranial adoptive transfer of autologous IL-13Rα2-specific CTL clones, safe antiglioma responses against antigen positive CD133$^+$glioma stem cells as well as antigen positive glioma cells were documented, but IL-13Rα2 antigen was not expressed in the eventually recurred tumor [165]. Immune escape like these antigen loss variants also can be presented in peptide vaccination targeting EGFRvIII in patients with glioblastoma [166], so antigen loss variants may be a major mechanism responsible for tumor progression.

In addition, there are safety concerns with regard to HER2-targeted T cell therapy. A patient administered T cells with a CAR recognizing ERBB2 died of respiratory distress probably due to cytokine storm by massive release from ERBB2 expressing T cells localized to the lung with recognition of low levels of ERBB2 on lung epithelial cells [167].

Genetic engineering can increase effector function of T cells by modification of tumor environment as well as enhanced T cell specificity to malignant gliomas. Other strategies for cancers to increase T cell effector function through genetic modification are described (Table 3).

4.2. Prolongation of T Cell Survival. Identification of T cell populations that can reproducibly survive *in vivo* for increased antitumor effect in ACT is also important. CD8$^+$ T cells have been described as naive cells and four antigen-experienced subtypes according to the differentiation status: T memory stem cell (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and differentiated effector T cells [168]. T cell differentiation is inversely correlated with antitumor effect in ACT for cancer [158, 169]. Preclinical studies in human T cells suggest that arrested differentiation via reducing IL-2 concentration in culture condition [170–172] and inhibitors of the WNT signaling pathway [173, 174] can lead to enrichment of less differentiated memory T cells with high replicative potential.

Recently isolated T_{SCM} cells in mouse model, the least differentiated memory subset, have a preferential intrinsic capacity for long-term *in vivo* persistence and for self-renewal, and a multipotent ability to derive T_{CM}, T_{EM}, and effector T cells in response to antigen reexposure [168, 175]. T_{SCM} cells have been shown to be more effective than T_{CM} cells which were more effective than T_{EM} cells in terms of ACT against tumors in various preclinical studies [135, 158, 169, 176]. T_{SCM} cells consistently express a surface marker typically found on naive T cells and also express stem cell antigen-1 (Sca-1), B cell lymphoma 2 (Bcl-2), the β chain of the IL-2 (IL-2Rβ), and the chemokine (C-X-C motif) receptor CXCR3 [168, 177]. The identification and *ex vivo* expansion to minimize corruption of a similar human stem cell-like memory T cells may be important in the development of ACT, and these cells may play a greater role in human future ACT strategies for patients with cancer.

5. Modification of the Host Environment

5.1. Lymphodepletion. Lymphoid cells have an independent homeostatic regulation of resting and memory cell compartments, so a rapid proliferation of remaining or infused lymphocytes happens to recover normal lymphocyte numbers after periods of lymphopenia [178, 179]. During homeostasis-induced T cell proliferation, naive T cells stably acquire the cell surface markers and functional properties of memory T cells capable of rapid and intense response to antigen, and these homeostasis-stimulated memory CD8$^+$ T cells

respond to lower doses of antigen than naive cells [180]. Considering that this recovery is mediated by MHC dependent recognition, that memory $CD8^+$ T cells respond in the reduced activation threshold of tumor-specific cells, and that proliferated T cells have effector functions, administration of tumor specific antigens in the form of a vaccine or *ex vivo* expanded adoptive T cell transfer during this recovery period can induce disproportionate enhancement of effector cell populations that have autoimmune responses against tumor-associated self-antigens, leading to increased antitumor effect of ACT [180–184].

The induction of immunodepleting condition in patients before T cell-based immunotherapy can be achieved by use of total body irradiation (TBI) or nonmyeloablative chemotherapy. Data in clinical trials using these approaches have been shown to enhance the efficacy of ACT [118, 119, 185–188] such as melanocyte-directed autoimmunity noted in some patients with metastatic melanoma treated by these approaches [118, 189].

Another therapeutic advantage of lymphodeletion prior to immunotherapy is the elimination of major immunosuppressive cellular elements within the tumor microenvironment such as MDSCs and Tregs. As described above, MDSCs are found in most patients with advanced cancers [103, 190–192], so elimination or blockade of the immunosuppressive functions of MDSCs can provoke an enhanced antitumor effect of immunotherapeutic strategies for tumors [193, 194]. MDSCs can also modulate the induction of Tregs [101, 102]. Tregs that play a two-directional role in controlling autoimmunity and T cell homeostasis can selectively suppress spontaneous lymphopenia-induced naive T cell proliferation [195] and actually enhance immune function by optimization of the conventional T cell diversity [196]. Tregs are increased after total body irradiation and inhibit the induction of effector T cells during recovery period from lymphopenia, whereas depletion of Tregs strongly inhibits tumor progression in animal study [197]. In a recent clinical pilot study, anti-IL-2Rα MAb daclizumab treatment combined with EGFRvIII-targeted peptide vaccination could deplete Tregs safely and significantly in patients with glioblastoma treated with lymphodepleting TMZ correlating with enhanced antitumor immunity [198].

Additionally, ACT can be enhanced by the increased depletion of endogenous cells that compete for homeostatic cytokines such as IL-7 and IL-15 [119], by the promotion of the expansion and function of adoptively transferred antitumor CD8 T cells through hematopoietic stem cells [199], and by the increased functionality of adoptively transferred T cells mediated by TBI-evoked microbial translocation [200].

5.2. Inhibition of Immunosuppressive Environment. Elimination or blockade of immunosuppressive molecules of human cancers can enhance the antitumor efficacy of ACT. The challengeable targets for the treatment of malignant gliomas can be TGF-β, Tregs, and signal transducer and activator of transcription 3 (STAT3).

TGF-β is a potent immunodepressant and blocking of TGF-β effects on T cells can improve antitumor efficacy of T cells after ACT for malignancies [153, 154, 201]. Administration of TGF-β receptor I kinase inhibitor increases tumor infiltration by NK, T cells and macrophage and increases survival in glioma-bearing mice [202, 203]. The most clinically advanced strategy to elicit TGF-β in gliomas is the use of intratumorally administered TGF-β2 antisense oligonucleotides using convection-enhanced delivery [204]. Phase II study that evaluated the efficacy and safety of trabedersen (TGF-β2 antisense oligonucleotides) administered intratumorally by convection-enhanced delivery compared with standard chemotherapy in patients with recurrent malignant gliomas showed a superior safety and a trend for superiority in 2-year survival rate of patients with anaplastic astrocytoma compared to chemotherapy [205]. However, further clinical study discontinued during the phase III trial unfortunately. TGF-β also influences the development, maintenance, and induction of Tregs, while disruption of TGF-β signaling prevents the generation of Tregs [206, 207].

Tregs have an important role in maintaining self-tolerance and in the prevention of autoimmunity physiologically, and increased Tregs fractions with $CD4^+$ T cell defects inducing decreased T cell responses are seen in patients with gliomas [208]. Characteristics of Tregs in both mice and humans are the high expression of surface markers CD25 (IL-2R-α-chain), constitutive expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), overexpression of glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), and the expression of the transcriptional regulator Foxp3 [209, 210]. These molecules can be therapeutic targets for depleting Tregs to improve ACT for gliomas.

Strategies such as anti-CD25 antibody and CD25-specific immunotoxin [211] employed to reduce Treg function target the constitutively expressed cell surface marker, CD25. IL-2Rα (CD25) blocking with anti-IL-2α (anti-CD25 antibody) daclizumab combining glioma antigen (CMV or EGFRvIII) specific vaccination during lymphopenia selectively depletes Tregs in mice and humans [198, 212].

Another possible approach to reduce Tregs in glioma is via CTLA-4 blockade. CTLA-4 is a transmembrane protein that binds to ligands B7-1 and B7-2 on APCs and is constitutively expressed on Tregs, acting as a potent negative regulator of T cell activation. Anti-CTLA-4 antibodies have shown potential therapeutics for gliomas [213], and combining sequential immunotherapy with GM-CSF expressing irradiated glioma cell vaccine synergistically prolongs survival in mice-bearing gliomas [214].

STAT3 is generally overexpressed in cancers including malignant gliomas and plays an important role in negative regulation of antitumor immunity. STAT3 regulates the expression of TGF-β and IL-10, cytokine related to the presence of Tregs in tumors, so STAT3 can be a target for depleting Tregs. Inhibition of STAT3 promotes the activity of NK and T cells on cancer cells [215, 216]. STAT3 inhibition was shown to reverse the immunosuppressive environment in malignant gliomas [217] and to promote the efficacy of ACT in a murine glioma model [216]. Furthermore, adoptive transfer of T cells that transfected miRNAs, gene transcripts modulating STAT3 signaling, exerts potent antiglioma

therapeutic effects in genetically engineered murine glioblastoma models and enhances effector responses in the local tumor microenvironment [218]. Additionally, a low dose metronomic TMZ therapy can induce Treg depletion [219] and inhibit trafficking of Tregs into the glioma microenvironment [220].

IDO is an intracellular enzyme that catalyzes oxidative catabolism of tryptophan [221, 222]. T cell proliferation is arrested when exposed to tryptophan shortage evoked by IDO, and most human tumors including gliomas evade cellular immune response through the constitutively expressed IDO [223]. Consequently, IDO expressing tumor cells are able to inhibit tumor specific T cell response [224]. Expression of IDO in APCs also allows macrophages and DCs to inhibit T cell proliferation [225] and expand potent autologous Tregs [226]. Inhibition of IDO can improve T cell therapy for cancers [227–229]. In addition, molecular targeted therapy with imatinib can potentiate antitumor cell responses in gastrointestinal tumor through the inhibition of IDO [230].

Recently, IDO emerged as therapeutic target for the treatment of gliomas [231]. IDO expression in glioma is associated with malignant progression [232] and a significant decrease of overall survival in patients [233]. IDO expression in brain tumors also increases the recruitment of Tregs in mouse model [233, 234].

6. Combining T Cell Therapy

Combining immunotherapy with cytotoxic chemotherapy or targeted therapy can promote the therapeutic potential for the treatment of cancers in comparison with the use of either treatment alone because abundant antigens can be released from the dying tumor cells and increased effector cell capacity to recognize and kill tumor cells can be induced by cytotoxic chemotherapeutic agents [235, 236]. This antigen processing can lead to the priming of adoptively transferred tumor-specific T cells as well as the activation of endogenous tumor-specific T cells. Chemotherapy can enhance tumor cell susceptibility to CTL-mediated cytotoxicity during cancer immunotherapy, increasing the efficacy of tumor-specific T cell activation in mice with advanced cancer [237, 238]. Furthermore, chemotherapy (dacarbazine, temozolomide, and cisplatin) induces intratumoral expression of T cell attracting chemokines [239]. Combined TMZ chemotherapy and immunotherapy with DC-based vaccines can lead to the enhancement of antitumor immunity through increased tumor-specific immune responses via the cross-priming of apoptotic tumor cell death as well as suppression of Tregs in glioma bearing mice [26] and showed to be beneficial for survival in a phase II trial in patients with newly diagnosed glioblastoma [27].

Oncogene addiction is a phenomenon in which the survival of cancer cells depends on an activated oncogene or inactivation of tumor suppressor gene and is an ideal potential target for molecular targeted therapy in human cancers [240, 241]. Tumor cell death after oncogene addiction may provide antigenic stimulation of T cells, and oncogene addiction may also reduce the production of immunosuppressive molecules by tumor cells, promising increased antitumor efficacy of combining ACT with molecular targeted therapy for cancers including gliomas [242]. Actually, BRAF inhibition can induce the enhanced T cell recognition and subsequent T cell response on melanoma cells [243], and BRAF inhibitor vemurafenib improves the antitumor activity of ACT for advanced melanoma in mice [244].

In vivo expansion of T cells by vaccination has limitation due to the immunosuppressive environment of the tumor, and clinical trials using vaccine alone do not have significant antitumor effect [245]. Combining T cell therapy and vaccination can also be an alternative approach to facilitate expansion and maintenance of T cells that survived in poor immunogenic tumor environment.

7. Future Directions

T cells used in ACT for malignant gliomas have been developed and will be more advanced to overcome immune evasion mechanisms and to survive in immunosuppressive environment employed by the tumor.

Future efforts will need to focus on identification of patient-specific tumor antigens through highly personalized approach, development of efficient lymphodepleting regimens prior to T cell transfer, and effective combination with other therapeutic modalities such as molecular agents targeting personalized oncogene addiction and potent host immune modulators.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Roles of Regulatory B Cells in Cancer

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Regulatory B cells (Bregs), a newly described subset of B cells, have been proved to play a suppressive role in immune system. Bregs can inhibit other immune cells through cytokines secretion and antigen presentation, which give them the role in the pathogenesis of autoimmune diseases and cancers. There are no clear criteria to identify Bregs; different markers were used in the different experimental conditions. Massive researches had described the functions of immune cells such as regulatory T cells (Tregs), dendritic cells (DCs), and B cells in the autoimmune disorder diseases and cancers. More and more researches focused on the roles of Bregs and the cytokines such as Interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) secreted by Bregs. The aim of this review is to summarize the characteristics of Bregs and the roles of Bregs in cancer.

1. Introduction

The relationship between immune system and cancer development has been well described over the past several decades. The immune system plays an important role in the prevention of tumors. It could specially identify and eliminate tumor cells through the tumor-associated antigens (TAA) or molecules expressed by the tumor cells [1]. This process was described as tumor immune surveillance or tumor immunoediting, which was divided into three essential phases: elimination, equilibrium, and escape. In the first phase, elimination, the transformed cells were recognized and eliminated by the innate immune response such as nature killer cells (NK) and macrophages before they became clinically apparent. The second phase was an equilibrium phase between the tumor cells and immunity. The antigen specific T cells could induce the adaptive immune response. The tumor cells would decrease the tumor-specific antigens and even lose the major MHC-I and MHC-II antigens along with the tumor progression. Finally, in the escape phase, the tumor cells became less immunogenic, escaped from immune attack, and suppressed antitumor immune response leading to the tumor production and growth [2]. Cancer genesis was the result of the immune escape. The immune effector cells and the cytokines played a key role in pursuing each phase. They had been clarified by the roles played in cancer immune surveillance [3]. In recent years, literatures have found some new subsets of the immune cells which are relevant to the tumor immune surveillance and promote the cancer production and progression, for example, the Tregs and Bregs. In this review, we focus on the relationship between the regulatory B cells and their functions in cancer.

2. Characteristics of Regulatory B Cells

2.1. Phenotypes and Markers of Bregs. In recent years, B cells have been demonstrated to downregulate inflammatory reactions and induce tolerance by production of IL-10 and/or TGF-β and interacting with pathogenic T cells to inhibit harmful immune responses.

The term "regulatory B cells" was introduced by Mizoguchi and collaborators, who identified Bregs as an IL-10-producing B cell subset in 2002 [4]. Those Bregs had been shown to ameliorate murine allergic and autoimmune diseases, such as contact hypersensitivity (CHS) [5], asthma [6], experimental autoimmune encephalomyelitis (EAE) [7], lupus [8], and collagen induced arthritis (CIA) [9]. Topical studies in CIA had identified the transitional 2 marginal-zone precursor (T2-MZP) cells that played an immunosuppressive function both *in vivo* and *in vitro* [10, 11]. To date, there are no precise unique phenotype markers to identify Bregs. Markers on mouse Bregs resembled those on $CD1d^{hi}$ $CD5^{+}$ B10 cells

[5], CD1d^{hi} MLN B cells (B220^{+} CD1d^{hi} CD21^{int} CD62^{low} IgM^{int} CD23^{int}) [12], CD1d^{hi} CD21^{hi} CD23^{-} CD24^{hi} IgM^{hi} IgD^{lo} marginal-zone B cells, CD19^{+} CD21^{hi} CD23^{hi} CD24^{hi} IgD^{hi} IgM^{hi} CD1d^{hi} T2-MZP cells [13], and Tim-1^{+} Bregs [14]. IL-15 coupled to granulocyte macrophage colony stimulating factor could convent naïve splenic B cells into IL-10-producing B cells. Those Bregs shared common markers with B10 cells and T2-MZP Bregs and acquired the expression of CD138 but lost the expression of CD19 [15]. Differing from above regulatory B cell subsets, the surface characteristics of adipose Bregs were CD1d^{lo} CD5$^{-/lo}$ CD11b^{lo} CD21/CD35^{lo} CD23$^{-/lo}$ CD25^{+} CD69^{+} CD72^{hi} CD185^{-} CD196^{+} IgM^{+} IgD^{+} [16]. These Bregs could maintain adipose tissue homeostasis and limit obesity-associated inflammation. The IL-10-producing B cell subset characterized in humans normally represents 1% to 3% of spleen B cells and <1% of peripheral blood B cells [17]. Human regulatory B cells were enriched in both transitional (CD24^{hi} CD38^{hi}) [18] and memory (CD24^{hi} CD27^{+}) [17] B cells. IL-10 production by CD24^{hi} CD27^{+} B cells regulated monocyte tumor necrosis factor alpha (TNF-α) production [17]. CD19^{+}CD24^{hi}CD38^{hi} B cells inhibited proinflammatory cytokine production by CD4^{+} T cells, dependent on IL-10, CD80, and CD86 but not TGF-β [18]. Human CD19^{+} CD25^{hi} CD86^{hi} CD1d^{hi} B regulatory cells could suppress the proliferation of CD4^{+}T cells and enhance Foxp3 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression in Treg cells by producing IL-10 and TGF-β [19]. Bregs did not belong to any clearly defined B cell subsets but they added value in both the CD27^{+} and the CD38^{hi} compartments [20]. Regardless of the different markers used to identify Bregs, the majority of protective effects of Bregs are dependent on IL-10 [4, 5, 7, 18, 21], a potent deactivator, which limits the intensity and duration of inflammatory responses. Thus, IL-10 secretion is still a vital standard in the identification of Bregs.

A few of signal pathways were under the responsibility of the production of IL-10 by Bregs. It had been demonstrated that the Breg response could be promoted by stimulation with Toll-like receptor 4 (TLR4) and Toll-like receptor 9 (TLR9) ligands [22]. LPS with PIM (PMA+ionomycin+monensin) could induce B10 cells *in vitro* [5]. Using a mouse model for multiple sclerosis, B10 cells maturation into functional IL-10-secreting effector cells that inhibited autoimmune diseases *in vivo* required IL-21 and CD40-dependent cognate interactions with T cells [23]. IL-21 induced GrB^{+} human Bregs expressing high levels of GrB, which thereby limited T cell proliferation by a GrB-dependent degradation of the T cell receptor ζ-chain [24]. Agonistic anti-CD40 specifically targeted T2 B cells and enriched Bregs upon short-term *in vitro* culture [11]. MyD88 was thought to be involved but not critical to the development of Breg, while played a considerate role in IL-10 expression [20]. B cell linker protein, as a signaling component for Bregs function, was essential for the suppression of CHS and EAE by mediating IL-10 production [25]. Nuclear factor κB-α-kinase (NFκB) and signal transducer and activator of transcription 3 (STAT3) were involved in the secretion of IL-10 by Bregs [26]. Matsumoto and colleagues had found that the production of IL-10 was reduced in Bregs from mice with stromal interaction molecule 1 (STIM1) and stromal interaction molecule 2 (STIM2) depleted [27].

2.2. The Roles of Bregs in Immune System. Regulatory B cells performed a diversity of mechanisms to regulate immune responses and target many different immune cell types, such as DCs [28] and macrophages [29] as well as T helper 1 (Th1) cells and T helper 2 (Th2) cells [30]. It has been demonstrated that Bregs were capable of suppressing the proliferation of CD4^{+} CD25^{-} T cells [31] and production of interferon gamma (IFN-γ) and IL-17 by Th1 and T helper 17 (Th17) cells, respectively [32, 33]. *In vitro* studies in human had further postulated the potential ability of Breg cells to influence innate immunity by abrogating mitogen-stimulated secretion of TNF-α by monocytes, macrophages, and T cells. Yet regulatory B cells had no impact on the secretion of IL-6 and IL-8 by CD4^{+} T cells [34]. Mean B10 and progenitor B10 cell frequencies from patients with autoimmune disease were significantly higher than controls after CD40L with LPS/CpG stimulation [17]. These suppressive effects were mediated by IL-10. A number of studies indicated that the production of IL-10 by Bregs in mice and human was important for generation of at least two regulatory T- cell subtypes and conventional Treg cells as well as type 1 regulatory T cells (Tr1) [32–34]. The lack of Bregs resulted in a decrease of Foxp3^{+} Tregs [33]. B cell deficiency caused a significant reduction in the number of peripheral but not thymic Tregs. Adoptive transfer of WT B cells into μMT mice restored both Treg numbers and recovery from EAE [35]. B cells isolated from donor MRL/lpr mice and stimulated with agonistic anti-CD40 *in vitro* converted autologous effector T cells into Tr1 cells [11]. Coculture of CD4^{+} T cells by IL-10^{+} B cells announced that IL-10^{+} B cells were in a position to induce CD4^{+}T cells to produce large quantity of IL-10, which mediated the immunosuppression and protection from development of cerebral malaria [31]. IL-10 produced by Bregs was essential for the generation and maintenance of the pool of Tregs. Bregs induced pulmonary infiltration of CD4^{+}CD25^{+}Foxp3^{+} conventional Tregs, which controlled allergic airway inflammation [13]. As a result, activated Bregs could directly or indirectly target immune cells to regulate immune responses.

3. Regulatory B Cells in Cancer

3.1. B Cells in Cancer. Both positive and negative roles of B cells during tumor immunity have been reported. Depletion of CD20-expressing B cells increased tumor burden in the lungs of mice intravenously injected with B16-F10 melanoma [36]. B cells facilitated T-mediated responses, which in turn impaired tumor development [36, 37]. The induction of CD4^{+} and CD8^{+} T cells was significantly impaired in B cell-depleted mice with B16 melanoma tumors [37]. Activated B cells could mediate significant tumor regression in an IgG2b-dependent manner [36, 38]. These studies highlighted

the effector function of B cells as a source of IgG2b which were highly cytotoxic toward tumor cells [38].

Despite these, negative regulatory functions of B cells during immune responses to tumors have also been proposed. As we know, the mutation and accumulation of B cells and the antibodies secreted by B cells may play decisive roles in the tumor formation. Studies on B cell-deficient μMT mice showed that B cell deficiency enhanced $CD4^+$ T cell priming and helped for $CD8^+$ T cell-mediated tumor immunity [39]. The repertoire of $CD4^+$ helper T cells was limited in the presence of B cells, resulting in reduced antitumor immune responses [40]. Upon stimulation by irradiated tumor cells, the production of IFN-γ from $CD8^+$ T cells and NK cells was found to be markedly increased in the B cell-deficient as compared with wild-type conditions [41]. Murine EMT-6 mammary tumors grew readily in immune competent mice but poorly in B-cell-deficient mice [42]. Syngeneic tumors progressed poorly in μMT mice deficient in B cells unless replenished with $B220^+$ B cells [40, 43]. B cells could exert lots of functions to promote cancer, such as producing immunoglobulins and cytokines that could induce Fc receptors (FcR) and complement mediated chronic inflammation, whereas the chronic inflammation was required for carcinogenesis [44–46]. B cells produced TGF-β, thereby mediating suppression of cellular immune responses. Previously, studies showed that B cells required IL-10 and TNF-α to promote tumorigenic. IL-10 and TNF-α probably could mediate Th2 activation and inhibition of cytotoxic activity of $CD8^+$ T cells and nucleus. The cytokines such as TNF-α produced by B cells could activate the inhibitor of κB-α-kinase (IκBα), signal transducer, and activator of STAT3 in tumor cells, which would get a relapse of the castration resistant prostate cancer [47]. Moreover, the studies of the tumor growth in a mouse model of skin carcinogenesis had shown that the immune cells infiltrating in the premalignant lesions would reduce in B cell deficiency mice, which resulted in the inhibition of skin carcinoma development. Antibodies secreted by B cells could form the immune complexes (ICs) that would stimulate myeloid cells recruitment at tumor sites, which promoted the tumor growth via binding to the Fcγ-activating receptors. Also, the macrophages would become protumor myeloid cells [44, 48]. A similar role of B cells was observed in a synthetic mammary cancer model. It was reported that the antitumor immune response by T cells was enhanced in mice with B cell deficiency in this cancer model [40]. It also supported that the tumor was reduced by treatment with anti-mouse IgM/IgG antibodies, which would deplete the mutation B cells [49].

3.2. Bregs in Cancer. Bregs, as a separate subpopulation of B cells, have been mainly researched in their roles in the autoimmune diseases and inflammatory diseases [50]. Considering the tumor promoting role of the B cells and the immune regulating role of the Bregs, recent studies have focused on the role of Bregs in cancer. Previous studies had illustrated that the tumor inhibition effect of T cells was regulated by IL-10 production, which threw out a point that Bregs might be involved in cancer [41].

Also, IL-10 was a persistent regulatory cytokine which could repress Th1 and Th2 cytokine expression [51]. A recent study showed that Bregs could still secrete antibodies especially with IgM subtype as the activated plasmablast/plasma B cells. Immunoglobulins could also mediate inflammation to promote cancer as described above [44, 52].

Prevacid B and coworkers had described a unique subset of Breg cells in the studies with the mouse 4T1 model of breast cancer. The Bregs belonged to the $CD19^+$ $B220^+$ $CD25^+$ B2 lymphocytes which were needed in the progress of 4T1 murine breast cancer cells metastasis lung [53]. Since the CD25 is highly expressed on all activated T cells, B cells, and the thymic Tregs, the authors described this novel subpopulation of Bregs as tBregs. This research had found that the proportion of tBregs was significantly increased in peripheral blood and secondary lymphoid organs. 4T1 murine cancer cells could directly induce the generation of tBregs that inhibited the proliferation of nonactive and preactivated T cells. Similar role of tBregs was proved in the human cancers *in vitro* [54]. tBregs highly expressing TGF-β as well as CD40, CD86, and MHC I and II molecules could promote the generation of $Foxp3^+$ Tregs. The conversion of $CD4^+$ T cells into Tregs was relying on cell contact between T and B cells and the TGF-β secretion. In the process of lung metastasis of mammary adenocarcinoma 4T1 cancer cells, Tregs were needed in order to inhibit antitumor defenses of NK cells [55]. In conclusion, the researcher had proved that the tBregs could transform nonregulatory $CD4^+$ T cells (non-Tregs) to active Tregs through secreting the TGF-β, which in turn inhibited T cells proliferation and increased tumor metastasis [53, 54]. Also, cancer cells could convert normal B cells into tBregs. So, as long as cancer persists, the cancer cells would induce tBregs generation and inhibit the antitumor immune process [43, 56]. Recently, it was reported that nonmetastatic cancer cells expressed and utilized metabolites of the 5-lipoxygenase (5-LO) pathway to induce tBregs generation [57]. The presence of inhibitors of 5-LO/FLAP (5-LO activating protein) significantly reduced the expression of almost every tBreg-associated marker, such as decreased expression of CD25, CD81, BAFFR, and B7-H1 and phosphorylation of STAT3. Functionally, these cells also failed to suppress proliferation of T cells and did not induce conversion of $FoxP3^+CD4^+$ Tregs from non-Treg $CD4^+$ T cells [57]. This was the first clearly defined example reported on the existence and function of Bregs in cancer.

The anti-CD20 antibody reduction that could deplete B cells was proved to be effective in the treatment of non-Hodgkin lymphomas and CLL, but some patients showed resistance to anti-CD20 therapy or eventually relapse. A recent study has revealed that the presence of Bregs and their IL-10 might inhibit the therapy efficacy [58]. B cell depletion by CD20 antibody would greatly enhance cancer progression and metastasis. Both murine and human tBregs expressed low levels of CD20 and, as such, anti-CD20 mostly enriched for these cells [59]. In the study of lymphoma, the model mice received anti-CD20 treatment and then, following transfer of Bregs, resulted in tumor burden significantly increased [58, 60]. Bregs could affect the phagocytic capacity of macrophages, both *in vivo* and *in vitro* [58]. Myeloid cells and macrophages were responsible for clearance of anti-CD20 bound to tumor cells [60].

Table 1: The immunophenotype and naming of the regulatory B cells in mouse and human.

Species	Marker/phenotype	Naming	References
Human	$CD24^{hi}$ $CD27^{+}$	B10 cells	[17]
	$CD19^{+}$ $CD25^{hi}$ $CD86^{hi}$ $CD1d^{hi}$	Bregs	[19]
	$CD19^{+}$ $CD24^{hi}$ $CD38^{hi}$	Bregs	[18]
	$CD19^{+}$ $CD38^{+}$ $CD1d^{+}$ IgM^{+} $CD147^{+}$ GrB^{+}	GrB^{+} Bregs	[24]
Mouse	$CD19^{+}$ $CD5^{+}$ $CD1d^{hi}$	B10 cells	[5]
	$B220^{+}$ $CD21^{int}$ $CD62^{int}$ IgM^{int} $CD23^{hi}$ $CD1d^{hi}$	$CD1d^{hi}$ MLN B cells	[12]
	$CD1d^{hi}$ $CD21^{hi}$ $CD23^{-}$ $CD24^{hi}$ IgM^{hi} IgD^{lo}	Marginal zone B cells	[13]
	$CD19^{+}$ $CD21^{hi}$ $CD23^{hi}$ $CD24^{hi}$ IgD^{hi} IgM^{hi} $CD1d^{hi}$	Transitional 2 marginal-zone precursor (T2-MZP)	[13]
	Tim-1^{+}	Tim-1^{+} Bregs	[14]
	$CD1d^{lo}$ $CD5^{-/lo}$ $CD11b^{lo}$ CD2 1/$CD35^{lo}$ $CD23^{-/lo}$ $CD25^{+}$ $CD69^{+}$ $CD72^{hi}$ $CD185^{-}$ $CD196^{+}$ IgM^{+} IgD^{+}	Adipose IL-10^{+} B cells	[16]
	$CD19^{+}$ $B220^{+}$ $CD25^{+}$	Tumor-evoked regulatory B cells (tBregs)	[53]

In the study of 7,12-dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced skin carcinogenesis, the cancer growth was reduced in B cell deficiency mice ($Rag2^{-/-}$ mice) [61]. However, it would be partially rescued with transfer of B cells. Nonetheless, transfer of B cells could not promote the skin cancers in $Rag2^{-/-}$ mice when the mice were deficient of TNF-α. TNF-α knockout mice transferred of B cells could not enhance the skin cancer either. The number of $CD19^{+}CD21^{+}$IL-10-secreting B cells which were defined as Bregs showed significant decrease in TNF-α KO mice. The results above suggested that Bregs could promote cancer growth and the secretion of TNF-α might lead to the generation and accumulation of Bregs in cancer.

Recently, study found that IL-21 induced GrB-expressing Breg cells resided within the microenvironment of different tumor types including breast, ovarian, cervical, colorectal, and prostate carcinomas [24]. GrB^{+} B cells might contribute to the modulation of cellular adaptive immune responses by Treg-like mechanisms, possibly allowing the escape of certain tumors from an efficient antitumor immune response [24].

4. Conclusion

Both cancer escape and autoimmune diseases belonged to the inappropriate regulatory immune process. B cells mediated humoral immunity by secreting antibody and also regulated T cell maturation and function via serving as APCs and providing regulatory molecules [62]. The functions of B cells in autoimmunity and cancer diseases were well described. Lots of evidences had suggested the promoting tumor role of B cells, probably through inhibiting the activation of T cells, especially the $CD8^{+}$ T cells. In recent years, a distinct subpopulation of B cells that performed significant immunosuppressive has been described. Despite the fact that more and more literatures focused on the regulatory B cells, their clear phenotype and characteristic markers are still not definite. Since all the Bregs could secrete IL-10 and the majority of protective effects of Bregs required IL-10, IL-10 secreting B cells was considered as Bregs. The phenotype and naming of human and mouse Bregs in different experimental conditions were displayed in Table 1.

Bregs, as a subset of B cells, could exert B cells function such as secreting antibody and cytokines, which induced the IC production and stimulated cell signal resulting in tumor progression. Bregs as well as total B cells could promote cancer growth mainly by inhibiting the cytotoxic activity of Th1/CD8+ cells. This process was actually mediated by their IL-10 and TGF-β production.

As we discussed above in all mouse cancer models, Bregs, together with other components of immune system, promoted tumor progression. IL-10 produced by Bregs could reduce the $Th1/CD8^{+}$ cells. Furthermore, TGF-β secreted by Bregs could convert $CD4^{+}$ T cells into Tregs that would promote tumor progression. In studies of anticancer therapies by anti-CD20 therapy, Bregs and their IL-10 should be responsible for the development of lymphoma resistance to anti-CD20 therapy.

There are still some subjects of Bregs that remain to be established. Whether Bregs can affect other immune cells and whether different subset of Bregs can differentially participate in immune modulation have not been illustrated. Also, further studies are needed to research on tumor treatment through targeting Bregs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yan He and Hongyan Qian contributed equally to this work.

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Research Article

IFN-γ-Secreting-Mesenchymal Stem Cells Exert an Antitumor Effect *In Vivo* via the TRAIL Pathway

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Mesenchymal stem cells (MSCs) can exhibit either prooncogenic or antitumor properties depending on the context. Based on our previous study, we hypothesized that MSCs engineered to deliver IFN-γ would kill cancer cells through persistent activation of the TRAIL pathway. Human bone-marrow (BM-) derived MSCs were isolated, amplified, and transduced with a lentiviral vector encoding the IFN-γ gene under the control of the EF1α promoter. The IFN-γ-modified MSCs effectively secreted functional IFN-γ, which led to long-term expression of TRAIL. More importantly, the IFN-γ-modified MSCs selectively induced apoptosis in lung tumor cells through caspase-3 activation within the target cells. The percentage of activated-caspase-3-positive tumor cells in IFN-γ-modified MSCs cocultures was significantly higher than in control MSCs cocultures. Treatment with anti-TRAIL antibody dramatically suppressed the caspase-3 activation observed in H460 cells. After injection into nude mice, the IFN-γ-modified MSCs inhibited the growth and progression of lung carcinoma compared with control cells. Collectively, our results provide a new strategy for tumor therapy that utilizes IFN-γ-modified MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) are derived from the mesodermal germ layer and can be easily isolated and cultured from many tissues, such as bone marrow, adipose tissue, and amniotic fluid [1–3]. Because MSCs exhibit low expression of MHC I and do not express MHC II molecules, these cells do not cause immunological reaction and rejection after they are infused into an allogenic body [4, 5]. In addition, other reports have indicated that MSCs exhibit tropism toward tumors [6]. Therefore, MSCs are considered a promising cellular carrier for tumor gene therapy. For example, IFN-α-engineered MSCs can halt tumor growth *in vivo* by activating immune cells [7], and MSCs modified with pigment epithelium-derived factor can inhibit hepatocellular carcinoma growth *in vivo* by decreasing angiogenesis [8].

In addition to isolation from normal tissue, MSCs have also been found in tumor tissue and may be a key source of tumor-associated stromal cells [9, 10]. The current evidence suggests that the role of MSCs in tumor survival and progression is complex and diverse [11]. In our previous report, IFN-γ-primed MSCs were shown to express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which causes apoptosis in tumor cells [12]. However, the expression of TRAIL only persisted for approximately seventy-two hours after one priming with IFN-γ. We therefore evaluated whether IFN-γ-secreting mesenchymal stem cells could exert a persistent antitumor effect via the TRAIL pathway.

In this study, we show that IFN-γ-modified MSCs continuously express TRAIL and induce apoptosis in lung cancer cells by activating caspase-3 within the target cells. *In vivo* models further confirm the antitumor effect of the IFN-γ-secreting MSCs.

2. Materials and Methods

2.1. Isolation, Expansion, and Differentiation of MSCs. According to a policy approved by our institutional ethics

committee, heparinized human bone marrow (BM) samples were obtained by iliac crest aspiration from healthy donors who had given informed consent. MSCs were isolated and cultured as previously described [12]. Briefly, BM aspirates were diluted with an equal volume of low-glucose, complete DMEM and then fractionated with Lymphoprep solution (Huajin, Shanghai, China). The mononuclear cells were collected, washed, plated in 75 cm^2 flasks, and incubated at 37°C under 5% CO_2. After 3 days of culture, the nonadherent cells were removed by replacing the medium, and the adherent cells were continuously cultured with a change of the medium every 3 days. The MSCs were digested and replated when they reached approximately 90% confluence.

Osteogenic differentiation of the MSCs was induced using low-glucose, complete DMEM supplemented with 0.1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO), 50 μg/L vitamin C (Sigma-Aldrich), and 10 mM β-glycerophosphate (Calbiochem, San Diego, CA). The medium was replaced every 2-3 days. Around day 15, the mineralization of the MSCs was assessed by staining with 0.5% alizarin red solution.

Adipogenic differentiation of the MSCs was performed as follows. First, the MSCs were incubated in adipogenesis-inducing medium (AIM) containing 1 μM dexamethasone, 0.2 μM indomethacin (Sigma-Aldrich), 0.5 mM IBMX (Sigma-Aldrich), 0.01 mg/mL insulin (Sigma-Aldrich), and 10% (v/v) FBS in high-glucose DMEM. After 72 hours, the AIM was replaced by adipogenesis-maintenance medium containing 0.01 mg/mL insulin (Sigma-Aldrich) and 10% (v/v) FBS in high-glucose DMEM; the cultures were maintained for 24 hours. This cycle was repeated 3 times. At day 13, the adipogenesis of the MSCs was assessed by staining with fresh 0.25% oil red O solution.

Chondrogenic differentiation of the MSCs was induced using a cell pellet culture system [13]. Briefly, 5×10^5 MSCs were suspended in 2 mL high-glucose, complete DMEM supplemented with 10 ng/mL TGF-β1 (PeproTech, Rocky Hill, NJ), 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 1 mM sodium pyruvate (Sigma-Aldrich), 37.5 μg/mL ascorbate-2-phosphate, and 0.1 μM dexamethasone and centrifuged at 500 g for 5 min in a 15 mL conical tube. The conical tube containing the compressed cells was placed in an incubator with a loosened cap. The medium was replaced every 3 days for 21 days. The chondrogenesis of the MSCs was assessed by staining with 1% alcian blue solution.

2.2. Construction of Lentiviral Vectors. The sequence encoding human IFN-γ (Funeng, Guangzhou, China) was first amplified using primer 1 (5′-GGGG ACA AGT TTG TAC AAA GCA GGCT GCC ACC ATG AAA TAT ACA AGT TAT ATC TTG GCT-TTT3′) and primer 2 (5′-GGGG ACC ACT TTG TAC AAG AAA GCT GGG T TTA CTG GGA TGC TCT TCG ACC TC-3′). After gel-purification, the PCR products were cloned into pDONR 221 through a BP recombination reaction following the manufacturer's instructions (Invitrogen, Carlsbad, CA) to generate the entry clone pDown-IFN-γ. Then, pUp-EF1α and pDown-IFN-γ were recombined into pDESTpuro through an LR recombination reaction to construct the expression vector pLV/Final-puro-EF1α-IFN-γ. Finally, PCR and sequencing were used to identify clones with the correct recombination events.

2.3. Lentivirus Construction and Transfection. The lentiviral particles carrying the IFN-γ or humanized Renilla GFP genes were prepared by transient cotransfection of pLV/Final-puro-EF1α-IFN-γ or pLV/Final-puro-EF1α-hrGFP with a lentiviral packaging mix (Invitrogen) into 293FT cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 hours, the viral particles were harvested, filtered through a 0.45 μm polyethersulfone membrane, and concentrated by ultracentrifugation. Human MSCs were transduced with the lentiviral particles carrying IFN-γ or hrGFP at a multiplicity of infection of 50. At the same time, the 3T3, 293FT, H460, H1299, A549, and MCF-7 cell lines were transduced with lentiviral particles carrying only hrGFP using the same protocol. After two rounds of infection, 1–5 μg/mL puromycin was added to the culture medium, and these cultures were maintained for 2-3 days. The isolated cell lines were defined as MSCs IFN-γ, MSCs hrGFP, 3T3 hrGFP, 293FT hrGFP, H460 hrGFP, H1299 hrGFP, A549 hrGFP, and MCF-7 hrGFP, respectively.

2.4. Real-Time PCR. Total RNAs were extracted from undifferentiated and differentiated MSCs using TRIzol reagent according to the manufacturer's instructions (Invitrogen). After digestion with DNase I (Fermentas, Burlington, Canada), 1 ug total RNA was reverse-transcribed using RevertAid first strand complementary DNA synthesis kit (Fermentas). All quantitative real-time PCR assays were performed on CFX Connect Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Green I Master Mix (TOYOBO, Osaka, Japan). All reactions were run in triplicate. Relative quantification (RQ) was performed by normalizing the expression of target gene to that of GAPDH, used as a reference. The minimally normalized detectable target gene expression level was assigned a value of unity, and the other values reflect fold changes compared with this minimal level. The primer sequences used in this study were as follows: osteocalcin: 5′-CAC TCC TCG CCC TAT TGG C-3′ and 5′-CCC TCC TGC TTG GAC ACA AAG-3′; αP2, 5′-AGC ACC ATA ACC TTA GAT G-3′ and 5′-CGT GGA AGT GAC GCC TTT C-3′; collagen I: 5′-CAG CCG CTT CAC CTA CAG C-3′ and 5′-TGT ATT CAA TCA CTG TCT TGC-3′; and GAPDH: 5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′.

2.5. ELISA. The supernatant media derived from the control MSCs, MSCs IFN-γ, and MSCs hrGFP were collected, and the IFN-γ in these media was measured using a human IFN-γ ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.6. Western Blots (WB). MSCs were first primed with IFN-γ (20 ng/mL). After 12 hours, the medium was replaced with normal low-glucose complete DMEM, and the cultures were maintained for 72 hours. Thereafter, the MSCs were

repeatedly primed with IFN-γ for 12 hours and then again maintained in normal medium for 24 hours. At different time points, the MSCs were washed with cold PBS and lysed in Laemmli buffer. The cell lysates were denatured at 100°C for 5 min and centrifuged at 12,000 g for 10 min at 4°C. The supernatants were recovered, separated by 12% SDS-PAGE, and transferred onto 0.45 μm PVDF membranes (Millipore, Bedford, MA). After blocking with TBS-Tween-20 (0.1%) containing 5% nonfat milk for 1 hour at room temperature, the PVDF membranes were incubated with the appropriate primary antibodies (anti-TRAIL or anti-GAPDH antibody) overnight at 4°C. Specifically bound primary antibodies were detected by peroxidase-coupled secondary antibodies and chemiluminescence (Cell Signaling Technologies, Beverly, MA). In addition, the expression of TRAIL in MSCs IFN-γ was also evaluated using this method.

2.7. Coculture Experiments. MSCs IFN-γ or MSCs were preplated in six-well plates at a density of 1×10^5 cells per well and incubated overnight. Then, 3T3 hrGFP (4×10^5), 293FT hrGFP (4×10^5), H460 hrGFP (4×10^5), H1299 hrGFP (4×10^5), A549 hrGFP (4×10^5), or MCF-7 hrGFP (4×10^5) cells were added to the wells. After 48 hours of coculture, the apoptotic level and percentage of green fluorescent cells were assessed by microscopic analysis after staining with DAPI (Sigma-Aldrich), and the total cell viability in the MSC IFN-γ cocultures was evaluated using a CCK-8 kit (Dojindo, Japan). These characteristics are reported as a percentage normalized to the values of the control MSC cocultures, which were set to 100%. Additionally, the levels of activated caspase-3 in the H460 hrGFP cells were measured using an antiactive caspase-3 antibody (Promega, Madison, WI) at different time points during coculture.

2.8. Xenograft Cancer Models. Six-week-old athymic nude mice were purchased from the Guangdong Medical Laboratory Animal Center and used in accordance with institutional guidelines under approved protocols. A total of 1×10^6 H460 cells combined with or without 3×10^5 MSCs or MSCs IFN-γ were suspended in 100 μL of PBS and subcutaneously injected into the flank region of nude mice. On the fourteenth day after the first injection, the same numbers of MSCs or MSCs IFN-γ were again injected at the same position. The mice were examined three times a week, and the sizes of the tumors were calculated as reported [14]: volume = length $\times$ width^2/2. After 60 days, the tumor masses were excised after the mice were sacrificed, and the tumors were removed, dissected, and characterized by hematoxylin and eosin staining.

2.9. Statistics. The data are expressed as the means ± SD. A two-tailed P value less than 0.05 from a Student t-test performed using SPSS version 12.0 (SPSS Inc., Chicago, IL) was considered statistically significant.

3. Results

3.1. Characterization of MSCs and TRAIL Expression Pattern after IFN-γ-Priming. First, the characteristics of MSCs were evaluated using differentiation assays. As shown in Figures 1(a)-1(b), MSCs could easily differentiate into osteoblasts, adipocytes, and chondrocytes under suitable induction conditions.

In addition, the TRAIL expression in MSCs only lasted approximately 72 hours after one IFN-γ-priming. However, these MSCs continued to express TRAIL if they were primed again with IFN-γ (Figure 1(c)).

3.2. MSCs Could Be Genetically Modified with IFN-γ to Express TRAIL. The lentiviral expression vector pLV/Final-puro-EF1α-IFN-γ was constructed using multisite gateway technology and then confirmed by PCR (Figure 2(a)) and DNA sequencing (data not shown). Lentivirus particles containing the IFN-γ or hrGFP genes were constructed in 293FT cells. MSCs were then transduced with the lentiviral particles. After puromycin selection, more than 90% of the MSCs were hrGFP-modified (Figure 2(b)). Because the transductions were performed in parallel, it could be inferred that the transduction efficiencies were similar to both the IFN-γ virus and the hrGFP virus. Moreover, ELISA results indicated that MSCs IFN-γ efficiently secreted IFN-γ (Figure 2(c)). Western blot results indicated that MSCs IFN-γ synthesized TRAIL at P2 and P5 passages, respectively (Figure 2(d)). On the other hand, the immunophenotype, differentiation potential, and proliferation property of MSCs IFN-γ were not dramatically changed, compared with those of MSCs (see Figure S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/318098).

3.3. IFN-γ-Secreting MSCs Selectively Induce Apoptosis in Tumor Cells In Vitro. As shown in Figures 3(a)–3(f), MSCs IFN-γ induced apoptosis in H460, H1299, A549, and MCF-7 cancer cells, but not in 3T3 and 293FT cells; apoptosis was evident by the detachment of GFP-positive target cells and the appearance of cellular debris and apoptotic bodies in GFP-positive cells. The observed cell viability, as detected by the CCK-8 kit, also indicated that MSCs IFN-γ inhibited the proliferation of the transformed tumor cells to a greater extent than control MSCs ($P < 0.05$) (Figure 3(g)).

3.4. IFN-γ-Secreting MSCs Kill Tumor Cells by TRAIL-Mediated Caspase-3 Activation. To further explore the mechanisms underlying MSCs IFN-γ-induced apoptosis, the activation of caspase-3 within target cells was measured. As shown in Figure 4, the percentage of H460 cells positive for activated caspase-3 was significantly higher for those cocultured with MSCs IFN-γ than those cocultured with control MSCs (27.1 ± 5.6% at 24 h and 41.7 ± 5.0% at 48 h compared with 1.6 ± 0.8% at 24 h and 1.6 ± 0.5% at 48 h, resp., $P < 0.01$). However, treatment with an anti-TRAIL antibody dramatically suppressed the observed caspase-3 activation in H460 cells (11.8±6% at 24 h and 27.9±5.7% at 48 h, $P < 0.05$). These results indicate that MSCs IFN-γ effectively induce apoptosis in tumor cells by activating caspase-3 within the target cells and reveal the TRAIL-mediated cytotoxic effect of MSCs IFN-γ.

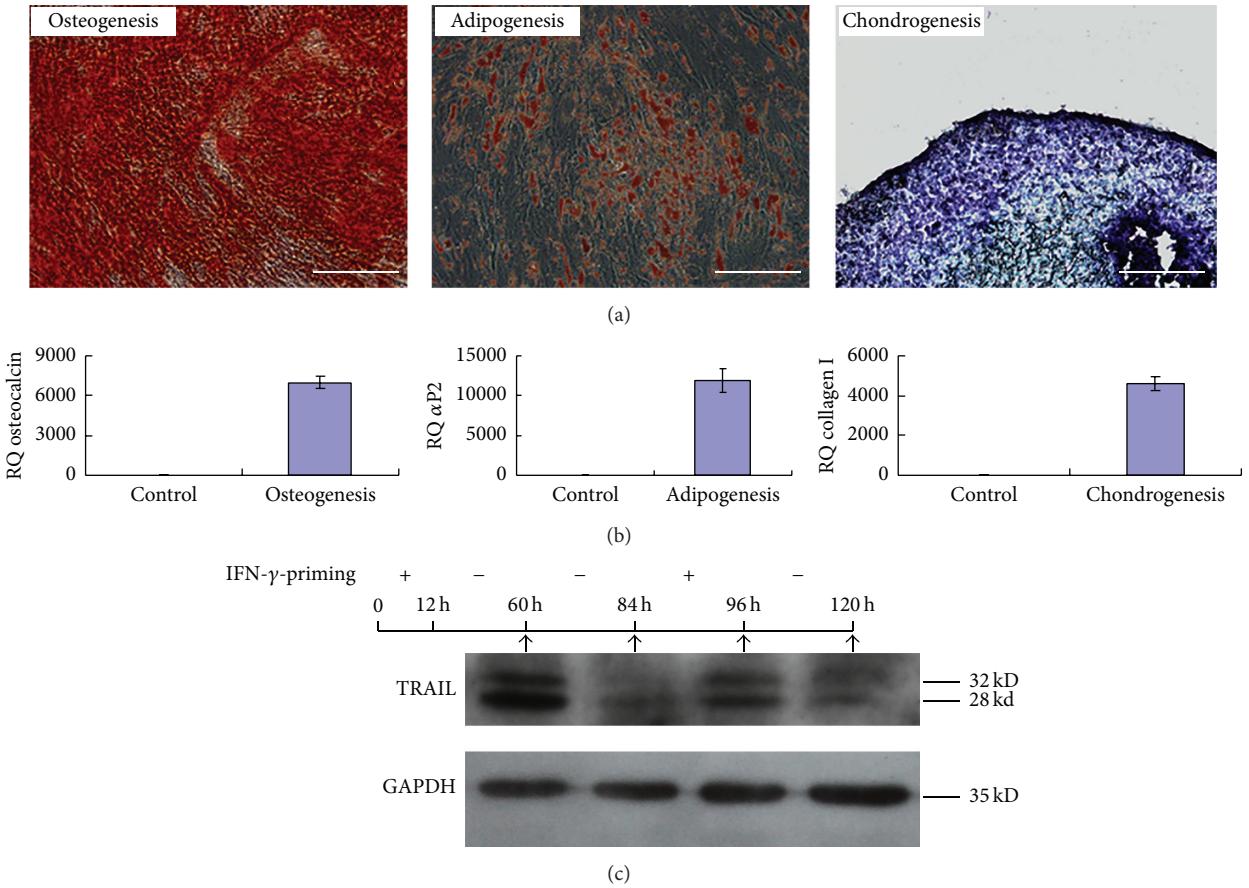

Figure 1: The characteristics of MSCs were analyzed. (a) The osteogenic, adipogenic, and chondrogenic differentiation of MSCs were identified by alizarin red S staining, oil red O staining, and alcian blue staining, respectively. Scale bar: 200 μm. (b) After 7 days of induction, the expression of genes involved in osteogenic, adipogenic, and chondrogenic differentiation was measured using quantitative real-time PCR and normalized to GAPDH expression. (c) TRAIL expression in MSCs undergoing IFN-γ-priming was analyzed by Western blotting. IFN-γ-primed MSCs showed reduced expression of TRAIL after the removal of IFN-γ but continuously expressed TRAIL when stimulated again with IFN-γ.

3.5. IFN-γ-Secreting MSCs Exhibit Antitumor Activity In Vivo. To evaluate the cytotoxicity of MSCs IFN-γ *in vivo*, xenograft tumor model of lung carcinoma was established, and the kinetics of tumor mass growth in nude mice were recorded. As shown in Figure 5(a), at approximately 20 days, tumor masses were observed in mice injected with H460 cells alone. The inclusion of MSCs IFN-γ delayed the appearance of tumors and inhibited the growth of the tumor mass. Finally, the tumor masses among the three groups had significantly different weights (0.45±0.09 g in the H460 group, 0.21±0.04 g in the MSCs IFN-γ group, and 0.72 ± 0.26 g in the MSCs group, $P < 0.05$). Correspondingly, hematoxylin and eosin staining indicated that tumor derived from the mixture of H460 and MSCs IFN-γ displayed dramatically tissue necrosis such as hemorrhagic region and fractured nuclei, compared with the other two groups (Figures 5(b)-5(c)).

4. Discussion

As mesoderm-derived progenitor cells, MSCs can home to tumor tissues through the chemotactic action of inflammatory factors [15]. However, the effects of MSCs on the growth and progression of tumors are still debated. For example, Kidd et al. reported that BM- and adipose-derived MSCs may be recruited by ovarian and breast tumors, are induced to become tumor-associated fibroblasts and vascular stromal cells, and promote tumor progression [16]. Luo et al. reported that BM-derived MSCs could promote prostate cancer metastasis via alteration of the CCL5-AR signaling pathway [15]. On the other hand, Zhu et al. reported that MSCs could inhibit K562 cell growth by secreting DKK1 [17], and Sun et al. reported that umbilical cord blood and adipose-derived MSCs could reduce lung metastasis and the growth of breast cancer cells by inducing apoptosis [18]. These results suggest that the effect of MSCs on tumors is variable and determined by the distinct tumor microenvironment and tumor type.

Alternatively, MSCs could produce variable responses under different induction conditions. For example, TLR4-primed MSCs have an antitumor effect, whereas TLR3-primed MSCs promote tumor growth and metastasis [19, 20]. Our previous report showed that IFN-γ induces MSCs to express TRAIL, which selectively mediates the apoptosis of tumor cells *in vitro*. However, the *in vivo*

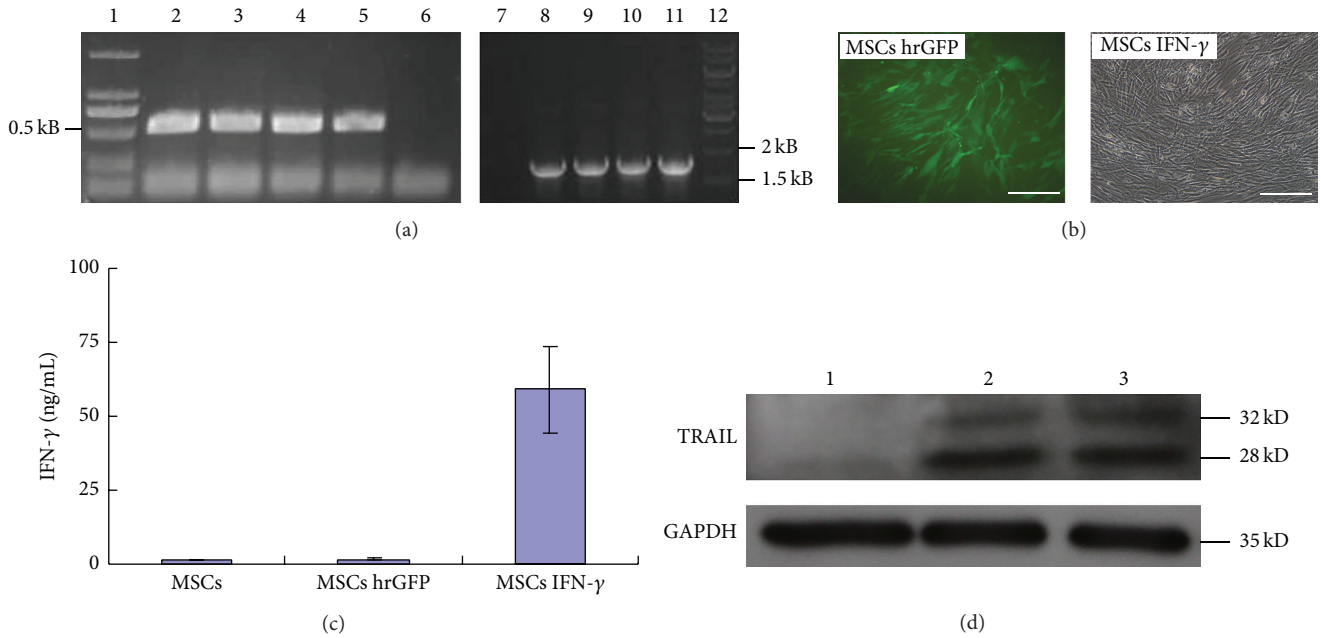

Figure 2: Construction of IFN-γ-modified MSCs. (a) The lentiviral vector pLV/Final-puro-EF1α- IFN-γ was identified by PCR. Lane 6/7: negative controls; lanes 2–5: amplification fragments of the IFN-γ coding sequence; lanes 8–11: amplification fragments of the EF1α promoter sequence. (b) The morphology of MSCs hrGFP and MSCs IFN-γ. Scale bar: 200 μm. (c) The expression of IFN-γ was assayed by ELISA. (d) TRAIL expression in MSCs IFN-γ was determined by Western blotting analysis. Lane 1: negative control; lane 2/3: MSCs IFN-γ at P2 and P5 passages.

effect of IFN-γ-primed MSCs on tumor growth is different than that observed *in vitro* [12]. A primary reason for this discrepancy may be that the MSCs stimulated only once with IFN-γ could not maintain TRAIL expression, weakening the cytotoxic effect of the IFN-γ-primed MSCs. Here, the assumption that TRAIL expression is reduced in IFN-γ-primed MSCs after the removal of IFN-γ was confirmed. However, these MSCs continued to express TRAIL if IFN-γ was again supplemented.

As a type II interferon, IFN-γ is mainly produced by lymphocytes and NK cells and plays an important role in the adaptive cellular immune response against tumors. In addition, because the IFN-γ receptor is ubiquitously expressed, IFN-γ can also influence a vast number of nonlymphoid cellular responses by upregulating the expression of a number of apoptosis-associated proteins [21, 22]. IFN-γ has been considered a promising antitumor drug. However, the clinical application of IFN-γ in protein form is hindered by serious side effects, which result from the high dose required to overcome the short half-life of the protein and to achieve significant therapeutic effects [23, 24].

Based on the work presented here, the benefits of IFN-γ may be exploited through the genetic engineering of MSCs, which are used as a cellular vector. In addition to their function as a cellular vector, MSCs also exhibit a cytotoxic response after priming with IFN-γ. To achieve the ideal transduction efficiency, a lentiviral vector system was chosen for our experiments. Compared to other genetic engineering systems, such as adenoviral or retroviral vectors, the HIV-based lentiviral vector system can stably transfect cells at different mitotic stages, which is critical for MSCs that are often quiescent [25, 26]. Several studies have reported that lentiviral vectors can effectively deliver target genes into MSCs [8, 27]. Correspondingly, our results also indicated that the IFN-γ gene could be stably inserted into MSCs, expressed, and secreted by the host cells using a lentiviral vector. At the same time, IFN-γ could induce MSCs to stably synthesize TRAIL in an autocrine or paracrine fashion.

Most importantly, our data indicate that IFN-γ-secreting MSCs exert a selective cytotoxic effect on different types of tumor cell lines, including lung cancer cells and breast cancer cells. This cytotoxicity of IFN-γ-modified MSCs is mediated by TRAIL via activation of the extrinsic apoptosis pathway. After injection into nude mice along with H460 tumor cells, IFN-γ-modified MSCs still maintained an antiproliferative effect. The antitumor effect of IFN-γ-modified MSCs is obviously enhanced compared with that of IFN-γ-primed MSCs, as previously reported [12]. However, similar to our report, this study showed that tumor growth in mice injected with H460 cells was more prominent when MSCs were included [12]. A possible reason for this phenomenon may be that the proapoptotic capacity of IFN-γ-modified MSCs is dominant over the tumor-supportive capacity of unmodified MSCs, resulting in the inhibition of tumors by IFN-γ-modified MSCs.

In conclusion, our data indicate that MSCs can be effectively modified with the IFN-γ gene using a lentiviral transduction system. The IFN-γ-modified MSCs inhibit tumor cell growth *in vitro* and *in vivo* through a TRAIL-mediated pathway. Thus, IFN-γ-modified MSCs may provide a new option for cancer therapy.

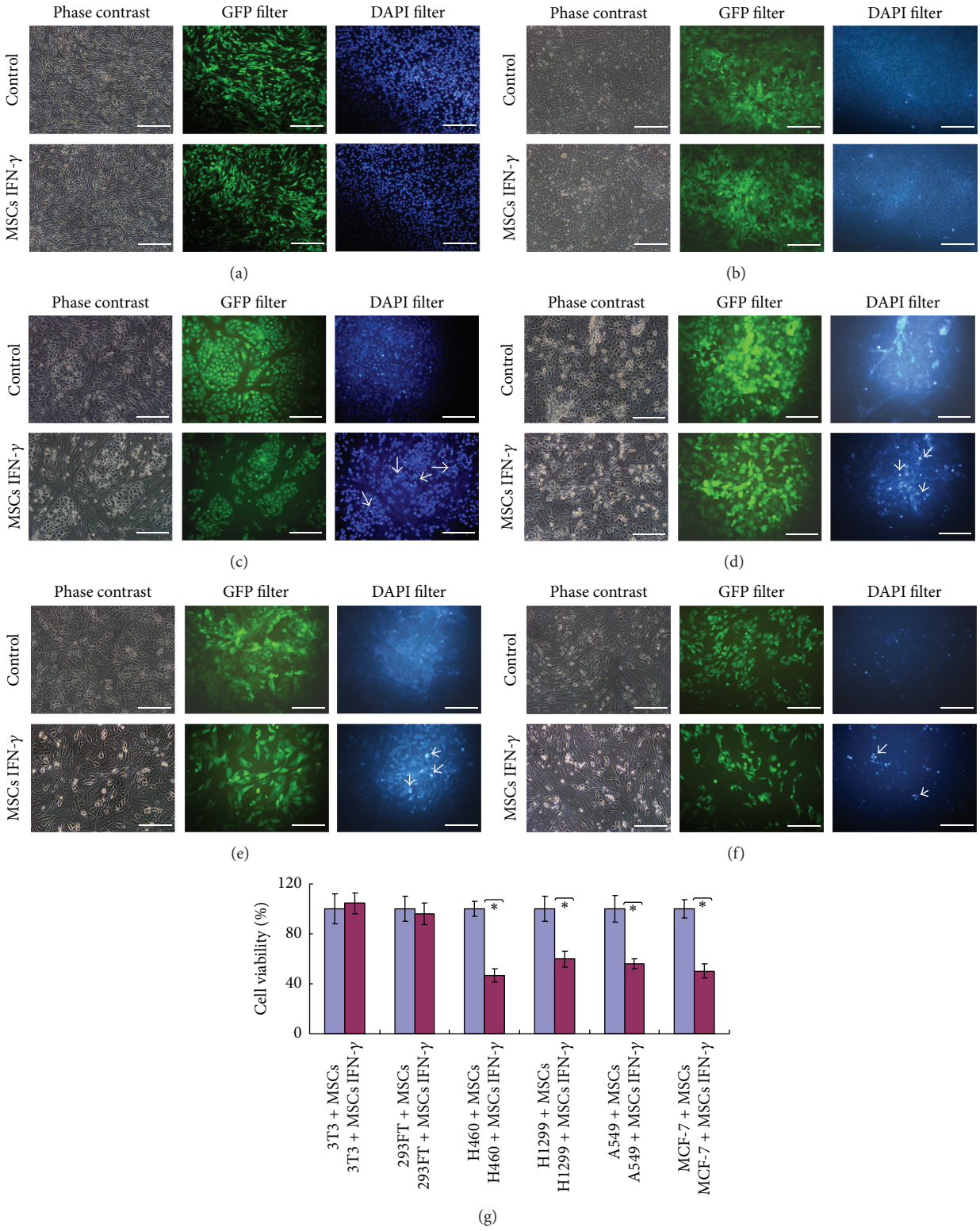

Figure 3: MSCs IFN-γ selectively induced apoptosis in cancer cells, but not in normal cells. MSCs IFN-γ were cocultured with 3T3 hrGFP (a), 293FT hrGFP (b), H460 hrGFP (c), H1299 hrGFP (d), A549 hrGFP (e), and MCF-7 hrGFP (f) cells for 48 hours. The mixed cells were observed using phase-contrast, GFP-specific, and DAPI-specific microscopy. The typical apoptotic and dead cells are marked. Scale bar: 200 μm. (g) The cell viability within the coculture system was measured using a CCK-8 kit and was shown as a percentage normalized to the viability of the control MSCs coculture group, which was set to 100%. The asterisks indicate $P < 0.01$. The reported results are representative of the results from three separate experiments.

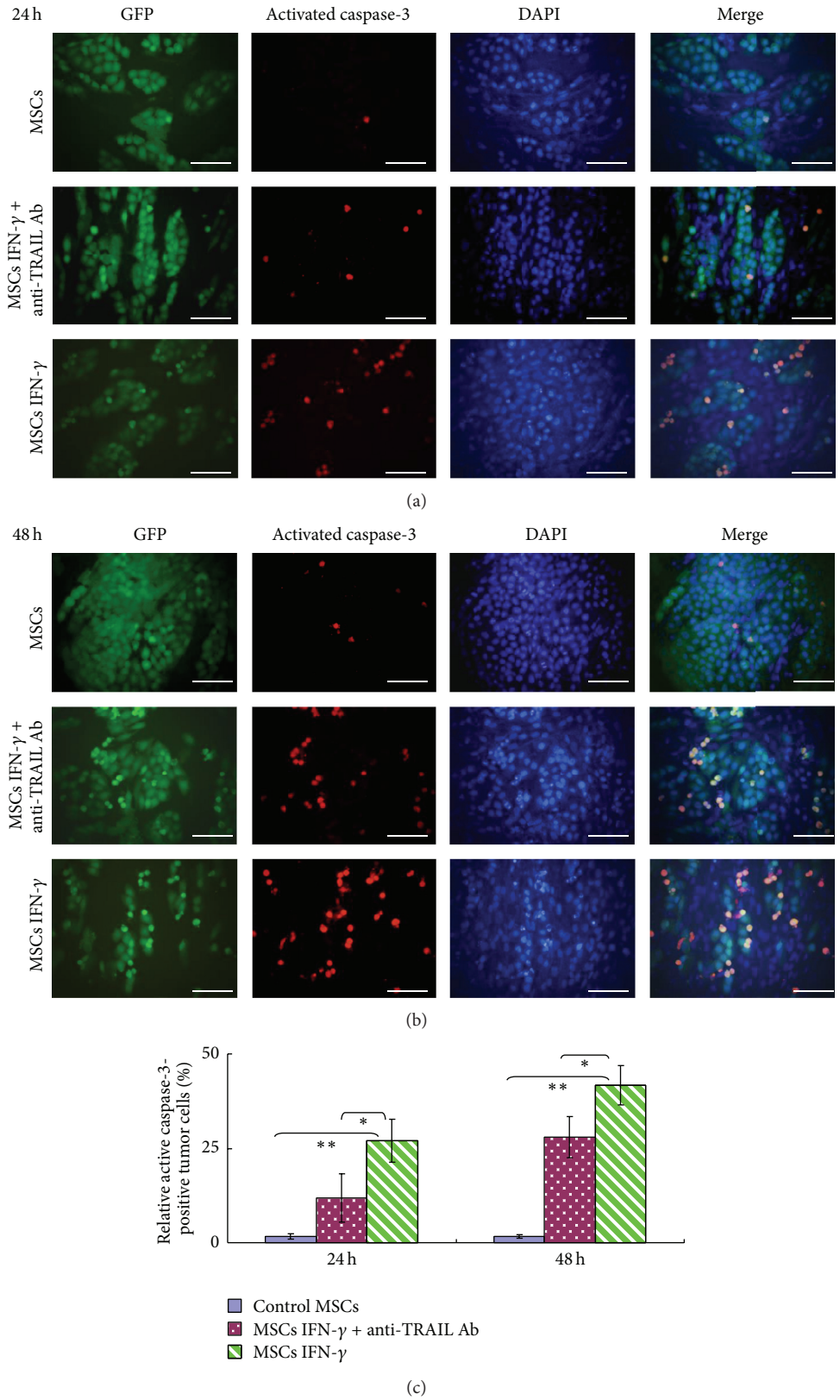

Figure 4: MSCs IFN-γ induced tumor cell apoptosis by activating caspase-3 within the target cells, a process which was mediated by TRAIL. MSCs IFN-γ were cocultured with H460 hrGFP cells for 24 h (a) and 48 h (b) in the presence or absence of an anti-TRAIL antibody. The activated caspase-3 was detected in the H460 hrGFP cells by immunofluorescence staining. (c) The percentage of activated caspase-3-positive H460 hrGFP cells was quantified using ImagePro 5.0 ($^{*}P < 0.05$, $^{**}P < 0.01$). The reported results are representative of the results from three separate experiments.

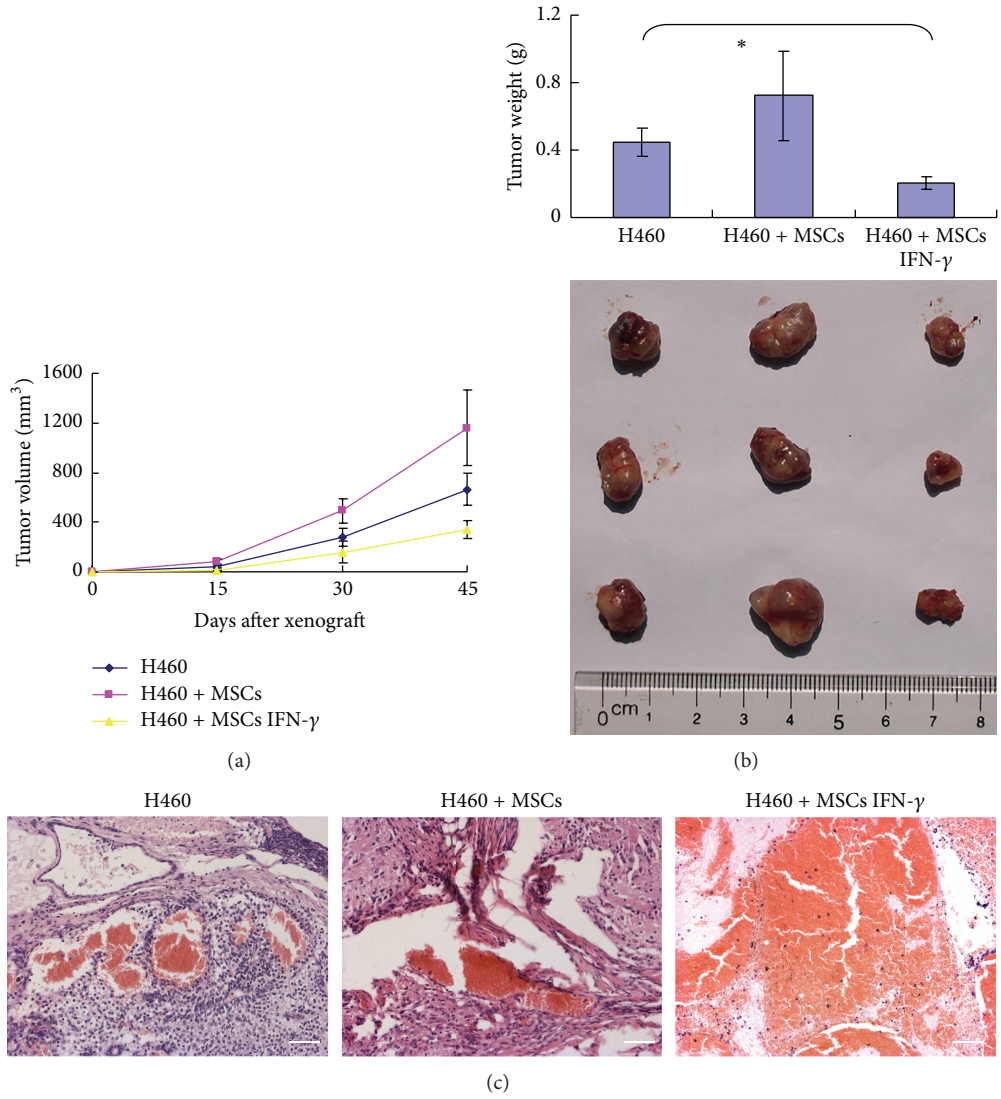

Figure 5: MSCs IFN-γ inhibit tumor mass growth in nude mice. (a) Tumor sizes were dynamically monitored after H460 cells combined with or without MSCs or MSCs IFN-γ were injected into nude mice ($n = 3$ mice/group, three separate experiments). (b) The tumor mass weight and morphology were compared at the end of the incubation period *in vivo*. The asterisks indicate $P < 0.05$. (c) Hematoxylin and eosin (H&E) staining of xenograft tumor. Tumor mass containing MSCs IFN-γ showed a serious extent of necrotic area, compared with the other two groups.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xinyuan Yang and Jingchun Du contributed equally to this work.

Acknowledgments

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Clinical Study

The Intensity of Radiotherapy-Elicited Immune Response Is Associated with Esophageal Cancer Clearance

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Radiation therapy is one of the standard therapeutic modalities for esophageal cancer, achieving its main antitumor efficacy through DNA damage. However, accumulating evidence shows that radiotherapy can substantially alter the tumor microenvironment, particularly with respect to its effects on immune cells. We hypothesized that the immune response elicited by radiotherapy may be as important as the radiation itself for successful treatment. More specifically, immunomodulatory cytokines may enhance the effectiveness of radiotherapy. To investigate this hypothesis, we measured changes in the serum interferon-gamma (IFN-γ) and interleukin-2 (IL-2) concentrations during radiotherapy and compared these modifications with outcomes. We found that serum concentrations of IL-2 and IFN-γ were positively associated with local response to radiotherapy in esophageal cancer. More generally, the intensity of the radiotherapy-elicited immune response was positively associated with local response to radiotherapy in esophageal cancer. Changes in serum IL-2 and IFN-γ concentrations were further associated with increased risks of acute hematologic toxicity and acute organ toxicity of the esophagus, lung, and skin. These results suggest that deciphering the mechanisms of radiotherapy-elicited immune response may help in the development of therapeutic interventions that would enhance the efficacy of radiotherapy and convert some ineffective responses to effective responses.

1. Introduction

Radiotherapy is one of the standard therapeutic modalities for patients with esophageal cancer. However, not all individuals respond equally to this therapy [1]. In practice, cases of esophageal cancer treated with radiotherapy can often be divided into 2 groups: those with effective responses, in which radiation can control or cure tumors, and those with ineffective responses, in which radiotherapy has little or no efficacy [2–4]. The underlying reasons for these differences in response are incompletely understood. Many studies of tissue and cellular responses to radiation have focused on the damage that is caused to proliferating malignant cells, leading to their deaths. Historically, topics of study related to radiotherapy have included the cell cycle, apoptosis, and DNA repair and survival [5, 6]. Recently, accumulating evidence has suggested that radiation also leads to significant alterations to the tumor microenvironment, particularly with respect to its effects on immune cells. It has been reported that radiotherapy can induce various tumor cell death modalities, releasing tumor-derived antigens and danger signals, either of which could trigger antitumor immune responses [7, 8].

The rate of macrophage infiltration is relatively high in tumor tissue [9]. Macrophage infiltration is present at the tumor margins and around necrotic foci. Macrophages exposed to Th1 cytokines, including interferon-gamma (IFN-γ) and interleukin-2 (IL-2), exhibit enhanced cytotoxic activity, production of proinflammatory cytokines, antigen presentation, and antitumor cellular immune response [10, 11]. Accordingly, IL-2 and IFN-γ have important roles in antitumor immunity. For example, IL-2 stimulates natural killer (NK) cell activity or cytotoxic T lymphocytes (CTLs) to kill tumor cells. IFN-γ is produced by the activation of T cells, and it can strengthen both the host T cell receptors'

dependence and human leukocyte antigen (HLA) restriction to cytotoxic T cells, increasing surface major histocompatibility complex (MHC) antigen expression, tumor necrosis factor concentrations, antitumor angiogenesis, and other antitumor responses [12, 13]. IFN-γ can be controlled by regulating the Fas/FasL expression of tumor cells and enhancing the sensitivity of tumor cells to the Fas-mediated apoptosis pathway, thereby reducing the ability of tumor cells to evade the immune system attack and, accordingly, inhibiting the malignant proliferation of tumor cells [14–16].

Therefore, we hypothesized that the immune response elicited by radiotherapy may be as important as the radiation itself for successful treatment. Further, we hypothesized that immunomodulatory cytokines may enhance the effectiveness of radiotherapy. However, few studies have investigated serum IL-2 and IFN-γ concentrations in effective and ineffective responses to radiotherapy. Particularly, there was not enough evidence to compare the changes in these concentrations during effective and ineffective responses to radiotherapy. In the present study, we sought to provide such evidence, specifically investigating correlations between radiotherapy outcomes and changes in the serum IL-2 and IFN-γ concentrations during radiotherapy for esophageal cancer.

Some studies have shown that the expression of cytokines (IFN-γ and IL-2) is associated with radiation-related tissue damage and inflammation [17, 18]. To clarify the relationship between cytokine profiles and acute toxicity induced by radiotherapy, we also examined the associations between serum concentrations of IL-2 and IFN-γ and radiotherapy-related acute toxicities (hematologic or of the esophagus, lung, or skin), which were graded prospectively.

2. Patients and Methods

2.1. Patient Eligibility and Treatment. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China), and all participating patients gave their written informed consent. The study included 63 patients diagnosed with histopathologically confirmed squamous cell carcinoma of the esophagus (cT1–4, any N, any M) at The First Affiliated Hospital of Xi'an Jiaotong University, from February 2013 to July 2013. In this study, clinical staging was performed using endoscopy, a barium swallow, computed tomography (CT) scans of the abdomen and chest, a pulmonary function test, a complete blood cell count, a serum chemistry profile, serum creatinine clearance, electrocardiography, complete history assessment and physical examination, and an assessment of Karnofsky performance status [19]. A treatment plan was then designed by a multidisciplinary tumor board. Eligible patients who were inoperable and were not fit for concurrent chemotherapy were treated with radiotherapy alone at the start of therapy. Here, inoperability was defined by the presence of distant metastases or an unfavorable preoperative risk score. Radiotherapy was administered in daily fractions of 2.0 Gy (5 times per week) with a total dose of 60–66 Gy. Linear particle accelerators (lilacs) were used with a 10 MV X-ray beam and a multiple field technique. All patients were treated using three-dimensional planning techniques.

2.2. Serum Collection and Enzyme-Linked Immunosorbent Assay. Blood was drawn once per week during radiotherapy; serum samples were separated by centrifugation at 3000 rpm for 5 min and stored at −80°C. Serum IL-2 and IFN-γ were measured with commercially available enzyme-linked immunosorbent assay kits (Westang Biotechnology Company, Shanghai, China) following the manufacturer's instructions. Samples were normalized to total protein as determined by a bicinchoninic acid assay (Westang Biotechnology Company).

2.3. Treatment-Related Toxicity. Acute treatment-related toxicity was evaluated weekly during radiotherapy and documented using the year 2009 National Cancer Institute's Common Toxicity Criteria (NCI-CTC, version 4.0). Supportive treatment was extensive and included pain management, dietary counseling, and leukocyte-stimulating agents administered orally or by injection, if necessary [15].

2.4. Efficacy Assessment. Short-term local efficacy in the esophagus was evaluated 4 weeks after finishing treatment and every 3 months for 1 year thereafter. The assessment included physical examination, history, endoscopy, a barium swallow, and thoracic/abdominal CT scans. In this study, short-term efficacy was analyzed according to the World Health Organization Response Evaluation Criteria in Solid Tumors [20]. Specifically, outcomes were classified as complete remission (CR), partial remission (PR), stable disease (SD), or progressive disease (PD), and the total effective response rate was defined as CR + PR.

2.5. Statistical Analysis. Fisher's exact test was used to compare categorical data. Between-group and between-sample differences in numeric data were assessed using the two-tailed Student's t-test. The Wilcoxon signed-rank test was used to assess the statistical significance of associations between radiation and cytokine expression, using aggregated median cytokine concentrations at baseline and during radiotherapy. The signed-rank test was also used to assess the difference between cytokine concentrations at baseline and during radiotherapy. For all analyses, $P \leq 0.05$ was considered significant.

3. Results

3.1. Patient and Treatment Characteristics. From February 2013 to July 2013, a total of 63 patients were enrolled in this study (Table 1). Radiotherapy could result in any one of the following 2 outcomes: effective response (CR + PR) or ineffective response (SD + PD). All 63 of the patients completed treatment with radiotherapy and were followed for at least 6 months. During the short-term follow-up, the local efficacy of radiation in the esophagus was assessed using physical examination, endoscopy, barium swallow, and thoracic/abdominal CT scans. Five patients (8%) showed CR

Table 1: Patient and treatment characteristics related to local short-term efficacy of radiotherapy for esophageal cancer.

Study population $N = 63$	CR + PR $N = 56$	SD + PD $N = 7$	P value
Age (years)			
Median	65	63	0.23
Range	43–82	55–67	
Sex			
Female	19	1	0.42
Male	37	6	
Stage			
I-II	6	1	0.43
III	48	5	
IV	2	1	
Tumor length			
<5 cm	15	3	0.34
≥5 cm	4	4	
Acute hematologic toxicity			
0/I-II	53	6	0.38
III-IV	3	1	
Acute organ toxicity			
0/I-II	48	5	0.31
III-IV	8	2	

CR + PR: patients with complete or partial response; SD + PD: patients with stable disease or progressive disease.

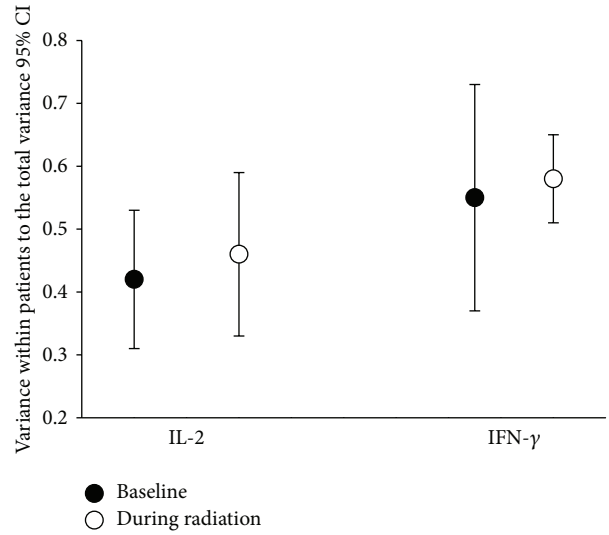

Figure 1: Cytokine variance within patients as a measure of heterogeneity ($n = 63$). We calculated the variance of 2 cytokines (interferon-gamma [IFN-γ] and interleukin-2 [IL-2]) within patients, as well as their total variances. The data points indicate the ratios of intrapatient to interpatient variances for the 2 cytokines at baseline (black circles) and during radiotherapy (white circles). The confidence interval is 95%. Based on these confidence intervals, there were no significant differences between the ratios at baseline and the ratios during radiotherapy for either of the cytokines.

and 51 patients showed PR (81%), constituting 56 patients (89%) with an overall effective response (CR + PR). Four patients had SD (6%) and 3 patients (5%) had PD, together amounting to 7 patients (11%) with an overall ineffective response. As presented in Table 1, the characteristics of the enrolled patients (including age, sex, and disease stage) were not significantly associated with the local short-term efficacy of radiation in the esophagus.

Acute hematologic toxicity (leukopenia, anemia, and thrombocytopenia) occurred in 60 of the 63 cases (95%). Four of these 60 toxicities (6.7%) were of degrees III-IV and 56 (93.3%) were of degrees I-II. Degree I-II nausea or vomiting occurred in 35 of the 63 cases (56%). Acute organ toxicity of the esophagus, lung, or skin occurred in 61 of the 63 cases (97%). Ten of these 61 cases (16.4%) were of degrees III-IV and 51 (83.6%) were of degrees I-II. The short-term local efficacy of radiation was not significantly associated with the rate of hematologic toxicities ($P = 0.31$).

3.2. Intrapatient Variation in Serum Cytokine Concentrations during Radiotherapy. Figure 1 presents the ratios of (a) the variance of the cytokine (IL-2 and IFN-γ) concentrations within each patient to (b) the total across-patient variance calculated for the baseline and during-radiotherapy measurements. It thereby compares intrapatient heterogeneity to interpatient heterogeneity for serum cytokine concentrations. For future studies, we suggest that this ratio of within-patient to between-patient variance should be reported as a measure of cytokine (IL-2 and IFN-γ) heterogeneity for the patient population. We observed that the ratios were similar at baseline and during radiotherapy for the 2 tested cytokines.

3.3. Changes in the Cytokine Profile Were Correlated with the Short-Term Efficacy of Radiotherapy. Recent results have suggested that the immune system mediates many of the antitumor effects of radiotherapy [21]. Macrophages exposed to Th1 cytokines, including interferon-gamma (IFN-γ) and interleukin-2 (IL-2), exhibit enhanced cytotoxic activity, production of proinflammatory cytokines, antigen presentation, and antitumor cellular immune response. Therefore, IL-2 and IFN-γ have important roles in antitumor immune activity. We examined the serum concentrations of immunomodulatory cytokines (IL-2 and IFN-γ) in the effective and ineffective local response groups (Figure 2). In the effective response (CR + PR) group, serum concentrations of IL-2 and IFN-γ increased with the number of radiotherapy fractions that had been administered, reaching a maximum after about 2-3 weeks (10–15 fractions of radiation) and gradually decreasing thereafter. In the ineffective response (SD + PD) group, serum concentrations of IL-2 and IFN-γ remained approximately steady throughout the course of radiotherapy. These results indicate that the intensity of the radiotherapy-elicited immune response was positively associated with local response to radiotherapy in esophageal cancer. As shown in Figure 2, we also found that serum IL-2 and IFN-γ concentrations in the CR + PR group were both higher than the corresponding concentrations in the SD + PD group at the same time during radiotherapy.

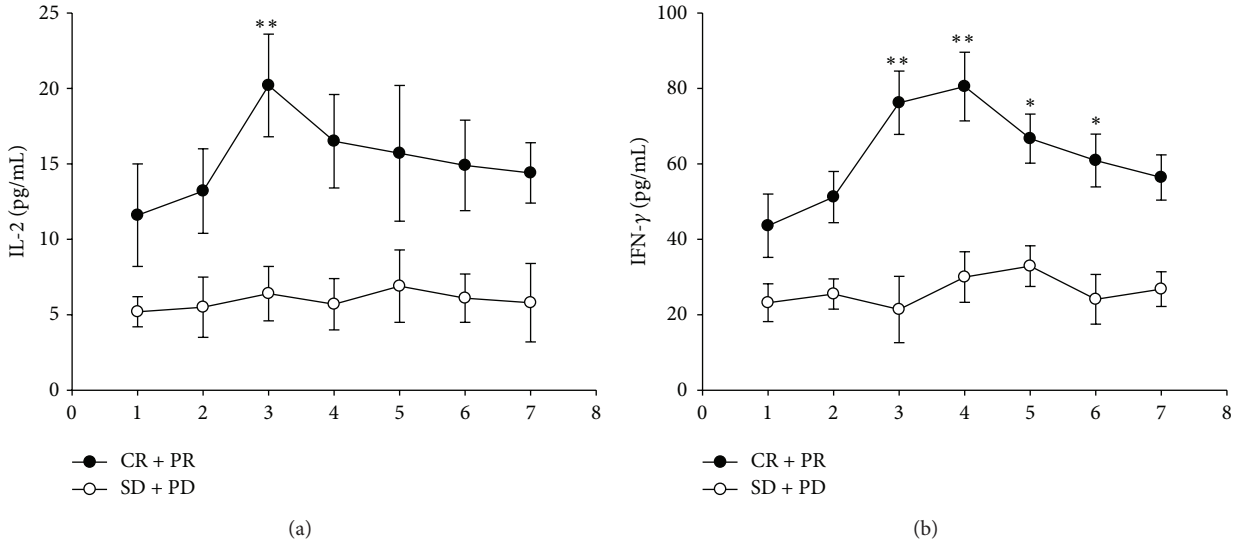

Figure 2: Changes in the cytokine profiles correlated with the short-term efficacy of radiotherapy. (a) Serum interleukin-2 (IL-2) comparisons for the effective response (CR + PR) and ineffective response (SD + PD) groups. (b) Serum interferon-gamma (IFN-γ) comparisons for the effective response (CR + PR) and ineffective response (SD + PD) groups. The total effective local response to radiotherapy relative to the serum concentrations of immunomodulatory cytokines (IL-2 and IFN-γ) is shown. In the effective response (CR + PR) group, serum concentrations of IL-2 and IFN-γ increased with the number of radiotherapy fractions that had been administered, reaching a maximum after about 2-3 weeks (10–15 fractions of radiation) and gradually decreasing thereafter. In the ineffective response (SD + PD) group, serum concentrations of IL-2 and IFN-γ remained approximately steady throughout the course of radiotherapy. Data are presented as mean ± 1 standard error of the mean. The asterisk indicates a statistically significant difference, as compared with the ineffective response group (Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$).

3.4. Change in Cytokine Profile Was Correlated with Acute Toxicity Induced by Radiotherapy. To clarify reported correlations between serum concentrations of IL-2 and IFN-γ and acute toxicity during radiotherapy, we compared the maximum cytokine concentration in each patient during radiotherapy with the maximum grade acute hematologic toxicity and acute organ toxicity (Figure 3). This comparison was performed for both IL-2 and IFN-γ, using separate statistical models. Our results suggest that changes in serum IL-2 and IFN-γ concentrations are associated with an increased probability of acute hematologic toxicity. Further, the results suggest that changes in serum IFN-γ concentrations are associated with an increased probability of acute organ toxicity of the esophagus and that changes in serum IFN-γ concentrations are associated with an increased probability of acute organ toxicity of the lung or skin.

4. Discussion

Radiotherapy is an important treatment modality for solid tumors. Accumulating evidence shows that radiotherapy eliminates tumor cells by changing the local tumor environment, in addition to its more direct effects. Radiation-elicited changes to local tumor microenvironment may produce inflammatory factors and enhance the antitumor immune responses locally [22]. To learn about the immune responses during cancer radiation therapy, it would be nice to detect regional lymph nodes of esophagus through immunohistochemical staining. But, in fact, it is difficult to obtain regional lymph nodes in patients during radiotherapy. Many studies have shown that IL-2 and IFN-γ have important roles in antitumor immune activity [23]. However, few studies have investigated serum IL-2 and IFN-γ concentrations in effective and ineffective responses to radiotherapy. Particularly, there was not enough evidence to compare the changes in these concentrations during effective and ineffective responses to radiotherapy. In the present study, we have provided such evidence, specifically detailing correlations between radiotherapy outcomes and changes in the serum IL-2 and IFN-γ concentrations during radiotherapy for esophageal cancer.

The present study enrolled 63 patients with esophageal cancer. All patients completed treatment with radiotherapy and were followed for at least 6 months. During the short-term follow-up, the local efficacy of radiation was assessed with physical examination, endoscopy, barium swallow, and thoracic/abdominal CT scans. Five patients (8%) exhibited CR and 51 patients exhibited PR (81%), constituting 56 patients (89%) with an overall effective response (CR + PR). Four patients had SD (6%) and 3 patients (5%) had PD, together amounting to 7 patients (11%) with an overall ineffective response. We found significant differences between patients with and without effective local response in terms of serum IL-2 and IFN-γ concentrations. In the effective response (CR + PR) group, serum concentrations of IL-2 and IFN-γ increased with the number of radiotherapy fractions that had been administered, reaching a maximum after about 2-3 weeks (10–15 fractions of radiation) and gradually decreasing thereafter. In the ineffective response (SD + PD)

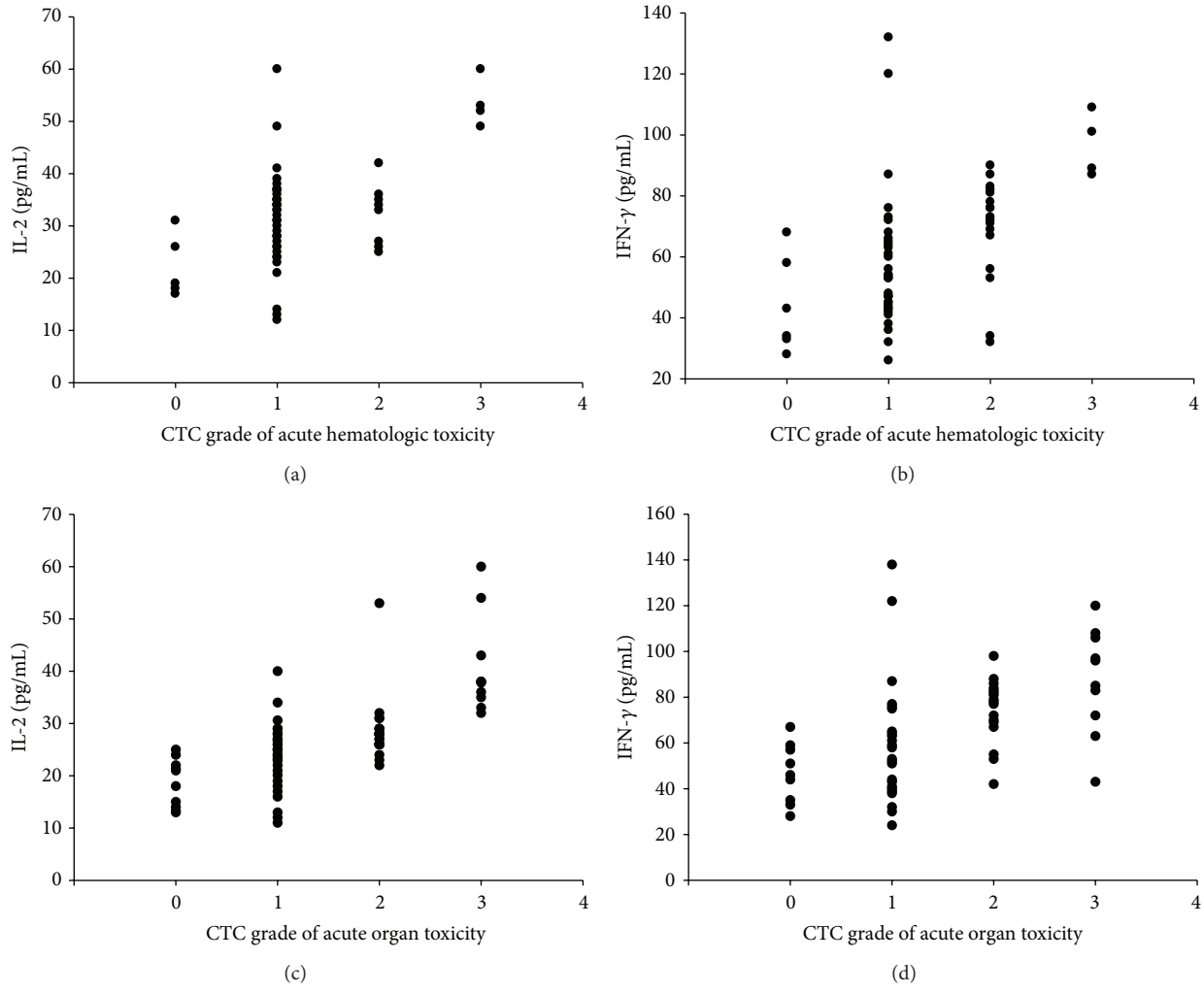

Figure 3: Relationship between serum concentrations of cytokines (interferon-gamma [IFN-γ] and interleukin-2 [IL-2]) and acute toxicity during radiotherapy. Maximum cytokine expression (IL-2 and IFN-γ) is presented as a function of maximum toxicity grade for the 63 patients with esophageal cancer. (a) presents the relationship between IL-2 expression and acute hematologic toxicity, (b) presents the relationship between IFN-γ expression and acute hematologic toxicity, (c) presents the relationship between IL-2 expression and acute organ toxicity, and (d) presents the relationship between IFN-γ expression and acute organ toxicity.

group, serum concentrations of IL-2 and IFN-γ remained approximately steady throughout the course of radiotherapy. Consistent with our hypothesis, results indicate that the intensity of the radiotherapy-elicited immune response was positively associated with the local response to radiotherapy in esophageal cancer.

Several studies have shown that cytokine (IFN-γ and IL-2) expression is associated with radiation-related tissue damage and inflammation. Increasing IL-2 and IFN-γ expressions were associated with an increased probability of acute toxicity induced by radiotherapy [17]. To examine the relationships between acute toxicity induced by radiotherapy and cytokine profiles, we examined associations between radiotherapy-related acute toxicities, which were graded prospectively, and serum IL-2 and IFN-γ concentrations. To clarify reported correlations between serum concentrations of IL-2 and IFN-γ and acute toxicity during radiotherapy, we compared the maximum cytokine concentration in each patient during radiotherapy with the maximum grade acute hematologic toxicity and acute organ toxicity (Figure 3). This comparison was performed for both IL-2 and IFN-γ, using separate statistical models. Our results suggest that changes in serum IL-2 and IFN-γ concentrations are associated with an increased probability of acute hematologic toxicity. Further, the results suggest that changes in serum IFN-γ concentrations are associated with an increased probability of acute organ toxicity of the esophagus and that changes in serum IFN-γ concentrations are associated with an increased probability of acute organ toxicity of the lung or skin.

In the current study, we demonstrated that serum concentrations of IL-2 and IFN-γ were positively associated with local response to radiotherapy in esophageal cancer. These findings suggest that the intensity of the radiotherapy-elicited immune response is positively associated with local response to radiotherapy in esophageal cancer. Further, changes in the IL-2 and IFN-γ serum concentrations were associated

with increased probabilities of acute hematologic toxicity and acute organ toxicity of the esophagus, lung, and skin. In fact, serum levels of IL-2 and IFN-γ have been found positively associated with patients' outcome [24]. IL-2 and IFN-γ have been used in immunotherapy in some tumors like malignant melanoma [25]. But the relationship between radiotherapy inducing acute toxicity and outcome is unclear. In our future research we will research the relationships of IL-2, IFN-γ, and other cytokines with radiotherapy acute phase response and outcome. These results suggest that deciphering the mechanisms of radiotherapy-elicited immune response may allow the development of therapeutic interventions that would enhance the efficacy of radiotherapy and convert some ineffective responses to effective responses.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Hapten-Induced Contact Hypersensitivity, Autoimmune Reactions, and Tumor Regression: Plausibility of Mediating Antitumor Immunity

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Haptens are small molecule irritants that bind to proteins and elicit an immune response. Haptens have been commonly used to study allergic contact dermatitis (ACD) using animal contact hypersensitivity (CHS) models. However, extensive research into contact hypersensitivity has offered a confusing and intriguing mechanism of allergic reactions occurring in the skin. The abilities of haptens to induce such reactions have been frequently utilized to study the mechanisms of inflammatory bowel disease (IBD) to induce autoimmune-like responses such as autoimmune hemolytic anemia and to elicit viral wart and tumor regression. Hapten-induced tumor regression has been studied since the mid-1900s and relies on four major concepts: (1) *ex vivo* haptenation, (2) *in situ* haptenation, (3) epifocal hapten application, and (4) antigen-hapten conjugate injection. Each of these approaches elicits unique responses in mice and humans. The present review attempts to provide a critical appraisal of the hapten-mediated tumor treatments and offers insights for future development of the field.

1. Introduction

Haptens are small molecules that elicit an immune response when bound to a carrier protein [1]. Haptens have been used to boost immune responses to antigens, to study ACD and IBD, and to induce autoimmune responses, viral wart regression, and even antitumor immunity. For years, haptenated protein (bovine serum albumin (BSA) or ovalbumin (OVA)) was mainly utilized to induce strong immune responses in animal models to help unravel the basics of T- and B-cell-mediated responses. Paul et al. [2] immunized BSA-tolerized rabbits with DNP-modified BSA producing antibodies to the dinitrophenyl (DNP)-BSA conjugate, BSA alone, and DNP alone, suggesting potential cross-reactive responses. Classically, B-cells are known to recognize the DNP-BSA conjugates *via* membrane bound IgM, process them, make antibody against the DNP, and present the BSA to CD4+ T-cells. These abilities of haptens have made them a tantalizing molecule for use in several settings. Haptens have been widely used to induce CHS, the animal model of ACD, a type IV delayed hypersensitivity reaction that is one of the most prevalent skin diseases in the world [3, 4]. CHS has two phases, a "sensitization" phase where the hapten is applied to skin for the first time, followed by an "elicitation" phase where the hapten is applied to a different skin area of the animal [3–5]. An in-depth analysis of the innate and adaptive immunologic mechanisms of CHS and ACD is covered in three recent reviews by Martin et al. [6], Christensen and Haase [5], and Honda et al. [4]. In this review, we will briefly cover these immune reactions to allow for a general understanding of how these reactions may apply to antitumor immunity.

Some hapten-mediated responses are correlated to drug-induced autoimmune reactions. When a drug is metabolized, its metabolites can form potent haptens, which bind self-protein and sometimes elicit autoimmune responses [7, 8].

Hapten-carrier conjugates have been used in the past as drug-abuse therapies [9, 10], inducing an immune response against the drug of interest. Haptens have also been used to create autoimmune models in mice, such as IBD [11–17], and to cause viral wart regression *via* epifocal hapten application [18, 19]. The ability of haptens to cause autoimmunity and wart regression is an important concept to consider when applying the use of haptens to cancer immunotherapy setting, as the immune response to cancer is similar to an autoimmune response [20]. Indeed, haptens have been tested as a treatment of cancer several times in the past. In this review, we examine the four main concepts of hapten-mediated antitumor treatment: (1) *ex vivo* haptenation [21–31], (2) *in situ* haptenation [32, 33], (3) epifocal hapten application [34–42], and (4) antigen-hapten administration [43–47]. Despite the wealth of experiments in this field, the mechanisms underlying these treatment approaches are largely unclear and require further study. We attempt to give a critical analysis of the use of haptens to induce tumor regression and suggest studies that must be done to fill the large knowledge gaps and further the field.

2. Haptens and Contact Hypersensitivity

Haptens are <1 kDa in size and elicit an immune response when bound to a carrier protein, including tolerized antigen. Haptens are not immunogenic by themselves, as they are too small to be recognized by the immune system. Most haptens are electrophilic compounds that covalently bind to nucleophilic residues creating new antigenic epitopes; an exception to this would be metal ions functioning as haptens [1]. Most haptens act as cutaneous allergens, eliciting ACD-like reaction on the skin. The most common haptens are urushiol (the toxin in poison ivy), fluorescein, nickel, oxazolone (Ox), DNP, and phosphorylcholine. Each hapten has a unique property that determines its allergenicity in terms of how quickly the hapten binds, how readily it can permeate the skin, and its electrophilicity, hydrophobicity, and subsequent bioavailability [1]. Varying mouse strains also greatly affect the immune stimulatory ability of the hapten. Contact hypersensitivity is usually measured through ear swelling, as the secondary challenge application is on the ear (elicitation phase); this is the widely used method to confirm sensitization of a hapten and unravel the immune mechanisms of haptens and CHS [3]. The body of literature on haptens and CHS reveals the use of several different animal models and haptens that lead to conflicting explanations of a certain step in the immune pathology of CHS, which should be considered when creating a general mechanism of CHS. While outlining our understanding of the mechanisms of CHS, we primarily focus on the aspects that will be important for hapten-mediated tumor regression.

2.1. The Sensitization Phase of Contact Hypersensitivity. The sensitization phase is when a hapten is first applied to the skin of an animal, typically the shaved abdomen, to prime the immune system toward the hapten. Figure 1 summarizes some of the cells and mechanisms thought to be involved in this priming event. Upon application to the skin, haptens immediately interact with keratinocytes (KC), langerhans cells (LC), and dermal dendritic cells (dDC). Hapten binding to KCs causes them to release IL-1β, IL-18, TNFα, and GM-CSF, activating LCs and dDCs and inducing their migration to the draining lymph node where they mature and present hapten-antigen to naïve T-cells [4–6, 48–52]. Dinitrofluorobenzene (DNFB) application to dermal dendritic cells *in vitro* upregulates MAPK and CD40, a coactivation signal for antigen-presenting cells (APCs) and T-cells [53]. Haptenation also causes the release of "danger signals," such as hyaluronic acid (HA), extracellular matrix ligands for Toll-like receptors, such as extradomain A+ fibronectin containing extra type III domain A (FnEDA+), prostaglandin E2 (PGE2), reactive oxygen species (ROS), heparin sulfate, tenascin, B defensins, and fibrinogen [4, 5, 54], from haptenated cells, which play an integral role in innate immune activation [6]. For instance, blocking HA degradation significantly reduces CHS sensitization [6], while the release of PGE2 activates LCs and induces their migration [55]. The *in vitro* formation of ROS in DCs is thought to cause degradation of the extracellular matrix, creating endogenous ligands for toll like receptors (TLRs)-2 and -4, as well as nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) [4, 6]. Keratinocytes are mainly stimulated by NLR-dependent mechanisms, specifically NLR family, pyrin domain containing 3 (NLRP3) [6]. NLRP3 stimulation triggers caspase-1 activation, which causes the release of IL-1β and IL-18 from keratinocytes and APCs. This NLR-dependent pathway is stimulated by adenosine triphosphate (ATP) efflux from haptenated and subsequently damaged cells. ATP binds to the purinergic receptor, P2RX7, a ligand gated ion channel that allows the release of K+ from the cell and provides further innate activation signals for LCs and dDCs, helping them mature [6].

Langerhans cells play a pivotal but controversial role in the sensitization phase; when LCs are depleted, the ear-swelling responses are reduced [50]. Further, LCs and dDCs work together to initiate CHS sensitization [56, 57]. The role of the LCs seem to be area and time of depletion dependent, for instance, LCs had a larger role in the flank than in the ear and LC depletion three days prior to hapten priming did not impair CHS but LC depletion 1 day prior did impair CHS [58, 59]. It was shown that only dDCs, not LCs, migrate to the draining lymph node (dLN) to activate and stimulate hapten-specific T-cells [52, 60]. Despite this controversy, LCs cells have been shown to play an important role in CHS sensitization.

Mast cells are also thought to play a role in CHS sensitization. Initial reports using mast cell deficient mice through a c-Kit mutation showed that CHS was enhanced, although this is hard to interpret as c-Kit mutation affects many cells [4, 60]. Diphtheria toxin-induced mast cell-deficient mice had reduced CHS and T-cell priming [4, 61, 62]. Mast cells also stimulate dDCs via intercellular adhesion molecule-1 (ICAM-1) or leukocyte function-associated antigen-1 (LFA-1) and TNFα [4, 61, 62]. Mast cells and dendritic cells are critical during the sensitization phase, causing DC migration and maturation [4, 5, 61, 62].

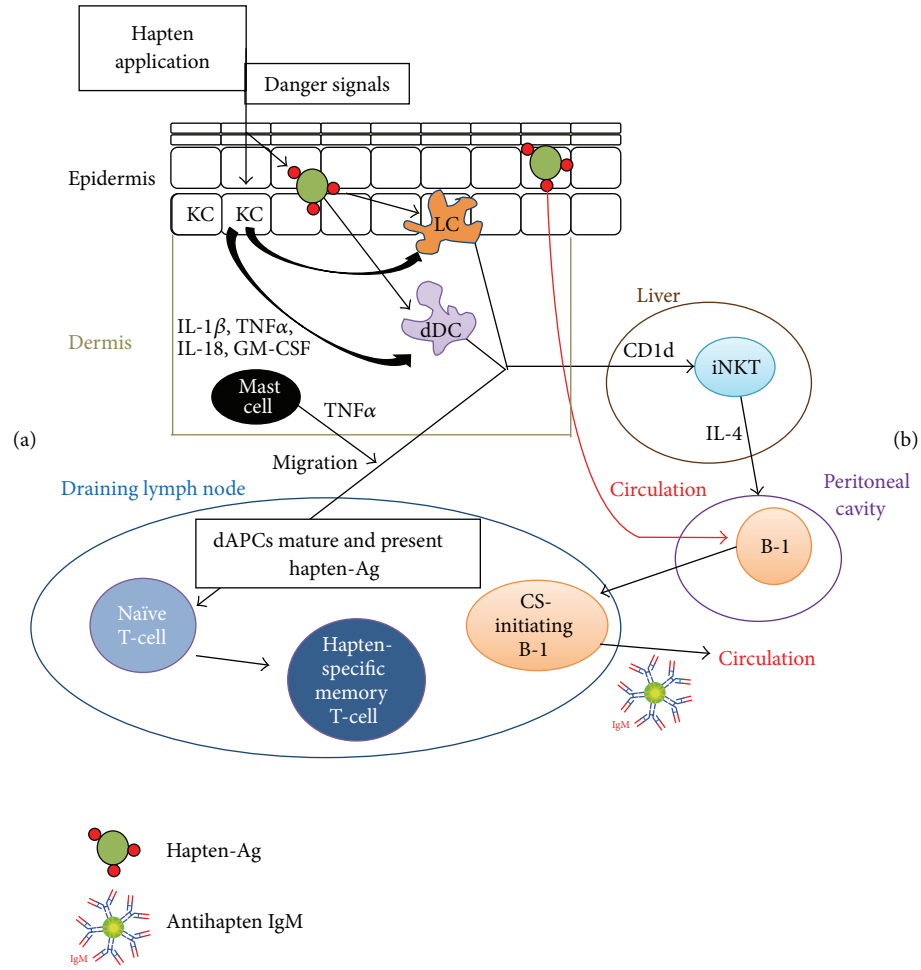

Figure 1: The likely pathway of the "sensitization" phase of contact hypersensitivity. (a) Hapten application induces strong innate immune mechanisms, causing cell death and the release of danger signals and endogenous ligands, leading to cytokine release, IL-1β, IL-18, TNFα, and GM-CSF, by keratinocytes (KC). This release will stimulate dermal antigen-presenting cells (dAPCs), langerhans cells, and dermal dendritic cells, to take up haptenated antigen and migrate to the dLN to activate naïve T-cells. Mast cells will aid in this migration by releasing TNFα. (b) iNKT cells in the liver will be activated by APCs presenting haptenated glycolipid by CD1d. This will cause cytokine release, IL-4, to stimulate naïve B-1 cells in the peritoneal cavity, along with the binding of hapten-antigen by membrane IgM. This will cause migration of these cells to the dLN, and subsequent maturation into CS-initiating B-1 cells, which release antihapten IgM into circulation.

Upon maturation by Keratinocyte stimulation, langerhans cells and dDCs migrate to the dLN. The dermal APCs activate naive T-cells and invariant natural killer T (iNKT) cells by presenting the haptenated antigen (peptide and lipid) via MHCI/II or CD1d, respectively. Peptide presentation depends on whether the haptenated protein becomes internalized and processed via the endosomal compartments, followed by MHC-I presentation [63], or whether the haptenated proteins are on the extracellular surface and cross presented via MHC-I to CD8-T-cells [64]. Many haptens enter the cells through passive diffusion and bind to intracellular proteins, which are presented by MHC-I, H-2K^b, to naive CD8+ T-cells [63]. Presentation to naive T-cells leads to the formation of hapten-specific memory T-cells with the capability to become hapten-specific effector T-cells (CD4+ and CD8+). Thus, these effector cells cause damage and regulate immune responses at the elicitation site [4, 5].

Haptenation also causes the release of endogenous glycolipids that are processed and presented via CD1d to iNKT cells in the liver [65]. In Balb/c and CBA/J mice iNKT-cells become stimulated within 30 minutes via "stimulatory" lipids in the liver and release IL-4 [65–68]. The IL-4, along with haptenated antigen in the circulation [66, 67, 69], stimulates naive B-1 cells within 1 hour to migrate to the draining lymph node and form "CS-initiating B-1 cells," a distinct class of B-1 cell, that creates hapten-specific IgM [70, 71]. In C57BL/6 mice, however, these iNKT-cells have an inhibitory role [72] as they release IL-4 and IL-13 which, along with T-regulatory cells that release IL-10, suppress the formation and function of the hapten-specific memory T-cells [73, 74]. The differences in function of iNKT-cells are most likely because Balb/c mice have a more Th2-based immune response, whereas C57BL/6 mice have a more Th1-like immune response [72]. Regardless, iNKT-cells play a large stimulatory or regulatory role in CHS.

O'Leary et al. [75] and Paust et al. [76] showed that natural killer (NK) cells induced CHS reactions in $RAG^{-/-}$ mice (devoid of T- and B-cells). Further experimentation [77] showed that liver NK cells are able to transfer CHS to naive animals in 1 hour. Currently, there is no literature on how these NK cells become activated, although one can infer that NK cells are more likely to become activated due to a lack of engagement of inhibitory receptors. Ly49C, found on these hapten-specific NK cells, is specific for $H\text{-}2K^b$ binding [78]. If the self-protein being presented is haptenated, it may no longer appropriately recognize or bind with the Ly49C, causing NK cells to recognize the cell as foreign. It is likely that DNP-bound MHC will affect Ly49C binding, but this requires experimental verification.

In summary, after hapten application, keratinocytes stimulate dAPC maturation and migration, leading to activation of hapten-specific memory T-cells, iNKT-cells, CS-initiating B-1 cells, and hepatic NK cells. The sensitization phase appropriately primes the immune system to the hapten, so that the elicitation phase can occur quickly and with optimal immune response.

2.2. Elicitation Phase of Contact Hypersensitivity. Upon secondary hapten challenge, the elicitation phase of CHS will occur as "early" and "late" events, resulting in swelling and severe damage of the challenged area. The early elicitation phase which peaks within 2 hours of challenge and dissipates by 4 hours seems to be antigen-independent [79], while the late elicitation phase occurs within 24 hours of the challenge and seems to be antigen-dependent [4]. Each of these concepts needs to be considered for understanding hapten-induced tumor-immunity.

2.2.1. Early Elicitation Phase. Figure 2 outlines the steps in the early elicitation phase. Upon hapten-challenge, there is antigen-nonspecific inflammation; iNKT-cells are restimulated by the stimulatory lipids released in the liver, causing them to once again produce IL-4. This release causes the restimulation of CS-initiating B-1 cells to produce IgM against hapten. The hapten-specific IgM and haptenated antigen will go into circulation, form complexes and activate complement C5a [65, 69, 80] through the classical complement pathway. The C5a will then bind to mast cells in the dermis, causing release of serotonin, TNFα, and CXCL2. TNFα and CXCL2 release will help recruit FasL+, neutrophil + neutrophils to the area. In combination with these neutrophils, TNFα and serotonin production by mast cells will cause the release of CXCL-10, CCL1, 2, and 5 from the surrounding tissue and the upregulation of ICAM-1, E- and P-selectin on endothelial cells in the vasculature, leading to hapten-specific T-cell recruitment [4, 61, 62, 81]. Neutrophils are also brought to the area by the release of CXCL1 and 2 from keratinocytes after hapten-challenge and elicit T-cell infiltration [4, 82]. FasL and perforin expression of neutrophils is essential to initiate proper T-cell infiltration, as administration of soluble FasL in the challenge area had similar responses [83]. Keratinocytes are known to release proinflammatory cytokines (IL-1β and TNFα) upon hapten stimulation [84], causing vascular endothelial cells to upregulate ICAM-1 and P- and E-selectins [4]. In the absence of IL-1 and TNFα, CHS is suppressed [85]. Keratinocytes also produce many chemokines that allow for hapten-specific T-cell entry into the challenged area, the most important being CXCL10, which will be bound by the CXCR3 on Th1 cells. The blockade or deficiency of IL-1β and TNFα reduces CHS by decreasing CXCL10 [4].

2.2.2. Late Elicitation Phase. Figure 3 outlines the steps in the late elicitation phase, which occurs within 24 hours of hapten-challenge. dDCs, LCs, KCs, and endothelial cells process haptenated antigen as previously described and present the antigen to hapten memory T-cells that have migrated to the dermis during the early elicitation phase [86]. Once stimulated in the dermis, memory T-cells will form hapten-specific CD4+ and CD8+ T-cells.

Typically, iNKT cells can either play a stimulatory or inhibitory role that depends on the mouse model used to study iNKT cells, C57BL/6 mice versus CBA/J mice, respectively. In CBA/J mice, iNKT cells can release IFNγ that helps to promote CD8+ effector development when working in conjunction with $\gamma\delta$ T-cells [65, 87]. In C57BL/6 mice, the iNKT-cells release IL-4 and IL-13, which suppress CHS reactions [72], possibly by stimulating a Th2 response. This is in contrast to other strains of mice wherein IL-4 release helps to stimulate CS initiating B-1 cells. $\gamma\delta$ T-cells seem to "collaborate" with iNKT-cells to elicit CD8+ T-cell-mediated damage during CHS [88]. Upon adoptive transfer with these two cell subtypes, there was a strong ear swelling response at 2 and 24 hours post-DNFB challenge, but if either one was depleted, the ear swelling significantly decreased. This collaboration of iNKT-cells and $\gamma\delta$ T-cells helps to activate $\alpha\beta$ TCR+ CS-effector cells [88].

Langerhans cells, once thought to be the main APC of haptenated-Ag, are thought to have more of a regulatory role in the elicitation of CHS. Depletion of epidermal LCs in hapten-sensitized mice elicited greater CHS responses [89] as LCs can suppress CHS responses via CD40-CD40L interactions with CD4+ T-cells causing the release of LC derived IL-10 [90]. Notably, LCs tolerize CD8+ T-cells by activating FoxP3+ T-regulatory cells (T-regs) in mice sensitized with a weak hapten and then challenged with a strong hapten [91]. It is likely that dDCs, endothelial cells, and KCs, not LCs, present antigen to memory T-cells in the dermis during the elicitation phase [5, 92].

Hapten-specific T-cells will traffic to the elicitation site by upregulation of chemokines, selectins, and adhesion molecules and differentiate into their appropriate effector or helper status by a multitude of cytokine signals (from the tissue and activated T-cells) and haptenated-antigen presentation [4, 5, 92]. Honda et al. [4] summarizes the roles of different cytokines in the elicitation phase of CHS and the large difference between the reactions elicited with the haptens trinitrochlorobenzene (TNCB), Ox, DNFB, and fluorescein isothiocyanate (FITC), all which are known to be Th1 haptens except for FITC, which is known to be a Th2 hapten. They further emphasize that the differing effect of cytokines reported in the literature is due to the hapten, animal model,

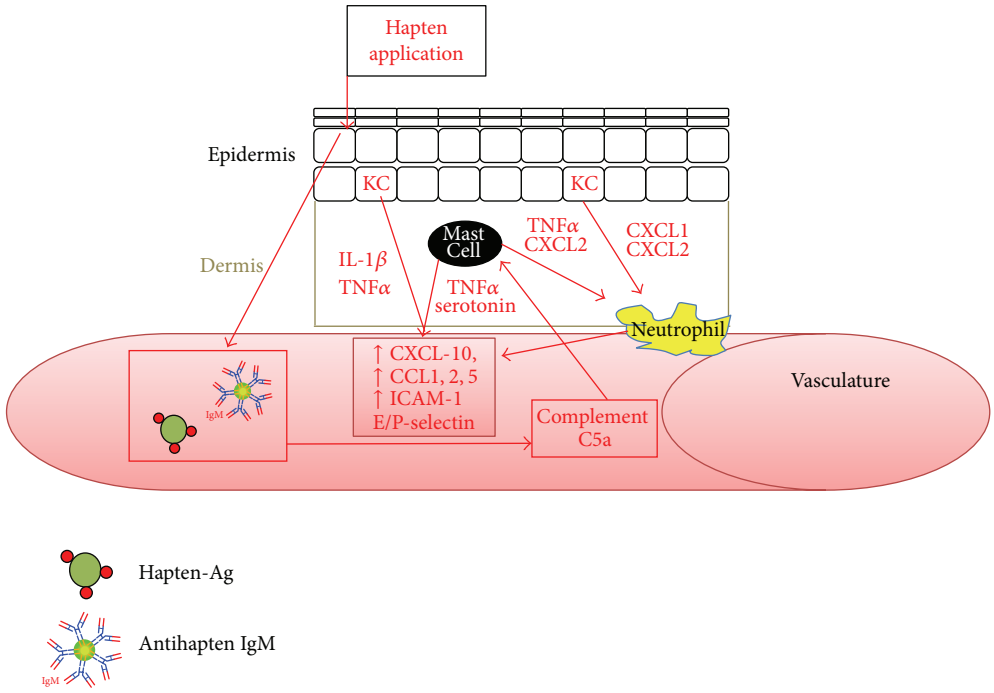

Figure 2: The likely pathway of the "early elicitation" phase of contact hypersensitivity. The red arrows and type indicate the early elicitation phase. Hapten challenge will restimulate iNKT cells to release IL-4, which along with hapten-antigen will stimulate CS-initiating B-1 cells as seen in Figure 1. These cells will release IgM, which will bind to hapten-antigen. This will cause formation of C5a, triggering activation of mast cells to produce TNFα and serotonin, increasing immune cell trafficking into the area and TNFα and CXCL2 to stimulate neutrophils in the dermis. Neutrophils will also be activated by CXCL1 and CXCL2 released from haptenation of the keratinocytes. Their activation will cause damage at the challenge site as well as more CXCL1 and CXCL2 release, inducing immune cell trafficking to the area as illustrated in Figure 3. Lastly, haptenated keratinocytes will release cytokines to induce immune cell trafficking to the area as depicted in Figure 3.

and possibly even the microbiota of the animals in the specific animal facility. We think that haptenation of microbiota will release multiple danger signals, haptenated bacterial proteins, and haptenated bacterial lipid, which can uniquely stimulate different types of CHS reactions through various innate immune responses, iNKT cell responses, and T-cell responses. This concept needs experimental verification.

The "Hapten Atopy Hypothesis," proposed by McFadden et al. [54], states that haptens delivered a few times by epifocal application will stimulate TLR4 through danger signal release, leading to a Th1 immune response, but repeated and prolonged exposure to haptens will likely shift the response from Th1 to Th2. When TLR4 is stimulated, it will weakly upregulate TLR2 expression to drive Th2 responses, possibly by heat-shock protein ligand upregulation. The repeated exposure of the haptens and weak stimulation of TLR2 will form Th2 cytokines, which will downregulate Th1 cytokines and suppress TLR4 function. This is known as the "danger limitation effect" [54]. Röse et al. [93] indirectly support this hypothesis by showing that different types of hapten challenges, acute (one challenge), subacute (three challenges), and chronic (5–13 challenges) result in different CHS responses. In the chronic exposure versus acute exposure, there is a decrease of Th1 cytokines (TNFα, INFγ, IL-2, and IL-12), an increase of Th2 cytokines (IL-4, IL-5, and IL-13), and an increase in T-regulatory cytokines (IL-10), indirectly giving support to the "Hapten Atopy Hypothesis".

There are multiple different T-cell subsets that are involved in the elicitation of CHS-related cellular damage. Classic delayed-type hypersensitivity is CD4+ regulated, and for many years it was assumed that CHS worked the same way. Now it is evident that both CD8+ and CD4+ T-cell subsets are involved in eliciting CHS [94]. The depletion of CD8+ T-cells greatly reduces CHS reactions [95]. Martin et al. [96] showed that CD8+ effector T-cells were the main cells that elicited CHS damage and CD4+ effector T-cells minimally acted as CHS effectors. Along with this notion, hapten-specific CD4+ T-cells are thought to consist of more CD4+ T-regs than effector cells, each having their own effect on CHS responses, inhibitory and stimulatory, respectively [94]. It is likely that both CD4+ and CD8+ effector T-cells work in tandem to elicit damage, as shown in CD4+ and CD8+ T-cell KO mice experiments where both subsets had great impact on CHS responses [97]. It seems that CD8+ T-cells are the main CHS-effector T-cells, and that CD4+ T-cells have a dual role, eliciting minimally the effector function and largely the regulatory function.

CD8+ T-cells elicit damage in the haptenated area during CHS elicitation phase by augmenting cytotoxicity with perforin and Fas/FasL interactions [98].

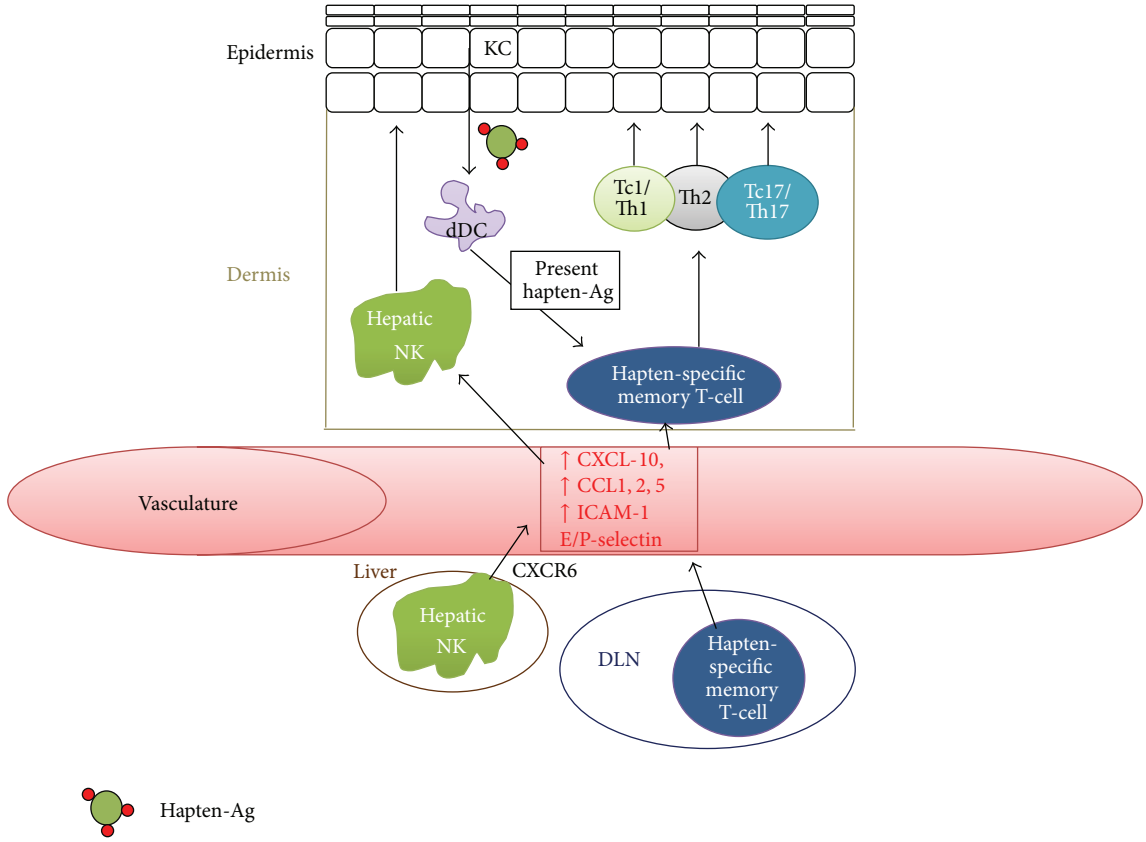

FIGURE 3: The likely pathway of the "late elicitation" phase of contact hypersensitivity. The red type indicates the "early" elicitation phase and the black arrows indicate the "late" elicitation phase. Hapten-specific memory T-cells will traffic to the hapten challenge site, where they will enter the dermis and divide into multiple different cells subsets. This will be initiated by dermal APCs presenting antigen as well as cytokine release from multiple different cell subsets. The multiple subsets will play different roles in the CHS reaction at the site. Lastly, CXCR6+ hepatic NK cells will traffic to the hapten challenge site and elicit damage.

This interaction seems to induce the apoptosis of KCs [99]. CD8+ T-cells have also been shown to release IFNγ and IL-17, which can stimulate neutrophils to draw more CD8+ T-cells to the area by keratinocyte-induced upregulation of chemokines [83, 100]. IL-17 release seems to play an important role in CHS and ACD [101, 102], as Th1/Th17 cells infiltrate ACD areas upon $NiSO_4$ application in human patients [103]. These results found in CHS and ACD models show that CD8+ T-cells and possibly Th17 cells are crucial players in CHS reactions.

T-regulatory cells down-regulate contact hypersensitivity by using the IL-2 produced from hapten-specific CD8+ effector cells [104]. CHS-associated T-regs traffic to the inflamed site during the elicitation phase [74] and likely inhibit CHS by CTLA-4 and CD86 interactions between T-regs and CD8+ T-cells, as treatment with anti-CTLA-4 antibody increased CHS responses [105]. They also inhibit CHS by IL-10 release, which is known to suppress CHS [106] and block entry of hapten-specific effector T-cells into the challenge site [73]. Taken together, T-regs play a large role in CHS regulation and are important when considering hapten-induced tumor regression.

Extensive studies were performed by Hans Ulrich Weltzien's group from 1992 to 1997 looking at the TCR specificities of CD4+ and CD8+ T-cells and the way in which haptenated protein is presented to T-cell receptors (TCRs). They showed that trinitrobenzene sulfonic acid (TNBS)-like haptens are H-$2K^b$ restricted [64]; haptenated Ag can be processed intracellular in the ER/Golgi to be presented by MHC I [63], and trinitrophenyl (TNP)-specific T-cell clones were able to recognize haptenated and unhaptenated portions of designed tryptic fragments of TNP-octapeptides [107]. TNP-specific CD4+ T-cell clones were able to recognize many different TNP-modified peptides, as long as TNP was present [108]. These papers suggest the ability of hapten-specific CD8+ clones to recognize unhaptenated portions of amino acid chains, whereas hapten-specific CD4+ T-cells only recognize haptenated protein.

$\gamma\delta$ T-cells and iNKT-cells were shown to work together to release IFNγ, which would stimulate a Tc1/Th1- like response [88]; however, they were shown to inhibit CHS reactions during elicitation by hindering the development of hapten-specific CD8+ T-cells [109]. $\gamma\delta$ T-cells played a role in eliciting dinitrochlorobenzene (DNCB)-induced CHS in lambs [110].

Recent unpublished work by Xiaodong Jiang et al., presented at "The American Association of Immunologists Conferences in May of 2013," focuses on the dermal $\gamma\delta$ T-cells in terms of how their depletion suppresses CHS reactions. It seems that IL-17 dermal $\gamma\delta$ T-cells are important in inducing CHS reactions. The involvement of dermal $\gamma\delta$ T-cells during elicitation is unclear and needs further study.

Recent studies have unraveled the ability of NK cells to induce CHS reactions. First described by O'Leary et al. [75] and Paust et al. [76], CHS was induced in a $RAG^{-/-}$ mouse (lacking B- and T-cells) with the assumption being that no ear swelling would be seen; these animals got an ear swelling reaction close to normal. The responsible cells were NK cells as seen by IL-$2R^{-/-}$ mice and antibody depletions. Using adoptive transfer systems, it was seen that these NK cells were hepatic, expressed Thy-1, Ly49c, and CXCR6 and could elicit CHS responses 4 months after sensitization. L-, P-, and E-selectins and NKG2D were found to play an important role in NK-mediated CHS reactions [75, 76]. These observations were furthered by Carbone et al., [111] who looked at a distinct CD3−, CD16−, perforin+, $CD56^{high}$, CD16−, and CD62L− (noncirculating) NK cell populations that produced IFNγ and TNFα in Nickel-challenged ACD regions of humans. Unexpectedly, these NK cells did not elicit a memory-like response as previously described but did contribute to keratinocyte apoptosis; this could be a mouse versus human phenomena [111]. Majewska-Szczepanik et al. [77] confirmed the presence of NK cell-mediated CHS in mice devoid of B- and T-cells, although the response was markedly diminished compared to wild-type (WT) mice. These cells produced IFNα, IFNγ, and IL-12, were Thy1+ and MAC1+ (fully licensed), CXCR6-dependent, and could elicit a CHS reaction in as little as 1 hour after transfer from a sensitized to naïve animal [77]. Likely uncertain of this body of results, Rouzaire et al. [112] did a comparison of T-cell-mediated to the NK cell-mediated reactions using the "classical" CHS protocol with DNFB; they showed that the NK cells failed to create a genuine CHS response in $RAG2^{-/-}$ mice, as the DNFB ear challenge did not require sensitization to elicit an ear swelling response. They confirmed O'Leary et al.'s [75] observations by performing similar adoptive transfer experiments of NK cells and showed that the responses were similar to transferred CD8+ T-cells. However, the recall response of these transferred NK cells upon a second hapten challenge was much weaker and short-lived than that of transferred CD8+ T-cells and there was little CD45.1+ T-cell infiltration into the challenged site in the NK cell-transferred mice [112]. It seems as though NK cells play some sort of role in CHS, although they may only be able to elicit true CHS reactions in adoptive transfer settings and may only help to elicit damage at the haptenation site.

3. Drug-Induced Autoimmunity versus Hapten-Induced Autoimmunity

There are many common allergens that cause CHS: metals likes Nickel or Gold, certain antibiotics like Neomycin, topical anesthetics, natural compounds such as Urushiol, the irritant in poison ivy, and many more. These all act directly as haptens, inducing a CHS-like reaction in the skin. There are instances where metabolizing a drug or chemical can lead to autoimmune-like responses, idiosyncratic drug reactions. This is when a drug's metabolite acts as a hapten and binds to cellular proteins, eliciting an immune response and antibody production to the metabolite-protein conjugate, the metabolite alone, and the protein alone [129]. These drugs are prohaptens, or chemicals that are not protein-reactive unless metabolically activated to the electrophilic state [1]. A common example of this is Penicillin-induced hemolytic anemia [7]. When the penicillin enters the body, it is metabolized in the liver and forms Penicillenic acid, similar to the hapten Oxazolone, which then covalently binds to red blood cells (RBCs) [7]. Antibodies (IgG) can form against the hapten-coated RBCs, which are then killed by antibody-dependent cellular cytotoxicity (ADCC) and cleared by macrophage opsonization. Hydralazine, a hypertension drug, is known to cause drug-induced lupus (DIL) through its metabolites. It was seen that hydrogen peroxide and other oxidants from the lungs react with hydralazine to produce metabolites that bind to self-protein. About 5% of the patients who take this drug develop DIL-like symptoms [130, 131]. There are several other examples of drug-induced autoimmunity in several different contexts, most involving the binding of a drug or its metabolite to self-protein inducing antibody production. In all cases, the drug or metabolite acts as a hapten to induce autoimmunity.

The autoimmune inducing capabilities of haptens have been shown experimentally. Paul et al. [2] showed proof of principle experiments that haptens could allow the immune system to overcome peripheral tolerance. By injection of haptenated-BSA, BSA-tolerized rabbits were able to induce the production of antibody towards the hapten, the BSA, and the conjugate. Haptens have been shown to induce hapten-specific CD8+ T-cell cross-recognition of haptenated and unhaptenated octapeptides as previously described [107]. Kang et al. [132] showed hapten-mediated autoimmunity experimentally in hen egg lysozyme (HEL)-transgenic (Tg) C57BL/6 (B6) mice that were immunized with HEL or hapten-modified (phosphorylcholine [PC]) HEL (PC-HEL). Hen egg lysozyme immunization failed to induce antibody responses against HEL in the transgenic animals, but the PC-HEL generated large amounts of anti-HEL antibody. This break in tolerance was by T-cells seen through T- and B-cell depletion and adoptive-transfer experiments. This concept is similar to that seen in CHS. Lastly, PC-HEL was better at generating HEL epitopes for T-cell recognition following antigen processing. They suggest that the "generation of new immunogenic epitopes of self-antigens may result in breaking self-tolerance and lead to the production of autoantibodies" [132]. Despite these examples, none of these papers showed the ability of these reactions to induce immune damage, as this would be indicative of autoimmune disease. Experimentally induced autoimmunity seems to be a hapten-dependent reaction that does not occur in the absence of the hapten.

Clearly, the main use of haptens is to study CHS. The unique property of haptens to induce immune reactions against self-peptide has been utilized in many other settings

besides CHS. Haptens have been commonly used to induce acute and chronic IBD in rats and mice using the haptens 2,4,6-trinitrobenzene sulfonic acid or 2,4-dinitriobenzene sulfonic acid (DNBS) to induce immune reactions in the intestine [11–15]. te Velde et al. [14] reviewed the models of TNBS-induced IBD, clearly stating many of the problems present in the field. IBD reactions seem to be hapten-dependent, and the hapten does not induce autoimmune reactions to the intestine once it is out of the animals' system. Haptens have been used to treat drug addiction. Ennifar et al. applied for a patent [9] for a novel hapten-carrier conjugate that stimulates the production of antibodies against nicotine. These antibodies could be used to treat nicotine-addicted patients, as they passively lower the nicotine levels in the serum and brain. A similar idea was tried using a novel hapten-conjugate, 6-glutarylmorphine-Keyhole Limpet Hemocyanin (KLH), conjugate that induced antibodies against morphine and heroin in rats. The treatment increased rat movement and attenuated other drug-induced behaviors, compared to the control group, in morphine and heroin addicted rats; this was associated with antibodies against the morphine and heroin. This treatment likely induced tolerance to the drugs [10]. These methods have not been extensively studied, making long-term dependence on the haptens unclear.

4. Applying Haptens and Contact Hypersensitivity to Antitumor Immunity

Clearly, haptens have been used in many contexts to study certain diseases and induce responses against certain malignancies. The properties of haptens to induce reactions are fascinating, although it seems as though these reactions may be hapten-dependent, and many will wane as the hapten is cleared. Despite this, the ability of haptens to induce reactions against self-protein, even if haptenated, is a unique property that make haptens tantalizing targets for cancer immunotherapy. In the following sections, we will review how haptens have been used to treat tumors, their advantages and disadvantages, the challenges present in the field, and possible directions of study to further the field.

4.1. The Four Concepts of Hapten-Mediated Antitumor Immunity. The use of haptens to induce tumor regression is not a new one, as many groups have attempted several different methods of hapten-mediated tumor regression. There are four overarching concepts involving the use of haptens to induce tumor immunity. (1) The tumor is removed, haptenated *ex vivo*, and injected back into sensitized animals or patients [21–31]. (2) The tumor is haptenated *in situ* (in the tumor) [32, 33]. (3) The tumor area is haptenated epifocally (at the tumor site) to induce a CHS-like reaction [34–42]. To note, this method has only been utilized for cutaneous skin cancers that can invade the epidermis or dermis, as CHS reactions require these. (4) ADCC reactions at the tumor site can be induced by intraperitoneal (i.p.) or subcutaneous (s.c.) administration of antigen-hapten conjugates in mice and patients, respectively with antigen-receptor high tumors [43–47]. These concepts (Table 1), the problems and holes present, and our interpretation of the possible antitumor mechanisms occurring are reviewed below.

4.2. Ex Vivo Haptenation to Mediate Tumor Regression. Many groups have utilized *ex vivo* haptenation to induce tumor regression in mice and humans. Hamaoka et al. [21] were the first group to use *ex vivo* haptenation as a cancer immunotherapy in mice. They used X5563 cells, a plasmacytoma cell line syngeneic to C3H/HeN mice previously shown to generate "killer" T-cell activity without inducing helper T-cell activity against tumor-associated transplantation antigen (TATA) and still grow. They immunized mice with hapten-modified X5563 cells to amplify helper T-cell activity, and augment killer T-cell responses to the TATA. They primed mice intraperitoneal (i.p.) with trinitrophenyl (TNP)-bound mouse gamma globulin (MGG) in order to generate TNP-specific T-cells. Six weeks later, they immunized mice i.p. with TNP-bound X5563 tumor cells, using TNBS, generating killer T-cells against X5563 and TNP-X5563 tumor cells; this did not occur in mice primed with unhaptenated tumors. They further amplified this response with a pretreatment of TNP-D-GL to ablate TNP-suppressor cell activity. Mice were given the full treatment (TNP-D-GL pretreatment, three days after TNP-MGG immunization, six weeks after immunized i.p. with TNP-X5563 cells once a week for five weeks) and then given a lethal dose of the X5563 cells. The tumor growth was greatly decreased and the mean survival time of the mice increased by 10 days posttreatment. This study only examined the tumor growth for 15 days, so it is likely that the tumor was able to proliferate and grow at further time points. This system is a nice proof of principle but has very little clinical application because it is a lengthy prophylactic treatment that minimally delays tumor growth and the effect of this treatment on an established tumor was not studied. Regardless of this, they showed that modification of TATA with hapten-induced TNP-reactive helper T-cells, which could amplify killer T-cell generation, resulting in slowed tumor growth and an antitumor immune response *in vivo*.

Fujiwara et al. [22] took Hamaoka's model and applied it to a BALB/c-LSTRA leukemia tumor system. They primed mice with TNP-D-GL, three days later, immunized mice with TNP-MGG, and six weeks later, i.p. injected TNP-LSTRA cells three times in two-week intervals. Syngeneic T-cells were stimulated *in vitro* by co-culturing them with TNP-LSTRA cells for five days. These cells showed significant lysis of LSTRA cells *in vitro*. The TNP-primed splenocytes were collected, mixed with viable LSTRA cells to perform *in vivo* tumor neutralization assays by intra-dermally (i.d.) injecting the mixture into TNP-sensitized Balb/c mice. This stopped tumor formation for at least 11 days after inoculation. Despite not showing the effect of this treatment on tumor cell challenges or established tumors, this study highlights the proof of a principle that anti-tumor immune responses can be generated with *ex vivo* haptenation of tumor cells.

Flood et al. [23] investigated *ex vivo* TNP-modification, using TNBS, of regressor and progressor tumors to cause

Table 1: Summary of the hapten-mediated tumor regression studies.

Hapten treatment	Author, year	Hapten used for treatment, alone and in combination	Tumor type/cell line used in animal and human studies	Route of administration of haptens and hapten-modified products	Observations
Ex vivo haptenation	Hamaoka et al., 1979 [21]	TNBS, TNP-MGG sensitization and TNP-D-GL pretreatment	X5563 cells in C3H/HeN mice	i.p. TNP-X5563 injection	Significantly delayed tumor growth for up to 15 days
	Fujiwara et al., 1980 [22]	TNBS, TNP-MGG sensitization and TNP-D-GL pretreatment	LSTRA cells in Balb/c mice	i.p. TNP-X5563 injection	Significantly delayed tumor growth for up to 10 days
	Flood et al., 1987 [23]	TNBS, N/A	Progressor and regressor fibrosarcomas in C3H/HeN mice	s.c. TNP-regressor/TNP-progressor injection	Significantly delayed tumor growth for up to 30 days
	Berd et al., 1993 [30]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stages III and IV metastatic melanoma in patients	i.d. DNP-autologous melanoma injection	5/46 patient responses for metastatic melanoma and 59% 2-year survival postnodal resection
	Sato et al., 1995 [29]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stages III and IV metastatic melanoma in patients	i.d. DNP-autologous melanoma injection	IFNγ producing CD8 T cells that killed DNP-melanoma only
	Sato et al., 1997 [27]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stages III and IV metastatic melanoma in patients	i.d. DNP-autologous melanoma injection	DNP-specific T-cells recognize only hapten-modified melanoma
	Berd et al., 1997 [28]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stage III metastatic melanoma postnodal resection in patients	i.d. DNP-autologous melanoma injection	5-year 45% relapse-free and 58% overall survival (62 patients)
	Berd et al., 2001 [26]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stage IV melanoma with pulmonary metastases in patients	i.d. DNP-autologous melanoma injection	11/83 patients had responses to treatment, only 2 had complete response
	Manne et al., 2002 [25]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stage III metastatic melanoma postnodal resection in patients	i.d. DNP-autologous melanoma injection	T-cell clones from DNP-vaccine patients with similar TCR VDJ peaks and CDR3 amino acid sequences
	Sojka et al., 2002 [31]	DNFB, CY pretreatment combined with BCG and nodal resection	410.1 cells in Balb/c mice	s.c. DNP-410.1 injection	40% relapse-free survival with DNP-vaccine versus 20% without DNP; CD4+, and CD8+ T cells, and IFNγ and TNFα important for survival.
	Berd et al., 2004 [24]	DNFB, DNFB sensitization and CY pretreatment combined with BCG and nodal resection	Stage III metastatic melanoma postnodal resection in patients	i.d. DNP-autologous melanoma injection	5-year 44% overall survival (214 patients)

Table 1: Continued.

Hapten treatment	Author, year	Hapten used for treatment, alone and in combination	Tumor type/cell line used in animal and human studies	Route of administration of haptens and hapten-modified products	Observations
In situ haptenation	Fujiwara et al., 1984 [32]	TNCB, TNCB sensitization and CY pretreatment	X5563 cells in C3H/HeN mice	Intratumoral injection of TNCB	>50% primary tumor regression and secondary tumor resistance. Helper T-cells crucial
	Fujiwara et al., 1984 [33]	TNCB, TNCB sensitization and CY pretreatment	X5563 cells, MCH-1-A1 cells, and MCA-induced tumors in C3H/HeN mice	Intratumoral injection of TNCB	>50% primary tumor regression and secondary tumor resistance. Helper T-cells crucial
Epifocal hapten application	Klein 1969 [34]	TEIB and DNCB, N/A	BCC and SCC in patients	Topical hapten application on tumor	Reviews various complete tumor regression cases in various different cancers and patients.
	Truchetet et al., 1989 [113]	DNCB, N/A	Metastatic melanoma in patients	Topical DNCB application on tumor	Reviews the use of DNCB to treat metastatic melanoma in the clinic and in case studies
	Strobbe et al., 1997 [35]	DNCB, DNCB sensitization on tumor and systemic DTIC	Recurrent melanoma in patients	Topical DNCB application on tumor	25% complete response with combined DNCB and DTIC treatment
	von Nida and Quirk, 2003 [36]	DNCB, DNCB sensitization	Metastatic melanoma in patients	Topical DNCB application on tumor	Tumor control for 7 years in metastatic melanoma patient with DNCB application
	Herrmann et al., 2004 [114]	DNCB, DNCB sensitization	Merkel cell carcinoma in patients	Topical DNCB application on tumor	Complete tumor regression on scalp and CD3+ T-cell and CD28+, KP-1+ Macrophage infiltration
	Damian et al., 2009 [39]	DPCP, DPCP sensitization	Metastatic melanoma in patients	Topical DPCP application on tumor	Of 7 patients, many had slow growing tumors or tumor regression at DPCP application site
	Martiniuk et al., 2010 [38]	DPCP, DPCP sensitization	Metastatic melanoma in patients	Topical DPCP application on tumor	Role of Th17 cells in tumor regression
	Kim 2012 [40]	DPCP, DPCP sensitization	Metastatic melanoma in patients	Topical DPCP application on tumor	Regression of melanoma nodules for 18 weeks
	Wack et al., 2001 [42]	DNCB, DNCB sensitization on tumor and systemic DTIC	B16F17 cells in C57BL/6 mice	Topical DNCB application on tumor	72% primary tumor regression and reduced pulmonary metastases
	Wack et al., 2002 [41]	DNCB, DNCB sensitization on tumor and systemic DTIC	B16F17 cells in C57BL/6 mice	Topical DNCB application on tumor	Repeat 2001 results, CD4+ and CD8+ T cells kill B16 *in vitro* and release IFNγ
	Lu and Low 2002 [46]	Folate-FITC conjugate, BSA-FITC sensitization with adjuvant GPI-0100 and systemic IL-2 and IFNα	M109 cells in Balb/c mice	i.v. and i.p. injection folate-FITC conjugate	FITC coating of tumors. 100% overall survival after optimization with combined treatment; survive secondary challenges
	Lu et al., 2005 [45]	Folate-FITC conjugate, BSA-FITC sensitization with adjuvant GPI-0100 and systemic IL-2 and IFNα	M109 cells in Balb/c mice	i.p. injection folate-FITC conjugate	NK-cell induced ADCC and Macrophage opsonization; CD4+ and CD8+ T-cells important. Complete tumor regression in 35 days

Table 1: Continued.

Hapten treatment	Author, year	Hapten used for treatment, alone and in combination	Tumor type/cell line used in animal and human studies	Route of administration of haptens and hapten-modified products	Observations
Antigen-hapten administration	Lu et al., 2006 [44]	Folate-FITC conjugate, BSA-FITC sensitization with adjuvant GPI-0100 and systemic IL-2 and IFNα	M109 cells in Balb/c mice	i.p. injection folate-FITC conjugate	Preclinical pharmacokinetic and tissue distribution studies
	Lu et al., 2007 [43]	Folate-DNP conjugate, KLH-DNP sensitization with adjuvant GPI-0100 and systemic IL-2 and IFNα	M109 cells in Balb/c mice	i.p. injection folate-DNP conjugate	60% cure-rate in mice
	Amato et al., 2013 [47]	EC17 folate-FITC conjugate, EC90 hapten fluorescein with adjuvant GPI-0100	Renal cell carcinoma in patients	s.c. injection folate-FITC conjugate	Phase-1 Study, 1/28 patients had partial response, 15/28 had stable disease; side effects

tumor rejection of unmodified progressive tumor cell lines in mice. They created a system of tumor inoculation rejection in C3H/HeN mice using primary s.c. immunization of TNP-bound 1591 regressor fibrosarcomas, followed 28 days later by a secondary immunization of a TNP-bound 3152 progresser fibrosarcoma and tertiary challenge of unmodified-3152 progressor cells. This resulted in slowed growth of 3152 progressor tumors for up to 30 days. The resistance to progressor tumor cells was adoptively transferred with total splenocytes to naïve animals. By antibody depletion, it was seen that Lyt-1-2+ T-cells and Lyt-1+2- T-cells expressing nonclassical helper T-cell phenotypes elicited the resistance. Thus, they established that haptenation could enhance immunity towards "weak" tumor-associated antigens by TNP-modification, despite the eventual progressor tumor growth. It would be interesting to see what would have happened if they had used a cytotoxic hapten, like TNCB for their immunizations, as hapten-mediated cell death may have elicited better immune response, or if they had sensitized the animals to TNP before vaccination, as this may have enhanced the immune response to the haptenated cells.

Berd et al. [24, 26, 28, 30] utilized the *ex vivo* haptenation as well as *in situ* haptenation mouse studies by Fujiwara et al. [32, 33] as the basis for clinical trials using *ex vivo* tumor cell haptenation as a primary treatment for metastatic melanoma or as an adjuvant treatment after surgical resection of nodal metastases in stages III and IV metastatic melanoma patients. Two weeks before vaccination, patients were pretreated with cyclophosphamide (CY) and 2 days later sensitized to 1% DNFB. Patients were treated with CY three days before the DNP vaccination; this was repeated every 28 days. Cyclophosphamide has long been known to enhance CHS-like responses as it decreases the percentage and number of CD4+ CD25+ T-regs that suppress the induction of CHS [133]. The DNP-vaccine was made by surgical resection of primary melanoma, irradiation, modification with DNFB, and intradermal injection back into patients along with Bacillus Calmette-Guerin (BCG), a known cancer immune adjuvant [134]. Forty-six patients with measurable metastases were treated, resulting in 20 patients with clinically evident inflammatory responses in nodal, subcutaneous, or intradermal tumors. These tumors had increased CD8+ T-cell infiltration, compared to prevaccination tumors, which strongly expressed HLA-DR and CD69 suggesting activation. In addition, 140 T-cells clones were created, 70 of which could kill autologous melanoma cells *in vitro*. It is commonly seen that tumor-infiltrating lymphocytes (TILs) are able to kill tumor cells *in vitro* once stimulated [135], so it is unclear if the DNP-vaccine was responsible for this cytotoxicity. Of the 40 evaluable patients, only five had clinical responses, four complete and one partial, with a median duration of 10 months. One patient remained melanoma free for 10 years after treatment. In the same publication [30], Berd et al. looked at the antitumor effects of DNP vaccination as a postoperative adjuvant therapy; they compared 41 patients treated with the vaccine after surgical resection to 22 patients who received surgical resection with administration of unhaptenated cells. They used the nodal melanoma metastases to prepare the vaccine. Patients received i.d. DNP vaccinations in 4-week intervals and CY was given 3 days before the first 2 vaccinations. The results correlated to a 3-year disease-free survival of 59% for the patients vaccinated with hapten-melanoma cells compared to about 24% for the patients that received the unhaptenated melanoma cells, suggesting that a good clinical response depended on the haptenation of the injected melanoma cells. Neither the immune-correlates nor tumor inflammation for this trial were fully corroborated. This

was only a short and small study, so it is hard to make concrete conclusions from this, although it indicates that DNP-vaccination is more useful as a postadjuvant therapy with less tumor burden. Of note, the control unhaptenated vaccine used in this study was not included for any of the subsequent trials [24–29].

Sato et al. [29] studied the immune response induced by the DNP-modified vaccine in these trials. They collected serum and peripheral blood lymphocytes (PBL) from 27 patients before DNFB sensitization (day 0), after DNFB sensitization (2 weeks), after two vaccinations (day 63), after four vaccinations (day 119), after six vaccinations (day 175), and after eight vaccinations (day 231) for immunologic study. There were DTH responses to DNP-modified autologous PBL and melanoma cells, although DTH responses to unmodified cells were not reported. They detected the development of anti-DNP antibody in 24 of 27 patients that was not induced by DNFB sensitization alone. Peripheral blood lymphocytes from 8 of 11 patients were stimulated with "DNP-modified autologous lymphocytes" *in vitro*; there was no response to unconjugated or TNP-conjugated autologous lymphocytes. CD8+ and CD4+ T-cells from these stimulated PBL were able to respond to DNP-modified lymphocytes, however, only CD8+ T-cells could respond to DNP-modified melanoma cells. None of these cells were able to respond to unmodified autologous PBL or TNP modified-autologous melanoma cells. These responding CD8+ T-cells produced high amounts of IFNγ and could kill DNBS-modified autologous melanoma cells; cytolytic activity to unmodified cells was not examined. In their discussion, the authors mention that they did not see an *in vitro* reaction to unmodified melanoma cells, but state that their clinical findings still hold true and that there is inflammation of distant tumor sites. They propose that in humans, the majority of T-cells are going to be reactive to DNP-melanoma, but there may be a small subset of cells that are able to react with the unmodified melanoma cells. Of note, this has yet to be demonstrated. In this regard, they showed no reaction of the responder T-cells to unmodified melanoma cells and did not study how these responder cells would specifically respond to modified or unmodified melanoma antigens (i.e., gp100 or HMW-MAA) that are known to elicit a T-cell response [29, 136].

Sato et al. [27] further observed that the DNP-specific T-cells from patients were able to respond to small DNP-modified peptides associated with the MHC, although these responses were limited to one HPLC peptide fraction of autologous melanoma. Of note, these T-cells did not respond to unmodified peptide fractions. This paper suggests that these T-cells are not going to respond to unmodified melanoma cells, which suggests that the hapten-specific T-cells are not affecting the tumor cells and may not be the only factor in the inflammation of distant metastases as concluded by Berd et al. [24, 26, 28, 30].

In 1997, Berd et al. [28] used the DNP-vaccine as a postsurgical adjuvant treatment after resection of nodal melanoma metastases in 62 patients. They observed 45% relapse-free survival in stage III melanoma patients compared to historical controls, stage III patients from an ECOG IFNγ+ resection study and an ECOG resection only study, which showed 34% and 22%, respectively. The HLA class I phenotype (A3+A2−), number of metastases (lower), age (>50 years old), DTH to unmodified autologous melanoma, and tumor inflammation seen in patients posttreatment were all positively correlated to overall survival. There were no experiments or discussion of the antitumor mechanism occurring in the patients except for histology of resected tumors posttreatment showing lymphocyte infiltration. The data is difficult to interpret as the controls groups were historical controls, albeit the fact that the inclusion of patients in the trial was based on poor prognosis and no patient was excluded that had extranodal extension of melanoma. However, the results would have been clearer if there had been a control group that only received unhaptenated tumor cells, as done in their earlier trials [30], to ascertain the importance of the haptenation in eliciting a response. Further immunogenic studies are necessary as well as studies with appropriate controls to unravel the efficacy of haptenation. In 2004, Berd et al. [24] extended the 1997 study to 214 patients with 5-year overall survival of 44%. Patients with DTH responses to unmodified autologous melanoma had a 5-year overall survival of 59%, double that of the DTH-negative group, whereas DTH to DNP-modified melanoma gave no overall survival benefit. They retrospectively observed that a baseline skin test with the DNP-vaccine before the start of treatment (on day −8 and −3) acted as an induction dose, which increased the overall survival of patients. As much of the data was based on clinical observations, there was no direct immune correlation between the vaccine and the tumor responses [24, 28].

Berd et al. [26] used the DNP-vaccine to treat pulmonary melanoma metastases in 97 stage IV patients. In this study, 11 responses out of 83 evaluable patients, two complete, four partial, and five mixed, were observed. The study describes several case reports of patients who had tumor regression of pulmonary metastases. Along with this, only 27 of 83 (33%) patients had tumor inflammation following the DNP-vaccine; lymphocytes and CD3+ cells infiltrated these tumors. Beside this, there were no immune correlates studied in this paper and it is difficult to know whether treatment caused the observed clinical outcome.

Manne et al. [25] studied the TCR rearrangement of the associated TILs in inflamed melanoma metastases after the DNP-vaccine. They observed that 9 of 10 inflamed tumor samples had dominant peaks in the same Vβ families. However, it was not tested if these TCRs were melanoma antigen-specific or if they could recognize unmodified melanoma cells.

The clinical trials using DNP-vaccine [24, 26, 28, 30] lack immunologic data linking the DNP-vaccine to an immunologic response at unmodified melanoma sites. The main focus of these papers seems to be T-cell responses, when it is now clear that multiple different cell subsets are involved in hapten responses; NK cells, iNKT-cells, Mast cells, B-1 cells, and neutrophils should have been considered in this study and could have been causing the distant tumor inflammation they observed. Along with this, there was no direct comparison of the DNP-treatment versus same the vaccine without DNP-modification after the first clinical trial, making it hard to

know the efficacy of the subsequent trials. Lastly, there is no data showing the efficacy of the *in vitro* haptenation, as it is likely that there were a small percentage of unmodified cells present in the vaccine that could have elicited the inflammation seen in the tumors.

Sojka et al. [31] extended these clinical trial protocols as a postsurgical adjuvant therapy for 410.1 mammary carcinoma-bearing Balb/c mice. Tumors were surgically excised before vaccination. Four to six days after excision, CY was i.p. injected followed by an s.c. injection (every 10 days for the duration of the experiment) of either unmodified or DNP-modified, irradiated 410.4 tumor cells with BCG. Importantly, the clinical trials by Berd's group injected the vaccine intradermally [24–30], whereas Sojka et al. [31] injected subcutaneously, which greatly alters the immune responses occurring. The DNP-modified treatment resulted in about 40% relapse-free survival of the mice, while the unmodified treatment was about 20%. They looked at multiple different parameters of the DNP vaccine to see what portions of the treatment were important and to study some immune correlates to the vaccine. There was a significant increase in relapse-free survival when using CY pretreatment. Relapse-free survival decreased with the depletion of CD4+ or CD8+ T-cells. The draining lymph node cells from mice showed a significant increase of IFNγ production when given DNP-modified versus unmodified vaccine. Lastly, there was a significant decrease in relapse-free survival when neutralizing IFNγ or TNFα. Surprisingly, the mice in this study were not sensitized to DNP before immunization, as done in Berd et al.'s clinical trials [24, 26, 28, 30] and shown to be crucial for antitumor responses. This study demonstrates a clear immunologic correlation between the hapten-modified vaccine and relapse-free survival of mice with mammary cancer, but does not fully explain the mechanism of this antitumor immune response. Importantly, this model is not representative of the clinical trials as it uses a different injection method than the clinical trials and does not use DNP-sensitization, likely eliciting a different response.

4.3. Plausible Immunologic Reactions Linked to Ex Vivo Haptenation. The immune responses occurring in *ex vivo* haptenation that elicit antitumor immunity are dependent on the injection site. Miller and Claman [142] and Mekori and Claman [143] showed that i.v. injection of DNP-modified cells induced tolerance to CHS-like reactions in mice. They further observed that the repeated i.v. injection of haptenated cells induced "desensitization" [143, 144]. Considering this issue, the anti-tumor immune studies dealt with administration of *ex vivo* haptenated-cells in three ways, i.p. (Hamaoka et al. [21] and Fujiwara et al. [22]), i.d., (Berd et al. [24, 26, 28, 30], Sato et al. [27, 29], and Manne et al. [25]), or s.c. (Flood et al. [23] and Sojka et al. [31]) injection, most likely to avoid tolerance and to take advantage of different immune responses. However, much of the mechanisms described below are not supported by experimentation, only by inference.

The mechanism of antitumor immunity after *ex vivo* haptenation by i.p. injection is probably similar to the classic hapten-protein response. B-cells in area of injection likely recognized the hapten-protein conjugates. Sensitization with the TNP-MGG conjugate causes initial recognition by B cells. The conjugate would have been taken up and processed, upon which the conjugate-protein would be presented to CD4+ helper T-cells causing cross-activation of both the T-cell and the B-cell. This would have caused the B-cell to produce antibodies against the hapten, the protein, and the conjugate [2] and would have caused the CD4+ T-cell to elicit hapten-antigen specific responses [108]. It is also possible that the antihapten/antitumor IgM and IgG bound to haptenated cells, inducing ADCC and/or opsonization by macrophages. In terms of the work by Hamaoka et al. [21] and Fujiwara et al. [22], the sensitization would form B-cells specific for the TNP, MGG, and TNP-MGG. Upon secondary stimulation with TNP-X5563, the TNP-specific B-cells would quickly recognize the TNP and produce hapten-specific IgM, binding TNP-X5563 cells and allowing for opsonization by macrophages or ADCC. This would have produced TNP-modified X5563 tumor antigens that could have been recognized and processed by the hapten-specific B-cells causing further cross-activation and the formation of CD4+ T-cells specific for X5563 cells. These CD4+ T-cells would have likely produced Th1 cytokines, like IFNγ and IL-2, stimulating X5563-specific effector T-cell clones already present in the animal allowing for cytotoxic responses to the tumor. It is also distinctly possible that one of the reasons their treatment was not very effective was because they modified the tumor cells with TNBS, which keeps cells viable [145]. This means that hapten-modified or unmodified protein was not immediately available for B-cells to process and elicit a quick reaction. Using a toxic hapten, like TNCB [146], may have made antigen more readily available for B-cells to process due to the tumor cell death.

The antitumor mechanism that was elicited from s.c. administration of *ex vivo* haptenated cells is more difficult to interpret as these studies used very different mouse models and delivery systems. Flood et al.'s [23] method likely induced a response similar to that described with the i.p. injections. When injected into the animal, the regressor tumor cells likely had cytotoxic T-cells that were specific for them and could clear the tumor cells when injected into the animal. If the regressor tumors were TNP-modified, it would have allowed for the release of TNP-bound proteins from these regressor cells that were being actively killed. This would have helped B-cell and CD4+ T-cell cross-activation as described with i.p. injections. Upon second immunization, hapten-specific B cells would have recognized the TNP-bound progressor cells and caused cross-activation with CD4+ T-cells, creating B-cells and CD4+ T-cells against the progressor tumor. The activation of tumor specific B-cells would have caused antibody formation against the tumor cells, potentially inducing ADCC or opsonization. The CD4+ T-cells would have provided costimulation to cytotoxic T-cells, which are otherwise unable to clear the progressor tumor. These in combination would have likely created the tumor resistance seen in Flood et al.'s [23] study. As stated above, using a toxic hapten may have made the antigen more readily available for B-cells to process due to the tumor cell death.

Sojka et al.'s [31] method of s.c. injection is much different, as it acts as an adjuvant therapy for any established metastases after surgical resection of the primary tumor. Importantly, the removal of the tumor could have been the priming step to the immune system as surgical resection of a primary tumor can reverse tumor-induced immunosuppression, even in the presence of metastases [147]. Their vaccination protocol killed the cells via irradiation and DNFB modification [146, 148], so it is likely that there would have been much DNP-modified protein available. The vaccine was also mixed with BCG, which stimulates the innate immune system. The actual vaccination protocol probably would have induced a similar response as Flood et al.'s [23] once the treatments were started. They delivered hapten-modified protein to the immune system, which would have stimulated a strong immune response due to repeated vaccination, hence the enhanced survival of mice with established tumor metastases. The sensitization occurred from DNP-modified tumor cell protein from the first injection, inducing cross activation of B- and CD4+ T-cells as described above and subsequent responses against the tumor [31].

The protocol of i.d. injection of hapten-modified tumor cells by Berd et al. [24, 26, 28, 30] appears to be the most appropriate *ex vivo* haptenated-vaccine administration as CHS-like immune responses will likely occur. In the clinical trials, patients were mostly sensitized before administration, allowing for the vaccination to induce CHS elicitation-like reactions (Table 2 and Figure 2). Importantly, these reactions will not be as strong as typical CHS reactions due to the lack of skin haptenation and subsequent innate immune responses, as the haptenated cells were intradermally injected. The danger signal release from skin haptenation would not have occurred; meaning restimulation of keratinocytes and dermal APCs would have occurred more slowly, causing less cytokine release. Also, no "early" elicitation of CHS-initiated mechanisms would have occurred, as iNKT-cells specific for haptens would not have become activated, implying that hapten-IgM from CS initiating-B-1 cells would not be produced. Decreased keratinocyte and CS-initiating B-1 activation would reduce stimulation of mast cells and neutrophils, lowering chemokine, selectin, and adhesion molecule upregulation in the vasculature and the trafficking of hapten-specific T-cells and NK cells to the area. Despite this, there would have been involvement of hapten-specific T-cells and hepatic NK cells, as the BCG will cause stimulation of the innate immune system allowing for recognition of haptenated-antigen. BCG likely initiated peripheral immune responses unrelated to the hapten vaccine, which might have slightly inhibited the response, as the immune system could have been "busy" mounting a new response. It may have served Berd et al. [24, 26, 28, 30] to epifocally apply DNFB to the site of the i.d. injection, eliciting a CHS reaction that could have exposed the vaccine to the immune system in a CHS context. Despite all this conjecture, it is hard to know how an antitumor response would have formed as i.d. injection would elicit a hapten-specific immune response and the DNP-vaccine trials did not show much experimental evidence of antitumor immune responses occurring from the vaccination.

Another important concept to consider is that haptenation in this fashion may not have induced a bystander effect (kill distant, unmodified tumor cells via immune responses) since the process seems to be hapten-dependent. Much of the justification for the work done was based on Weltzien's group's papers between 1992 and 1997, as earlier described [63, 64, 107, 108]. In this work, they saw the ability of hapten-specific CD8+ T-cell clones to recognize and respond to hapten bound and unbound portions of small tryptic fragments of proteins suggesting some cross-reactivity of the cells. An overarching assumption is that this will be true for naturally processed proteins, like that present in the clinical trial treatments using *ex vivo* haptenation. Sato et al. [27, 29] show that DNP-specific TILs from DNP-vaccinated patients (that were not present before vaccination) were specific for only two peptide fragments from a melanoma peptide library and these fragments had to be DNP-modified. To note, no stimulation occurred with unmodified cells. Despite clinical observations of bystander effects, it is very hard to decipher what is occurring since there is not much experimental evidence in support of this claim. As stated before, it is possible that unmodified melanoma cells injected into patients with this vaccine induced an immune response along with the DNP-protein response, leading to tumor inflammation and some antimelanoma immune response. Despite all the work done on *ex vivo* haptenation, as alluded above, there is little experimental evidence to suggest that the vaccination induces direct antitumor effects even though the DNP-vaccine trials show survival impacts in patients. Along with that, the treatment is expensive and very time consuming and relies on the removal of a tumor mass, making it an untenable option.

4.4. In Situ Haptenation to Mediate Tumor Regression. Fujiwara et al. [32] seemingly abandoned their *ex vivo* tumor cell haptenation immunization for *in situ* haptenation of established tumors. They created a tumor regression model in C3H/HeN X5563 plasmacytoma tumor-bearing mice (dermal) by intratumoral injection of TNCB in TNCB sensitized C3H/HeN mice. As before, they suggested that haptenation would augment TATA helper T-cell responses to generate more powerful killer T-cell responses. They established the following method of tumor regression; pretreatment of CY, 2 days later TNCB sensitization, 5 weeks later implantation of tumor cells, ~6 day after implantation intratumoral injection of 0.15 mL 0.5% TNCB into tumor masses between 7 and 10 mm in diameter. Importantly, splenocytes from sensitized mice caused *in vitro* lysis of TNP-X5563 cells, while unprimed mice splenocytes did not. TNCB ear challenge after 5 weeks induced a response, suggesting appropriate sensitization. The spleen cells from tumor-bearing mice, stimulated *in vitro* with irradiated TNP-X5563 tumor cells, along with the addition of TNP-helper cells, resulted in appreciable augmentation of anti-X5563 cytotoxic T lymphocyte (CTL) responses. Of the fully treated mice, >50% of them had complete tumor regression, as measured by the absence of myeloma protein from the blood serum 45 days after treatment. Of these animals, 90% of them rejected a secondary tumor challenge of

Table 2: Contact hypersensitivity immune mechanisms that may lead to tumor regression.

CHS immune cell	CHS immune reaction	Plausible direct and indirect mechanisms of tumor regression
Hapten modification of epidermal cells → release of danger signals	ATP release → P2RX7 → NLRP3 activation	IL-18 and IL-1β → protection against colorectal tumorigenesis [115]
	ROS	Inhibit MDSC maturation [116]
		Induce cell death in established tumor [117]
Dermal APCs	Stimulation by haptenization	Possibly stimulate exhausted CD8+ T-cells [118, 119]
Keratinocytes	IL-18 release	Protection against colorectal tumorigenesis [116]
	IL-1β release	Polarization of IFNγ CD8+ T-cells [115]
iNKT cells	IFNγ production	Protective role dependent on Th1 cytokines [140] and antitumor activity [150]
Mast cells	TNFα and CXCL2 release	Neutrophil activation [4]
	TNFα and serotonin release	Chemokine, selectin and adhesion molecule upregulation for hapten-specific T-cell trafficking
Neutrophils	KC damage (FasL and perforin)	Potential tumor damage, although neutrophils not known to directly kill tumor cells in the first 24 hours [121, 122]
	CXCL1 and CXCL2	Chemokine, selectin and adhesion molecule upregulation for hapten-specific T-cell trafficking
CS initiating B-1 cells	Hapten-antibody production	Hapten-tumor IgM → ADCC
CD8+ T-cells	IFNγ	TIL activation [125] and antitumor activity [150]
	Hapten-specific CD8+ T-cells	Haptenated-tumor cell killing
	Infiltration into CHS site	Tumor-infiltrating lymphocytes [125]
CD4+ T-cells	Hapten-specific	Rescue exhausted CD8+ T-cells [123]
Tc17/Th17	IL-17 CD4+ and CD8+ Cells	Antitumor immune responses [126, 127]
Hepatic NK cells	Hapten-specific NK-cells	Hapten-tumor cell killing [128]

→: Leads to ...

1/10th the original tumor cells, although the data is not shown. An issue of this study is that 0.15 mL of solution was injected into tumors regardless of their size, meaning that smaller tumors would have increased haptenation and *vice versa*. It is possible that the animals that responded all had smaller tumors, although this was not recorded or mentioned in the study. Large injection volumes could potentially cause the tumor microenvironment to be destroyed, causing tumor cell spillage into the animal. The destruction of tumors sites could have also induced enhanced DNP-tumor reactions by the animal due to better availability of the tumor cells. Although this was the first model of *in situ* haptenation of a tumor and subsequent tumor regression, the mechanism remains unclear.

Fujiwara et al. [33] furthered their method by showing secondary challenge and neutralization data as well as repeating it in multiple model tumor systems. They repeated their results in the X5563 system, showing that 4 of 5 mice had tumor regression. Myeloma protein was not present in their serum for up to 2 months after regression. They challenged mice with only 10^5 X5563 cells (1/10 of the primary inoculation) intradermally showing that 11 of 12 of the mice could resist the tumor, compared to 0 of 10 in naïve mice or 2 of 10 in surgically resected mice (this data was not shown in their previous paper). Conversely, they do not show the tumor growth in these injections and use the word "resistance," which would imply that the tumors still grew after the challenge, even if the treatment slowed their growth. This is supported by Winn assays at low E : T ratios that shows slight tumor growth 12 days after secondary tumor challenge. In addition, Fujiwara et al. [32] established TNP-mediated tumor regression in mice with methylcholanthrene (MCA)-induced transplantable tumor cells (MCH-1-A1) and MCA-induced autochthonous tumors using similar methods. The MCH-1-A1 showed similar primary tumor regression as that of the X5563 model. For the inducible system, 11 of 25 of the animals had tumor regression for up to 12 weeks. To note, many of the regressed tumors stayed at a constant size or slowly decreased in size for about 5 weeks after TNCB injection, there after dramatically increasing or decreasing in size. The reproducibility of tumor regression in different tumor models is encouraging, but the fact that the secondary tumor challenges were only resisted and not rejected suggests that this method may not induce strong antitumor immune responses and may be hapten-dependent [33].

4.5. Plausible Immunologic Reactions Linked to In Situ Haptenation. In situ haptenation offers the most challenging explanation of what occurs, as it relies on the immune cells present inside the tumor microenvironment to elicit responses. It is likely that the haptenation of tumor cells will cause massive amounts of cell death, as typically seen from haptenation [146], of not only the tumor cells but any

of the stromal cells associated with the tumor. This will cause the release of many danger signals and haptenated protein, which will stimulate APC present in or near the tumor, tumor-infiltrating dendritic cells. These dendritic cells may migrate to the dLN where it is possible that it will stimulate a T-cell response to the tumor antigen [149]. Fujiwara et al. [33] concluded that two mechanisms might have occurred to cause tumor regression: (1) a DTH response to the TNP-modification of tumor cells, eliciting anti-TNP CTL, B cells, and DTH responses in the tumor site or (2) the bystander effect of anti-TNP CTL by amplification of anti-TNP helper T-cell activity. Neither of these mechanisms has been confirmed, but the extensive mechanisms of CHS were not as clear in 1984, so it is likely that the mechanisms are far more complicated than that, and that there are a slew of CHS-effectors involved in the tumor regression. As highlighted before, there is no experimental or mechanistic explanation of a bystander effect, only observational.

The mechanisms of contact hypersensitivity are hard to apply to this context, as the reactions are being induced in a tumor suppressive environment, which may not include many immune cell types [150]. On top of this, the induction of hapten-mediated cell death must be considered, as it likely induces tumor regression and immune responses (Table 2). It is very possible that the tumor regression is due to cell death of all the tumor cells or some combination of cell death and haptenation of the tumor cells. When speculating in this context, it is important to remember that tumor cell death in the tumor can elicit antitumor immune responses, although the type of cell death necessary to mediate immunity remains unclear. As seen in Table 2, it has been shown that in some systems, autophagy from chemotherapy induced the release of HMGB1 and ATP, causing the recruitment and activation of dendritic cells and T-cells [120]. The ATP release may be similar to that seen in CHS, where hapten modification causes ATP release, stimulation of PSRX7 on dendritic cells, and NLRP3 activation. This leads to IL-18 and IL-1β release, which can activate dendritic cells in the area. Along with this, haptenation of the tumors may induce the upregulation of CHS chemokines, selectins, and adhesion molecules in the tumor vasculature, causing recruitment of hapten-specific T and NK cells. This could aid in primary tumor regression. Fujiwara et al. [32, 33] used a relatively high concentration of TNCB in large injection volumes, so it is plausible that many of the cancer cells were going to be TNP-bound and died. Low concentrations of haptens induce apoptosis, and higher concentrations, like used in Fujiwara's work, seem to cause necrosis [146, 148]. Hapten-mediated cell death must be considered as a viable mechanism for *in situ* haptenation-induced tumor regression. Theoretically comparing hydrophobic and hydrophilic haptens, such as TNCB and TNBS, respectively, could test this, where TNCB kills bound-cells and TNBS allows further proliferation and growth of bound-cells. A tumor regression experiment using *in situ* haptenation injection with these two haptens (separately) in hapten-sensitized mice would show if it is the TNP haptenation leading to antitumor immune responses, the hapten-mediated cell death that is eliciting tumor regression, or some combination of both.

4.6. Epifocal Hapten Application Leading to a CHS-Like Immune Reaction at the Tumor Site

4.6.1. Use of Epifocal Hapten Application to Induce Viral Wart Regression. The contact allergens for topical treatments of various dermatological problems, such as alopecia areata, viral warts, and some cutaneous tumors, have been used since the 1960s. Buckley and Vivier [18] reviewed many of the clinical trials using contact sensitizers to induce viral wart regression. They pointed out that very few of these studies had the proper control groups or randomization, making many of the observations biased and hard to gather conclusions from. The sensitizers mainly used for these trials were DNCB, a potent contact allergen and mutagen first used in 1912, squaric acid dibutyl ester (SADBE), a potent contact allergen first used in 1979, nonmutagenic, and commonly used to treat viral warts in Europe and Southeast Asia, and Diphencyprone (DPCP), a potent contact allergen in humans and animals, nonmutagenic, and commercially available in the UK. All patients given this treatment were usually sensitized under the armpit with ~2% solutions of the hapten. The hapten was then applied to the warts at a concentration of 0.1% (depending on location) and was increased depending on the reaction seen. Application was stopped when there were no visible warts. The mechanism of action for these contact allergens affecting viral warts is not well investigated, although it is theorized that the allergen application induces alterations in cytokine levels, nonspecific inflammation causing wart regression, and haptenation inducing hapten-specific immune responses [18]. It is likely that CHS/ACD-like reactions are occurring in the wart site, although there is little evidence for this. It was seen that CD8+ T-cells infiltrate into warts upon DPCP application, and DNCB application can increase complement-binding wart virus-specific antibodies. Overall, the clearances of warts ranged from 7 to 100% in the trials with a median clearance rate of 62%. It was also seen that long-term, hapten-dependent treatment was needed to cause regression [18].

Upitis and Krol [19] conducted a clinical trial using the hapten diphenylcyclopropenone (DPC) to treat recalcitrant palmoplantar and periungual warts. The study had 154 patients, all of which were sensitized to DPC; 135 of which had complete clearance of warts with an average of 5 treatments over 6 months. There were very few side effects to the treatments, leading the authors to the conclusion that DPC should be considered as a first line treatment for warts. However, the mechanism of action is not well explained. A more recent clinical study [124], treated six facial wart patients, who were not responding to other treatments, with DPCP. Patients were sensitized to 2% DPCP as described above, and various concentrations of DPCP were applied to the warts of interest in 8–10 sessions. Four of six patients had complete disappearance of the warts with no recurrence for a year and the other two patients had improved warts. Once again, the mechanism of action is unknown in this study [124]. Both of these studies seemed to be hapten-dependent phenomena.

Despite the evidence suggesting that contact allergen application can treat warts, warts are known to spontaneously

regress and disappear. Many of these studies were over one year, and very frequently, warts will spontaneously regress within a one- to two-year period. Along with this, the mechanism of this viral wart regression remains largely unknown and needs further elucidation, although it is likely that a hapten-dependent CHS-like immune response would have occurred, as most patients were sensitized to the hapten prior to use.

4.6.2. Use of Epifocal Hapten Application to Induce Tumor Regression. Epifocal hapten application at cutaneous tumor sites to elicit CHS-like immune reactions and primary tumor regression is a long-established and appealing concept. Edmund Klein reviewed multiple clinical uses of epifocal hapten application for the treatment of cutaneous cancers [34]. He assessed studies on cutaneous neoplasms, where treatment of epitheliomas using chemotherapy was compared to hapten-induced (2,3,5-triethyleneiminobenzoquinone) [TEIB] and DNCB cutaneous hypersensitivity reactions at the tumor site. These cutaneous hypersensitivity reactions at the tumor site resulted in the regression of superficial basal cell carcinomas (BCC), squamous cell carcinomas (SCC) *in situ*, and premalignant keratosis. In particular, multiple studies on patients with BCC where hypersensitivity was induced by topical application of cream containing 0.05% TEIB were described. A case study was done on one patient receiving this treatment, who had regression of several hundred basal cell carcinomas after 3 weeks of daily topical application. The tumors would become eurythmic, exudated, and necrotic within 24 hours of application. The patient had no recurrence of regressed lesions for 5 years after treatments. Whenever the patient developed new lesions in different sites, the cream was applied and the tumors would disappear. There were also several studies performed on squamous cell carcinoma. The carcinomas *in situ* responded very well to topical challenge with TEIB or DNCB and the reaction was similar to that seen in the basal cell carcinomas. More than 90% of the lesions underwent regression following the hapten challenge, although the deeper lesions responded poorly and did not fully regress, needing secondary treatment with the hapten, chemotherapy, or other standard treatment to eradicate it. These studies clearly demonstrate the powerful ability of haptens to cause CHS reactions in epidermal tumor sites to cause local tumor regression. To note, the hapten-mediated tumor regression did not cause regression of untreated tumors suggesting that hapten-dependent tumor regression was mediated by cell death and/or CHS-like reactions [34].

Epifocal hapten application has been used to topically treat metastatic cutaneous melanoma since 1973. Truchetet et al. [113] reviewed the use of DNCB in the treatment of metastatic melanoma in the clinical settings. Most of these studies used epifocal DNCB application at a concentration of 1–10% in acetone, some using sensitization and some not. In 1978, Loth and Ehring [151] tried the treatment in 35 patients, nine of whom had a favorable response. In 1981, Picrard et al. [152] described 86 cases of primary melanoma with or without metastases treated with DNCB after sensitization. The tumors were excised at multiple time points after treatment. All the patients benefitted from the epifocal applications of DNCB on tumor and normal skin between the primary melanoma and excision of metastases. The 5-year survival was 77% with DNCB application before and after resection versus 70% with DNCB application only after resection. There was no survival benefit seen when the disease had spread to the lymph node. They state that DNCB treatments are only useful for local recurrences and skin metastases, not surgically inaccessible regions. This would imply that the reaction is directly hapten-dependent and a bystander effect is not occurring in a majority of patients as the reactions may be limited to the skin lesions. The mechanism of tumor regression and whether it is mediated by hapten-cell death or CHS like immune reactions was not studied.

Strobbe et al. [35] treated 59 recurrent melanoma patients with a combination of topical DNCB and systemic dacarbazine (DTIC). Patients were sensitized to 2% DNCB on their cutaneous metastasis on day 1 and day 8, followed by additional treatment on day 15. Topical treatments were administered three times per week for 2 weeks. DTIC treatment was started 4 weeks after the first DNCB application with 3 consecutive doses of 400 mg/m^2, a single dose of 800 mg/m^2, or 5 consecutive doses of 250 mg/m^2 and repeated every 3-4 weeks. Of the 59 patients, 15 (25%) had a complete response, 7 (12%) had partial response or stable disease, and 37 (65%) had tumor progression. The overall 5-year survival was 15%, with a median survival of 10 months. The median survival of the group with complete response was 50 months. The presence of severe local reaction to topical DNCB application correlated with improved overall survival. Of the 15 complete responders, 5 patients exhibited a 5-year durable response. Besides these observations, there are no immune correlates reported in this study. This study does not compare the data collected to DTIC only treated patients, which is reported to have a 10.2% response rate in stage IV melanoma patients [153]. DNCB treatment only was also not studied, making it difficult to determine which treatment had an effect. However, they did state that no DNCB-treated lesions disappeared until the start of DTIC treatment. Along with this, they sensitized patients at the tumor site, potentially diminishing the immune reactions as tumors are immune-suppressive. It would have made more impact if the hapten sensitization was given elsewhere as done in many other clinical settings using contact sensitizers to treat metastatic melanoma. Although this study shows a few patients responding to the treatment, the data is not strong enough to suggest a positive response to the treatment.

There have been many case studies using epifocal DNCB or DPCP treatments for melanoma metastases [36, 38–40]. von Nida and Quirk [36] described a patient who was sensitized to 2% DNCB on normal skin and once the appearance of low-grade eczema appeared at that site, the patient was instructed to apply 2% DNCB to the tumor nodules. Within 2 weeks, eczema-like reactions appeared at each site and tumors were all regressing. Tumor nodules continued to appear and regress with treatment for the next 2 years. This

went on for 7 years until the patient had liver metastasis and succumbed to the disease. The DNCB treatment in this case seemed to slow the progression of disease by treating cutaneous lesions in a hapten-dependent manner but did not ultimately stop the disease from metastasizing [36]. Damian et al. [39] described seven case studies of metastatic melanoma patients who were sensitized with 2 drops of 2% DPCP in acetone on the upper inner arm for 48 hours. Two weeks after sensitization, DPCP aqueous cream was applied weekly to all cutaneous melanoma metastases. All of them had either slowing of tumor growth or regression of tumors where the DPCP was applied. Three of the patients succumbed to the disease due to metastases within 5 weeks to 19 months, but four were alive at the time of publication. In a follow-up study, the role of Th17 cells in one patient who remained free of cutaneous and regional disease for 4.5 years after DPCP and DTIC treatment of the disease was reported [38]. They observed lymphocyte infiltration into the tumor after treatment marked by "cells [that] display typical morphologic characteristics of melanophages." However, no specific immunologic stains were performed. RNA expression analysis revealed upregulation of the human Th17 genes (L-17A/B/C/D/E/F; CD27; CD70; PLZF-1; CTLA-4 FoxP3 and RORγT) in the posttreatment tissue sections. This was not confirmed by looking at the presence of Th17-associated protein or increased Th17 cell infiltration [38]. Lastly, another group [40] reported a patient treated with the same method as Damian et al., [39] which had regression of melanoma nodules on the ankle for up to 18 weeks. This area was dry and eczematous with the appearance of numerous eosinophils (determined by H&E statin, no specific eosinophil markers) and no melanoma (HMB-45 stain).

There was a case report by Herrmann et al. [114] showing complete regression of Merkel cell carcinoma in the scalp 1 year after treatment using a topical DNCB treatment. The patient was sensitized to 2% DNCB and DNCB was applied to the lesions for 4 subsequent weeks. H&E immunostaining of biopsied specimens showed infiltration of CD3+ T-cells and CD28+, KP-1+ Macrophages. To note, mitoses of the tumor cells were still present, but much less frequent than before treatment.

Although these case studies [36, 38–40] suggest a beneficial aspect of the DNCB or DPCP treatment, it is difficult to interpret these results, as case reports are typically the best-case scenario and are from rare patients that have a response. Along with this, it is challenging to compare the study by Strobbe et al. [35] and the case studies [36, 38–40], as Strobbe et al. [35] sensitized patients at the tumor site, which is immune-suppressive and may have dampened sensitization, while the case studies sensitized patients at distant skin sites, allowing for appropriate sensitization. Something that all these studies do show is that the tumor regression seems to be hapten-dependent and seems to not induce a bystander effect, evident from metastases formation. There were very few immune correlations made in any of these studies, only visual observations, making it difficult to interpret how these treatments are inducing tumor regression. It would be interesting to expand the observations by Klein [34] and perform a controlled trial in BCC or SCC patients to establish if this method can indeed induce tumor regression, decrease recurrence of metastatic disease, and potentially increase the patient survival.

Wack et al. [42] created a mouse melanoma model based on Strobbe et al.'s [35] work utilizing DTIC and DNCB and examined the tumor regression mechanisms in B16F17, slow growing B16 substrain, bearing C57BL/6 mice. Seven days after subcutaneous tumor inoculation, when the tumor was 25 μL in volume, mice were treated with i.p. injection of DTIC and/or epifocal (on the skin of the tumor site) DNCB application (25 μL in acetone and olive oil, 4 : 1) 24 hours later. The concentration of both DTIC and DNCB was optimized to be 50 mg/kg DTIC on days 7, 12, 16, and 20 and 3% DNCB on day 8 (to mimic CHS sensitization) and 1% DNCB on days 12, 16, and 20. This treatment regimen resulted in tumor regression and tumor-free mice for up to 150 days in 72% of mice. Lastly, whether or not this treatment would cause tumor regression or resistance of B16F17 lung metastases injected i.v. on day 7 was tested. The combined treatment of DTIC and DNCB was started on Day 9. DTIC and DNCB combination treated mice had significantly less lung metastases than the control and untreated groups 30 days after inoculation. Interestingly, there was no single treatment controls used in many of these experiments, making it difficult to see the effect of the combined treatment compared to the individual treatment effects.

This work has three large issues. (1) The animals were not sensitized to DNCB using normal sensitization procedures. Typically, for CHS reactions, animals are sensitized to the hapten five days before challenge and are sensitized on the distant area (usually the abdomen) from the challenge. This ensures that any reaction being elicited is truly an immune response. The effective sensitization time for DNCB (5 days) was not given and moreover, the sensitization was elicited on the tumor, which is immune suppressive. These two factors probably reduced the sensitization efficacy significantly. The authors mention that they tested the sensitization of different percentages of DNCB using the ear-swelling test, but it is unclear if the DNCB in this setting was applied on the tumor or in a different area of the animal. If the ear-swelling test was performed after sensitization at the tumor site, it would have been prudent to compare the ear swelling to mice sensitized at a nontumor site to see if the sensitization was affected by doing it at the tumor site. (2) All of the tumor measurements here are mean tumor volumes, yet there are no standard deviation or error bars on any of the points. It is difficult to tell what the range of data is and its relative significance. (3) Appropriate controls were not used for each experiment; DTIC treatment alone or DNCB treatment alone was given in the first figure and did not reflect in any subsequent figure. This makes the results difficult to interpret because it is unclear if it is the combination treatment or just a single treatment that caused the observed primary or pulmonary tumor regression.

Wack et al. [41] performed a follow-up study using this model to look into the antitumor immune responses elicited by the DTIC/DNCB combination treatment. Once again, there were no single treatment controls in any of their experiments. They first repeated their previous results

showing that 5 of 7 mice underwent complete tumor regression in the 35-day observation period. They looked at the incidence of pulmonary tumors after 7 treatments (the last study used only 4 treatments [42]) and observed that there were only 7 ± 4 tumors in the combination group versus 133 ± 31 in control mice. Splenocytes from treated animals that underwent primary tumor regression were tested for their ability to kill B16-melanoma cells *in vitro* using ^{51}Cr-release assay. The cytotoxicity of splenocytes from treated animals toward B16s was 3 times higher than control animals; these splenocytes also released more IFNγ. MACS isolated and *in vitro* restimulated CD4+ and CD8+ T-cells each from treated splenocytes had higher killing than the control, whereas the NK cells had similar killing as the control. The similar NK cell killing was expected, as NK cells involved in CHS are derived from the liver [75] and not the spleen. Ability of TILs from the primary B16 tumor to kill B16 melanoma cells and release IFNγ *in vitro* was higher in the treated versus untreated animals. These cells also had high mRNA levels of IFNγ, TNFα, and IL-6. Using Rag$^{-/-}$ mice, the paper also showed that tumor regression was dependent on T-cells and that this model was repeatable with another hapten, Oxazolone [41].

However, this study also has three large issues. (1) As highlighted before, the single treatment controls were not looked at for any experiment, making it hard to tell if the ability of immune cells to kill or produce cytokines *in vitro* is from the combination of treatments or just one treatment alone. (2) For the cytotoxicity studies using CD4+ T-cells, CD8+ T-cells, and NK cells, they only stimulate these cells with melanoma *in vitro*, not stimulating the cells with DNP-modified melanoma to see if this has an ability to cause cytotoxicity. It is very likely that NK cells will not kill unhaptenated cells because of inhibitory molecules binding to MHC, as previously described. It is hard to draw conclusions from these cytotoxicity assays as stimulation with melanoma and DNP-bound melanoma was not compared. (3) The study used immune cells from the spleen, even though it is commonly known that CHS-related T-cells mature and preside in the draining lymph nodes and CHS-related NK cells reside in the liver. It is very possible that the collected cells had nothing to do with the treatment.

Despite the highlighted issues, these two papers establish the only mouse-model of tumor regression utilizing epifocal hapten application. However, these papers do not elucidate how the tumor regression is being mediated. To further elucidate the validity of this method, these experiments would need to be repeated with all the appropriate single treatment controls taking into consideration the extensive issues present in each paper.

4.6.3. Plausible Immunologic Reactions Linked to Epifocal Hapten Application. When considering the use of epifocal hapten application to induce CHS-like immune reactions at the tumor site, two aspects must be taken into account: (1) haptens will induce cell death and CHS-like immune reactions that may be able to cause tumor regression by utilizing the extensive immune cell milieu (Table 2). (2) Haptens will induce CHS-like immune reactions that may lead to tumor cell growth and increased immune suppression (Table 3).

It is likely that epifocal hapten application induces tumor regression through CHS-like mechanisms (Table 2). First, epifocal hapten application would induce massive cell death in the tumor as any haptenated tumor cell would likely die. In a hapten presensitized animal, tumor haptenation and cell death will cause the release of danger signals, ATP, and ROS. These signals will help induce immune cells in the surrounding tissue. ATP release will induce P2RX7, which will cause the activation of NLRP3 on APCs, eliciting the production of IL-18 and IL-1β; these elicit protection against colorectal tumorigenesis by polarizing IFNγ+ CD8+ T-cells against tumors in the context of chemotherapy [115]. Release of ROS has the ability to inhibit myeloid derived suppressor cell (MDSC) maturation, known to suppress immune responses against tumors by releasing IL-10 [116], and induce cell death of tumor cells in the established tumor [117]. The stimulation of APCs by danger signals could potentially reactivate exhausted CD8+ T-cells in the tumor microenvironment as DCs are linked to T-cell exhaustion [118, 119] or help APCs traffic to the lymph node to establish new CD8+ effector T-cells. iNKT-cells, activated by CD1d presentation of haptenated tumor glycolipids, and $\gamma\delta$ T-cells will work together to produce IFNγ, which has an antitumor protective role as a potent Th1 cytokine [140] and mediates antitumor activity [150]. iNKT-cell activation will also lead to IL-4 release causing the activation of CS-initiating B-1 cells to produce Hapten-Tumor IgM. This antibody could potentially lead to the coating of cancer cells and subsequent ADCC. This hapten-tumor IgM will also lead to the activation of mast cells which will release TNFα and CXCL2, causing cause FasL+, perforin+ neutrophil cell infiltration. These neutrophils may be able to kill the tumor cells in the first 24 hours [121, 122] and provoke release of CXCL1 and CXCL2 from the surrounding tissue, helping T-cells traffic to the tumor site. The mast cells will also release TNFα and Serotonin, causing upregulation of chemokines, selectins, and adhesion molecules and subsequent hapten-specific T-cell to trafficking to the tumor. Hapten-specific CD8+ T-cells will enter the area and produce IFNγ, which can help to stimulate other effector TILs in the area [125] and cause antitumor activity [150]. Along with this, the entry of hapten-specific CD4+ T-cells could potentially rescue exhausted CD8+ T-cells, as seen in chronic viral infections [123]. The entry of Tc17 and Th17 cells could elicit multiple antitumor immune responses, as CD4+ and CD8+ IL-17 producing T-cells have been shown to elicit tumor regression in melanoma mouse models [126, 127]. Lastly, hapten application could induce the infiltration of CXCR6+ Hepatic NK cells, which may be able to cause tumor cell death once in the site [128]. Despite all the possible reactions that could occur, it is difficult to say if and how these responses would lead to a bystander tumor effect, as there is little evidence for the functionality of hapten-effector cross-reactivity. The only process that could lead to a bystander effect is the massive amount of cell death that occurs from haptenation, causing the release of tumor antigens into the animal and potential immune recognition against these antigens.

Table 3: Contact hypersensitivity reactions that may lead to tumor growth.

CHS immune cell	CHS immune reaction	Plausible direct effect on tumor	Plausible immune suppression that may lead to tumor growth
Hapten modification of epidermal cells → release of danger signals	Prostaglandin E2 (PGE2) release	Colon cancer growth [137]	MDSCs activation [116]
	ROS release	Angiogenesis through VEGF [138]	Nitration of T-cell-peptide-MHC interaction → T-cell suppression [116]
	ATP release → P2RX7 → NLRP3 activation	N/A	Decreased tumor responsiveness to vaccination [115]
LCs and dDCs	TLR4 and 2 Stimulation	N/A	Immune evasion and myeloid cells to promote metastases [115, 116]
Keratinocytes	IL-1β, IL-6, IL-18, and TNFα	N/A	MDSCs recruitment and infiltration → IL-10 production in tumor site [116]
	CXCL10 Upregulation	Angiogenesis [139]	N/A
iNKT cells	IL-4 and IL-13	N/A	MDSCs and M2MΦ recruitment and infiltration → IL-10 and TGFβ production in tumor site [116]; Suppression of tumor-specific CD8+ T-cells [140]
Mast cells	CCL2 and CCL5 upregulation	N/A	TAMs (IL-10 high, IL-12 low, IL-1Rα high, and IL-1decoyR high) → IL-10, angiogenesis, tumor metastasis stimulation, TGFβ, TNFα, IL-1α [116]; MDSCs recruitment and infiltration → IL-10 production in tumor site [116]
	TNFα	Oxygen delivery to hypoxic tumor cells [116]	N/A
	CXCL2	Melanoma cell proliferation [139]	N/A
Neutrophils	CXCL1 and CXCL2	Melanoma cell proliferation [116, 139]	N/A
Hapten-specific T-regs	IL-10	N/A	Effector T-cell suppression [141]
	CTLA-4	N/A	CD8+ T-cell exhaustion [118]

→: Leads to . . .

There are many aspects of CHS-like reactions that could cause tumor immune suppression and promote tumor cell growth, instead of regression. Bock et al. [154] looked at the ability of continuous DNFB exposure to cause toxicity and tumor formation in multiple different mouse strains. In this study, the animals were exposed to one dose of 7,12-dimethylbenz[α]anthracene (DMBA), a known cancer causing agent, and then applied 0.1% DNFB to the site 5 times a week for 14–50 weeks starting 21 days after the DMBA. This caused 35/50 Swiss, 6/30 C57BL/6, and 5/30 Balb/c mice to form tumors. DMBA treatment alone resulted in very low incidence of tumors, 2/50 Swiss, and 0/30 C57BL/6 and Balb/c mice, respectively. There were no tumor formations in Swiss mice (0/50) that were treated with only DNFB. The data suggest that although DNFB is not a causative agent of cancers, it is a tumor-promoting agent and can possibly cause tumor formation in predisposed conditions or already growing tumors with repeated exposure. It is important to note that massive amounts of DNFB were given to these animals over very long periods of time and the mechanism of hapten-mediated tumor promotion was not discussed.

An extensive 24-year study, between 1984 and 2008, by Engkilde et al. [155] looked at the association between contact allergy by small chemicals and cancer incidence. The group patch tested, a way of identifying whether a small molecule causes skin inflammation upon contact, 16,922 patients (6,113 men and 10,809 women), 35.8% of which had a positive reaction to at least one allergen. These results were linked to the Danish Cancer Registry, where the group saw that 3,200 (18.9%) of the dermatitis patients had some type of cancer and that 1,207 (37.7%) of these patients had a positive patch test. The group found significant correlations between contact allergy and bladder, breast, and skin (nonmelanoma) cancer regardless of sex. There was also an inverse correlation between a positive patch test and brain/CNS cancer in women. This study underscores that the reactions causing ACD, like those involved in CHS, may be associated with cancer in certain cases.

We have conceptualized some of the possible mechanisms of hapten-induced CHS promoting tumor immune suppression and tumor growth (Table 3). Epifocal application of a hapten will cause the release of danger signals, such as PGE2, ROS, and ATP. PGE2 release has been seen to induce

colon cancer growth [137] and cause MDSC activation in the tumor site. ROS release is known to upregulate VEGF, promoting angiogenesis in tumor sites [138], and possibly cause the nitration of T-cell-peptide-MHC interactions, inducing T-cell suppression [116]. ATP release will induce P2RX7, which will cause the activation of NLRP3 on dermal APCs, eliciting the production of IL-1β and IL-18 which has been shown to decrease the tumor responsiveness to certain vaccinations [115]. The danger signal release will cause TLR4 and TLR2 stimulation of dermal APCs, which has been shown to elicit immune evasion by helping myeloid cells establish metastases via TGF-β [115, 116]. Haptenation will also cause keratinocytes to release IL-1β, IL-6, IL-18, and TNFα, which have been shown to cause MDSC recruitment and infiltration at the tumor site, subsequently causing IL-10 release and immune suppression [116]. Keratinocytes will also cause CXCL10 upregulation, which has been shown to elicit angiogenesis [139]. iNKT-cell activation will cause release of IL-4 and IL-13, which are both known to elicit MDSC recruitment and infiltration [116] as well as direct suppression of tumor-specific CD8+ T-cells [140]. Mast cell activation by complement C5a will cause CCL2 and CCL5 upregulation, which has been to shown to induce Tumor Associated Macrophages (TAMs) to release IL-10, promote angiogenesis, and stimulate tumor metastasis [116]. Mast cells will also release TNFα, known to help deliver oxygen to hypoxic areas of the tumor allowing for tumor growth [116], and release CXCL2, seen to induce melanoma cell proliferation [139]. Lastly, the induction of CHS at the tumor site could cause the infiltration of hapten-specific T-regs, which could potentially release IL-10 to suppress effector T-cells [141] or elicit CD8+ T-cell exhaustion by expression of CTLA-4 [118].

It is likely that the antitumor immunity or tumor-mediated immune suppression and tumor growth due to elicitation of CHS from epifocal hapten application will have much to do with the (a) type of tumor treated (b) growth rate of the tumor, and (c) timing of the administration. It is suggested, by hapten-specific T-cell migration data, that no antigen presentation occurs outside of the dermis in the CHS elicitation phase [86]. This finding makes it likely that epifocal hapten application will only be useful for treating cutaneous cancer. The mechanisms of hapten-induced tumor regression using epifocal hapten application still remain unclear and need to be further studied. It is also essential to figure out the situations in which a hapten will induce tumor regression versus tumor growth by testing several different haptens in well-defined systems, which have yet to be created. If all this is done, it can be understood if epifocal hapten application is useful in eliciting tumor regression and antitumor immune responses.

4.7. Antigen-Hapten Conjugate-Mediated Antibody-Dependent Cellular Cytotoxicity. From 2002 to 2013, Philip S. Low's group used a unique approach to hapten-mediated tumor treatment. They synthesized folate-hapten conjugates and used them to treat folate receptor high cancers. The concept is that the folate would bind to folate receptors on the tumors coating the tumors in haptens, which could lead to ADCC and complement system activation, effectively killing the tumor in hapten-sensitized animals. In their work, they utilized the haptens FITC and DNP, and treated folate high M109 lung carcinomas. This treatment is not directly cytotoxic like direct haptenation. It is important to note that the immune mechanisms occurring here are wildly different than what has been described earlier (Sections 1, 2, and 3 of the hapten-mediated tumor treatments) having little to do with CHS mechanisms, and mostly mediated by hapten-induced ADCC. These studies present a good mechanistic view of how the tumor regression is occurring.

Lu and Low [46] conjugated the Th2-hapten FITC to folate [46]. They treated cancer cells *in vitro* with the Folate-FITC conjugates, ensuring the FITC coating of M109 cells. Balb/c mice were inoculated with M109 cells and sensitized to BSA-FITC, inducing a strong anti-FITC antibody response. Intravenous injection of Folate-FITC coated s.c. M109 tumors within one day. They observed slight increase in survival in mice with peritoneal M109 tumors with the IL-2 or Folate-FITC alone (i.p. administration), but large increase in survival with the combination of the therapies. They added IFNα treatment to the IL-2 + Folate-FITC, which showed a very significant increase in survival, from a maximum of 30 days up to over 80 days in 20% of the animals. After immense optimization of folate-FITC, IL-2, and IFNα concentrations, they were able to find a curative treatment that gave 100% survival of mice for 100 days. They rechallenged long-term survivors with the same number followed by 3x as many M109 cells and saw that the mice were able to survive the rechallenges, suggesting long-term immunity in these mice; this was only shown as survival curves, so it was unclear if the tumors grew or not. Of note, many cells in the body express the folate receptor and this treatment could cause FITC coating and ADCC at distant, folate receptor expressing sites [156]. Realizing this, the authors submitted cured animals for toxicological analysis where it was determined that the treatment was not toxic and that there was no opsonization or damage of organs [46]. Along with that, IL-2 and IFNα treatments are known to cause side effects in clinical use, so combining them with the folate-FITC conjugate could increase any potential side effects [157]. Despite these worries, they clearly showed that this method coated tumors cells *in vivo* with FITC and significantly increased mouse survival in combination with cytokine treatment.

Lu et al., [45] then studied the immune mechanisms of folate-FITC-mediated tumor regression. They observed a bimodal plot of folate-FITC at various concentrations; this is commonly seen in treatments that do not directly kill tumor cell. There was no complement-mediated lysis of folate-FITC-labeled tumor cells occurring. NK cells showed direct lysis of folate-FITC coated tumor cells in the presence of anti-FITC antibody, suggesting ADCC. Macrophages engulfed the folate-FITC-bound tumor cells opsonized with FITC antiserum and ~34% of these cells were engulfed after a 30-minute coculture. These data suggest that both NK cells and macrophages are involved in killing and clearing folate-FITC/anti-FITC antibody marked tumor cells. Using the

complete treatment *in vivo*, they compared the survival of treated control mice and NK cell-depleted mice, showing a decrease in the overall survival, back to the basal level without NK cells. Depletion of CD4+ T-cells and CD8+ T-cells alone and in combination and depletion of macrophages significantly decreased the overall survival of the mice, close to that of the untreated mice, but not as extreme a decrease as the NK cell depletion. CD8+ T-cells were removed from cured animals and were seen to kill M109 cells better than T-cells from untreated animals, suggesting that this treatment is eliciting T-cell memory against the tumor. However, they did not perform adoptive transfer experiments to see if these cells could clear M109 tumors in naïve animals. Lastly, they showed that the optimized treatment was able to fully regress the tumor for 35 days, whereas the controls (PBS and PBS + IL-2/IFNα) had little effect.

These papers provide strong evidence for folate-FITC-mediated tumor regression and underlying immune mechanisms of this regression. However, it must be determined what the role of CD4+ and CD8+ T-cells is in this treatment and how the animals are clearing secondary tumor challenges. It is likely that macrophages are presenting tumor antigens after opsonization, causing the formation of tumor-specific T-cells. This is likely the reason CD4+ and CD8+ T-cells are important for animal survival.

Lu et al. [44] performed preclinical pharmacokinetics and tissue distribution studies. They utilized a radioactive folate-FITC conjugate to track the movement of the conjugate *in vivo* and saw that it was rapidly eliminated in naïve mice but formed immune complexes with FITC-specific antibodies in FITC sensitized animals, causing an extended duration of folate-FITC in the animal (173-fold increase in drug exposure). Extremely high doses of the folate-FITC were shown to cosaturate the tumor cell's folate receptors and the circulating FITC-specific antibodies, hindering immune recognition of the tumor and thereby lowering the antitumor activity.

Lu et al. [43] also established folate-DNP conjugates (EC57, EC63, EC0293, and EC0294) that showed similar results to the folate-FITC conjugate when using similar treatment regimens. One (EC0294) of four tested-conjugates, in combination with IL-2 and IFNα, markedly improved survival of M109 tumor bearing mice for more than 100 days; two of the treatments, EC0293 and EC0294, gave 40 and 60% cure rates, respectively, among these mice. They did not include tumor regression data. The cured mice all rejected the secondary tumor inoculation of M109 cells, suggesting an antitumor immune response. They looked into the risk of an allergic response, passive cutaneous anaphylaxis assay, to the treatment and saw that the conjugates that gave allergic responses were the ones that cured mice. These results show that the folate-DNP conjugates can elicit prolonged survival, secondary tumor rejection, and autoimmune side effects; however, they do not show direct tumor regression results. This study shows that the concept of antigen-hapten treatment is a very effective treatment for folate receptor high cancers as it can be done with different haptens (FITC and DNP) and potentially elicits long-term tumor immunity. It would be interesting to know if other antigen-receptor targets could elicit similar results.

Recently, Low's group [47] published a phase I clinical study using the folate-FITC treatment alone in patients with renal cell carcinoma. Patients were given EC90, the hapten fluorescein, with the adjuvant GPI-0100 to stimulate the production of anti-FITC antibodies followed by EC17, the folate-FITC conjugate treatment. 39 patients got at least one dose of the EC90, and 33 received at least one dose of the EC17 treatment. Of the 33 patients that received the EC17 treatment, 28 patients had baseline and at least one had follow-up tumor assessment. Of 28 patients, 1 (4%) patient achieved partial response, 15 (54%) patients achieved stable disease, and 12 (43%) had progressive disease. Of the 16 patients that completed 2 cycles of the EC17 therapy, 12 (75%) had stable disease and 4 (25%) had progressive disease and of the 11 patients that completed 3 cycles of the therapy, 6 (55%) had stable disease and 5 (45%) had progressive disease. There was no apparent relationship found between the anti-FITC antibody titer and the best response to the therapy. Although many patients had stable disease, only one had partial regression and no patients had complete regression.

These results are not unexpected, as the mouse treatment required the use of IL-2 and IFNα treatments to be fully effective. In the clinical study, patients were also not sensitized to the hapten, likely affecting the results. This trial was likely performed to see the side effects of the folate-FITC conjugate alone on patients. As stated in the phase I study, Low's group has completed a phase II trial of the EC17 treatment in combination with cytokine treatment and we hope those results will be published soon. It still needs to be determined how tumor challenges are rejected using this method.

5. Conclusions

Evidently, the field of contact hypersensitivity is still expanding, as there are many conflicting reports on several different aspects of the mechanism. The use of different mouse strains, different haptens, and different administrations or concentrations of haptens greatly impacts the immune responses seen. It would be paramount to attempt to standardize the methods of inducing CHS, so that more clear mechanisms can be established between different haptens and mouse strains. There is much work to be done to fill in the gaps and confirm parts of the pathway that remain unclear. Obviously, the use of haptens and haptenation as a tumor treatment needs further research to determine its efficacy. Much of the work with hapten-inducing tumor regression was done before the field of CHS was developed to its present state, and without in-depth immunologic mechanism depiction. This leaves much speculation about all the results found, as we underscored in this review.

Of the four concepts, antigen-hapten delivery seems to be the most appealing, but it uses completely different tumor clearance than the other treatment mechanisms, as it is mediated by ADCC. The work done by Low's group [43–47]

is detailed in explaining the tumor regression mechanism; however, further research is needed to understand if the treatment of folate-FITC along with IL-2 and IFNα can be effective. Along with this, it must be understood how tumor rechallenges are rejected after treatment.

For the field of hapten-mediated tumor regression to move forward, we propose that each model of hapten-mediated tumor regression be fully studied so that the mechanisms of primary and secondary tumor regression become clear. In this regard, we urge that the field must also consider the effect of hapten-mediated cell death, as the dead cells, like irradiated cells, may elicit antitumor immunity; it needs to be determined if hapten modification alone (on the surface) or hapten modification followed by cell death is needed to mediate antitumor immune responses. It also must be determined whether or not hapten-induced tumor regression can induce bystander effects or if it is hapten-dependent.

Lastly, it is very important to realize that no hapten treatment has been effective without the combination of another immune- or tumor-modulating agent(s), suggesting that haptens may never be able to elicit complete tumor regression by themselves. If this is true, haptens may be considered as adjuvants to possibly increase tumor regression and antitumor immunity by combining them with other tumor treatments that have measurable efficacy. Much of the data on hapten-mediated tumor treatments is observational; thus more mechanistic studies using similar mouse models and haptens as well as more stringently-controlled clinical trials are essential to determine if haptens are appropriate as cancer immunotherapies.

Conflict of Interests

Both authors declare that they have no financial or any other conflict of interests.

Authors' Contribution

Dan A. Erkes performed exhaustive literature searches, interpreted research in the field, and prepared the draft of the review. Senthamil R. Selvan conceived the idea for this review and overall approach, interpreted research in the field, and contributed to writing and critical revision of the paper. Both authors have read and given their approval of the final paper.

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Review Article

The Treg/Th17 Paradigm in Lung Cancer

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Pathogenic mechanisms underlying the development of lung cancer are very complex and not yet entirely clarified. T lymphocytes and their immune-regulatory cytokines play a pivotal role in controlling tumor growth and metastasis. Following activation by unique cytokines, CD4+ T helper cells differentiate into Th1, Th2, Th17, and regulatory T cells (Tregs). Traditionally, research in lung cancer immunity has focused almost exclusively on Th1/Th2 cell balance. Recently, Th17 cells and Tregs represent an intriguing issue to be addressed in lung cancer pathogenesis. Tregs play an important role in the preservation of self-tolerance and modulation of overall immune responses against tumor cells. Th17 cells directly or via other proinflammatory cytokines modulate antitumor immune responses. Notably, there is a close relation between Tregs and Th17 cells. However, the possible interaction between these subsets in lung cancer remains to be elucidated. In this setting, targeting Treg/Th17 balance for therapeutic purposes may represent a useful tool for lung cancer treatment in the future. The purpose of this review is to discuss recent findings of the role of these novel populations in lung cancer immunity and to highlight the pleiotropic effects of these subsets on the development and regulation of lung cancer.

1. Introduction

Lung cancer is the second most frequent cancer worldwide and continues to be the leading cause of cancer deaths [1]. Lung cancer is occurring in high frequencies in many economically developing countries; in the west the incidence is now declining which reflects changing cigarette smoking habits in the second half of the 20th century [1]. Only 15% of the patients survive for more than 5 years after primary diagnosis [1, 2]. Cigarette smoking and other noxious particles and gases that favor chronic lung inflammation have been established as risk factors for lung cancer development [2–4]. In particular, cigarette smoking with chronic inflammatory infiltrates in lung parenchyma [5], cigarette smoking with chronic obstructive pulmonary disease [6], and pulmonary tuberculosis [7] have been described as critical risk factors of lung cancer. In addition, tumor microenvironment consisting of immune cells is also identified as an indispensable participant of tumor immune pathogenesis [8].

Histologically, lung cancer is divided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC represents about 80% of all lung cancer cases and includes three histological subtypes: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. About 80–90% of NSCLCs are directly related to tobacco smoke [9] while SCLC represents about 20% of lung cancers and nearly all SCLCs are associated with smoking [9, 10]. Several studies have demonstrated that tumor microenvironment consisting of immune cells is an indispensable participant of the neoplastic process by favoring tumor cell proliferation, survival, and metastasis [11, 12]. Lung cancer is more and more common and receiving increasing attention; however, lack of methods for early diagnosis and lack of systemic therapies are the main reasons why the prognosis for many patients is still poor. There is a need, therefore, to elucidate the immune mechanisms to develop new therapeutic strategies such as immunotherapy. However, the precise regulatory mechanisms of the disease are poorly understood.

Various studies have demonstrated that tumor-infiltrating lymphocytes, especially CD4+ helper T cells, are present in the lungs of patients with non-small cell lung cancer [13]. CD4+ T helper cells are significantly important in removing cancerous tissue or cells. CD4+ helper T cells can be functionally divided into Th1, Th2, Th17, and regulatory T cells (Tregs) based on the secretion of cytokines [14, 15]. They perform different biological functions in antitumor immunity and tumor immune evasion and play an important role, respectively, in tumor tolerance mechanisms, tumor immune microenvironment, and immune homeostasis [16, 17]. Traditionally, research in lung cancer immunity has focused almost exclusively on Th1/Th2 cell balance [18]. Recently, the identification of Th17 cells and Tregs not only changes the classical Th1/Th2 paradigm of T helper cell differentiation but also markedly facilitates our understanding of human immunity under both physiological and pathological conditions [15, 19, 20]. Notably, there is a close relation between Tregs and Th17 cells. With time, the relationship has become increasingly complex and more closely intertwined. Several studies showed that Th17 and Tregs are present in lung cancer [21, 22]; however, the possible interaction between these subsets in lung cancer remains to be elucidated. The aim of this review is to discuss recent findings of the role of these novel populations in lung cancer immunity and to highlight the pleiotropic effects of these subsets on the development and regulation of lung cancer. Targeting Treg/Th17 balance for therapeutic purposes may represent a useful tool for lung cancer treatment in the future.

2. Th1-Th2 Paradigm in Lung Cancer

Traditionally, naïve CD4+ T cells become activated and differentiate into two effector T cell subsets after encountering a specific antigen. Th1 cells, which produce interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α), are the major effectors of phagocyte-mediated host defense, protective against intracellular pathogens, and Th2 cells, which produce IL-4, IL-5, IL-6, IL-13, and IL-10, are important in allergic responses and protection against infection of helminthic parasites [23, 24]. The key transcription factors driving Th1 cell differentiation are T-bet, STAT-1, and STAT-4, whereas the transcription factors STAT-6, c-maf, GATA-3, and NFAT are master regulators of Th2 development and function [14, 25]. IL-12 has been shown to induce the differentiation of Th1 cells and to enhance autoimmune disease in certain animal models [26]. Th1 cells secrete IL-2 and IFN-γ that suppress Th2 responses, whereas Th2 cells secrete IL-4 and IL-10 that inhibit Th1 responses [23]. Concerning Th1/Th2 paradigm in lung cancer, the hypothesis of Th1 predominance and downregulation of the Th2 response was reinforced by evidence from both murine studies and the clinical course of Th1 and Th2 based conditions in lung cancer. A study by Ito et al. [18] addressed the expression of Th1 and Th2 cells in lung cancer. It was found that the percentage of Th1 cells in tumor-infiltrating lymphocytes was significantly higher than in the corresponding peripheral blood and the proportion of Th2 cells was significantly lower than that of Th1 cells. This indicates that Th1 cells are dominantly tumor infiltrators. In addition, some studies showed that the mRNA and protein expression levels of INF-γ and IL-12 were significantly increased, whereas the expression level of IL-4 and the frequencies of the IL-4 variant −590T/C were decreased in lung tumor tissue [27–30]. All these studies demonstrated that these Th1 cells develop in the presence of tumor antigens and Th1 polarizing cytokines such as IL-12 and INF-γ in the lung microenvironment. Additionally, the Th1-to-Th2 ratios were significantly elevated in the tumor-infiltrating lymphocytes (TIL) of the patients with early stage lung cancer, while the Th1-to-Th2 ratios were significantly depressed in the PBL of the patients with tumor recurrences [18]. Moreover, the mRNA expression of IL-4 and IL-10 in tumor tissue and pleural effusion of NSCLC patients was significantly higher than that of IL-2, IL-12, and INF-γ [31]. These findings suggested that the imbalance and conversion of Th1 and Th2 cells might be responsible for both the occurrence and the progression of lung cancer.

Although the above findings have suggested that Th1 and Th2 cells are involved in lung cancer development, the functional role of Th1/Th2 tumor-infiltrating T cells is not clear. Experiments performed on tumor bearing mice showed that T-bet knockout mice have significantly higher tumor load associated with reduced Th1 cells, suggesting that T-bet expressing Th1 cells protect from lung tumor growth [32]. IL-10 transgenic mice are unable to limit the growth of immunogenic tumors; however, administration of blocking IL-10 mAbs restored in vivo antitumor responses [33]. IFN-γ may exert potent antitumor effects on lung cancer and metastasis, as this cytokine boosts natural killer cell activity, induces macrophage activation and antigen presentation, and activates tumor-specific CD8+CTLs which are required for the elimination of cancer cells [34–36]. IL-10 inhibits the generation of cell-mediated antitumor immunity by inhibiting a broad array of immune parameters including antigen presentation, antigen-specific T cell proliferation, and type 1 cytokine production [37]. Together, these findings support the implication of enhanced Th1 cells in augmenting antitumor responses but enhanced Th2 cells in downregulating antitumor immunity.

Of note, although the Th1 and Th2 responses can be seen as discrete responses in lung cancer development, there is considerable cross-talk and overlap between the functions of the two subsets. Previous studies have shown that peripheral blood lymphocytes from NSCLC patients with recurrence showed an unfavorable imbalance between Th1 and Th2 cells, with significantly depressed Th1-to-Th2 ratios [18]. Similarly, tumor cells from patients with advanced lung cancer express some type 2 cytokines such as IL-10 and transforming growth factor-β (TGF-β), while little or no levels of type 1 cytokines such as IL-2 and IFN-gamma were noted [38, 39]. With the progression of a tumor, including malignant effusion and distant metastasis, the cell-mediated immunity of lung cancer patients is impaired and tumor cells produce type 2 cytokines to suppress the differentiation of T cells into Th1 cells. In addition, these immunosuppressive cytokines IL-10

and TGF-β might not only suppress Th1 cell responses by tumor-infiltrating T cells but also favor the development of regulatory T cells [40–42].

3. Treg Cells in Lung Cancer

Th17 and regulatory T cells (Tregs) have replaced the 20-year-old Th1-Th2 paradigm [43]. This new paradigm has significantly improved our understanding on the differentiation of functional CD4+ T helper cell subsets and T cell regulation of inflammation and autoimmunity. Studies ongoing for more than a decade have provided firm evidence for the existence of a unique CD4+CD25+ T-cell population of "professional" regulatory/suppressor T cells that actively and dominantly prevent both the activation and the effector function of autoreactive T cells that have escaped other mechanisms of tolerance [44–46]. TGF-β1 and IL-2 are the two crucial cytokines involved in the differentiation of naïve T-cells into Tregs, which express the forkhead lineage-specific transcription factor FoxP3 protein. TGF-β1 together with IL-2 is also needed for Tregs expansion. IL-2-induced STAT5 has a prominent role in promoting Foxp3 expression [47]. All-trans retinoic acid (atRA), an active metabolite of retinoic acid, markedly enhances TGF-β-induced Foxp3 expression and stability in mice [48] and the expansion of these Tregs [49]. IL-2-deficient mice have a reduced number of nTreg-cells, indicating the importance of Treg cell proliferation [50]. Multiple lines of evidence have indicated that Tregs are involved in the control of the local immune response and in the growth of human lung cancer [21, 51, 52]. A higher level of TGF-β1 in the BALF of patients with primary lung cancer compared with the healthy subjects has been found [53]. In addition, IL-2 levels are higher in patients with NSCLC [54] compared with healthy controls. The findings of higher levels of both TGF-β1 and IL-2 suggest that these proinflammatory cytokines might promote the generation and differentiation of Tregs in lung cancer. Furthermore, it was found that there were increased proportions of tumor-infiltrating CD4+CD25+ T cells in patients with early stage NSCLC, and these CD4+CD25+ T cells were found to secrete immunosuppressive cytokine TGF-β, which may play a role in cancer progression [55, 56]. In addition, these regulatory T cells have been shown to express cytotoxic lymphocyte-associated antigen-4 CTLA-4 (CD152) in mice [57], and triggering of CTLA-4 has been shown to induce TGF-β secretion [58]. Similarly, Chen et al. [59] also found that increased proportions of CD4+CD25+ T cells in malignant pleural effusion (MPE) were regulatory T cells as they express high levels of Foxp3 transcription factor and CTLA-4. Moreover, pleural CD4+CD25+ T cells could potently suppress the proliferation of CD4+CD25- T cells and CTLA-4 was involved in the suppressive activity of pleural CD4+CD25+ T cells. In a recent study, Ganesan et al. [60] demonstrated that tumor-infiltrating Tregs partially repressed CD8+ T cell responses in mouse models of lung adenocarcinoma. Thus, Treg cells in NSCLC appear to selectively inhibit host immune responses and therefore might contribute to cancer progression. What underlies the enrichment of Treg cells within tumor tissue or PE of lung cancer patients? There is considerable evidence to suggest that increased CD4+CD25+ T cells in tumor site might be due to either active recruitment or local differentiation. A study by Zhao et al. [61] showed that there were increased levels of TGF-β1 and IL-2 in serum in patients with NSCLC compared with healthy controls. TGF-β induces Treg expansion in lung cancer microenvironment [48]. In addition, other studies identified that CCL22 in MPE might be related to the accumulation of Treg cells in MPE. Indeed, an in vitro migration assay further confirmed that MPE could induce the migration of Treg cells and that either anti-CCL22 mAb significantly inhibited the ability of the MPE to stimulate Tregs chemotaxis [62]. Further analysis of Tregs and related cytokines in lung cancer patients and tumor bearing animals clearly demonstrated the relationship between the stage of the disease and the relative proportion and number of Treg cells. For instance, experimental results suggested that Tregs were shown to inhibit NK cell-mediated suppression of tumor growth and metastases largely by a TGF-β-dependent mechanism [63]. Petersen et al. [64] reported that patients with stage I NSCLC who have a higher proportion of tumor Treg cells had a significant risk of recurrence. Similarly, Shimizu et al. [65] also showed that tumor-infiltrating FoxP3+ Tregs correlate with cyclooxygenase-2 (COX-2) expression and increased tumor recurrence in stage I to stage III NSCLC. Furthermore, Liu et al. [66] showed that a high FoxP3+ Treg/CD8+ T cell ratio is a risk factor for poor response to platinum-based chemotherapy in advanced NSCLC. This means that Tregs are able to inhibit antitumor immunity and mediate immune tolerance favoring tumor growth. However, to the best of our knowledge, although the elevated frequency of Tregs in tumor tissues correlated with the prognosis, whether or not Tregs directly contribute to tumor growth remains unclear. Further research should be done by using adoptive transfer of Treg cells and IL-2 or TGF-β-null (or IL-2 or TGF-β deficient) mice.

4. Th17 Cells in Lung Cancer

The subset of CD4+ T cells that produce both IL-17A and IL-17F is now defined as a separate subset of Th17 cells. Distinct from Th1 and Th2 cells, Th17 cells are reported to be generated from naïve T cells by IL-6, IL-1, and IL-21, with or without TGF-β, and are expanded and stabilized further by IL-23 [67–70] and by virtue of expressing the orphan nuclear receptors RORgt and ROR α as critical transcription factors [71]. STAT3 regulates IL-6-induced expression of RORgt and RORa and IL-17 production [72]. In the mouse, naïve CD4+ T cells stimulated by TGF-β and IL-6 differentiate into Th17 cells [73]. However, whether TGF-β plays a decisive role in the differentiation of naïve human CD4+ T cells into Th17 cells is controversy. Acosta-Rodriguez et al. [74] found that human Th17 cells originate in response to the combined activity of IL-1β and IL-6, whereas Wilson et al. [75] found that the activity of IL-1β or IL-23 alone was critical. In some human studies, the addition of TGF-β to human naïve or memory CD4+ T cells was even found to be inhibitory on the development

of Th17 cells [76]. Many studies have demonstrated the importance of Th17 cells in clearing pathogens during host defense reactions and in inducing tissue inflammation in autoimmune disease; however, the role of Th17 cells and related cytokine IL-17 in lung cancer immunity has been confusing.

Besides IL-17 and IL-17F, Th17 cells also secrete other cytokines such as IL-21, IL-22, and CCL20 [76]. IL-17 (also known as IL-17A) was first cloned in 1993 and identified as cytotoxic T lymphocyte-associated antigen (CTLA)-8 [77]. IL-17F was later discovered and closely related to IL-17A [78]. They are mainly produced by activated memory CD4+ T cells but can be induced in CD8+ T cells, NK T cells, DC cells, and possibly other cells [79–84]. An induction of IL-17A mRNA and protein expression was noted in lung CD4+ T cells in patients with NSCLC as compared to healthy controls, suggesting IL-17 is involved in lung cancer [22, 31]. However, the role of IL-17 in tumor immunity remains undefined. Functionally, overexpression of IL-17 in tumor cell lines promotes angiogenesis and tumor growth when the tumors are implanted in immune-compromised mice [85]. Intranasal treatment of mice with a neutralizing anti-IL-17A antibody in experimental lung adenocarcinoma caused a significant reduction of tumor growth as compared to control treated mice [32]. Similarly, IL-17A deficiency or IL-17A blockade led to suppression of lung metastasis in tumor models [86]. Further, IL-17 could directly promote the invasion of NSCLC cells both in vitro and in vivo. Furthermore, the elevated expression of IL-17 in peripheral blood was associated with the TNM stage [87]. These reports indicate that IL-17-mediated responses promote tumor development through the induction of tumor-promoting microenvironments at tumor sites and that IL-17-mediated regulation of myeloid-derived suppressor cells (MDSCs) is a primary mechanism for its tumor-promoting effects [88]. In contrast, recent reports indicate that tumor growth in subcutaneous tissue and lung tumor metastasis are enhanced in IL-$17^{-/-}$ mice and that the mechanism is associated with reducing IFN-g-producing tumor-infiltrating NK and T cells [89, 90]. It implicates that IL-17-mediated responses are protective against tumor development. With regard to IL-22, several studies have showed that high expression concentrations of IL-22 were detected in tumor tissue, MPE, and serum of patients with lung cancer and that the overexpression of IL-22 was correlated with occurrence and progress of lung cancer [91, 92]. IL-22 might also play a role during tumor genesis because IL-22 stimulates signaling pathways that are involved in the cell proliferation, cell apoptosis, and cell cycle control [93–96]. So far, however, additional roles for the Th17-derived cytokines in lung cancer remain largely unexplored. Additionally, further research should be done by using adoptive transfer of Th17 cells in mice to demonstrate the cellular mechanisms.

Concerning Th17 cells in lung cancer, recent data from humans and mice clearly support the role of Th17 cells in lung cancer pathogenesis. It was found that the expression of Th17 markers ROR c and ROR γt was significantly induced in NSCLC [31], and a larger Th17 cell was included in SCLC [97]. Consistently, Th17 numbers in malignant PE from patients with lung cancer were much higher than numbers in peripheral blood. The overrepresentation of Th17 cells in MPE might be due to Th17 cell differentiation and expansion stimulated by pleural proinflammatory cytokines IL-1β, IL-6, IL-23, or their various combinations and to recruitment of Th17 cells from peripheral blood induced by pleural chemokines CCL20 and CCL22 [98]. In spite of the above findings highlighting the expression and differentiation of Th17 cells in lung cancer, their physiological functions in cancer immunity still remain largely unknown. It has been suggested that Th17 cells themselves do not have direct in vitro killing activity to tumor cells. Th17 cells stimulate tumor residential cells to produce CCL2 and CCL20, which provokes the recruitment of dendritic cell, granulocyte, CD4+ T cell, CD8+ T cell, and NK cell to the tumor site [99]. Increased numbers of DCs after Th17 cell transfer enhance tumor antigen in the lung and migrate to the lymph nodes where they activate CD8+ T cells against the tumor [100]. The tumor-specific CD8+ T cells may kill tumors independent of IFNγ, possibly via perforin pathway [28]. Taken together, these reports demonstrate that Th17 cells participate in antitumor immunity by facilitating T cell recruitment to the tumor site and CD8+ T cell priming and suggest a new avenue for developing Th17 cell-based therapy for lung cancer.

5. The Balance and Correlation between Th17 Cells and Treg Cells in Lung Cancer

Besides the above-mentioned difficulty to clarify the effective role played by Th17 and Treg cells in lung cancer pathogenesis, recent studies made this matter even more complex providing the clue of these T-cell subsets [31, 101, 102]. Recently, it became increasingly clear that CD4+ T cell subsets are not stable and display plasticity during differentiation and maintenance [47]. There is a close relation between Tregs and Th17 cells. In mice these cells originate from a common precursor, with the differentiation of which is dependent upon the production of dendritic cells activated by microorganisms [103]. Moreover, the progenitor cells differentiate to Th17/Treg intermediate cells, which express both RORC and Foxp3 [104]. Additionally, Tregs and Th17 cells share common chemokine receptors (CCR6, CCR4) and homing properties (CCL20) [105]. In human a differentiation link between Th17 cells and Tregs has been reported, in which TGF-β is essential for the generation of both cells [103]. The differentiation of Th17 cells is inhibited by high TGF-β concentrations but requires IL-1β and IL-6. Retinoic acid, which is a key regulator of TGF-β dependent immune responses, is able to inhibit ROR-γt in Th17-inducing conditions and simultaneously promote Tregs differentiation [103, 104]. Besides, there is also an inverse correlation between ROR-γt and FoxP3 [106]. Indeed, in the presence of proinflammatory cytokines and low concentration of TGF-β, ROR-γt expression is further upregulated, whereas FoxP3 expression and function are inhibited. This evidence shows the importance of cytokines environment in the differentiation of CD4+ T-cell subsets, depending upon the balance of expression of

the transcriptional factors ROR-γt and FoxP3. Recently, the relationship has taken a further twist, with the surprising finding that Tregs are able to convert to Th17 cells in the context of inflammatory signals, such as IL-1β, IL-6, IL-21, and IL-23 [107–109].

Many studies have revealed that lung cancer may occur as a consequence of cytokine imbalance and eventually of the Th17/Treg ratio. For instance, SCLC as well as NSCLC cells overexpress TGF-β [110, 111]. Serum levels of TGF-β were increased in lung cancer patients with lymph node metastasis compared with patients who were without lymph node metastasis, and the TGF-β levels were significantly higher in patients with stage III disease compared with patients who had stages I and II disease [112]. Activated TGF-β promotes tumor metastases [113]. Notably, TGF-β has suppressive activity in early tumorigenesis but may become tumor-promoting in the later stages of the disease [113]. Similarly, IL-17A may elicit pro- as well as antitumor properties. Additionally, this cytokine induces IL-6 production to interfere with Tregs development. Studies by Zhou et al. [108] have shown that there is a significant increase in Tregs and FoxP3 expression and a decrease in Th17 cells, ROR ct and IL-17 expression in peripheral blood of NSCLC patients while compared to that in healthy patients, and Foxp3 levels correlated with levels of RORc and IL-17. In particular Th17/Treg ratio is negatively correlated with the TNM stages. As a consequence, tumor-derived low TGF-β may synergize with IL-6 and IL-21 to promote Th17 cell differentiation in early stage lung cancer, while in late stage disease tumor-derived high TGF-β may induce overproduction of Treg cytokines and, in turn, promotes a shift in the Th17/Treg balance toward a Treg response and inhibiting the Th17 response [108, 114]. Further study by Ye et al. [101] provides functional evidence that regulatory T cells from malignant pleural effusion in lung cancer were found to inhibit generation and differentiation of Th17 cells via the latency-associated peptide LAP. Thus, Tregs and Th17 arise in a mutually exclusive fashion, depending on tumor microenviroment. Taken together, these results suggest that Tregs and Th17 cells are involved in the perpetuation of the inflammatory immune response in lung cancer, and restoring an adequate cytokine network and Th17/Treg balance may help to achieve a better clinical response.

6. Closing Remark and Prospective

T lymphocytes and related cytokines modulate immune responses in the tumor microenvironment during progression/metastasis, and the balance between destructive inflammation and protective immunity determines the direction of the malignant process [115–119]. Th17 and Treg cells are two mutually contradictory T cell subsets. The differentiation of Th17 cells depends on the concomitant action of IL-6 and the suppressive cytokine TGF-b which is also necessary for the induction of Tregs. IL-6, in turn, inhibits the development of Tregs suggesting that IL-6 plays a pivotal role in dictating the balance between the generation of Tregs and Th17 cells. Maintaining an appropriate balance between Th17 and Treg cells can ensure effective immunity while avoiding inflammatory and tumor immunosurveillance. Accumulated evidence has demonstrated quantitative or functional imbalance between Th17 and Tregs and these subsets' expression correlation with prognosis in lung cancer, suggesting that Th17 and Tregs represent important key pathogenic players in lung cancer pathogenesis. Th17 cells dominantly act to induce antitumor immunity. In contrast, Tregs better enable inhibition of antitumor immunity. However, the molecular mechanisms underlying the involvement and regulation of these two subsets in lung cancer immunopathology remain largely unknown. In addition, a number of crucial questions remain to be answered. What precise roles do TGF-β, IL-6, IL-17, and IL-22 play in lung cancer immunopathology? How might Th17/Treg imbalance be induced and lead to lung cancer immune pathogenesis? Is the Th17/Treg imbalance in early stages of tumor development the same as in late stage? Are there complementary roles for Th17 and Treg responses in lung cancer? Further understanding of the mechanisms of Th17/Treg-mediated inflammatory immune responses, in tilting the balance between destructive inflammation and antitumor immunosurveillance, may open new lines of investigation for lung cancer treatment in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Selective Depletion of Regulatory T Cell Subsets by Docetaxel Treatment in Patients with Nonsmall Cell Lung Cancer

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Regulatory T (Treg) cells are potent suppressors that maintain immune homeostasis. Accumulation of Treg can inhibit effective immune responses in cancer patients, leading to tumor development and progression. Despite direct cytotoxicity, several chemotherapeutic drugs have been reported to deplete Treg cells for better prognosis for cancer patients. Treg cells are a heterogenous population with at least three different subsets, nonsuppressive, resting, and activated Treg cells. However, the characteristics of Treg cell subsets in lung cancer patients and how chemotherapy affects Treg cells remain elusive. In this study, we first analyzed Treg cell subsets in peripheral blood samples from 40 nonsmall cell lung cancer (NSCLC) patients and 20 healthy donors. Treg cells, specifically activated Treg cell subset, significantly increased in patients with NSCLC. Compared to nonsuppressive Treg cells, activated Treg cells expressed higher level of CD39 and predominantly produced inhibitory cytokines. *In vitro* assay showed that docetaxel reduced all three subsets of Treg cells. More importantly, we found docetaxel-based chemotherapy significantly decreased all three Treg subsets after 4 cycles of treatment in 17 NSCLC patients. Taken together, this study revealed dynamic changes of various Treg cell subsets in NSCLC patients before and after chemotherapy, providing activated Treg cells as a potential target for chemotherapy.

1. Introduction

Regulatory T cells (Treg cells) are a key member to maintain self-tolerance and immune homeostasis [1, 2]. They play crucial roles in a variety of human diseases, such as autoimmune disease, allergy, chronic infection, and cancers [3–6]. Treg cells can suppress the immune response of $CD4^+$ and $CD8^+$ T cells mainly by secretion of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF-β) [7, 8]. Foxp3 is the most specific marker for $CD4^+$ Treg cell development and function [9–11]. Sakaguchi's group confirmed that human $CD4^+Foxp3^+$ Treg cells can be divided into three subsets: $CD45RA^+Foxp3^{lo}$, $CD45RA^-Foxp3^{hi}$, and $CD45RA^-Foxp3^{lo}$ cells [12]. $CD4^+CD45RA^+Foxp3^{lo}$ Treg cells as antigen-experienced cells are referred to as resting Treg cells (rTreg) [13–15]. $CD4^+CD45RA^-Foxp3^{hi}$ Treg cells which are activated with highly suppressive function and proliferating ability *in vivo* are defined as activated Treg cells (aTreg) [13–15]. $CD4^+CD45RA^-Foxp3^{lo}$ Treg cells include a remarkable amount of nonregulatory, cytokine-secreting T cells (nonsuppressive T cells or non-Treg cells) [13–15].

Elevated Treg cells reduce immune responses against tumor and induce excessive tumor progression [16, 17].

$CD4^+CD25^+$ Treg cells are augmented in tumor tissue as well as in circulation in patients with malignant melanoma, Hodgkin lymphoma, and lung, gastric, ovarian, pancreatic, and breast cancer [18–20]. Traditionally, the aim of chemotherapy is direct cytotoxicity to induce tumor cell death. Taxanes containing docetaxel or paclitaxel have been used to treat a variety of malignancies such as lung, prostate, and breast cancers. They have also been reported to modulate components of the immune system in mice by disrupting intracellular microtubular networks [21]. In those studies, docetaxel showed clear antitumor effects and further enhanced antitumor effects by modulation of immune cell subsets or regulatory T cells. However, no study has demonstrated the effect of docetaxel on the frequency and function of individual Treg cell subsets.

In this report, we characterized three subsets of $CD4^+Foxp3^+$ Treg cells in NSCLC patients. Only aTreg cells have been found to increase in NSCLC patients, especially in patients with advanced NSCLC. We also identified the relationship between three Treg subsets and pathological characteristics. Finally, our data demonstrate that docetaxel modulates different subsets of Treg cells both in *in vitro* analysis and *in vivo* clinical settings.

2. Materials and Methods

2.1. Patients and Blood Samples. From February 2013 to November 2013, 40 patients with NSCLC from the First Affiliated Hospital of Zhengzhou University were enrolled. The patients have not been treated with anticancer drugs, radiotherapy, or surgery in the beginning of the study and have no other systemic diseases. Peripheral blood was collected from 40 patients with NSCLC and 20 healthy donors with similar gender and age distribution, respectively. All patients gave written informed consent. The whole consent procedure was in accordance with the standard defined by Institutional Review Boards of the First Affiliated Hospital of Zhengzhou University. Patient characteristics were summarized in Table 1.

2.2. Antibodies and FACS Analysis. Fresh human peripheral blood mononuclear cells (PBMCs) were stained with anti-CD4 (PerCP-Cy 5.5 or APC-Cy7-conjugated from BD Bioscience), anti-CD25 (APC-Cy7 or APC-conjugated from BD Bioscience), and anti-CD45RA (FITC-conjugated from BD Bioscience). Intracellular detection of Foxp3 with anti-Foxp3 (PE-conjugated from BD Bioscience) was performed on fixed and permeabilized cells with the Foxp3 staining buffer set (Biolegend, USA) according to the manufacturer's instructions. The following fluorescence-conjugated antibodies were also used: CD39 (APC), Interferon-γ (IFN-γ) (PE-Cy7 or APC), and TGF-β (APC) obtained from BD Biosciences. PBMCs were stained according to the manufacturer's recommendations. The appropriate isotype-matched control antibodies were purchased from BD Bioscience. Cells were analyzed using a FACSCantoII flow cytometer (BD, USA) and Diva analysis software (BD, USA).

Table 1: Clinical and pathologic characteristics of patients ($n = 40$).

Characteristics	Number of patients	Proportion (%)
Sex	40	
Male	24	60
Female	16	40
Age (years)		
<60	10	25
≥60	30	75
Pathology		
Adenocarcinoma	20	50
Squamous	20	50
Stage		
I-II	8	20
III-IV	32	80

2.3. Intracellular Staining. Intracellular staining for IFN-γ and TGF-β was performed as follows: PBMCs were freshly isolated and stimulated with 1 mg/mL PMA (Sigma, USA) and 1 mg/mL ionomycin (Sigma, USA) in the presence of Brefeldin-A (BFA, Biolegend, USA) for 5 h. Cells were stained for cell surface markers and then fixed and permeabilized with anti-human Foxp3 Ab for intracellular cytokine staining. FACSCanto II flow cytometer (BD, USA) was used to determine fluorescence intensity and Diva analysis software was used to analyze the data.

2.4. Cell Isolation and Sorting. PBMCs were isolated by density gradient centrifugation (Tianjin HY, China) within 2 h after sample collection. There is a linear correlation between CD25 and Foxp3 levels expressed on $CD4^+CD25^+$ T cells [7]. To isolate live Treg subsets for functional assays, the PBMCs were stained with CD4 and CD25 Abs and sorted using Moflo-XDP (Beckman Coulter, USA) according to the manufacturer's instructions. The purity of $CD4^+CD25^+$ T cells was >90%, confirmed by flow cytometry (data not shown).

2.5. In Vitro Assay of Docetaxel Effect on the Treg Subsets. The purified $CD4^+CD25^+$ T cells were resuspended in RPMI1640 (Gibco, USA) containing 10% fetal bovine serum (FBS, Sigma, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. After 24 h of incubation in the atmosphere with 5% CO_2 at 37°C, 100 IU/mL IL-2 (Beijing SL, China) and 1 μg/mL docetaxel (Zhejiang WM, China) were added. Assay cultures after 36 h were harvested and ready for phenotype and cytokine analysis of the three subsets of Tregs, aTreg, rTreg, and non-Treg cells being, respectively, defined as $CD4^+CD45RA^-CD25^{hi}$, $CD4^+CD45RA^+CD25^{lo}$, and $CD4^+CD45RA^-CD25^{lo}$ T cells.

2.6. Therapeutic Regimen. Of these 40 NSCLC patients, 17 received cisplatin (75 mg/m^2) plus docetaxel (30 mg/m^2 on

day 1 and day 8) every three weeks. All patients were treated for 4 cycles.

2.7. Statistical Analysis. Differences between groups were assessed using Student's t-test and paired t-test. The correlation between Treg cell subsets and clinical characters was determined by one-way ANOVA. The change of Treg cells treated with docetaxel was determined by randomized block design ANOVA. P values were considered significant at $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Statistical analyses were performed in SPSS version 17.0.

3. Results

3.1. Only aTreg Cells Increased in NSCLC Patients. The combination of Foxp3 and CD45RA staining of $CD4^+$ T cells in PBMCs of NSCLC patients revealed the existence of three subsets of Treg cells (Figure 1(a)). Notably, these three $CD4^+Foxp3^+$ populations could be distinctly separated into $Foxp3^{lo}CD45RA^+$ cells (rTreg cells), $Foxp3^{hi}CD45RA^-$ cells (aTreg cells), and $Foxp3^{lo}CD45RA^-$ cells (non-Treg cells). As shown in Figure 1(b), the percentage of $CD4^+Foxp3^+$ Treg cells from PBMCs increased in NSCLC patients compared to healthy donors ($1.76 \pm 0.17\%$ versus $1.01 \pm 0.16\%$, $P < 0.01$). We further analyzed three subsets of $CD4^+Foxp3^+$ cells in total $CD4^+$ T cells. Our data showed that only aTreg cells but not rTreg or non-Treg cells increased in NSCLC patients compared to healthy donors ($1.07 \pm 0.16\%$ versus $0.25 \pm 0.04\%$, $P < 0.001$), indicating that aTreg cells might play an important role in the pathogenesis of lung cancer.

3.2. Activated Treg Cells Expressed Higher Levels of CD39 and Inhibitory Cytokines in Patients with NSCLC. To evaluate the suppressive function of Treg subsets, we further detected the phenotypes of different Treg subsets in patients with NSCLC. CD39 is an ectonucleotidase and has been defined as an additional important marker for Treg cells, which converts extracellular ATP into immunosuppressive adenosine [22]. CD39 has been defined as an additional important functional marker for Treg cells [23]. So, CD39 expression was detected in the three Treg cell subsets. We found that CD39 was enriched in aTreg and rTreg cells in comparison to non-Treg cells in PBMCs (Figure 2(a)). We also studied the cytokine pattern in Treg cell subsets in PBMCs from NSCLC patients after *ex vivo* stimulation. As shown in Figure 2(b), aTreg cells secreted significant amount of TGF-β ($P < 0.05$) but very little INF-γ ($P < 0.001$) compared to non-Treg cells. In contrast, non-Treg cells predominantly secreted INF-γ but not TGF-β. Activated Treg cells also secreted more TGF-β than rTreg cells, but the difference is not significant. These characteristics suggest that aTreg cells were the major Treg subset with inhibitory function in NSCLC patients.

3.3. Activated Treg Cells Correlated with Advanced Pathological Stages in NSCLC Patients. The clinical relevance of Treg cell subsets with tumor stages and other pathological factors was examined. In thirty-two NSCLC patients at stages III-IV, the percentage of $CD4^+Foxp3^+$ Treg cells in PBMCs was significantly higher than that in patients at stages I-II ($2.01 \pm 0.23\%$ versus $0.98 \pm 0.25\%$, $P < 0.05$, Figure 3(a)). We also evaluated whether the subsets of Treg cells correlated with tumor stages. The frequency of aTreg cells was much higher in patients with NSCLC at stages III-IV ($1.30 \pm 0.18\%$ versus $0.38 \pm 0.09\%$, $P < 0.05$, Figure 3(a)). However, there were no significant differences in the subsets of Treg cells between different types of histology (Figure 3(b)).

3.4. Effect of Docetaxel on Three Subsets of Treg Cells. Previous studies have shown that docetaxel induced tumor cell death and also increased the number of $CD4^+$ and $CD8^+$ T cells [24]. We investigated if docetaxel had different effects on each Treg subset. To address this issue, we treated purified $CD4^+CD25^+$ T cells derived PBMCs from NSCLC patients with docetaxel *in vitro*. Because the degree of Foxp3 was proportional to CD25 expression (Figure 4(a)), we isolated and defined aTreg, rTreg, and non-Treg cells as $CD4^+CD45RA^-CD25^{hi}$, $CD4^+CD45RA^+CD25^{lo}$, and $CD4^+CD45RA^-CD25^{lo}$ T cells. Three subsets of Treg cells were all decreased after docetaxel treatment. More interestingly, aTreg cells secreted more INF-γ and less TGF-β after docetaxel treatment ($P < 0.05$, Figure 4(b)). But there were no significant differences for cytokine production in rTreg and non-Treg cells after docetaxel treatment.

To further confirm the clinical effect of docetaxel on Treg subsets, we collected peripheral blood from NSCLC patients 1 day before the first cycle and 2 weeks after each cycle of docetaxel treatment. As shown in Figure 5, three subsets of Treg cells were reduced after four cycles of chemotherapy ($P < 0.05$). The trend we observed coincided with the results observed *in vitro*.

4. Discussion

The adaptive immune system plays an important role in control of tumor development. Treg cells increased in most human solid tumors and can suppress antitumor immune responses by inhibition of tumor-specific CD8 T cells [24]. More and more reports showed that the increased number of Treg cells in solid tumors was related to greater tumor progress and poorer survivals [25]. Recently, $CD4^+Foxp3^+$ Tregs in tumor tissue were reported to have significantly increased compared with normal lung tissue [26]. In this study we reported significant increase of aTreg cells in peripheral blood of NSCLC patients. Consistent with previous studies, we confirmed that Treg cells increased in PBMCs. Furthermore, our results characterized three distinct subsets of Treg cells in NSCLC patients and revealed the relationship between Treg subsets and several pathological factors. The conversion of non-Treg cells to Treg cells is one of the mechanisms to promote the accumulation of Treg cells in suppressing antitumor immune response [27]. Activated Treg cells are highly proliferative *in vivo* but rapidly died, and rTreg cells can differentiate to aTreg cells under stimulation. However, we did not detect the translation of rTreg in NSCLS. Our results showed that depleting Treg cells might

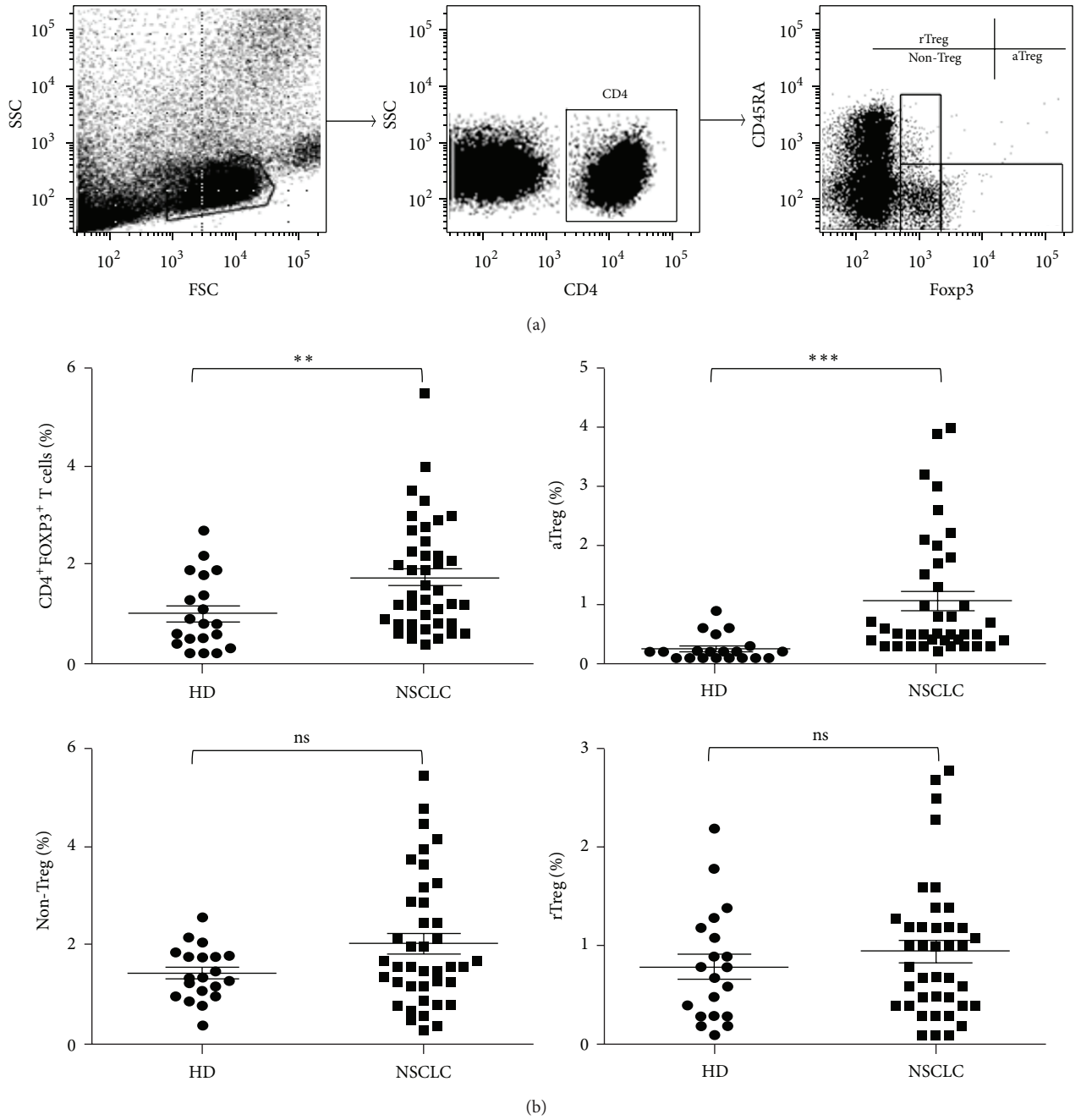

Figure 1: aTreg but not rTreg and non-Treg cells increased in NSCLC patients. (a) $CD4^+Foxp3^+$ T cells and three subsets of Treg cells from PBMCs were isolated and analyzed by FACS. (b) The percentages of Treg cells ($CD4^+Foxp3^+$ T (total Treg) cells, $CD4^+CD45RA^-Foxp3^{hi}$ (aTreg) cells, $CD4^+CD45RA^-Foxp3^{lo}$ (non-Treg) cells, and $CD4^+CD45RA^+Foxp3^{lo}$ (rTreg) cells) in $CD4^+$ T cells were calculated after FACS analysis. HD: healthy donor, $n = 20$; NSCLC: nonsmall cell lung cancer, $n = 40$. Each dot represents one individual sample. $^{**}P < 0.01$ and $^{***}P < 0.001$ for statistical analysis by Student's t-test.

be therapeutically beneficial for the tumor immunotherapy [13].

Multiple mechanisms have been reported for Treg cells implicating in the immune suppression of human cancer, which may be potential target for depleting Tregs for immunotherapy [28]. In order to explore the potential factors contributing to the conversion in Treg cells, we identified the differences of cytokines and other cell surface makers among three Treg subsets. Autocrine IFN-γ was reported to regulate TGF-β-driven Foxp3 expression in induced regulatory T cells (iTreg) and suppress the conversion of naïve CD4 T cells into $CD4^+Foxp^+$ T cells [29]. We found lower IFN-γ in aTreg cells compared with non-Treg cells, suggesting that aTreg cells produce lower level of IFN-γ to suppress

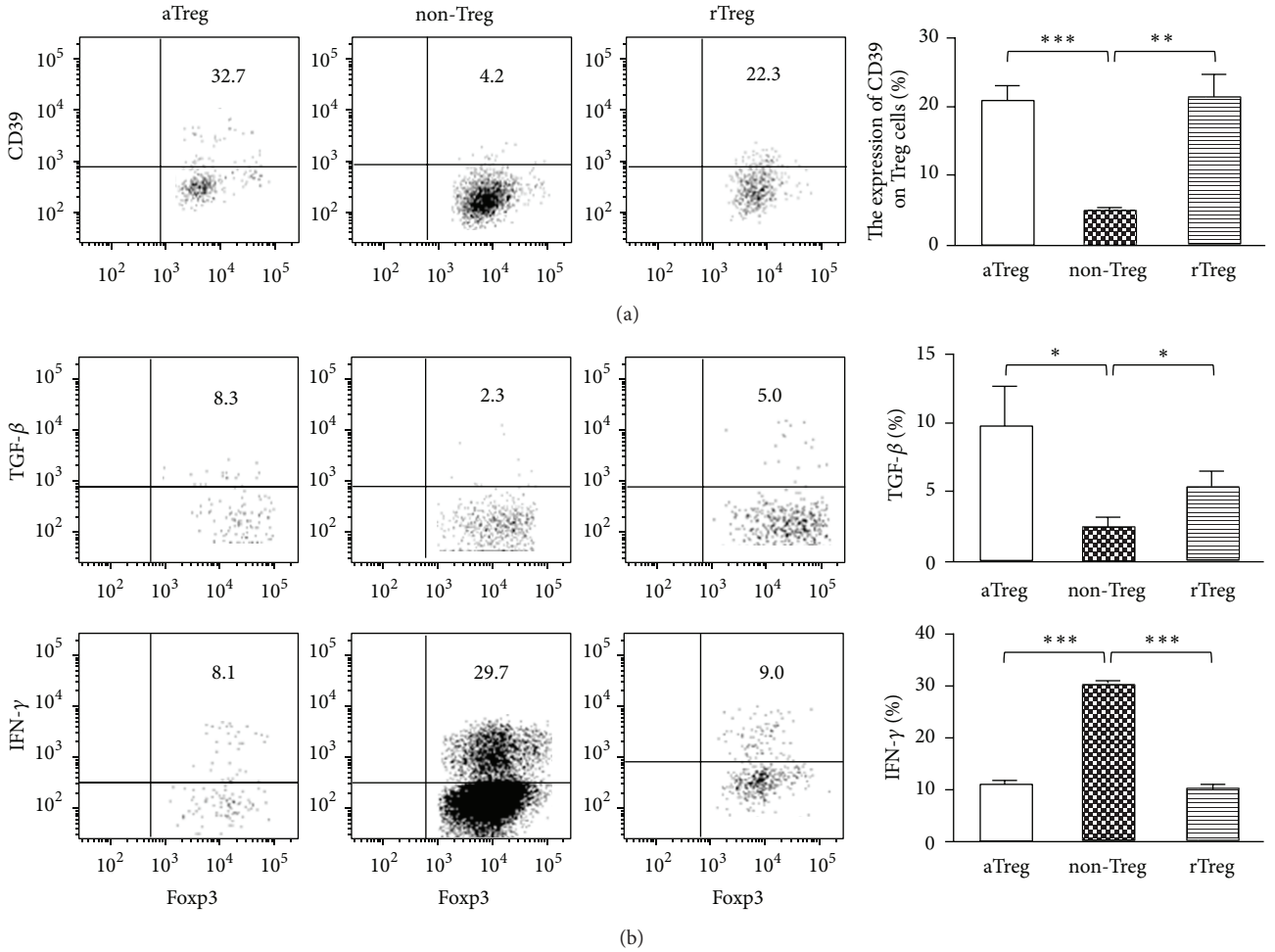

Figure 2: aTreg cells expressed higher immunosuppressive marker CD39 in NSCLC patients and secreted suppressive cytokines. (a) PBMCs were collected from NSCLC patients. The phenotype marker of CD39 was evaluated in the three subsets of $CD4^+Foxp3^+$ Treg cells, including aTreg, non-Treg, and rTreg cells. The dot plots (left) represent the expression of CD39 in each group. The bar figures (right) represent the mean percentage of each population ± standard error of mean. (b) These cells were also stimulated *in vitro* and the cytokine profiles including TGF-β and IFN-γ were analyzed. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by paired t-test.

the antitumor immune responses. TGF-β serves as a pleiotropic regulator of essential functions in immune cells [30]. TGF-β signaling pathway can inhibit Tregs proliferation in thymus and promote $CD8^+$ T cell maturation as well as NKT cell development [31]. But TGF-β is also required for Tregs development and survival. And no response to TGF-β can decrease the number of Tregs [31–33]. In this study, we found higher expression of TGF-β in aTreg cells. Among three subsets of Treg cells, aTreg which performed high proliferation, corresponding to HLA-DR-expressing and suppressing the proliferation of responder cells, was the main functional population of Tregs. It has been showed that the TGF-β pathway related genes have dysregulation between different Treg cell subsets [34], indicating that the high expression of TGF-β in aTreg cells was important for the Treg cells function. The high expression of TGF-β can induce a series of molecular events regulation which contributed to cell cycle, apoptosis, and others. Our findings supported the essential role of TGF-β for Tregs development and differentiation. CD39 can catalyze the conversion of extracellular ATP or ADP to AMP and act as another key mechanism of Tregs in suppressing antitumor immune response [35]. CD39 expressed on Tregs was reported to inhibit NK activity and promote hepatic metastatic tumor growth [36]. The increased expression of CD39 in $CD4^+$ T cells was related to poorer prognosis [37]. We also found high expression of CD39 in aTreg cells from NCSLS patients. Polyoxometalate-1, an inhibitor of nucleoside triphosphate diphosphohydrolase activity, can effectively inhibit the Treg cells activity *in vitro* and the tumor growth *in vivo* [36]. Therefore inhibition of CD39 may promote the antitumor immune responses by suppression of Tregs and act as component of immunotherapy for cancer.

Lung cancer is the most common cause of cancer-related mortality worldwide, with 85% being of the NSCLC histological subtype [38]. Since we found the association of aTregs and the clinical stage of NSCLC, next we detected the portion of Tregs in NSCLC patients after chemotherapies. We found Treg cells decreased after treatment with docetaxel *in vitro*, but only aTreg cells decreased with more IFN-γ and

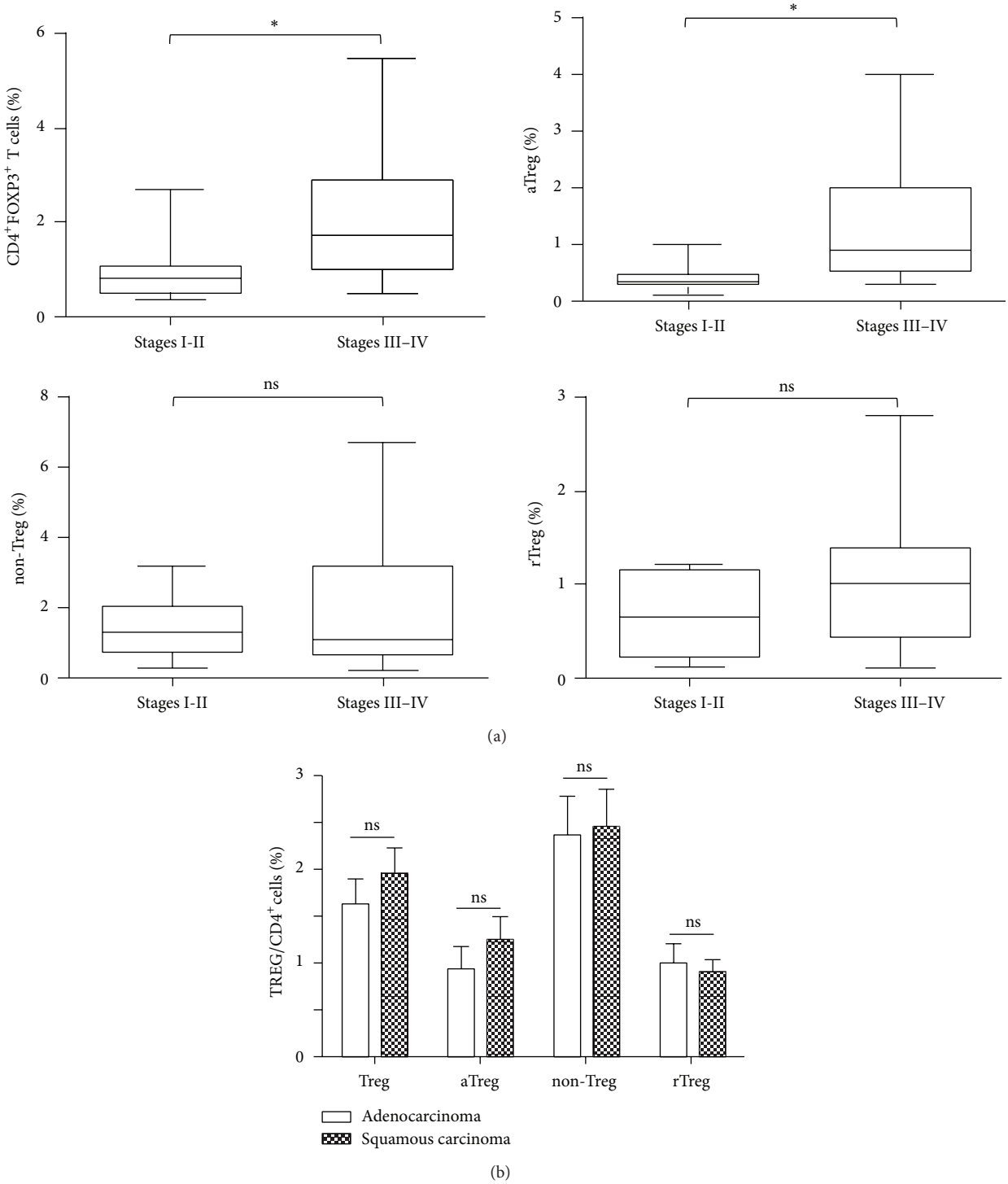

Figure 3: aTreg cells had a higher level in patients with advanced NSCLC. NSCLC patients were grouped according to clinical stage and pathology. (a) The percentages of $CD4^+Foxp3^+$ T cells, aTreg cells, non-Treg cells, and rTreg cells were compared in PBMCs of NSCLC patients at stages I-II and III-IV. (b) The 4 groups of Treg cells were compared in PBMCs of NSCLC patients between adenocarcinoma and squamous carcinoma. Statistical analysis was determined by one-way ANOVA. $^*P < 0.05$.

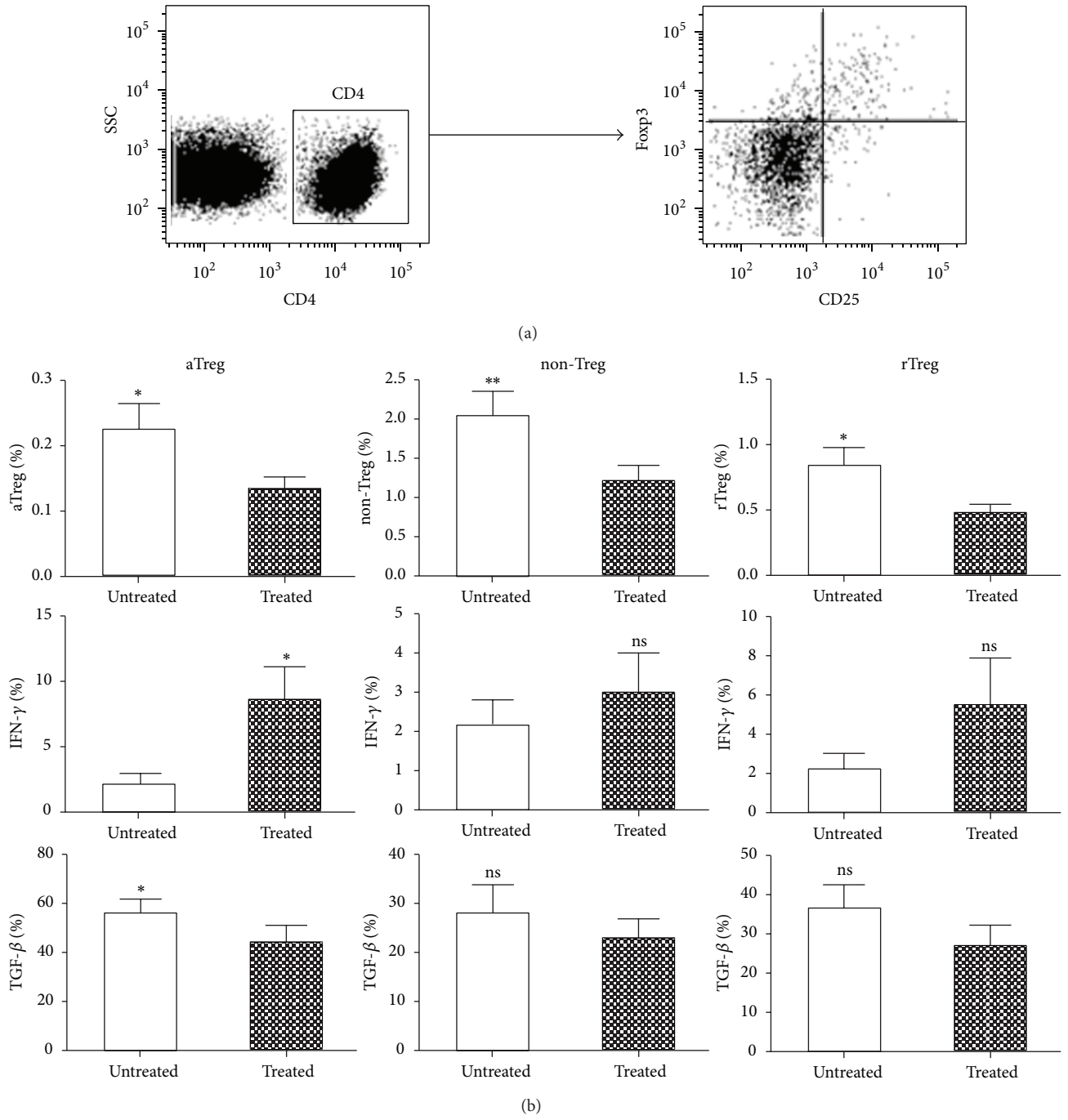

Figure 4: The three Treg subsets reduced after being treated with docetaxel *in vitro*. (a) PBMCs from NSCLC patients were staining with CD25 and Foxp3 antibody and analyzed by flow cytometry. (b) The peripheral blood of NSCLC patients was collected and $CD4^+CD25^+$ T cells were isolated by flow cytometric sorting. Three Treg subsets were defined with $CD4^+CD45RA^-CD25^{hi}$ (aTreg) cells, $CD4^+CD45RA^-CD25^{lo}$ (non-Treg) cells, and $CD4^+CD45RA^+CD25^{lo}$ (rTreg) cells. The differences of three subsets with or without docetaxel were analyzed. $^*P < 0.05$; $^{**}P < 0.01$ by paired t-test.

less TGF-β. We also found that the three subsets of Treg cells were greatly reduced after 4 cycles of chemotherapy. This study of NSCLC patients agreed with our findings *in vitro*. Although the portion of aTregs was extremely lower after 4 cycles of chemotherapy, there are still aTreg and rTregs remaining in peripheral blood. It is already known that rTreg cells can differentiate to aTreg cells after stimulation if aTreg cells die. We considered this may contribute to the recurrence and poor survivals of NSCLC. The depletion of Treg cells can significantly prolong survival in combination with chemotherapy in preliminary studies; on the other hand, chemotherapy can decrease Treg cells to improve

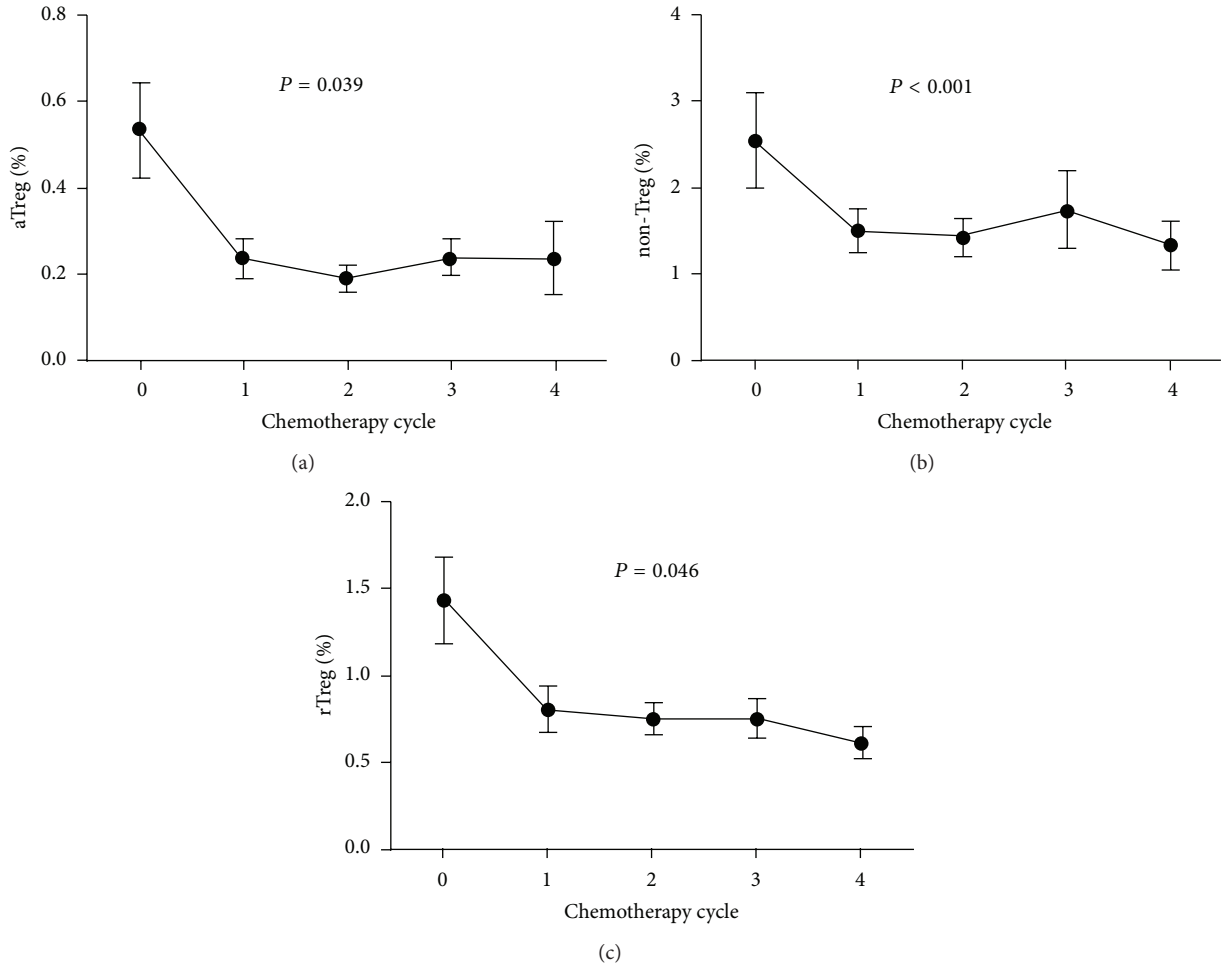

Figure 5: Treg cell subsets of NSCLC patients were decreased after chemotherapy. The patients with NSCLC received 4 cycles of chemotherapy and the peripheral blood was collected 1 day before the first cycle and 2 weeks after each cycle. Three subsets were analyzed by FACS. Statistical analysis was determined by randomized block design ANOVA. $^{*}P < 0.05$.

antitumor immunity [26, 39]. Our results combined with others shed light on the development of new therapeutic schedules combining chemotherapy with immunotherapy.

5. Conclusion

In conclusion, we found that aTreg cells significantly increased in NSCLC patients and associated with late clinical stages. In this study, we also found aTreg significantly decreased after effective chemotherapy. In molecular events, it showed that aTreg cells showed lower levels of IFN-γ and higher level of TGF-β and CD39. The chemotherapy drug docetaxel can decrease aTreg cells and change the expression of IFN-γ and TGF-β. This study indicates that the inhibition of TGF-β and CD39 to suppress Tregs may act as a component of immunotherapy for cancer. It also provides the potential of combination with chemotherapy and immunotherapy in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jie-Yao Li and Xiu-Fang Duan contributed equally to this work.

Acknowledgments

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Research Article

Identification of Osteosarcoma-Related Specific Proteins in Serum Samples Using Surface-Enhanced Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry

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Osteosarcoma (OS) is the most common malignant bone tumor. To identify OS-related specific proteins for early diagnosis of OS, a novel approach, surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS) to serum samples from 25 OS patients, 16 osteochondroma, and 26 age-matched normal human volunteers as controls, was performed. Two proteins showed a significantly different expression in OS serum samples from control groups. Proteomic profiles and external leave-one-out cross-validation analysis showed that the correct rate of allocation, the sensitivity, and the specificity of diagnosis were 100%. These two proteins were further identified by searching the EPO-KB database, and one of the proteins identified as Serine rich region profile is involved in various cellular signaling cascades and tumor genesis. The presence of these two proteins in OS patients but absence from premalignant and normal human controls implied that they can be potential biomarkers for early diagnosis of OS.

1. Introduction

Osteosarcoma (OS), characterized by the production of osteoid material by malignant osteoblastic cells, is a primary malignant bone tumor deriving from primitive bone-forming mesenchymal cells [1]. Overall, the treatment strategies are preoperative chemotherapy and surgical resection followed by postoperative chemotherapy and adjuvant therapy for several years [2]. The 5-year survival rates for patients with localized osteosarcoma have dramatically improved from less than 15% in the 1950s to greater than 60% since the 1980s [3]. Despite advances in diagnostic and treatment regiments, progress since the 1980s has been minimal with 5-year survival rates still in the 60–70% range [4]. One of the reasons could be the presence of mutated oncogenes, which confer drug resistance of the cancer cell.

Currently, the diagnosis of OS is generally dependent on a comprehensive examination including clinical symptoms, imaging, laboratory examinations, biopsy, and immunohistochemistry. Clinically, OS was always diagnosed at the middle or even at a late stage. Therefore, the long-term survival rate of OS has not been improved in the past 20 years [2]. The histological examination of the biopsy specimens is still preferred by many orthopedic oncologists for the diagnosis of OS. Despite its invasive procedure, the accuracy of diagnosis may vary among different sample collection and different observers, making the clinical prediction questionable. Thus, we have focused our attention on the development of a noninvasive method for the early diagnosis of osteosarcoma.

Many studies have identified that antigenic changes in cells can be recognized by the immune system of patients. Some early studies demonstrated the presence of serum

autoantibodies to a panel of known tumor associated antigen (TAA) in various human cancers [5]. The study indicated that autoantibody reactivity to individual TAA rarely exceeded 20% in the cancer patient populations compared with normal human sera (NHS) which are usually less than 5%. It is conceivable that specific autoantibody profiles can be identified with help for discriminating autoantibody reactivity between cancer patients and control individuals and distinguishing between some types of cancer. So far, only a few studies have been performed in the field of using autoantibodies as diagnostic markers in osteosarcoma.

Several different approaches based on mass spectrometry (MS) have been applied in the search for cancer biomarkers [6]. Recently, technological improvements in MS have greatly increased their exploits in biomarker discovery. Direct analysis of serum samples using MS is becoming more popular due to its high-throughput nature and increased sensitivity. One of these proteomic approaches, surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS), is a rapid and sensitive proteomic technique to identify biomarkers in various forms of cancers [7, 8]. This technology has been effectively used in the validation of serum antigens for early-stage detection of various cancers, such as prostate [9], ovarian [10], and breast cancers [11]. Therefore, exploitation of SELDI-TOF-MS for screening biomarkers in OS is applicable. The searching database tool, empirical proteomic ontology knowledge base (EPO-KB), including tens of thousands of mass to charge ratio (M/Z) protein function, was used to identify proteins characterized from SELDI-TOF-MS. Using SELDI-TOF-MS combined with EPO-KB makes it possible to directly separate and identify the proteins from human osteosarcoma sera [12]. The major aim of this study using SELDI-TOF-MS combined with EPO-KB is to identify serum markers from human osteosarcoma sera as potential OS-associated markers for early diagnosis of OS.

2. Materials and Methods

2.1. Serum Samples. Sera from 25 Chinese patients, Han ethnic (fourteen males and eleven females, mean age 19.3 years, and range 2–50), with high-grade OS were collected at the time of diagnosis before biopsy and chemotherapy were analyzed. All samples were provided by Luoyang Orthopedic-Traumatological Hospital and Luoyang Institute of Orthopedic and Traumatology between Jan. 2008 and Dec. 2010. We randomly collected 26 human serum samples from normal age-matched donors in the same hospital as a normal control and 16 samples from osteochondroma (OC) (nine males and six females, mean age 16.8 years, and range 4–48 years, Chinese patients, Han ethnic) as benign tumor control. All blood samples (5 mL from each patient or normal person) were collected in EDTA-containing tubes at room temperature and immediately centrifuged at 1000 rpm for 10 min. The serum supernatant was collected and divided into aliquots and stored at −80°C. The study was approved by the ethics committee of Luoyang Orthopedic-Traumatological Hospital and Luoyang Institute of Orthopedic and Traumatology. The written informed consent for participation was obtained from all subjects.

2.2. Sample Preparation. Serum samples were diluted to a certain concentration and immediately centrifuged (20,000 rpm) for 10 min at 4°C. Twenty microliters of each serum was denatured by adding 40 mL of U9 buffer containing 9 mol/L uric acid, 2% CHAPS (pH 9.0), and 50 mmol/L Tris-HCl (pH 9.0) and shaken for 20 min at 4°C. Twenty microliters of the denatured samples were fractionated by 240 μL of U1 buffer and shaken for 30 min. All these samples were used in proteomic profiling.

2.3. SELDI-TOF-MS and Database Search. SELDI-TOF-MS was performed on NP20 chips. Each chip was added with bluestone (50 μL, 100 mmol/L) and shaken (200 rpm) for 5 min at room temperature, and the bluestone solution was immediately poured out. After being washed using ionized water for 5 times, the chip was added with 50 μL of CM Low Stringency Buffer (0.1 M sodium acetate, pH 4.0), shaken (200 rpm) for 5 min. Then, each chip was added with 150 μL of standard buffer containing 100 mol/L sodium phosphate and 500 mmol/L sodium chloride (pH 7.0) and incubated on the oscillator for 5 min at room temperature, and the buffer solution was removed. This process was repeated one more time. Each serum sample (50 μL) was randomly assigned to a spot on each protein-chip array and incubated on the oscillator for 60 min at 4°C. The samples were denatured by 150 μL of standard buffer, washed and oscillated for 3 times each for 5 min, and quickly washed with 1 mmol/L HEPES (pH 7.0). Chip EAM (sinapinic acid (SPA)) was added with 100 mmol/L acetonitrile (75 μL) and trifluoroacetic acid (TFA) (75 μL). The dissolved SPA was centrifuged for 1 min. 2 μL of SPA was spotted twice, 1 μL each time. The chip was air-dried before spotting. The reagents such as uric acid, acetonitrile, TFA, and SPA were purchased from Sigma. The PBSIIc and IMAC3-Cu were calibrated externally by using the all-in-one peptide and protein II molecular mass standards (Ciphergen Biosystems). Data were collected with Ciphergen protein-chip software. Proteins were randomly spotted on the chip. Each sample was analyzed in duplicate to minimize the effects of intra-assay variation. Proteomic profiles on the protein-chip arrays were detected by a Protein Biology System (Model PBSIIc).

2.4. Statistical Analysis. Data analysis was performed initially with Ciphergen Express Software 3.0 (Ciphergen Biosystems). Statistical signification peaks were recorded by $\overline{x} \pm s$ and identified by using parametric t-test with P value of 0.001 with Biomarker Wizard and Biomarker Pattern System software. The diagnostic accuracy was measured by external leave-one-out cross-validation, which is one of the feature selection steps. Other statistical analyses were performed using SPSS 11.5.

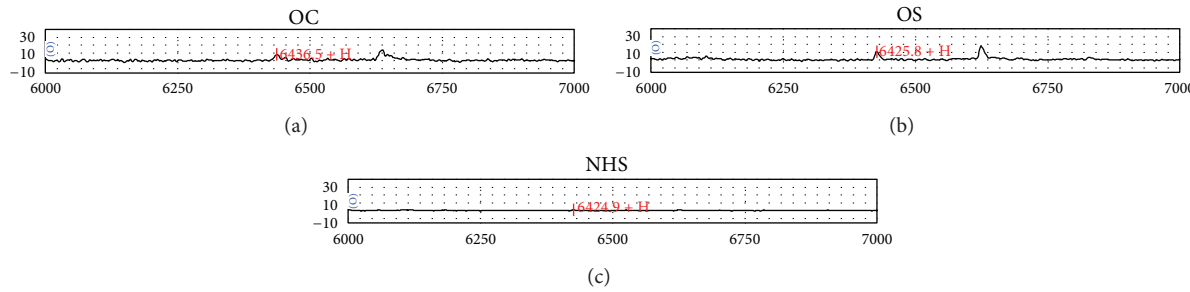

Figure 1: The SELDI-TOF-MS profiles of osteochondroma (OC), osteosarcoma (OS), and normal human serum (NHS) at the 6438 Da. The SELDI-TOF-MS profiles of the serum samples from OC (a), OS (b), and NHS (c).

Table 1: The M/Zs of 2 special serum proteins in three groups.

M/Z	NHS (mean ± SD)	OS (mean ± SD)	OC (mean ± SD)	P value	VI (score)
3954.00 Da	1.75 ± 0.35	−0.78 ± 0.08	1.47 ± 0.35	0.001	100
6438.00 Da	0.45 ± 0.21	0.88 ± 0.13	5.78 ± 1.12	0.001	100

M/Z: mass to charge ratio; NHS: normal human serum; OS: osteosarcoma; OC: osteochondroma; VI: variable importance.

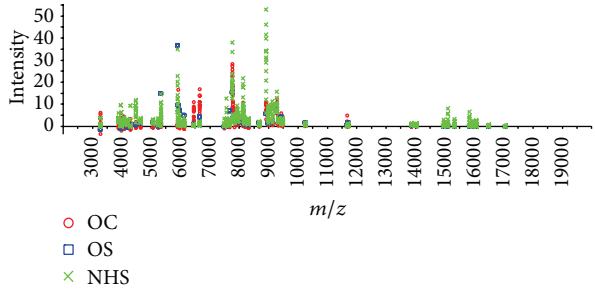

Figure 2: The protein intensity of osteochondroma (OC), osteosarcoma (OS), and normal human serum (NHS). The protein intensity of the serum samples from NHS (green panel), OS (blue panel), and OC (red panel).

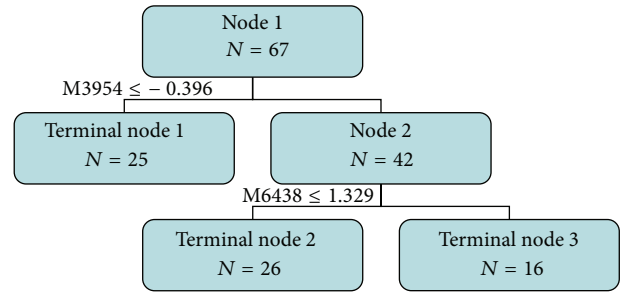

Figure 3: Classification tree topology for GROUP. Node 1 total sample (25 OS, 26 normal, and 16 OC), M3954 ≤ −0.396 (including 25 OS) divided to terminal node 1; M3954 > −0.396 (including 26 normal and 16 OC) divided to node 2; M6438 ≤ 1.329 (including 26 normal) divided to terminal node 2; M6438 > 1.329 (including 16 OC) divided to terminal node 3.

3. Results

3.1. Identification of Two Statistically Significant Proteins of OS. Sixty-seven serum samples were assayed by SELDI-TOF-MS. From a total of 67 serum samples, 25 serum samples are from OS patients compared with the control group of 42 serum samples (16 from osteochondroma and 26 from normal human serum). Interestingly, two protein peaks differed significantly in the OS patient group. One protein peak at 3954 Da was overexpressed and another one at 6438 Da (Table 1). The mass spectrum shows the comparison of three serum samples from patients with OS and OC as well as a serum from a normal individual (Figures 1 and 2).

3.2. Classification Tree Topology for GROUP and Analysis of Specificity and Sensitivity. In order to take a marked contrast, we apply the Biomarker Pattern software to analyze protein differences in the template group to m/z, respectively, 3954 Da and 6438 Da proteins composed of two different diagnostic classification tree models (Figure 3). The 67 samples were repeated sampling in the learning mode, and the diagnostic sensitivity was 98.51% and specificity was 98.51%, while, in test mode, the diagnostic sensitivity was 98.51%, and specificity was 100.00%. It was verified by leave-one-out and 3-fold cross-validation that correct grouping rate was 100% (67/67), good response rate was 100% (25/25), and specificity was 100% (42/42).

3.3. Database Search Results. We searched the M/Z of 3954 Da and 6438 Da in the first classification tree models and got related results in the EPO-KB database. The relevant information of 6438 Da protein was retrieved; however, the 3954 Da protein needs further identification (http://www.expasy.org/cgi-bin/prosite-search-ac?PDOC50099 and http://us.expasy.org/prosite/PS50324). It is serine rich protein sequences involved in cell signal transduction and carcinogenesis. In some microtubule plus end tracking proteins, there are some basic amino acid serine rich sequences. These basic amino acids serine rich sequences are often mediated by the microtubule plus end tracking proteins with microtubules

and EB protein and the role of other proteins. Protein peak of M/Z at 6438 Da in the B-cell lymphoma is highly expressed and, in the low expression of T cell lymphoma, it is closely related to the human immune system regulation.

4. Discussion

SELDI-TOF-MS is a relatively new proteomic technology that has impacted many areas of biological research [13, 14]. The high-throughput nature of this technology allows the processing of many samples simultaneously in a relatively short period of time. It requires only small amounts of samples for profiling, which makes it particularly suitable for clinical or translational studies. SELDI-TOF-MS has been successfully applied in identifying early detection biomarkers in multiple cancers, including ovarian, prostate, and breast cancers. However, SELDI-TOF-MS technology is not widely used in bone oncology to identify biomarkers and perform molecular classification, partly due to the limited sample size when compared to other cancers. The current study shows that patients with OS and OC and normal individuals can be distinguished from each other by the special proteins with different M/Z characters screened by SELDI-TOF-MS. The results showed three samples determined by the difference of the following standards (M3954 $\leq$ -0.396 (normal), M6438 $\leq$ 1.329 (OS), and M6438 $>$ 1.329 (OC)). It was verified by leave-one-out and 3-fold cross-validation that correct grouping rate was 100% (67/67), which proved that the results is reliable.

The diagnosis of OS generally depended on a comprehensive way including clinical symptoms, signs, image, laboratory examinations, biopsy, immunohistochemistry, and other methods. The surgical biopsy is also the terminal way for final diagnosis, which is an invasive procedure and has risks of enhancing tumor dissemination and metastases. If we can perform specific markers with high sensitivity and specificity using blood specimens from the patients, it will be more convenient and noninvasive to the patients at the initial diagnosis. These specific markers combined with X-ray exam could be used to monitor OS development in coordination with chemotherapy or radiography. And this combination would greatly improve the early-stage detection and treatment of OS. The present study has found the M/Z of 3954 Da and 6438 Da proteins in the classification tree models and gotten 6438 Da in the EPO-KB database, which is a serine rich protein sequences involved in cell signal transduction and carcinogenesis. These basic amino acids serine rich sequences are often mediated by the microtubule plus end tracking proteins with microtubules and EB protein and the role of other proteins. Since protein peak of M/Z at 6438 Da showed high expression in the B-cell lymphoma and low expression in T-cell lymphoma, it is closely related to the human immune system regulation. We thought that the 6438 Da protein identified by SELDI-TOF-MS may potentially be used for early detection, diagnosis, therapeutically monitoring, and prognosis of OS. In addition, randomized clinical trials are required to investigate its effect (or lack thereof).

SELDI-TOF-MS technology is still more constraining in clinical research, because the only result we can get is m/z, rather than the molecular weight. After finding the different proteins by SELDI-TOF-MS, further studies with chromatography, two-dimensional electrophoresis, spectroscopy, and other techniques need to be performed to identify these proteins immensely. However, these laboratory approaches could not be used for large-scale screening of biomarkers in clinical application. Additionally, its wide acceptance is still in question due to some concerns regarding reproducibility and reduced sensitivity at high molecular weight range [15].

These findings suggest that the M/Z of 3954 Da and 6438 Da proteins could potentially be biomarkers for early diagnosis of osteosarcoma. It would be interesting and important to conduct other independent studies in large populations for comparison. Future functional studies are warranted to verify these findings and to improve our understanding of the underling molecular mechanism of genetic contribution to OS carcinogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jianli Gu and Jitian Li contributed equally to this work.

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Research Article

Evaluation of Diagnostic Value in Using a Panel of Multiple Tumor-Associated Antigens for Immunodiagnosis of Cancer

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To determine whether a panel of multiple tumor-associated antigens (TAAs) would enhance antibody detection, the diagnostic value of autoantibodies to a panel of multiple TAAs in cancer has been evaluated. The TAAs used in this study was composed of eight TAAs including Imp1, p62, Koc, p53, C-myc, Cyclin B1, Survivin, and p16 full-length recombinant proteins. Enzyme-linked immunosorbent assay and immunoblotting were used to detect antibodies in 304 cancer sera and also 58 sera from normal individuals. The antibody frequency to any individual TAA in cancer was variable but rarely exceeded 20%. With the successive addition of TAAs to a final combination of total of eight antigens, there was a stepwise increase of positive antibody reactions reaching a sensitivity of 63.5% and a specificity of 86.2% in the combined cancer group. In different types of cancer, the ranges of positive and negative likelihood ratio were 4.07–4.76 and 0.39–0.51, respectively, and the ranges of positive and negative predictive values were 74.2–88.7% and 58.8–75.8%, respectively. Agreement rate and Kappa value were 67.1% and 0.51, respectively. These results further support our previous hypothesis that detection of anti-TAAs autoantibodies for diagnosis of certain type of cancer can be enhanced by using a miniarray of several TAAs.

1. Introduction

Many studies demonstrated that cancer sera contain antibodies which react with a unique group of autologous cellular antigens generally known as tumor-associated antigens (TAAs) [1, 2]. The types of cellular proteins which induce autoantibody responses are quite varied and include tumor suppressors such as p53 [3] and p16 [4], mRNA-binding proteins such as p62 [5], cell-cycle control proteins such as cyclin B1 [6, 7], and other cancer-related proteins. The immune systems of certain cancer patients are able to sense these aberrant tumor-associated proteins as unknown antigens and have the capability to respond by producing autoantibodies [8, 9]. Although the mechanism underlying the production of such autoantibodies in cancer patients is not completely understood, these autoantibodies can be used as reporters identifying aberrant cellular mechanisms in tumorigenesis and also serve as immunodiagnostic markers for cancer detection [1, 2, 10].

Many investigators have been interested in the use of autoantibodies as serological markers for cancer diagnosis, especially because of the general absence of these autoantibodies in normal individuals and noncancer conditions. Enthusiasm for this approach has been tempered by the low sensitivity. We have observed that this drawback can be overcome by using a panel of carefully selected TAAs and that different types of cancer may require different panels of TAAs to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis [11–15]. This feature is one of the innovative notions we have proposed in our study. For example, a previous study showed that the frequency of antibodies to any individual antigen rarely exceeded 15–20%, but with the successive addition of TAAs to a final combination of total seven antigens, there was stepwise increase in the percentage of positive reactors between 44% and 68% against a combined panel of seven antigens [16]. In addition, breast, lung, and prostate cancers showed separate and distinctive profiles of antibody

responses. It is conceivable that tailor-made TAA panels or arrays could be developed for different cancers and that TAA miniarrays might provide another approach to tumor detection and diagnosis.

In the present study, we determine whether a miniarray of multiple TAAs would enhance autoantibody detection and be a useful approach to cancer detection and diagnosis. In addition, this study also carries out evaluation of the diagnostic value of autoantibodies to a panel of multiple TAAs in different types of cancer.

2. Materials and Methods

2.1. Serum Samples. Sera from 304 patients with different types of cancer (98 lung cancer, 50 hepatocellular carcinoma, 46 colorectal cancer, 41 gastric cancer, and 69 other cancers including 15 bladder cancer, 14 pancreatic cancer, 12 breast cancer, 8 esophageal cancer, 7 ovarian cancer, 7 renal carcinoma, and 6 prostate cancer) and 58 normalhuman sera were obtained from the Department of Clinical laboratory Technology of Dalian Municipal Central Hospital (Liaoning Province, China). All cancer sera were collected at one time of cancer diagnosis when the patients had not yet received treatment with any chemotherapy or radiotherapy; 58 normal human sera were collected from adults during annual health examination in people who had no obvious evidence of malignancy. Due to regulations concerning studies of human subjects, the patient's name and identification number were blinded to investigators. This study was approved by the Institutional Review Boards of Dalian Municipal Central Hospital and collaborating academic institutions.

2.2. Recombinant TAAs. All TAAs used in this study, including Imp1, p62, Koc, p53, C-myc, Cyclin B1, Survivin, and p16, were constructed and purified from our previous studies [12, 14]. The reactivities of these selected TAAs were determined with either polyclonal or monoclonal antibodies against the respective proteins.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA). Purified recombinant proteins (Imp1, p62, Koc, p53, C-myc, Cyclin B1, Survivin, and p16) were individually diluted in phosphate buffered saline (PBS) to a final concentration of 0.5 μg/mL, and 200 μL was pipetted into each well to coat onto microtitre plates (Gibico, USA) overnight at 4°. The human serum samples were diluted in serum diluent at 1 : 200, incubated with the antigen-coated wells at 37° for 90 minutes followed by washing with PBS containing 0.05% Tween-20 (PBST) and then incubated with horseradish peroxidase- (HRP-) conjugated goat anti-human IgG (Caltag Laboratory, USA) as a secondary antibody diluted in anti-immunoglobulin diluent at 1 : 4,000 for 90 minutes followed by washing with PBST. The solution of 3,3′,5,5′-TMB (3,3′,5,5′-tetramethyl benzidine, TMB) was used as the detecting agent. The OD of each well was read at 450 nm. Each sample was tested in duplicate. The cut-off value for determining a positive reaction was designated as the mean absorbance of the 58 normal human sera plus 2 standard deviations (mean + 2SD). Because several hundreds of tests with sera were analyzed at different time periods, each run of ELISA included 4 NHS samples and 2 positive control samples. Four NHS samples representing a range of 2SD above and below the mean of the 58 normal human sera were always used in each experiment and the average value of these 4 NHS samples was used in each run to normalize all absorbance values to the standard mean of the entire 58 normal samples. In addition, all positive sera were confirmed with repeat testing, as were some negative sera. The detailed protocol of ELISA has been described previously [5, 16].

2.4. Western Blotting. Serum samples that were determined, using ELISA method, to contain autoantibodies were further tested by western blotting to confirm the immunoreactivity to TAAs. In brief, the purified recombinant proteins (Imp1, p62, Koc, p53, c-myc, cyclin B1, survivin, and p16) were electrophoresed by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. After blocking in TBST with 5% nonfat milk for 2 hours at room temperature, the PVDF membrane was incubated for 90 minutes with patient's sera diluted in serum diluent at 1 : 100 and then incubated with HRP-conjugated goat anti-human IgG diluted at 1 : 3000 for 90 minutes followed by washing with TBST solution. The ECL-kit was used to detect immunoreactive bands according to the manufacturer's instructions (Kangwei Biological Technology Company, Beijing, China).

2.5. Statistical Analysis. To determine whether the frequency of autoantibodies to eight TAAs in each cohort of patients' sera was significantly higher than that in sera from normal cohort, the data were analyzed using the χ^2 tests with Yates' correction. Two statistically significant levels (0.05 and 0.01) were used. The comprehensive evaluation of testing result for each anti-TAA antibody including the methods for calculating the sensitivity, specificity, Youden's index, positive and negative likelihood ratios, positive and negative predictive values, agreement rate, and Kappa value was based on the methodology provided in Epidemiology textbook [17].

3. Results

3.1. Frequency of Autoantibodies to the Miniarray of Eight TAAs. In order to evaluate the diagnostic values of antibodies to multiple TAAs in immunodiagnosis of cancer, eight purified recombinant TAAs were used as coating antigens in ELISA to detect autoantibodies against these eight TAAs in different types of cancer in this study. A positive test for anti-TAAs antibodies was taken as an absorbance reading above the mean + 2SD of the 58 normal human sera. As shown in Table 1, the frequency of autoantibodies to a miniarray of eight TAAs in sera from 304 patients with different types of cancer was variable, ranging between 4.9% and 26.8%. The highest frequency was cyclin B1 (26.8%) in gastric cancer. The cumulative autoantibody frequencies to eight TAAs were 64.3%, 66.0%, 65.2%, 56.1%, and 63.8% in different types of cancer, significantly higher than the frequency in sera from normal individuals (13.8%). The ELISA results were

Table 1: Frequency of antibodies to eight TAAs.

Autoantibodies to	Number (%) of autoantibodies in different types of cancers						
	Lung (98)	HCC (50)	Colorectal (46)	Gastric (41)	Others (69)	Total (304)	NHS (58)
Koc	9 (9.2)[a]	9 (18.0)[b]	7 (15.2)[b]	2 (4.9)	8 (11.6)[a]	35 (11.5)[b]	0
p62	15 (15.3)[b]	10 (20.0)[b]	10 (21.7)[b]	4 (9.8)	9 (13.0)[a]	48 (15.8)[b]	1 (1.7)
Imp1	17 (17.3)[b]	8 (16.0)[b]	10 (21.7)[b]	6 (14.6)[b]	9 (13.0)[a]	50 (16.4)[b]	0
Cyclin B1	17 (17.3)[b]	10 (20.0)[b]	8 (17.4)[a]	11 (26.8)[b]	12 (17.4)[b]	58 (19.1)[b]	1 (1.7)
p16	18 (18.4)[b]	3 (6.0)	9 (19.6)[a]	4 (9.8)	13 (18.8)[b]	47 (15.5)[a]	2 (3.4)
Survivin	19 (19.4)[b]	6 (12.0)	7 (15.2)[a]	6 (14.6)[a]	10 (14.5)[a]	48 (15.5)[b]	1 (1.7)
C-myc	24 (24.5)[b]	7 (14.0)	10 (21.7)[b]	7 (17.1)[a]	17 (24.6)[b]	65 (21.4)[b]	2 (3.4)
p53	25 (25.5)[b]	10 (20.0)[a]	11 (23.9)[b]	5 (12.2)	14 (20.3)[b]	65 (21.4)[b]	2 (3.4)
Cumulative to eight antigens	63 (64.3)[b]	33 (66.0)[b]	30 (65.2)[b]	23 (56.1)[b]	44 (63.8)[b]	193 (63.5)[b]	8 (13.8)

Cutoff value: mean +2 SD of NHS; P values to NHS were calculated to be <0.05 ([a]) or 0.01 ([b]).
HCC: hepatocellular carcinoma; NHS: normal human sera.

Table 2: Sequential addition of antigens to the miniarray of eight TAAs.

Number of different TAA panels	Number and percentage of autoantibodies in different types of cancers						
	Lung (98)	HCC (50)	Colorectal (46)	Gastric (41)	Others (69)	Total (304)	NHS (58)
(1) Koc	9 (9.2)[a]	9 (18.0)[b]	7 (15.2)[b]	2 (4.9)	8 (11.6)[a]	35 (11.5)[b]	0 (0)
(2) Koc and p62	20 (20.4)[b]	17 (34.0)[b]	15 (32.6)[b]	6 (14.6)[a]	15 (21.7)[b]	73 (24.0)[b]	1 (1.7)
(3) Koc, p62, and Imp1	31 (31.6)[b]	22 (44.0)[b]	20 (43.5)[b]	10 (24.4)[b]	18 (26.1)[b]	101 (33.2)[b]	1 (1.7)
(4) Koc, p62, Imp1, and cyclin B1	41 (41.8)[b]	26 (52.0)[b]	22 (47.8)[b]	16 (39.0)[b]	29 (42.0)[b]	134 (44.1)[b]	2 (3.4)
(5) Koc, p62, Imp1, cyclin B1, and p16	47 (48.0)[b]	27 (54.0)[b]	24 (52.2)[b]	19 (46.3)[b]	36 (52.2)[b]	153 (50.3)[b]	4 (6.9)
(6) Koc, p62, Imp1, cyclin B1, p16, and survivin	54 (55.1)[b]	29 (58.0)[b]	25 (54.3)[b]	21 (51.2)[b]	38 (55.1)[b]	167 (55.0)[b]	5 (8.6)
(7) Koc, p62, Imp1, cyclin B1, p16, survivin, and C-myc	58 (59.2)[b]	32 (64.0)[b]	28 (60.9)[b]	23 (56.1)[b]	42 (60.9)[b]	183 (60.2)[b]	7 (12.1)
(8) Koc, p62, Imp1, cyclin B1, p16, survivin, C-myc, and p53	63 (64.3)[b]	33 (66.0)[b]	30 (65.2)[b]	23 (56.1)[b]	44 (63.8)[b]	193 (63.5)[b]	8 (13.8)

All P values relative to NHS were calculated to be <0.05 ([a]) or 0.01 ([b]); HCC: hepatocellular carcinoma; NHS: normal human sera.

also confirmed by immunoblotting analysis. Figure 1 shows a miniarray analysis of eight antigens with 18 representative sera using western blot.

Differences in the reactions of different cancers to TAAs were observed and variations in the frequency of anti-TAAs antibodies were observed for any antigen. Positive reaction to Koc was detected in HCC, lung, colorectal, and other cancers but there was no significant difference between gastric cancer and normal individuals. For anti-C-myc antibody, a significant increased frequency was found in lung, colorectal, gastric, and other cancers, but there was no increased frequency in HCC. It is apparent from the data in Table 1 that different profiles of anti-TAAs antibodies could be observed using this array of eight TAAs.

An interesting feature we have observed in this study was that the highest frequencies of anti-TAAs antibody were 26.8% (anti-cyclin B1) in gastric cancer and 25.5% (anti-p53) in lung cancer. As noted previously by many investigators, the likelihood that antibodies would be detected against any individual TAA did not reach the level of high sensitivity, which would be useful as diagnostic biomarker. However, using a miniarray of eight TAAs, the number of anti-TAAs positive reactions increased to 56.1% in gastric cancer and 66.0% in HCC. For a total of 304 cancer patients, the sensitivity of the eight TAA arrays was 63.5% and the specificity was 86.2%. These data indicate that the use of the multiple TAAs can increase the sensitivity of anti-TAAs antibody detection in cancer sera.

3.2. Stepwise Increase in Rate of Anti-TAAs Antibody Positivity with Successive Addition of TAAs. Table 2 shows that the sequential addition of antigens to the array resulted in a stepwise increase in the number of positive reactions. With the successive addition of TAAs to a final combination of total eight antigens, this varied from one cancer to another and also from one antigen to another. The addition of CyclinB1 to Koc, p62 and Imp1 in the antigen array increased the number of positive reactions in gastric cancer (24.4–39.0%) and other cancers (26.1–42.0%), but there were lower increases in colorectal cancer (43.5–47.8%). In gastric cancer, the addition of p53 did not further increase the number of positive reactors compared with other types of cancers. These observations suggest that, for certain types of cancer, some antigens may turn out to be more specific while others may not.

3.3. Evaluation of the Diagnostic Values of a Panel of Eight TAAs in the Immunodiagnosis of Cancer. The validity of

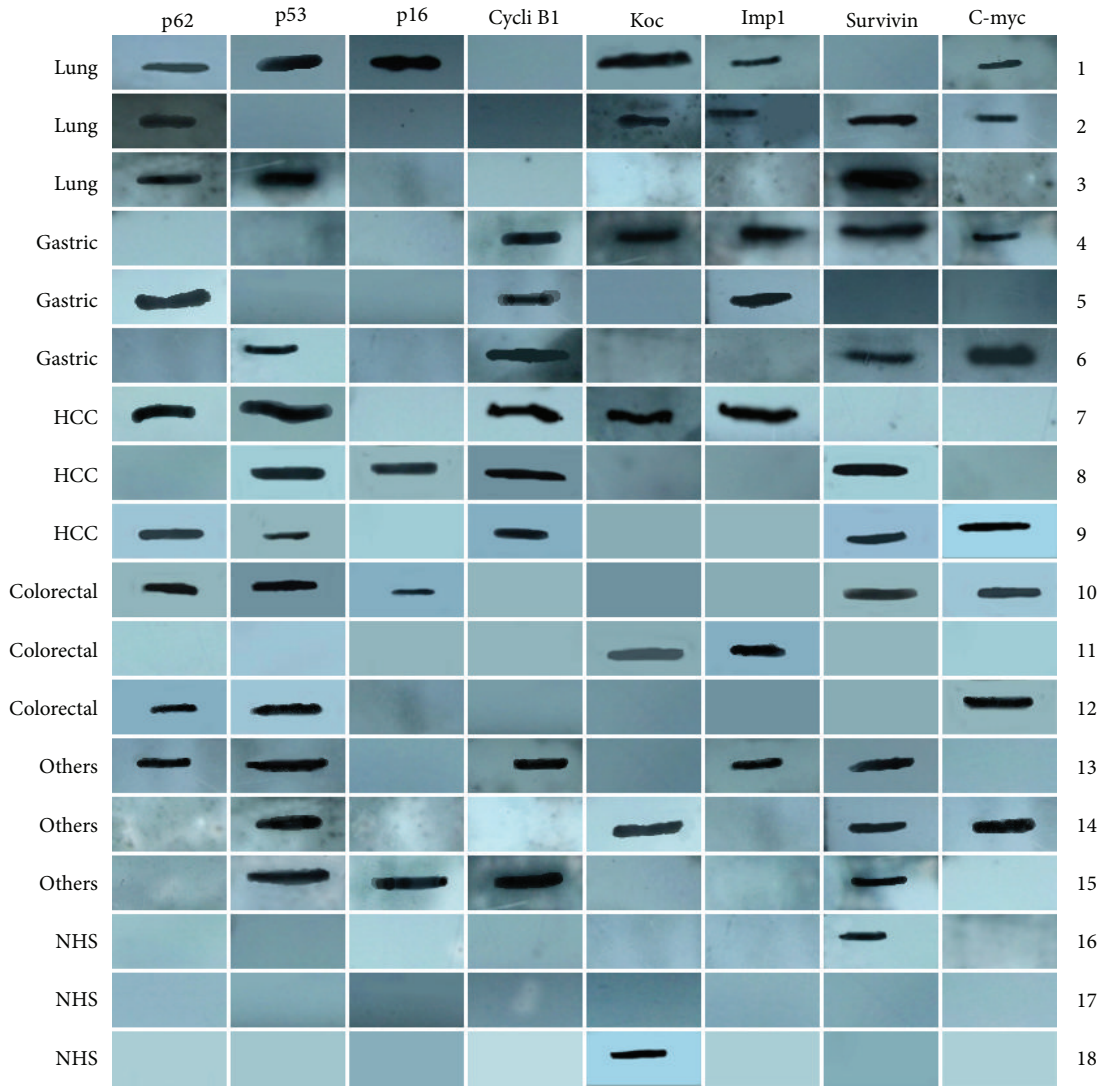

Figure 1: Miniarray of multiple TAAs with representative cancer sera using western blot analysis. Lanes 1–3 are three representative lung cancer sera; lanes 4–6 are three representative gastric cancer sera; lanes 7–9 are three representative hepatocellular carcinoma (HCC) sera; lanes 10–12 are three representative colorectal cancer sera; lanes 13–15 are three representative other cancers sera, showing different antibody profiles with the eight TAAs; and lanes 16–18 are three representative normal human sera (NHS), showing positive reactivity to Koc and survivin but not with other TAAs.

a test method is defined as its ability to distinguish between individuals with a disease and those without the disease. In order to assess the diagnostic value of this approach using a miniarray of multiple TAAs in separating individuals with and without cancer, a group of parameters, such as the YI, sensitivity/specificity, and PPV/NPV, were calculated and are shown in Tables 3 and 4.

Table 3 shows the comprehensive evaluation of antibodies to a panel of eight TAAs. With the successive addition of TAAs to a total of eight antigens, there was a stepwise increase of positive antibody reactions up to 63.5% and there was also a slight decrease of specificity from 100.0% with one TAA to 86.2% with a panel of eight TAAs. It is consistent with the results of two other parameters (PPV/NPV). The PPV/NPV were also variable in different combinations of TAAs. In the panel of eight TAAs, the ranges of the PPV and the NPV in different types of cancer were 74.2–88.7%, and 58.8–75.8%, respectively. Youden's index also increased from 0.049–0.180 with one TAA to 0.423–0.522 with eight TAAs. The ranges of positive and negative likelihood ratios were, respectively, 4.07–4.76 and 0.39–0.51 in different cancer cohort and in total cancer group were 4.60 and 0.42, respectively, which showed that the clinical diagnostic value of parallel assay of eight TAAs was high. Positive and negative predictive values were 74.2–88.7% and 58.8–75.8%, respectively. It indicated that parallel assay of eight TAAs raised the diagnostic accuracy greatly. Agreement rate and Kappa were 72.4–74.0% and 0.44–0.53, respectively, which indicated the observed value of this assay had middle range of coincidence with actual value. These data suggest that using a miniarray of multiple TAAs can increase the clinical diagnostic quality and value in cancer.

Table 3: Evaluation of diagnostic values of different TAA panels in the diagnosis of cancer.

		Number of different TAA panels[a]							
		1	2	3	4	5	6	7	8
NHS	Positive % (number)	0 (0)	1.7 (1)	1.7 (1)	3.4 (2)	6.9 (4)	8.6 (5)	12.1 (7)	13.8 (8)
Lung (98)	Positive % (number)	9.2 (9)	20.4 (20)	31.6 (31)	41.8 (41)	48.0 (47)	55.1 (54)	59.2 (58)	64.3 (63)
	Se/Sp	9.2/100.0	20.4/98.3	31.6/98.3	41.8/96.6	48.0/93.1	55.1/91.4	59.2/87.9	64.3/86.2
	YI	0.092	0.187	0.299	0.384	0.411	0.465	0.471	0.505
	PPV/NPV	100.0/39.5	95.2/42.2	96.9/46.0	95.3/49.6	92.2/51.4	91.5/54.6	89.2/56.0	88.7/58.8
HCC (50)	Positive % (number)	18.0 (9)	34.0 (17)	44.0 (22)	52.0 (26)	54.0 (27)	58.0 (29)	64.0 (32)	66.0 (33)
	Se/Sp	18.0/100.0	34.0/98.3	44.0/98.3	52.0/96.6	54.0/93.1	58.0/91.4	64.0/87.9	66.0/86.2
	YI	0.180	0.323	0.423	0.486	0.471	0.494	0.520	0.522
	PPV/NPV	100.0/58.6	94.4/63.3	95.7/67.1	92.9/70.0	87.1/70.1	85.3/71.6	82.1/73.9	80.5/74.6
Colorectal (46)	Positive % (number)	15.2 (7)	32.6 (15)	43.5 (20)	47.8 (22)	52.2 (24)	54.3 (25)	60.9 (28)	65.2 (30)
	Se/Sp	15.2/100.0	32.6/98.3	43.5/98.3	47.8/96.6	52.2/93.1	54.3/91.4	60.9/87.9	65.2/86.2
	YI	0.152	0.309	0.418	0.444	0.453	0.457	0.488	0.514
	PPV/NPV	100.0/59.8	93.8/63.3	95.2/68.7	91.7/70.0	85.7/71.1	83.3/71.6	80.0/73.9	78.9/75.8
Gastric (41)	Positive % (number)	4.9 (2)	14.6 (6)	24.4 (10)	39.0 (16)	46.3 (19)	51.2 (21)	56.1 (23)	56.1 (23)
	Se/Sp	4.9/100.0	14.6/98.3	24.4/98.3	39.0/96.6	46.3/93.1	51.2/91.4	56.1/87.9	56.1/86.2
	YI	0.049	0.129	0.227	0.356	0.394	0.426	0.440	0.423
	PPV/NPV	100.0/59.8	85.7/62.0	90.9/64.8	88.9/69.1	82.6/71.1	80.8/72.6	76.7/73.9	74.2/73.5
Others (69)	Positive % (number)	11.6 (8)	21.7 (15)	26.1 (18)	42.0 (29)	52.2 (36)	55.1 (38)	60.9 (42)	63.8 (44)
	Se/Sp	11.6/100.0	21.7/98.3	26.1/98.3	42.0/96.6	52.2/93.1	55.1/91.4	60.9/87.9	63.8/86.2
	YI	0.116	0.200	0.244	0.386	0.453	0.465	0.488	0.500
	PPV/NPV	100.0/48.7	93.8/51.4	94.7/52.8	93.5/58.3	90.0/62.1	88.4/63.1	85.7/65.4	84.6/66.7
Total (304)	Positive % (number)	11.5 (35)	24.0 (73)	33.2 (101)	44.1 (134)	50.3 (153)	55.0 (167)	60.2 (183)	63.5 (193)
	Se/Sp	11.5/100.0	24.0/98.3	33.2/98.3	44.1/96.6	50.3/93.1	55.0/91.4	60.2/87.9	63.5/86.2
	YI	0.115	0.223	0.315	0.407	0.434	0.464	0.481	0.497
	PPV/NPV	100.0/17.7	98.6/19.8	99.0/21.9	98.5/24.8	97.5/26.3	97.1/27.9	96.3/29.7	96.0/31.1

[a]Number of different TAA panels, corresponding to TAA panels shown in Table 2.
Se: sensitivity; Sp: specificity; YI: Youden's index; PPV: positive predictive value; NPV: negative predictive value.

Table 4: Summary of diagnostic value of antibodies to a panel of eight TAAs.

	Lung	HCC	Colorectal	Gastric	Others	Total	NHS
Any TAA positive	63 (A1)	33 (A2)	30 (A3)	23 (A4)	44 (A5)	193 (A6)	8 (B)
All TAA negative	35 (C1)	17 (C2)	16 (C3)	18 (C4)	25 (C5)	111 (C6)	50 (D)
Youden's index	0.505	0.522	0.514	0.423	0.500	0.497	—
+Likelihood ratio	4.66	4.76	4.72	4.07	4.62	4.60	—
−Likelihood ratio	0.41	0.39	0.45	0.51	0.42	0.42	—
+Predictive value	88.7	80.5	78.9	74.2	84.6	96.0	—
−Predictive value	58.8	74.6	75.8	73.5	66.7	31.1	—
Agreement rate	72.4	76.9	76.9	73.7	74.0	67.1	—
Kappa value	0.46	0.53	0.52	0.44	0.49	0.51	—

Note: Calculation Formulas used in Table 4 were as follows.
Youden's index = sensitivity + specificity − 1.
Positive (+) likelihood ratio = sensitivity/(1 − specificity).
Negative (−) likelihood ratio = (1 − sensitivity)/specificity.
Positive (+) predictive value = $A/(A + B) \times 100\%$.
Negative (−) predictive value = $D/(C + D) \times 100\%$.
Agreement rate = $(A + D)/(A + B + C + D) \times 100\%$.
Kappa value = $[N(A + D) - (R_1C_1 + R_2C_2)]/[N_2 - (R_1C_1 + R_2C_2)]$.

4. Discussion

Numerous studies indicate that no single marker can completely identify and differentiate the cancer groups from healthy controls. However, a combination of multiple markers may provide a promising way for the early detection of cancer. On the other hand, the multifactorial and multistep nature of the molecular pathogenesis of human cancer can also be considered in the design and interpretation of studies to identify biomarkers useful for the early detection of cancer. Our previous studies showed that combinations of multiple antigen-antibody systems might acquire higher sensitivity for diagnosis of cancer [13, 18]. Wang et al. used a phage display library derived from prostate cancer tissue to develop a phage protein microarray for the analysis of autoantibodies in serum samples from 119 patients with prostate cancer and 138 individuals with no history of prostate cancer [19]. In this study, a 22-phage-peptide detector was constructed for prostate-cancer screening, with 81.6% sensitivity and 88.2% specificity. These studies strongly support the hypothesis that "customized" TAA arrays enhance autoantibody detection in cancer and constitute promising and powerful tools for the immunoserological diagnosis of certain cancer.

In the present study, eight TAAs were used as coating antigens in 304 sera from patients with different types of cancer and 58 sera from normal individuals. With the successive addition of TAAs to a final combination of total eight antigens, the sensitivity in detecting autoantibody in any type of cancer increased from 20% to 26% when one antigen was used, while it ranged from 56% to 66% when eight antigens were used. In the combined cancer group, positive predictive value was 96.0%. It indicated that parallel assay of eight TAAs raised the diagnostic quality greatly. In addition, positive likelihood ratio was 4.60, which showed that the clinical diagnostic value of parallel assay of eight TAAs was high, and Kappa value was 0.51, which indicated the observed value of this assay had middle range of coincidence with actual value.

Our aim is to increase the sensitivity and specificity of anti-TAA antibodies as diagnostic markers in cancer detection by expanding the TAA array, including TAAs which may be more selectively associated with one specific type of cancer and not with others. For future studies, we propose that certain selected antibody-antigen systems may be unique to one type of cancer and others may not. Optimum candidates for inclusion in a miniarray of multiple TAAs and the specific panels of TAAs should be developed for different cancers. We stress the notion that panels of "customized" TAAs should be used for different types of cancer and that these customized panels should be rigorously tested for sensitivity and specificity not only against other cancers but also against other disease conditions. For instance, in the case of lung cancer, the nature of precondition would be heavy cigarette smokers. It has been well known that cigarette smoking is the major cause of lung cancer and is currently estimated to cause 85% of all lung cancer deaths. In the case of HCC, the natural conditions would be chronic hepatitis and liver cirrhosis. Cancer-associated antigen panels might conceivably be used for early detection of tumors in high-risk individuals. Anti-p53 antibodies were detected in two heavy smokers before clinical detection of lung cancer, and, in one patient, early treatment resulted in good response, which correlated with total disappearance of p53 antibodies [20, 21]. The basis for the notion of the customized panels of TTAs is to identify a specific panel of TAAs for one type of cancer and compare this with antigen panels associated with the natural conditions or high-risk individuals.

In conclusion, this study further supports our previous hypothesis that a customized miniarray of multiple carefully selected TAAs might acquire higher sensitivity for the diagnosis of cancer. TAA arrays provide promising and powerful tools for enhancing cancer detection, but their utility in a clinical setting is currently still in its infancy. Before TAA arrays could be widely implemented in screening programs for cancer diagnosis or as tools for monitoring cancer progression and guiding therapeutic interventions, it would be important to maximize their sensitivity and specificity by defining systematically the optimal combination of TAAs.

5. Conclusion

This study demonstrates that detection of autoantibodies for diagnosis of certain types of cancer can be enhanced by using a miniarray of several TAAs as target antigens. These results also indicated that the design of unique TAA panels for different cancers would help to determine whether using a miniarray of multiple TAAs is a clinically useful noninvasive approach in cancer detection and diagnosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Peng Wang and Chunhua Song contributed equally to this work.

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Research Article

Serum Level of Antibody against Benzo[a]pyrene-7,8-diol-9,10-epoxide-DNA Adducts in People Dermally Exposed to PAHs

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Some specific antibodies indicate the presence of antigenic structures on DNA (DNA adducts) that can play an important role in the process of mutagenesis and/or carcinogenesis. They indicate the presence of increased genotoxic potential (hazard) prior to the formation of disease (primary prevention). The present study was focused on the serum level of benzo[a]pyrene 7,8-diol-9,10-epoxide-DNA adducts antibodies (anti-BPDE-DNA) in psoriatic patients ($n = 55$) dermally exposed to different levels of polycyclic aromatic hydrocarbons (PAHs). The general goal of the study was to contribute to better understanding of the value of the assumed biomarker (anti-BPDE-DNA) for evaluation of the organism's answer to genotoxic exposure to PAHs. Elevated level of exposure to PAHs resulted in the increased level of anti-BPDE-DNA. However, almost all levels of anti-BPDE-DNA ranged within the field of low values. Both variants of GT (CCT-3% and CCT-5%) induced higher expression of anti-BPDE-DNA in the group of nonsmokers. Significant relations between the level of anti-BPDE-DNA and PASI score, total duration of the therapy, or time of UVR exposure were not found. Further studies are needed to reduce interpretation uncertainty of this promising bioindicator.

1. Introduction

The immune response to the antigenic changes in cancer cells includes expression of serum antibody against these cellular antigens (tumor-associated antigens, TAAs). The serum antibody against TAAs can be used as biomarker in cancer immunodiagnosis. In this case, we can talk about the biomarkers in early secondary prevention [1].

Other specific antibodies indicate the presence of antigenic structures on DNA (DNA adducts) that can play an important role in the process of mutagenesis and/or carcinogenesis. They indicate the presence of increased genotoxic potential (hazard) prior to the formation or development of disease. Here we can talk about the biomarkers in primary prevention. The persistence and stability of given antibodies in the serum is an advantage over other potential markers which are rapidly degraded due to reparation processes (for example chromosomal aberration) [2].

Polycyclic aromatic hydrocarbons (PAHs) are recognized as potential environmental mutagens/carcinogens, requiring bioactivation [3]. Typical representative of the group of PAHs is benzo[a]pyrene (BaP). BaP and its ultimate

metabolite benzo[a]pyrene 7,8-diol 9,10-epoxide (BPDE), are classical DNA damaging carcinogens which produce DNA adducts [4]. Formation of DNA adducts is generally one of the assumed mechanisms of PAHs induced mutagenesis/carcinogenesis. In this sense, increased levels of DNA adducts can represent an increased genotoxic potential of exposure. Adducted DNA becomes antigenic and induces immune response by production of antibodies against BPDE-DNA adducts (anti-BPDE-DNA). Anti-BPDE-DNA has been found in serum of PAHs exposed subjects (occupational exposures, smokers) [1, 5]. Accordingly, the presence of circulating anti-carcinogen antibody has been proposed as a biomarker of genotoxic exposure (DNA damage) [6, 7]. However, the use of this bio-indicator is still associated with considerable uncertainty concerning the interpretation of results.

Psoriasis is a chronic, relapsing and remitting immune-mediated inflammatory skin disease that has a prevalence of 2-3% in the world's population, whence 1-2% in Europe [8, 9]. In 1925, William H. Goeckerman from the Mayo Clinic reported the successful use of topical crude coal tar (CCT) and broad-spectrum of UV radiation (UVR) in the treatment of psoriasis [10]. This medical procedure is known as Goeckerman therapy (GT). Despite the availability of newer treatments, classical topical treatments for psoriasis still have an important position for selected patient populations [11]. Topical treatment, including GT, is now applied in approximately 75% of cases which are classified as light to moderately severe forms [12, 13]. Fundamental mechanism of the therapeutic effects of CCT is based on immunosuppression (caused by high portion of PAHs) without evidence of systemic immuno-toxicity [14].

The use of GT has recently decreased for several reasons, including supposed genotoxicity of CCT [14–17]. The CCT is rich in PAHs and GT therefore presents heavy dermal exposure to mutagenic/carcinogenic PAHs. The mutagenicity/carcinogenicity of CCT has been shown in animal studies and studies in occupational settings [18, 19] but there was no clear evidence of an increased risk of skin tumors or internal tumors after the therapy of CCT [3, 20].

Presented study is focused on the serum level of anti-BPDE-DNA in psoriatic patients dermally exposed to PAHs (CCT). General goal is to contribute to better understanding the value of assumed biomarker (anti-BPDE-DNA) for evaluation of the organism's reaction to genotoxic exposure (BaP) and for evaluation of the protective capacity of the immune system (against BPDE-DNA adducts). During the study, we investigated (1) whether changes in the level of genotoxic exposure (CCT/BaP) affect the level of anti-BPDE-DNA and (2) other important factors which could affect exposure the level of anti-BPDE-DNA.

2. Material and Methods

2.1. Study Groups. The monitored group consisted of patients with chronic stable plague psoriasis, treated by GT at the Clinic of Dermal and Venereal Diseases, University Hospital Hradec Kralove (Czech Republic). Over the period of four years we collected data from 55 adult patients. Of this number 23 patients were treated with dermatological ointment containing 3% of CCT (CCT-3% group) and 32 patients were treated with ointment containing 5% of CCT (CCT-5% group). The CCT-3% group consisted of 12 women and 11 men, the average age of 40 years, age variance 18–75 years, 11 smokers and 12 nonsmokers. The CCT-5% group consisted of 13 women and 19 men, average age of 57 years, age variance 18–75 years, 14 smokers and 18 nonsmokers.

With the use of the questionnaire, we checked the patients for previous excessive exposure to PAHs and artificial UVR. The patients who had prior excessive exposure to PAHs and/or artificial UVR were excluded from the monitored group. The study was approved by the Ethics Committee of the University Hospital in Hradec Kralove, Czech Republic. Informed written consent was obtained from each patient.

2.2. Goeckerman Therapy. Dermatological ointment containing 3% and 5% of CCT (pharmaceutical grade crude coal tar; CCT-3% and CCT-5%) was administered daily overnight on psoriatic lesions (10–75% of total body surface in monitored group). The content of BaP in the sample of used pharmaceutical grade crude coal tar was 0.008 mg/g CCT. In morning, the residue of tar ointment was removed from the body (using oil bath) and the patient was whole-body irradiated by UVR. The irradiation was individual according to the disease activity (1–15 min). The density of radiation was 248.17 μW/cm^2 for UV-B and 132.1 μW/cm^2 for UV-A (controlled by Sola-Scope 2000 spectrometer; Solatell, UK). Duration of the therapy was determined by the status of the disease and with regard to the treatment efficacy (average duration of 13 days; range of 10–22 days). The efficacy was calculated from the actual state of erythema, desquamation, and skin infiltration by using of PASI score (Psoriasis Area Severity Index) [21]. The therapy was ceased when 50% decrease of PASI was achieved.

2.3. Serum Level of Anti-BPDE-DNA. Samples of heparinized venous blood were obtained by venipuncture of the cubital vein before first treatment and again after completion of GT (at the day of dismissal from the hospital) using BD Vacutainer sampling tubes. Blood sera were isolated by centrifugation. Serum samples were stored under −70°C until they were analyzed. Repeated thawing and freezing were avoided. Serum level of anti-BPDE-DNA (IgG/IgM) was analyzed by ELISA method (ELISA-VIDITEST anti-BPDE-DNA human, VIDIA, Jesenice, Czech Republic). The results were expressed in the form of Evaluation Index (EI = absorbance of evaluated serum/absorbance of high positive control serum). Samples with EI less than 0.5 are termed as the serum with low levels of anti-BPDE-DNA. Analogously, serum samples with EI greater than 0.5 are referred to as the serum with high level of anti-BPDE-DNA (ELISA-VIDITEST anti-BPDE-DNA human, VIDIA, Jesenice, Czech Republic).

2.4. Statistical Analysis. Data were analyzed by using MATLAB rel. 2013b software (Mathworks, Inc., Massachusetts, USA). Because the Lilliefors test of normality had rejected the

Table 1: The serum level of anti-BPDE-DNA before and after the therapy (all patients).

CCT content	Anti-BPDE-DNA		Statistical significance of differences
	Before GT	After GT	
CCT-3% ($n = 23$)	0.22 (0.18–0.29)	0.28 (0.20–0.34)	$P <$ **0.01**
CCT-5% ($n = 32$)	0.31 (0.25–0.45)	0.37 (0.28–0.48)	$P <$ **0.001**
Statistical significance of differences	$P <$ **0.01**	$P <$ **0.05**	

Anti-BPDE-DNA values (expressed as the EI) are presented as median (lower–upper quartile) because of nonnormal data distribution; *n*: number of subjects; *P*: Wilcoxon matched-pairs test.

Table 2: The serum level of anti-BPDE-DNA before and after the therapy (nonsmokers).

CCT content	Anti-BPDE-DNA		Statistical significance of differences
	Before GT	After GT	
CCT-3% ($n = 12$)	0.23 (0.17–0.30)	0.28 (0.19–0.43)	$P <$ **0.001**
CCT-5% ($n = 18$)	0.34 (0.28–0.47)	0.38 (0.30–0.50)	$P <$ **0.05**
Statistical significance of differences	$P <$ **0.05**	**NS**	

Anti-BPDE-DNA values (expressed as the EI) are presented as median (lower–upper quartile) because of nonnormal data distribution; *n*: number of subjects; *P*: Wilcoxon matched-pairs test.

hypothesis of normal distribution, nonparametric tests were used. Data were analyzed by the Wilcoxon signed rank test. The effect of smoking was evaluated by the Wilcoxon rank sum test. The association between serum level of anti-BPDE-DNA after the therapy and selected parameters was evaluated by Spearman Rank Order Correlations.

3. Results

After the statistical processing of results stated in Tables 1–3, we have found that both variants of the therapy (group with CCT-3% and group with CCT-5%) significantly increased serum level of anti-BPDE-DNA. The therapy with CCT-5% increases the level of anti-BPDE-DNA at a higher level of significance ($P < 0.001$) than the therapy with CCT-3% ($P < 0.01$). However, it must be stated, that majority of anti-BPDE-DNA values (approx. 85% of all samples) ranged in the field of low values (EI < 0.5), regardless whether it was collected before or after therapy (Figure 1).

Both variants of the therapy significantly improved the status of the disease (decreased PASI score; $P < 0.001$) which generally confirmed high effectiveness of the therapy. In comparison to CCT-3% variant, the effectiveness of the CCT-5% variant, expressed as PASI score, was higher by about 12% (Table 4).

The serum levels of anti-BPDE-DNA in smokers and non-smokers (before and after the treatment) were comparable (Tables 2 and 3). However, it seems that both variants of the treatment induced higher expression of anti-BPDE-DNA in the group of non-smokers (Tables 2 and 3 and Figures 2 and 3).

The therapy increased the level of anti-BPDE-DNA and decreased PASI score; however, we found no significant relationship between these two parameters (CCT-3%, $r = -0.17$; CCT-5%, $r = -0.22$). As well, the relationships between final level of anti-BPDE-DNA and total duration of the therapy (CCT-3%, $r = 0.21$; CCT-5%, $r = 0.10$), and between final level of anti-BPDE-DNA and time of UVR exposure (CCT-3%, $r = 0.18$; CCT-5%, $r = 0.25$) were not significant.

Scatter plots (Figures 1, 2, and 3) depicts anti-BPDE-DNA values before and after the GT therapy. Each dot belongs to one patient. The open dots mark patients from the group treated by CCT-3%, the black filled dots mark patients treated by CCT-5%. Values above the dotted diagonal line reflect increase of the anti-BPDE-DNA after the therapy. Probability of difference between the pre- and the post-treatment medians was evaluated by Wilcoxon signed rank test. The top histogram shows distribution of the anti-BPDE-DNA before treatment; the white color corresponds to the patients with CCT-3%, added black bars represent the patients with CCT-5%. The right side histogram corresponds to the after treatment distribution of anti-BPDE-DNA values.

4. Discussion

After exposure to BaP, antibodies (IgG and IgM) against BaP can be detected in human serum. These antibodies are capable of scavenging BaP from the environment and thereby block its metabolic activation in cells [1]. This immune response may decrease the risk of cancer.

The situation is different with antibodies (IgG and IgM) against BPDE-DNA adducts. Elevated levels of these antibodies were found in the population of individuals exposed to higher concentrations of PAHs and corresponded with increased levels of DNA adducts at a group level. The serum

Table 3: The serum level of anti-BPDE-DNA before and after the therapy (smokers).

CCT content	Anti-BPDE-DNA		Statistical significance of differences
	Before GT	After GT	
CCT-3% ($n = 11$)	0.21 (0.18–0.28)	0.28 (0.20–0.31)	**NS**
CCT-5% ($n = 14$)	0.30 (0.24–0.38)	0.35 (0.25–0.45)	$P <$ **0.05**
Statistical significance of differences	**NS**	**NS**	

Anti-BPDE-DNA values (expressed as the EI) are presented as median (lower–upper quartile) because of nonnormal data distribution; *n*: number of subjects; *P*: Wilcoxon matched-pairs test.

Table 4: The effectiveness of the therapy (PASI score).

CCT content	PASI score		Statistical significance of differences	Efficiency (%)
	Before GT	After GT		
CCT-3% ($n = 23$)	16.20 (12.70–20.85)	7.80 (6.60–10.70)	$P <$ **0.001**	50.9 (41.9–60.58)
CCT-5% ($n = 32$)	17.10 (14.80–20.60)	6.05 (4.30–7.95)	$P <$ **0.001**	62.7 (52.4–74.0)
Statistical significance of differences	**NS**	$P <$ **0.05**		$P <$ **0.01**

The effectiveness of the therapy is expressed as the PASI score; the values are presented as median (lower–upper quartile) because of nonnormal data distribution; *n*: number of subjects; *P*: Wilcoxon matched-pairs test.
Calculation of the efficiency of the treatment: (100 − [PASI after GT/PASI before GT] × 100).

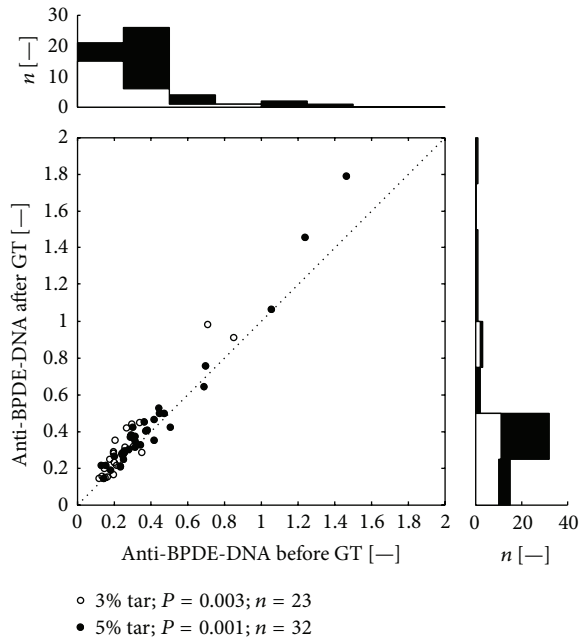

Figure 1: Distribution of values of anti-BPDE-DNA (all patients). Legend: Scatter plot depicts anti-BPDE-DNA values before and after the GT therapy. All together 55 dots represent 110 measurements, each dot belongs to a one patient.

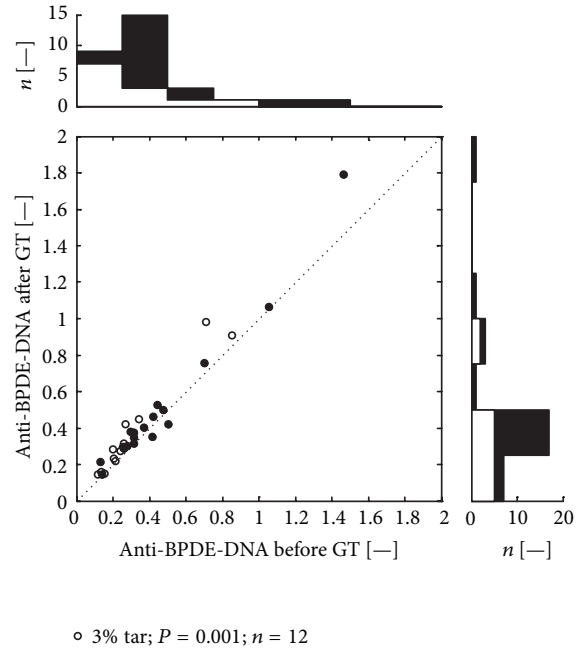

Figure 2: Distribution of values of anti-BPDE-DNA (non-smokers). Legend: Scatter plot depicts anti-BPDE-DNA values before and after the GT therapy. All together 30 dots represent 60 measurements, each dot belongs to a one patient.

level of anti-BPDE-DNA reflects either the previous exposure of the individual to BaP and/or the immune status and protective capacity of the immune system against BaP induced cancer [5].

Biological monitoring includes indicators of exposure, effect and susceptibility. BPDE-DNA adducts can be indicative of both exposure and genotoxic effects of BaP. Circulating anti-BPDE-DNA signals the presence of BPDE-DNA adducts. The intensity of that signal depends (among others) on the level of BPDE-DNA adducts. The level of BPDE-DNA adducts is determined by the character of BaP

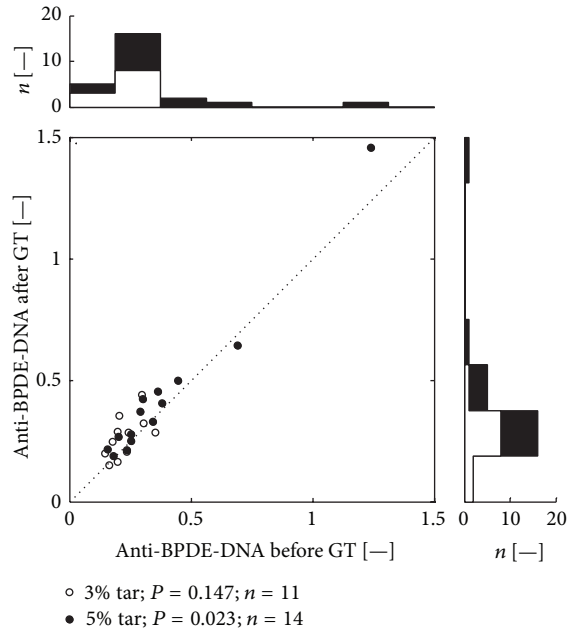

Figure 3: Distribution of values of anti-BPDE-DNA (smokers). Legend: Scatter plot depicts anti-BPDE-DNA values before and after the GT therapy. All together 25 dots represent 50 measurements, each dot belongs to a one patient.

metabolism and by the degree of adaptation and reparation processes.

There are only few epidemiological studies of PAHs exposed population to assess the impact of carcinogen-specific antibodies on their risk of tumor development and on their relation to other indicators of genotoxic exposure [1, 5]. Also, few attempts have been made in vivo or in vitro to understand the implications of an antibody response to metabolic activation of carcinogens and carcinogenesis. Recent study provided evidence that specific humoral immunity may modulate the genotoxic effect induced by subsequent carcinogen exposure, however, the mechanisms involved remain largely unexplored [1].

Scientific data concerning the level of anti-BPDE in the treatment of CCT are very limited. In one study, a group of psoriatic patients (treated by CCT) was used as a model for evaluation the suitability of immunoassays for the biomonitoring of exposure to BaP and related PAHs. The assays included measurement of PAH diol epoxide-DNA adducts in white blood cells, PAH-albumin adducts and serum levels of antibodies recognizing BP diol epoxide-DNA adducts. PAH-DNA adducts were elevated in patients: mean 6.77 ± 12.05/10(8) compared to controls: 4.90 ± 8.81/10(8), ($P = 0.12$), however there was no difference in PAH-albumin adducts between patients (mean 0.61 ± 0.31 fmol/micrograms) and controls (0.63 ± 0.30 fmol/micrograms). About 30% of patients and controls had measurable titer of antibodies recognizing BPDE-DNA adducts [22].

In presented study we have found that both variants of the therapy (group with CCT-3% and group with CCT-5%) significantly increased serum level of anti-BPDE-DNA (Table 1, Figure 1). Nevertheless, it must be stressed that majority of anti-BPDE-DNA values (85% of all samples) ranged within the field of low values (EI < 0.5).

A person is exposed to PAHs from many sources daily. These substances are virtually ubiquitous and contaminate air, water and food [2, 14]. From this perspective, it is understood that a certain level of antibodies will be present in all individuals. Smoking increases the level of BPDE-DNA adducts [23]. Serum anti-BPDE-DNA can be detected in smokers, and its persistence for months after smoking cessation suggests its usefulness for relatively long-term surveys [24]. On the other hand, it seems that long term chronic exposure to PAHs (for instance, by smoking) reduces body immune response and presumably reduces levels of anti-PAHs antibodies [1, 25].

Similar situation can be seen at GT. Application of CCT (PAHs) increases the level of BPDE-DNA adducts and consequently increases the production of anti-BPDE-DNA. However, PAHs have also immunosuppressive effect and can reduce the production of anti-BPDE-DNA in this way. Therefore, the final level of anti-BPDE-DNA is probably the result of these conflicting processes.

Before the therapy we found in the group of smokers lower levels of anti-BPDE-DNA. It may be related to the above-described immunosuppressive effect of chronic exposure to tobacco smoke which can reduce expression of anti-BPDE-DNA. This assumption is also supported by the fact that in non-smokers, we found more significant increase of the anti-BPDE-DNA level than in smokers (Tables 2 and 3 and Figures 2 and 3). In addition, adaptation mechanisms (in smokers) will undoubtedly reduce the answer of the organism per unit dose of mutagenic stimulus (BaP).

State of repair mechanisms can significantly affect the level of BPDE-DNA adducts (and consequently anti-BPDE-DNA). Smoking may induce the repair mechanism but also it can cause their overload.

GT is used mainly for chronic stable plaque psoriasis and has a high benefit for patients with refractory psoriasis [13]. Also, in the present study, both variants of the therapy significantly improved the status of the disease (decrease of PASI score; $P < 0.001$) and confirmed generally high effectiveness of the GT.

5. Conclusion

The serum level of anti-BPDE-DNA in psoriatic patients (dermally exposed to different level of CCT/PAHs) has been observed. Elevated level of genotoxic exposure (BaP) resulted in an increased level of anti-BPDE-DNA. However, almost all levels of anti-BPDE-DNA ranged in the field of low values. Both variants of GT (CCT-3% a CCT-5%) induced higher expression of anti-BPDE-DNA in the group of non-smokers. Significant relationships between the level of anti-BPDE-DNA and PASI score, total duration of the therapy or time of UVR exposure were not found. Further studies are needed

to reduce interpretation uncertainty of this promising bioindicator.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Targeted Inhibition of mTOR Signaling Improves Sensitivity of Esophageal Squamous Cell Carcinoma Cells to Cisplatin

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mTOR is an evolutionarily conserved serine-threonine kinase with a central role in cell growth, invasion, and metastasis of tumors, and is activated in many cancers. The aims of this study were to investigate the expression of mTOR in ESCC tissues and its relationship with progression of ESCC and measure the changes of sensitivity of ESCC cells to cisplatin after cells were treated with mTOR siRNA by WST-8 assays, TUNEL, RT-PCR, and western blots in vitro and in vivo. The results showed that the expression of mTOR was higher in ESCC specimens than that in normal esophageal tissues and its expression was closely correlated with the TNM stage of ESCC. mTOR siRNA significantly increased the sensitivity of the EC9706 cells to cisplatin at proliferation in vitro and in vivo. The growth of ESCC xenografts was significantly inhibited by mTOR siRNA or cisplatin, and the cell number of apoptosis was obviously increased after xenografts were treated with mTOR siRNA or cisplatin alone, especially when mTOR siRNA combined with cisplatin. The present study demonstrates that the expression of mTOR has important clinical significance and inhibition of mTOR pathway by mTOR siRNA can improve the sensitivity of ESCC cells to cisplatin.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in developing countries, especially in Northern China [1], and patients with ESCC have a poor prognosis with a dramatic decreased 5-year survival rate [2, 3]. It is thus imperative to find new therapeutic targets underlying initiation and progression of ESCC to improve therapy for ESCC.

Mammalian target of rapamycin (mTOR) is a member of the phosphoinositide 3-kinase-related kinase (PIKK) family with homologs in all mammalians and its activity has been linked with cell growth, proliferation, survival, protein translation, and other cellular metabolic processes [4–6]. Activation of mTOR occurs via a multistep process that includes upstream phosphoinositide 3-kinase (PI3K) and Akt activation [7, 8]. Activation of mTOR regulates a number of its downstream effectors important in cellular growth, such as p70S6 kinase (S6K) and elongation initiation factor 4E (eIF4E) binding protein-1 (4EBP1), resulting in enhanced translation of subset of genes that are required for protein synthesis and cell growth [9–11]. Accumulating evidences have demonstrated that mTOR has a central role not only for cell growth but also for invasion and metastasis of cancers [7]. Rapamycin is the special inhibitor of mTOR; more and more reports have shown that rapamycin and its anologs temsirolimus (CCI-779) and everolimus (RAD001) exert antiproliferative effects through the inhibition of mTOR by binding to FKBP12 [12, 13]. The inhibition of mTOR decreases phosphorylation and activation of p70S6K and 4EBP1, which results in the inhibition of translation of critical mRNA involved in tumorigenesis [4, 6]. Activation of mTOR pathway occurs in many cancers and has recently been shown to be correlated with more aggressive disease behavior [14, 15]. It has been assumed that this may be because mTOR at the crossroad of a network of molecular pathways

regulates the synthesis of proteins required for growth of cancer cells [16]. At present, rapamycin and its analogs have been used in numerous clinical trials for solid tumor, such as prostate, breast, and pancreatic cancers, and they display encouraging antitumor activity with minimal toxicity and no immunosuppression over a broad of dose level [17].

In this study, the expression level of mTOR was examined by immunohistochemistry in human ESCC specimens, and the effects of mTOR siRNA and cisplatin alone or combined on cell proliferation, tumor growth, and cell apoptosis were, respectively, investigated in EC9706 cells and xenografts.

2. Materials and Methods

2.1. Patients and Tissue Samples. 35 ESCC tissue samples (16 men and 19 women with the mean age of 61.3 ± 9.1 years) from Chinese patients were collected from Cancer Hospital of Anyang City, China. No patients had undergone chemotherapy or radiotherapy prior to surgery. Among them, histopathology classification was 9 (I), 14 (II), and 12 tissues (III), and the infiltration appeared in mucosa, muscle layer, and fiber membrane of 7, 15, and 13 tissues, respectively. Furthermore, lymph node metastasis existed in 16 of 35 patients, and TNM phase was I-II of 13 and III-IV of 22, respectively. After the tissues were fixed in 10% formalin and embedded in paraffin wax and 4 μm thick sections were cut, the expressions of mTOR in them were measured immunohistochemically and the relationship between expression levels of mTOR protein and differentiation degree, depth of infiltration, lymph node metastasis, and TNM stage was analyzed, respectively.

2.2. Cell Culture and Animal Treatment. EC9706 cells purchased from the Chinese Academy of Medical Sciences, Beijing, China, were cultured in RPMI/1640 medium (Gibco-BRI, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in the presence of 5% CO_2 as described previously [18].

All animal studies were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of Henan Province, China. Male athymic BALB/c nude mice (Vital River Animal Ltd., Beijing, China) at 4-5 weeks of age were used in the study. Five mice per cage were housed in wire-top cages with sawdust bedding in an isolated, clean, air-conditioned room at a temperature of 25-26°C and a relative humidity of ~50%, lit 12 hours/day.

2.3. Immunohistochemical Analysis. The tissue sections were deparaffinized in xylene, hydrated through graded ethanols and distilled water, and washed thoroughly with PBS. For antigen retrieval, the slides were put in a container having 10 mmol/L citrate buffer (pH 6), and then the container was placed in boiled water for 20 minutes. After the slides were put at room temperature (RT) for 30 minutes, they were rinsed thrice with PBS. Subsequently the slides were incubated in 3% hydrogen peroxide in PBS for 30 minutes to quench the endogenous peroxidase. The sections were washed in PBS and then incubated in the blocking solution (10% rabbit serum in PBS) for 30 minutes in a chamber with saturated humidity at RT. Excess solution was discarded and the sections were incubated with primary antibody rabbit polyclonal anti-mTOR of 1 : 200 (Santa Cruz Biotechnology, USA) and PBS as negative control, respectively, at 4°C overnight. The slides having been washed with PBS were subsequently incubated with the biotinylated secondary antibody of 1 : 8,000 (Santa Cruz Biotechnology, USA) for 30 minutes, followed by being incubated with the HRP-linked streptavidin biotin complex in a box with saturated humidity for 10 minutes at RT. Finally, the slides were washed and developed with 3,3-diaminobenzidine (DAB) solution for ~3 minutes, and then the sections were counterstained with hematoxylin, dehydrated and cleared in xylene, and mounted. Immunohistochemical evaluation was performed by a pathologist without knowledge of the clinical and pathologic characteristics of these patients. The tumor cells were scored further according to the intensity (I), distribution (D), and pattern (P) reported by Dong et al. [19]: I score: 0, negative; 1, weak; 2, moderate; and 3, strong; D score (%): 0, negative; 1, 10–50%; 2, 51–90%; and 3, >90%; P score: 0, no staining; 1, sporadic positive staining; 2, focal positive staining; and 3, diffuse positive staining. The total scores of each tissue = I × D × P; the 0 score was negative, and ≥1 score was positive.

2.4. Cell Proliferation Assay. Cell proliferation was determined using WST-8 dye (Beyotime Inst Biotech, China) according to the manufacturer's instructions. Briefly, EC9706 cells transfected with mTOR siRNA (sc-35409, Santa Cruz) for 24 hours were harvested and seeded in a 96-well flat-bottomed plate (5×10^3 cells/well) and cultured at 37°C for 24 hours. Subsequently, cells were treated with cisplatin at increasing concentrations in the presence of 10% FBS for 24 hours. After 10 μL WST-8 was added to each well, cells were incubated at 37°C for 2 hours and the absorbance was finally determined at 450 nm using a microplate reader (Bio-Rad Laboratories, USA). Each sample was assayed in triplicate for each group.

2.5. Tumor Xenografts in Athymic Nude Mice. EC9706 cells were treated with mTOR siRNA as indicated in our reported data [20]. Twenty athymic mice were divided into two groups of 10 mice each, in which one group was subcutaneously inoculated with EC9706 cells transfected with mTOR siRNA for 24 hours and another without mTOR siRNA. Briefly, cells grown at logarithm phase were harvested, washed, and resuspended in PBS at 2×10^7 cells/mL. A cell resuspension of 200 μL (4×10^6 cells) was inoculated s.c. into the right flank of athymic mice each. For tumor growth analysis, the tumor size was measured every day with a sliding caliper, and the tumor volume was defined as (longest diameter) × (shortest diameter)^2/2 [21]. The cisplatin solution was prepared as described previously [18]. Further, tumor-bearing animals of the two groups were randomly subdivided into 2 groups of 5 animals each [22, 23], respectively. The treatment schedule was as follows. The groups without or with mTOR siRNA were injection i.p. with cisplatin (1 mg/kg) and PBS as

controls, respectively, every day for 2 weeks. Inhibition rate = [(tumor volume of control group − tumor volume of experimental group)/tumor volume of control group] × 100%.

After being treated for two weeks, tumor-bearing mice were sacrificed and the tumors were removed, weighed, and then cut into 3 pieces, one of which immediately was fixed in 4% buffered paraformaldehyde overnight for TUNEL assay and two of which were put into liquid nitrogen solution for protein or RNA analysis.

2.6. Western Blots. Small pieces of fresh xenografts were immediately homogenized in protein lysis buffer and centrifugated at 12,000 rpm for 20 minutes, and then the supernatant was harvested as the total cellular protein extracts. The protein concentrations were determined using Bradford method [24]. Equivalent amounts of proteins (50 μg) were separated by SDS-PAGE and electrotransferred to supported nitrocellulose membranes (Amersham, Sweden) by a semidry transferor. After the membranes were blocked for 2 hours in blocking buffer (5% skimmed milk in PBS-T containing 0.05% Tween 20) at RT, they were incubated with the different primary antibodies: rabbit polyclonal anti-mTOR and p70S6K, mouse monoclonal anti-4EBP1 and p-4EBP1 of 1 : 200 and β-actin of 1 : 400 (Santa Cruz Biotechnology, USA), and mouse monoclonal anti-p-p70S6K of 1 : 2,000 (Cell Signaling Technology, USA) diluted in 1% skimmed milk in PBS-T, respectively, at RT for 2 hours, followed by being incubated with the appropriate HRP-linked secondary antibodies. Finally, the bands of specific proteins on the membranes were visualized with chemiluminescent substrate (Santa Cruz Biotechnology, USA) according to the manufacturer's instructions. The membranes were rinsed three times with PBS-T between the incubations described above [18].

2.7. Semiquantitative RT-PCR. Total RNA was prepared from the xenograft tissues with trizol reagent and reversely transcribed to cDNA using AMV First Strand DNA Synthesis Kit (Biotech Co., Shanghai, China). Briefly, a 1 μg of the isolated RNA was reversely transcribed to cDNA at 37°C for 1 hour in a 20 μL of reaction mixture containing 1 μL AMV reverse transcriptase, 1 μL random hexamer, 4 μL 5 × AMV buffer, 1 μL RNase inhibitor (20 U/μL), and 2 μL dNTP (10 mM). The PCR amplification mixture of 25 μL contained 0.5 μL cDNA mixture, 0.5 U Taq DNA polymerase, 2.5 μL of 10 × PCR buffer, 2.5 mM dNTP mixture, and 50 pM sense and antisense primers each. The used oligonucleotide primers of mTOR, p70S6K, and 4EBP1 and the PCR conditions were as described previously [18]. The amplified products were subjected to electrophoresis on 1% agarose gels containing 0.2 μg/μL ethidium bromide and visualized under a UV light [18].

2.8. TUNEL Assay. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was carried out using the *in situ* cell death detection kit (KeyGen Biotech Ltd., China) according to the manufacturer's instructions. Briefly, xenograft tissues were embedded in paraffin and then sectioned for the TUNEL assay. The tissue sections were deparaffinized in xylene and rehydrated in graded ethanol for dehydration. After being washed with PBS and incubated in 3% H_2O_2 solution for 20 minutes, the sections were treated with proteinase K (20 μg/mL in PBS) for 20 minutes at RT and rewashed with PBS. Subsequently, the sections were treated with a biotin-dNTP reaction mixture labeled by TdT at 37°C for 1 hour and treated with streptavidin-HRP solution for 5 minutes at RT. Finally, the slides were washed and developed with DAB solution and counterstained with hematoxylin. The results were determined by counting 1,500 cells in 5 randomly selected fields.

Table 1: Expressions of mTOR proteins in different tissues.

Tissue type	n	mTOR			P
		−	+	Positive rate (%)	
Normal	15	12	3	20.0	
Dysplasia	15	8	7	46.7	0.021
Cancer	35	13	22	62.9	

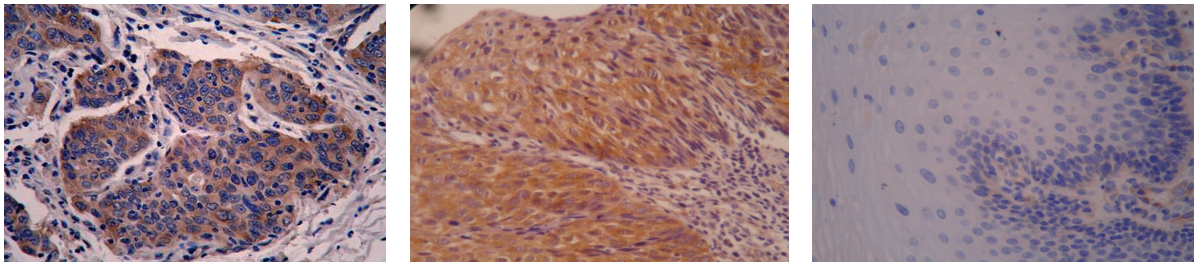
2.9. Statistical Analysis. The results of all experiments were performed by standard chi-square test and one-way analysis of variance, respectively, using SPSS version 20.0 (SPSS, Chicago, USA). All summary statistics were expressed as means ± SD but tumor volumes were expressed as means ± SE. In all statistical analyses, a P value <0.05 was considered statistically significant.

2.10. Study Ethics Approval. The study was approved by the Ethics Committee of Zhengzhou University, Henan, China.

3. Results

3.1. Expression of mTOR in ESCC Tissues. To examine the potential role of the mTOR pathway in ESCC, the expression of mTOR was examined immunohistochemically in ESCC tissues, and the results showed that mTOR was mainly expressed in the cytoplasm (Figure 1). The expression rates of mTOR were 20% (3/15), 46.7% (7/15), and 62.9% (22/35) in normal esophageal, dysplasia, and cancer tissues, respectively. As shown in Tables 1 and 2, there was a statistical significance among them ($P < 0.05$). The expression of mTOR was not related to the histologic type, the depth of infiltration, and lymph node metastasis (all $P > 0.05$) but closely related to the TNM stage ($P < 0.01$).

3.2. Effect of mTOR siRNA on Sensitivity of Cell Proliferation to Cisplatin. To investigate the changes of sensitivity of cells to cisplatin after being transfected with mTOR siRNA, the EC9706 cells with or without mTOR siRNA were seeded in a 96-well flat-bottomed plate, cultured at 37°C for 24 hours, and treated with cisplatin of different concentrations of 0.05, 0.1, 0.6, and 1 μg/mL for 24 hours. As shown in Figure 2, the proliferation of cells with or without mTOR siRNA became slower and inhibitory effects of cisplatin on proliferation of EC9706 cells were in a dose-dependent manner. The ratio of alive cells with mTOR siRNA was lower ($P < 0.05$) than that



(a) (b) (c)

Figure 1: Expression of mTOR in human normal esophageal and ESCC tissues by immunohistochemical analysis. (a) Negative expression of mTOR in normal tissues of the esophagus. (b) Moderate positive expression of mTOR in dysplasia tissues. (c) Positive expression of mTOR in ESCC tissues (×400).

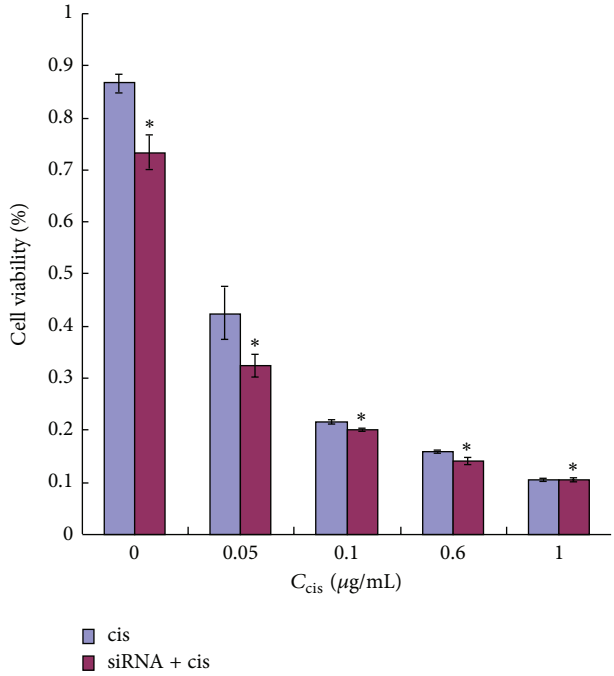


Figure 2: Effects of mTOR siRNA on cell proliferation and sensitivity to cisplatin. To determine the effects of mTOR siRNA on cell proliferation and sensitivity of ESCC cells with mTOR siRNA to cisplatin, proliferations of the cells with or without mTOR siRNA treated with cisplatin at different concentrations for 24 h were detected with WST-8 dye. Results pooled from three independent experiments were expressed as mean ± SD. $^*P < 0.05$, compared to untreated cells.

Table 2: Clinical significance of mTOR protein expression.

Pathological features	n	mTOR	
		Positive n (%)	P
Histology classification			
I	9	6 (66.7)	0.917
II	14	9 (64.3)	
III	12	7 (58.3)	
Depth of infiltration			
Mucosa	7	3 (42.9)	0.308
Muscle layer	15	9 (60.0)	
Fiber membrane	13	10 (76.9)	
Lymph node metastasis			
No	19	10 (52.6)	0.172
Yes	16	12 (75.0)	
TNM phase			
I, II	13	4 (30.8)	0.003
III, IV	22	18 (81.8)	

without mTOR siRNA at the same concentration of cisplatin. But the proliferations revealed no difference between cells with and without siRNA in presence of 1 g/mL cisplatin.

3.3. Inhibition Effects of Cisplatin and mTOR siRNA on the Growth of Xenografts. The sensitivity of xenografts with or without mTOR siRNA to cisplatin was evaluated in the transplantable tumor of ESCC. As shown in Table 3 and Figure 3, the volumes of tumors in all groups were progressively increased during the experiment, of them the volume of tumors in the control group on day 15 after the treatment was 17-fold bigger ($P < 0.01$) than that on day 1. Antitumor effect of mTOR siRNA and cisplatin alone or combined with each other emerged from days 7 to 15 at termination of the treatment, which had statistic difference between the three experimental groups and the control group (day 7: $P < 0.05$; days 9–15: all $P < 0.01$). The volume of tumors on day 15 after the treatment was 9-fold bigger than that on day 1 in mTOR siRNA or cisplatin alone group ($P < 0.05$), while the volume of tumors in mTOR siRNA combined with cisplatin group on day 15 after the treatment was 5-fold bigger than that on day 1 ($P < 0.01$). Additionally, compared to control group, the inhibition rate of tumor growth was 58.27% after mTOR siRNA alone treatment and 63.18% after single-agent cisplatin, respectively, while combination of mTOR siRNA with cisplatin significantly enhanced the inhibition effect of tumor growth with 76.70% on ESCC in vivo at termination of the experiment. Obviously, the treated groups each showed significant growth inhibition compared to control group ($P < 0.05$ or < 0.01) during the same period, especially the mTOR siRNA + cisplatin group, indicating that mTOR siRNA

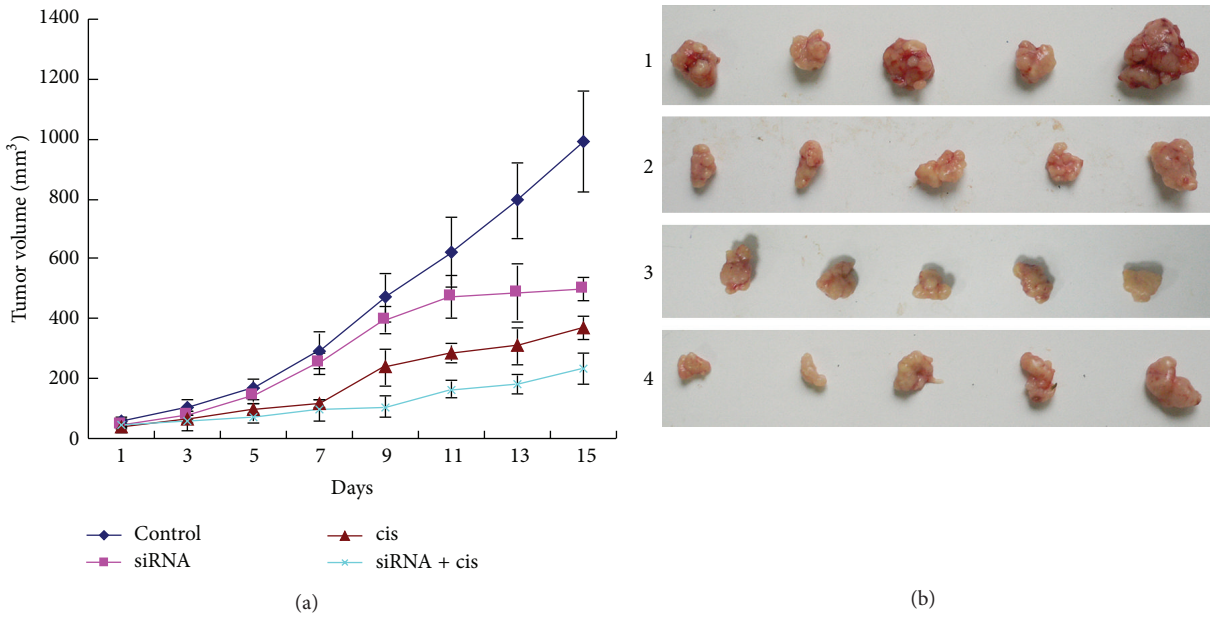


Figure 3: Tumor regression observed in EC9706 xenografts treated with different ways. (a) Tumor volumes from the xenografts of groups each were assessed every day, as described in Materials and Methods, and the results were expressed as means ± SE (mm^3). The tumor growth of treated groups each became slow, in which group treated with mTOR siRNA combined with cisplatin was the slowest. (b) Tumors from the xenograft treated with different ways for two weeks. 1, 2, 3, and 4 represent control, mTOR siRNA, cisplatin, and mTOR siRNA + cisplatin groups, respectively.

Table 3: Effects of mTOR siRNA and cisplatin alone or combined on growth of human ESCC xenograft in nude mice ($n = 5$).

Group	Animal weight	Tumor volume at beginning[1]	Tumor volume at termination	Inhibition rate of tumor (%)
Control	20.54 ± 1.23	57.03 ± 10.45	999.28 ± 167.30	—
siRNA	20.52 ± 2.08	43.62 ± 10.85	416.97 ± 38.12*	58.27
cis	20.38 ± 1.42	39.09 ± 2.08	367.91 ± 61.21*	63.18
siRNA + cis	20.66 ± 1.79	46.28 ± 25.56	232.86 ± 54.23*	76.70

[1]Tumor volume is expressed as mm^3. *$P < 0.05$, compared to control group.

combined with cisplatin has the strongest inhibition of tumor growth.

3.4. Actions of mTOR siRNA and Cisplatin on Effectors in mTOR Pathway. mTOR and the phosphorylation status of its downstream targets, p70S6K and 4EBP1, in the xenografts were examined by western blots. As shown in Figure 4, mTOR siRNA alone and mTOR siRNA combined with cisplatin downregulated the protein levels of mTOR, p-p70S6K, and p-4EBP1 but upregulated the protein levels of p70S6K and 4E-BP1 ($P < 0.05$). Compared to the control group, the protein expression levels of mTOR, p-p70S6K, and p-4EBP1 had no obvious changes in cisplatin group ($P > 0.05$), while they decreased by ~4-fold, ~5-fold, and ~2-fold, respectively, in the mTOR siRNA group, and 1.4-fold, 4.5-fold, and 1.5-fold, respectively, in mTOR siRNA + cisplatin group, demonstrating that the protein expression of mTOR between the mTOR siRNA group and mTOR siRNA + cisplatin group has a difference ($P < 0.05$) but not p-p70S6K and p-4EBP1 ($P > 0.05$).

To investigate the effect of mTOR siRNA and cisplatin alone or combined with each other on the mRNA expressions of the effectors in mTOR pathway, their mRNA expressions in all groups were measured by RT-PCR. Compared to the control group, the mRNA level of mTOR was downregulated while the levels of p70S6K and 4EBP1 mRNA were upregulated in mTOR siRNA alone or siRNA combined with cisplatin groups ($P < 0.05$). It had no obvious changes in cisplatin alone group ($P > 0.05$, Figure 5), suggesting that cisplatin has no effect on mTOR pathway.

3.5. Effects of mTOR siRNA Alone or Combined with Cisplatin on Cell Apoptosis. The apoptosis of tumor cells of ESCC xenografts in groups each was determined by an *in situ* TUNEL assay and the results showed that there were 63, 54, and 102 apoptotic cells/1,500 cells in mTOR siRNA, cisplatin, and mTOR siRNA + cisplatin groups, respectively, compared to control group (6 apoptotic cells/1,500 cells) ($P < 0.05$ or <0.01, Table 4). The number of apoptosis cells in mTOR siRNA + cisplatin group was the highest among the three experimental groups ($P < 0.05$), while the number of apoptosis cells in mTOR siRNA group had no statistical difference from that of cisplatin group ($P > 0.05$). The

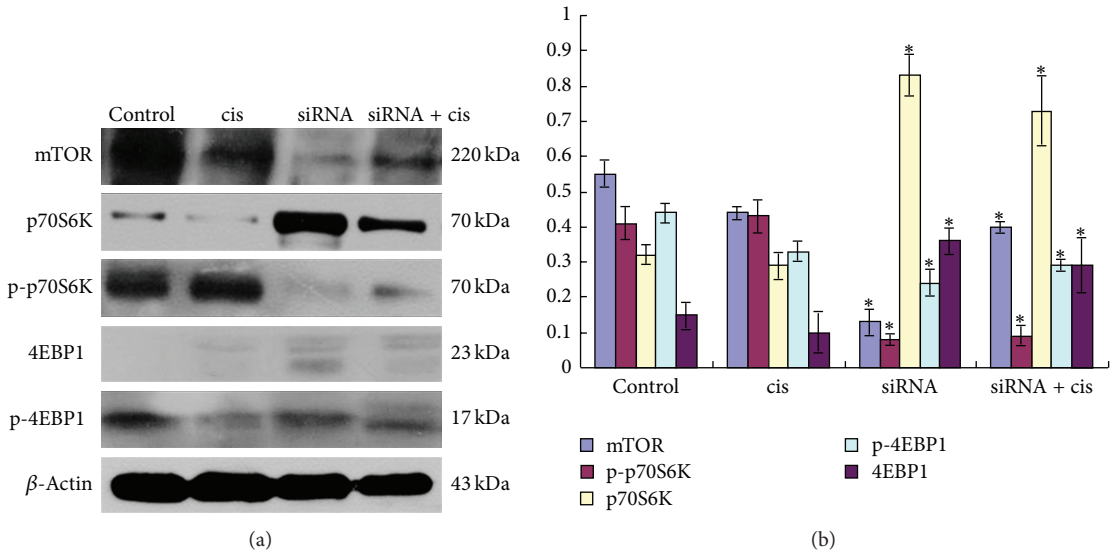


Figure 4: Protein expression of effectors in mTOR pathway of EC9706 xenografts treated with different ways. (a) Antibodies to mTOR, p70S6K, p-p70S6K, 4EBP1, and p-4EBP1, respectively. (b) Semiquantitative values of bands from three independently repeated experiments, which were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. Reduced expressions of mTOR, p-p70S6K, and p-4EBP1 and elevated expressions of p70S6K and 4EBP1 were observed in groups treated with mTOR siRNA and mTOR siRNA combined with cisplatin. β-Actin was used as loading control. $^*P < 0.05$, compared to control group.

Table 4: Effects of mTOR siRNA alone or combined with cisplatin on cell apoptosis (TUNEL).

Group	The counted cell number	The number of apoptosis cells
Control	1500	6 ± 1
siRNA	1500	63 ± 2*
cis	1500	54 ± 5*
siRNA + cis	1500	102 ± 4*

$^*P < 0.05$, compared to control group.

results above indicate that mTOR siRNA promotes apoptosis of ESCC cells and the effect of inducing apoptosis is stronger when it is combined with cisplatin.

4. Discussion

Since mTOR was identified and cloned in 1994 [25], it has been examined in a wide array of cancer types and aberrantly activated mTOR pathway plays an essential role in the growth of different types of tumors including ESCC [26, 27]. So far, several statuses of the effectors on the upstream and downstream of mTOR pathway such as amplification of a catalytic subunit of PI3K and loss or mutation of PTEN gene have been detected in many malignant tumors [28, 29]. In some malignancies, proteins lying downstream of the mTOR pathway have been also altered, for example, eIF4E, which promotes the phosphorylation of 4EBP1 (p-4EBP1), expression levels of which correlate with tumor progression [28].

The activation of mTOR has been shown in ESCC cell lines in our previous study [20]. In this study, we examined the expression of mTOR in ESCC specimens and investigated the relationship of the expression level of mTOR with infiltration, lymph node metastasis, and TNM phase. As shown in Figure 1 and Tables 1 and 2, the expression rate of mTOR of cancer tissue was the highest (62.9%) and there were statistically significant differences among normal esophageal, dysplasia, and cancer tissues ($P < 0.05$). Moreover, the expression of mTOR was closely related to the TNM phase (all $P < 0.01$). The above-mentioned results indicate that the aberrantly activated mTOR may be a clinical diagnostic mark in ESCC.

As the special inhibitors of mTOR, rapamycin and its anologs have been evaluated in many tumors including ESCC and have shown the marked inhibition effects on the mTOR pathway and tumor growth [12, 13, 17]. RNA silencing including both short interfering RNA (siRNA) and short hairpin RNA (shRNA) has been used in cancer research in vitro and in vivo [30–32]. In this study, we found that mTOR siRNA alone could lead to slow growth of tumors and the volume of tumors at termination of the experiment on day 15 was only 9-fold bigger than that on day 1 in the group of mTOR siRNA alone, but 17-fold in the control group, and there was a significant difference ($P < 0.05$) in comparison of mTOR siRNA alone with control groups. As demonstrated by TUNEL assay, moreover, the number of apoptotic cells in mTOR siRNA group was 10-fold more than that in control group, suggesting that mTOR siRNA alone may induce cell apoptosis of ESCC.

Cisplatin is a widely used chemotherapeutic agent that exerts its cytotoxic effects by disrupting the DNA structure in cells through the formation of intrastrand adducts and interstrand cross-links [33]. It has been proven to be one of the most clinically active agents for the treatment of a variety of

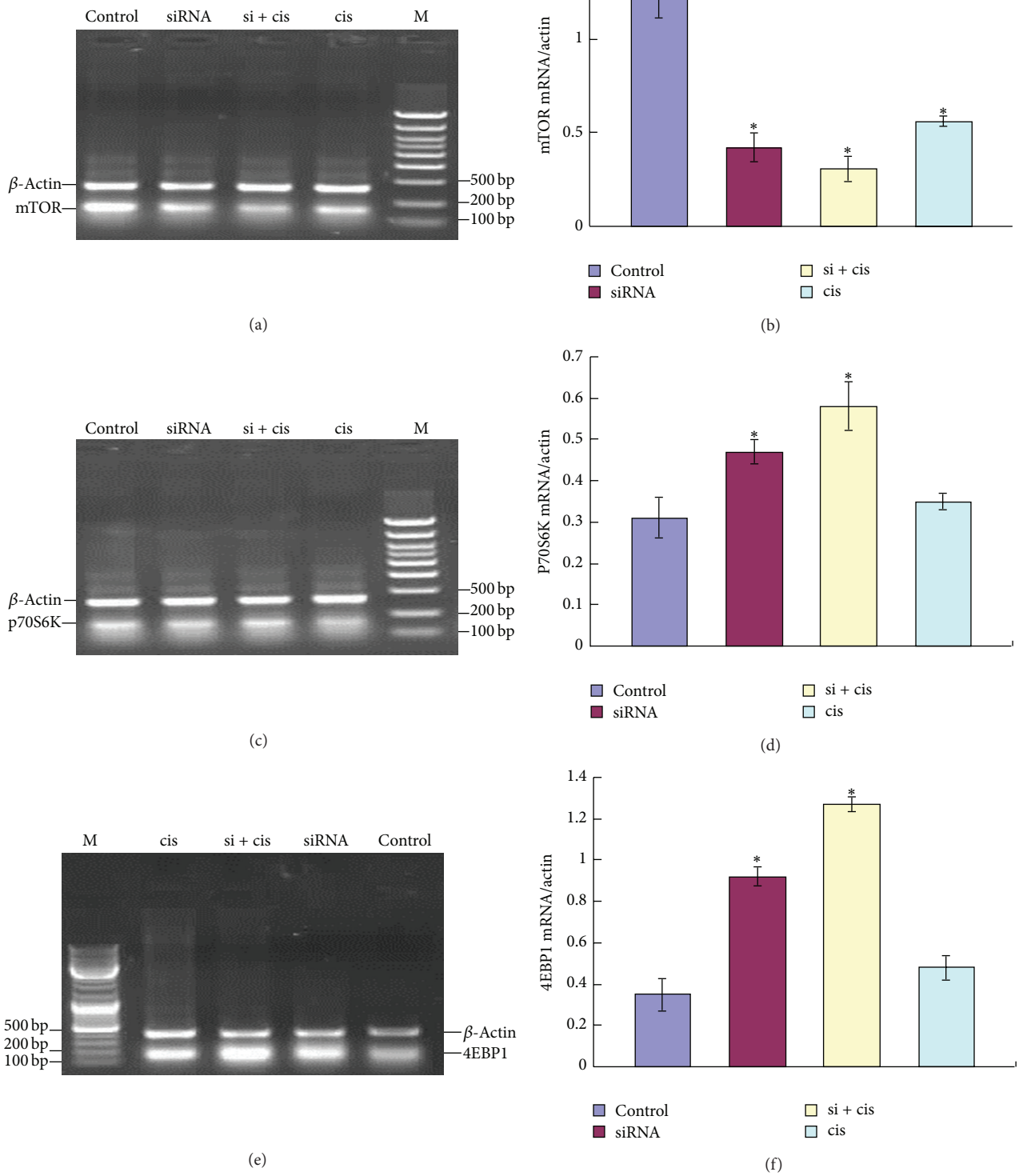


Figure 5: Analysis of total mRNA expressions of mTOR (a), p70S6K (b), and 4EBP1 (c) from EC9706 xenografts treated by different ways. ((d)–(f)) Semiquantitative values of mRNA levels of mTOR, p70S6K, and 4EBP1 to β-actin, respectively. Results from three independently repeated experiments, which were statistically analyzed by densitometry using BandScan 5.0 software, are expressed as means ± SD. The mRNA level of mTOR was downregulated while mRNA levels of p70S6K and 4EBP1 were upregulated after being treated with mTOR siRNA and siRNA + cisplatin. $^{*}P < 0.05$, compared to control group.

solid tumors, including ESCC [34]. However, its clinical therapeutic effect is often limited by intrinsic or acquired tumor cell resistance. In addition, cisplatin's associated nephrotoxicity and neurotoxicity, especially when administered at higher doses, have been further obstacles to the success of this treatment [30]. Thus, we investigated the combination effect of mTOR siRNA and cisplatin to determine whether mTOR siRNA would potentiate the effects of cisplatin on ESCC. The results of cells proliferation showed that cisplatin that inhibited the proliferation of EC9706 cells was in a dose-dependent manner and the inhibition effects of cisplatin became stronger at the same concentration after cells were transfected with mTOR siRNA ($P < 0.05$). But the proliferations of cells with/without siRNA revealed no difference in presence of 1 g/mL cisplatin, which may be because the inhibition ratio of cisplatin at this concentration was very high, nearly reached 90% and made the role of mTOR siRNA seem to be not obvious. The results of nude mice showed that the tumors growth became slow when cells were treated with mTOR siRNA and cisplatin alone or combined compared to control group from day 7 to 15 after treatment, which had statistic differences between the three experimental groups and the control group; while the tumor growth was the slowest, when mTOR siRNA combined with cisplatin, the volume of tumor only was 5-fold bigger in the mTOR siRNA combined with cisplatin group but 17-fold bigger in the control group at the termination than at day 1 of the treatment ($P < 0.01$). The inhibition rates of tumors in mTOR siRNA, cisplatin, and mTOR siRNA + cisplatin groups were 58.27%, 63.18%, and 76.70%, respectively, indicating that mTOR siRNA combined with cisplatin has the strongest inhibition of tumor growth. Additionally, the number of apoptosis cells was the most in mTOR siRNA + cisplatin group (102 cells/1,500 cells) compared to that in mTOR siRNA (63 cells/1,500 cells) and cisplatin (54 cells/1,500 cells) alone group ($P < 0.05$). A potential explanation for the result is that mTOR siRNA inhibits the phosphorylation of p70S6K and 4EBP1, the key factors in cell proliferation and growth, so cisplatin-induced cell proliferation and apoptosis are enhanced. Above all, mTOR pathway has important effects on the tumorigenesis and progression of ESCC and inhibition of mTOR pathway by mTOR siRNA promotes the sensitivity of cells to cisplatin.

In this study, mTOR was overexpressed in ESCC specimens compared to normal esophageal tissue and the expression level of mTOR had close relationship to the TNM phase of ESCC. Transient inhibition of mTOR by mTOR siRNA significantly increased the sensitivity of the EC9706 cells to cisplatin in vitro and in vivo. Besides, cisplatin significantly inhibited in vivo growth of ESCC xenografts and increased the number of apoptotic cell after mTOR pathway was inhibited by mTOR siRNA. The above results indicate that mTOR may be a potential therapeutic target and inhibition of mTOR pathway can improve the sensitivity of chemotherapeutics in ESCC.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Tumor-Activated TCR$\gamma\delta^+$ T Cells from Gastric Cancer Patients Induce the Antitumor Immune Response of TCR$\alpha\beta^+$ T Cells via Their Antigen-Presenting Cell-Like Effects

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Human $\gamma\delta$ T cells display the principal characteristics of professional antigen-presenting cells (APCs), in addition to playing a vital role in immunity through cytokine secretion and their cytotoxic activity. However, it is not clear whether $\gamma\delta$ T cells perform APC-like functions under pathological conditions. In this study, we showed that, in contrast to peripheral-derived $\gamma\delta$ T cells directly isolated from PBMCs of gastric cancer patients, tumor-activated $\gamma\delta$ T cells not only killed tumor cells efficiently but also strongly induced primary $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells proliferation and differentiation. More importantly, they abrogated the immunosuppression induced by $CD4^+CD25^+$ Treg cells and induced the cytotoxic function of $CD8^+$ $\alpha\beta$ T cells from patients with gastric cancer. In conclusion, tumor-activated $\gamma\delta$ T cells can induce adaptive immune responses through their APC-like functions, and these cells may be a potentially useful tool in the development of tumor vaccines and immunotherapy.

1. Introduction

$\gamma\delta$ T cells are a distinct subset of $CD3^+$ T lymphocytes characterized by the presence of T cell receptors (TCRs), which are encoded by Vγ- and Vδ-gene segments [1]. In human peripheral blood, $\gamma\delta$ T cells typically represent only 3–5% of all T lymphocytes and are V$\delta2^+\gamma\delta$ T cell subset predominant; however, they are common in the organs and mucosa, and, here, they are V$\delta1^+\gamma\delta$ T cell subset predominant, acting as the first defense system against the entry of foreign organisms. In contrast to conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells express a limited repertoire of TCR V-region genes. Stimulated $\gamma\delta$ T cells undergo activation, which results in a plethora of poorly defined changes, including proliferation, proinflammatory cytokine, and chemokine secretion, and altered cell surface phenotypes [1]. $\gamma\delta$ T cells participate in the immune response by direct cytolysis, development of memory phenotypes, and modulation of immune cells, and they have been implicated in autoimmune disorders, immune deficiencies, infections, and tumor diseases.

$\gamma\delta$ T cells recognize and kill a range of tumor cells with multiple tissue origins [2, 3], and the genetic absence of $\gamma\delta$ T cells rendered mice significantly more susceptible to tumor growth in vivo [4–6]. The antitumor properties of $\gamma\delta$ T cells have been exploited as a potential target for tumor immunotherapy [2, 7]. It has been reported that the most common subtype of these cells in human blood is Vγ9Vδ2, which recognizes a group of nonpeptide phosphoantigens (PAg)—of which isoprenyl pyrophosphate (IPP) has been well characterized—that are known to be upregulated in infection or cancer [8, 9]. Further, PAgs may be presented to $\gamma\delta$-TCRs via a surface molecule (i.e., F1-ATPase) in a manner

somewhat analogous to MHC-mediated antigen presentation [10], suggesting that Vγ9Vδ2 cells can function as professional antigen-presenting cells (APCs) [11–13]. Human $\gamma\delta$ T cells exhibit a potent cytotoxicity against various tumor cells as cytotoxic T cells [2, 14–17]. However, the significance of $\gamma\delta$ T cells expressing the APC-like phenotype and the mechanisms by which they fight tumor cells remains largely unknown. In this study, we showed that $\gamma\delta$ T cells from patients with gastric cancer could not only serve as targets for $\gamma\delta$ T-mediated antitumor activity but also display the APC-like phenotype and functions.

2. Materials and Methods

2.1. Patient Subjects. Human peripheral blood and fresh tumor tissue samples were obtained from gastric cancer patients (16 men and 4 women; age: 47–69 years; median age: 58.1 ± 6.4 years) newly diagnosed on the basis of clinical history, gastroscopic examination, and pathological diagnosis. Healthy controls (8 men and 2 women; age: 39–63 years; median age: 54.4 ± 8.7 years) were also enrolled, based on normal results from laboratory and physical examinations. Ethics approval for this study was granted by the Ethics Committee of the Affiliated Hospital of Jiangsu University, and written informed consent was obtained from all patients enrolled.

2.2. Flow Cytometric Assays. Cells (1×10^5) were suspended in PBS containing 2% FBS for 10 min to block nonspecific binding sites and then were incubated at 4°C for 30 min to determine the percentages of subsets of lymphocyte cells with a combination of antibodies as follows: CD3-APC (UCHT1), CD8-PE (B9.11), CD4-FITC (13B8.2), CD80-FITC (MAB104), CD83-PE (HB15a), CD86-PE (HA5.2B7), HLA-DR-PE (IM0464), CD25-PE (B1.49.9), pan $\gamma\delta$-PC5 (IMMU510), and CD45-APC (J33); all were purchased from Beckman Coulter. Cell apoptosis was stained with the Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. For indirect staining, cells were washed twice with PBS and then incubated for 20 min at 4°C with PE-conjugated goat anti-mouse IgG. Compensation was set up with single-stained samples; low forward scatter elements (RBC and debris) were excluded from analysis, and 10,000 events were collected and analyzed by FACSAria cytometer (BD Biosciences).

2.3. Cell Isolation and Purification. Fresh peripheral blood was collected in sodium-heparin vacutainer tubes. Periphery mononuclear cells (PBMCs) were isolated by Ficoll density gradient (Sigma Aldrich) centrifugation. $CD8^+$ T, TCR$\gamma\delta^+$ T, $CD4^+CD25^-$ T, and $CD4^+CD25^+$ Treg cells were isolated and purified from fresh PBMCs of patients with gastric cancer by magnetic cell separation. In brief, $CD8^+$ T (>95% purity) and TCR$\gamma\delta^+$ T cells (>95% purity, named as peripheral-derived $\gamma\delta$ T cells) were firstly separated by positive selection using human blood TCR$\gamma\delta^+$ T and $CD8^+$ T Cell Isolation Kits (Miltenyi Biotec) according to the manufacturer's instructions. From the fraction of remaining cells further isolation of $CD4^+CD25^-$ T cells (>90% purity) and $CD4^+CD25^+$ T cells (>95% purity) was accomplished by negative selection and positive selection using a human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec).

2.4. Generation of Tumor-Activated $\gamma\delta$ T Cells. Gastric cancer tissues were minced and digested with a triple enzyme mixture comprising collagenase type IV, hyaluronidase, and deoxyribonuclease for 2 h at room temperature. After digestion, the cells were washed twice in RPMI 1640 and then irradiated (30 Gy) and preserved. Peripheral-derived $\gamma\delta$ T cells (6×10^5 cells/mL) were then cocultured with the irradiated tumor tissue cells (3 : 1 ratio) in RPMI 1640 containing 10% human serum supplemented with l-glutamine, 2-mercaptoethanol, IL-2 (200 U/mL; R&D Systems), and IL-15 (20 ng/mL; R&D Systems) for generation and expansion of tumor-activated $\gamma\delta$ T cells.

2.5. Proliferation Assay of $\gamma\delta$ T Cells. Irradiated (30 Gy) PBMCs or tumor tissue cells (2×10^4 cells/well) seeded in 96-well plates with 200 μL RPMI 1640 medium containing 10% FBS and IL-2 (100 U/mL) were added to purified autologous $\gamma\delta$ T cells (6×10^4 cells/well) and incubated at 37°C 5% CO_2 for 3 days. Cells were pulsed with 1 μCi/well of [^{3}H]TdR and harvested after 12 h. The incorporation of [^{3}H]TdR was determined using a liquid scintillation counter (LS6500; Beckman Coulter, Brea, CA, USA).

2.6. In Vitro Functional Assay. To determine the functional effect of tumor-activated $\gamma\delta$ T cells on adaptive immune T cells, an in vitro functional assay was performed as previously described [18]. In brief, autologous $CD4^+CD25^-$ T cells or $CD8^+$ T cells (1×10^6 cells/mL) were labeled for 15 min with 4.5 μM carboxyfluorescein succinimidyl ester (CFSE; Sigma Aldrich). Labeled $CD4^+$ or $CD8^+$ T cells (2×10^5 cells/mL) were cocultured with $\gamma\delta$ T cells alone or together in the indicated ratios in 24-well plates containing 10% FBS-RPMI 1640 medium at 37°C in 5% CO_2. To determine the functional effect of the tumor-activated $\gamma\delta$ T cells on $CD4^+CD25^+$ Treg cells, autologous $CD4^+$ T cells (2×10^5 cells/mL) were cocultured with $CD4^+CD25^+$ Treg cells (2×10^5 cells/mL) in the absence or presence of $\gamma\delta$ T cells, anti-CD3 (OKT3; eBioscience), and anti-CD28 (CD28.2; eBioscience). Proliferation of $CD4^+$ or $CD8^+$ T cells was determined by fluorescence correlation microscopy (FCM) to assess CFSE dilution on day 3. Transwell experiments were also performed with 24-well plates with a pore size of 0.4 μm (Corning Costar, Cambridge, MA, USA). To determine whether the effect of tumor-activated $\gamma\delta$ T cells could be blocked by specific antibodies, T cell activity was assessed in the absence or presence of various antibodies such as those against IL-1β (AF-201-NA; R&D Systems), IL-6 (AF-206-NA; R&D Systems), IL-12 (MAB1510; R&D Systems), CD80 (MAB140; R&D Systems), CD86

(MAB141; R&D Systems), and IFN-γ (AB-285-NA; R&D Systems).

2.7. Cytotoxic Assay. The cytotoxic activity of effector T cells was determined by measuring the amount of lactate dehydrogenase (LDH) released from target cells. The commercial LDH Cytotoxicity kit (Beyotime, China) was used according to the manufacturer's instructions. Maximum LDH release of target cells was determined by lysing target cells for 45 min (lysis buffer provided within the assay) and subsequently measuring the LDH from the culture medium. Absorbance values after the colorimetric reaction were measured at 490 nm with a reference wavelength of 655 nm, using a Bio-Rad Model 550 microplate reader (Bio-Rad, Hercules, CA, USA).

2.8. Measurement of Cytokines by ELISA. The supernatants from $\gamma\delta$ T cells culture were collected and stored at -80°C until analysis. Cytokines of IFN-γ, IL-1, IL-4, IL-6, IL-10, and IL-12 concentrations were measured using commercial enzyme-linked immunosorbent assay (R&D systems) according to the manufacturer's instructions.

2.9. Confocal Microscopy. Tumor cells were labeled with CFSE and incubated with tumor-activated $\gamma\delta$ T cells stained with mouse antihuman HLA-DR-PE-Cy5 before analysis. Images were captured using confocal microscopy (Leica TCS SP5; Leica Microsystems, Bannockburn, IL, USA) using the LAS AF confocal software. Protein antigen capture between cell-cell interactions was visualized using time-lapse confocal microscopy as described previously [19].

2.10. Statistical Analysis. Unless indicated otherwise, data are expressed as mean ± SD. Comparison between two groups was performed by Student's t-tests. Data from more than two groups were compared using one-way ANOVA with the Tukey-Kramer multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Tumor-Activated γδ T Cells Display Characteristics of APCs. $\gamma\delta$ T cells were directly isolated from the peripheral blood of gastric cancer patients (defined as peripheral-derived $\gamma\delta$ T cells) and cultured with irradiated autologous PBMCs in the presence of IL-2 and IL-15. The results showed no obvious proliferation of the peripheral-derived $\gamma\delta$ T cells. In contrast, when the peripheral-derived $\gamma\delta$ T cells were cultured with irradiated autologous tumor cells in the presence of IL-2 and IL-15, they showed markedly increased proliferation (defined as tumor-activated $\gamma\delta$ T cells) (Figure 1(a); $n = 9$, $P < 0.01$).

To evaluate the potential functions of tumor-activated $\gamma\delta$ T cells, we assessed the pattern of surface expression of immune-costimulatory molecules and the profile of cytokines secreted. The results showed no detectable expression of CD80, CD83, or CD86 on the surface of $\gamma\delta$ T cells from PBMCs of normal donors (control), peripheral-derived $\gamma\delta$ T cells, and $\gamma\delta$ T cells directly isolated from tumor tissues (defined as tumor tissue-derived $\gamma\delta$ T cells). In addition, the antigen-presenting molecule HLA-DR was strongly expressed on $\gamma\delta$ T cells, and the expression in cells from patients was higher than that in cells from normal donors. Interestingly, we found that the T cell costimulatory molecules CD80 and CD86 and the mature molecule CD83 were substantially upregulated on the tumor-activated $\gamma\delta$ T cells, along with the obvious increased expression of HLA-DR (Figure 1(b); $n = 9$). To explore the phenomenon that the factors from tumor tissue alone were particular to induce APC-like phenotypes in autogenetic $\gamma\delta$ T cells, a group by using normal $\gamma\delta$ T cells activated by tumor tissue was set up. We found that the $\gamma\delta$ T cells proliferated but lacked changes of APC-like phenotypes (similar to control), suggesting that the $\gamma\delta$ T cell proliferation was caused by an allogeneic response. Taking together, the factors from tumor tissue alone could contribute to induce APC-like phenotypes in autogenetic $\gamma\delta$ T cells.

Confocal microscopy results showed that, when $\gamma\delta$ T cells from patients were cocultured with autologous tumor cells for 2 h, the antigen component from the tumor cells was colocalized on the $\gamma\delta$ T cells (Figure 1(c); $n = 3$), suggesting that $\gamma\delta$ T cells could capture some antigens from the tumor cells. Analysis of the profiles of cytokines in the culture supernatants showed that the tumor-activated $\gamma\delta$ T cells secreted substantially more Th1-prone cytokines such as IFN-γ, IL-1, IL-6, and IL-12, but not IL-4 and IL-10, than peripheral-derived $\gamma\delta$ T cells did (Figure 1(d); $n = 5$).

To investigate the immunostimulatory properties of the tumor-activated $\gamma\delta$ T cells, the ability to induce expansion of effector $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells was examined using in vitro functional assay. The results showed that, in contrast to peripheral-derived $\gamma\delta$ T cells, tumor-activated $\gamma\delta$ T cells induced significantly higher proliferation of $CD4^+$ T cells (Figures 2(a) and 2(b); $n = 3$, $P < 0.01$) and $CD8^+$ T cells (Figures 2(c) and 2(d); $n = 3$, $P < 0.01$) in a number-dependent pattern, as assessed by the reduction in CFSE signals. Further, the proliferation of $CD4^+$ or $CD8^+$ T cells induced by the tumor-activated $\gamma\delta$ T cells was blocked in a transwell experiment and anti-CD80/CD86 antibody blocking experiment (Figure 2(e); $n = 3$, all $P < 0.01$), suggesting that the effect of tumor-activated $\gamma\delta$ T cells on $CD4^+$ or $CD8^+$ T cells is dependent on cell-cell contact and costimulatory molecule CD80/CD86.

3.2. Tumor-Activated γδ T Cells Abrogate Immunosuppression Induced by $CD4^+CD25^+$ Treg Cells. Our previous study demonstrated that $CD4^+CD25^+$ Treg cells isolated from tumor patients inhibited the proliferation of autologous effector $CD4^+$ T cells [20]. To determine the effect of tumor-activated $\gamma\delta$ T cells on $CD4^+CD25^+$ Treg cells, the peripheral-derived $\gamma\delta$ T cells or tumor-activated $\gamma\delta$ T cells were cocultured with $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Treg cells, respectively. As shown in Figures 3(a) and 3(b), the proliferation of $CD4^+CD25^-$ T cells was inhibited in the presence of $CD4^+CD25^+$ Treg cells isolated from gastric

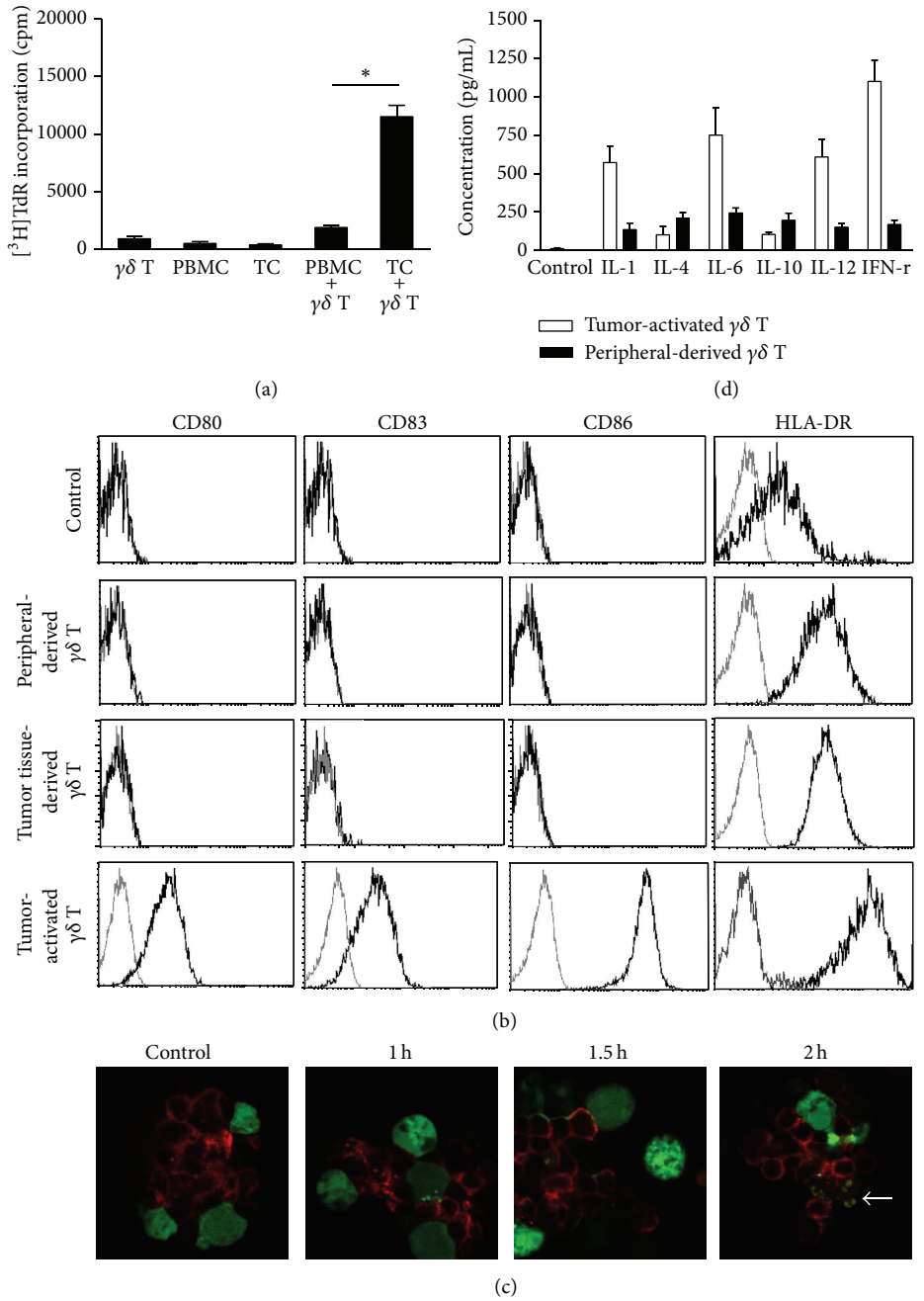

Figure 1: APC-like characteristics of tumor-activated $\gamma\delta$ T cells. (a) The proliferation of $\gamma\delta$ T cells was analyzed from [^{3}H] thymine deoxyriboside (TdR) incorporation. Irradiated (30 Gy) PBMCs or tumor tissue cells (2×10^4 cells/well) were cocultured with peripheral-derived $\gamma\delta$ T cells (6×10^4 cells/well) from patients with gastric cancer in 96-well plates for 3 days in the presence of IL-2 (200 U/mL) and IL-15 (20 ng/mL). [^{3}H]TdR incorporation was measured during the last 12 h of the incubation. Results are expressed as means ± SD (cpm) of three wells. The data are representative of at least nine independent experiments. (b) FCM was used to analyze gating for the $CD3^+TCR\gamma\delta^+$ cell population; phenotypes including CD80, CD83, CD86, and HLA-DR on the $\gamma\delta$ T cells from PBMCs of healthy donors (control), peripheral-derived, tumor tissue-derived, and tumor-activated $\gamma\delta$ T cells. Results are representative of nine independent experiments. (c) Uptake and sorting of soluble proteins in tumor-activated $\gamma\delta$ T cells. $\gamma\delta$ T cells were cocultured with CFSE-prelabeled tumor cells (green fluorescence) for the indicated time. The cells were then collected, cytocentrifuged, and stained with TCR$\gamma\delta$-PECY5 mAb (red fluorescence). The interaction between $\gamma\delta$ T cells and tumor cells was observed using confocal microscopy. The arrow shows the colocalization of the TCR of $\gamma\delta$ T cells with some components of tumor cells. Results are representative of three independent experiments. (d) Tumor-activated or peripheral-derived $\gamma\delta$ T cells (1×10^6 cells/mL) were cultured in RPMI 1640 containing IL-2 (200 U/mL) and IL-15 (20 ng/mL) for 12 h, and the culture supernatants were collected and assayed for IFN-γ, IL-1, IL-4, IL-6, IL-10, and IL-12 using ELISA. Data are representative of five independent experiments. $^*P < 0.01$.

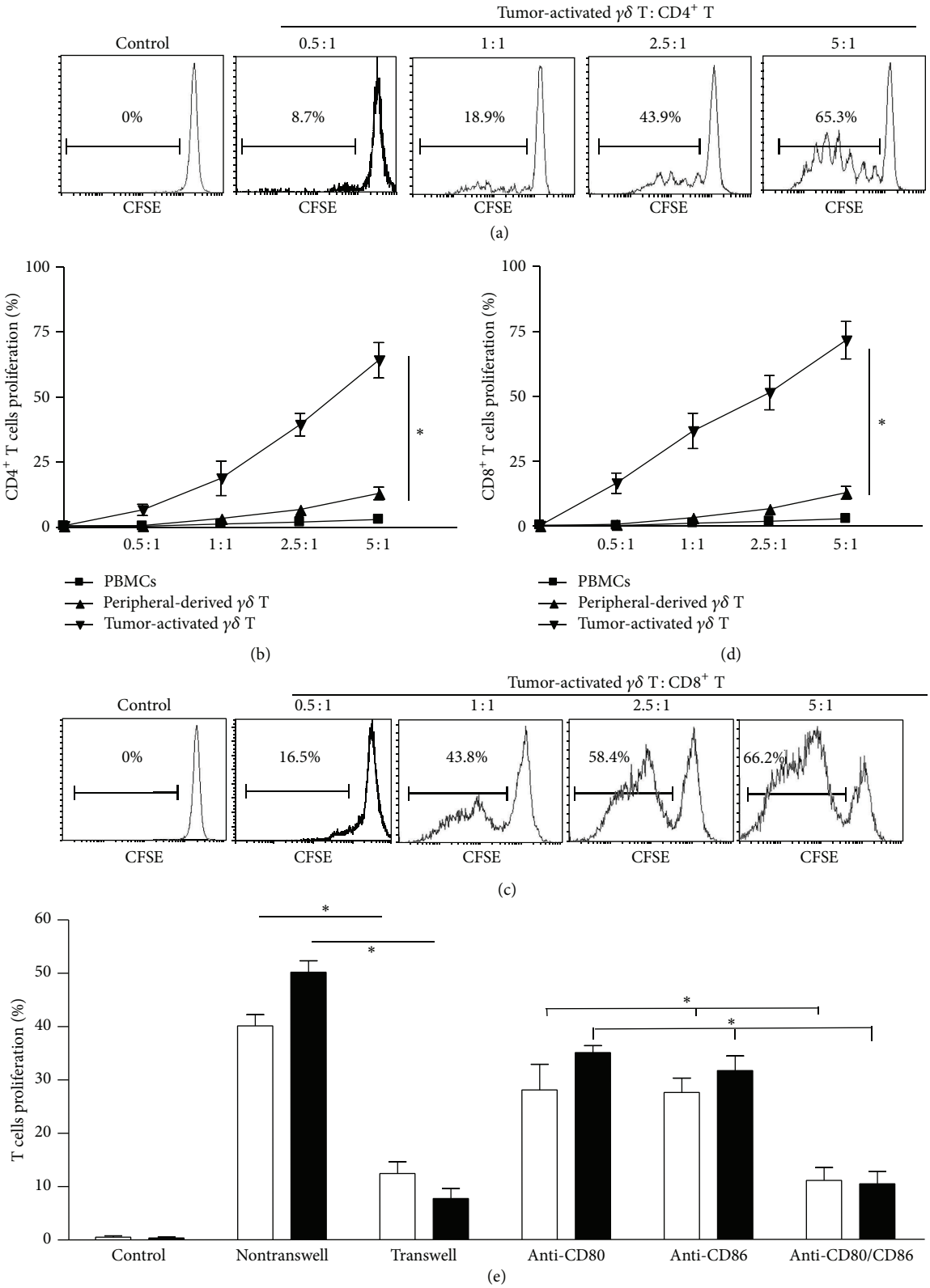

Figure 2: Stimulatory effect of tumor-activated $\gamma\delta$ T cells on primary $CD4^+$ and $CD8^+$ T cells. For this test, 2×10^5 cells/mL CFSE-prelabeled primary $CD4^+$ T cells ((a) and (b)) or $CD8^+$ T cells ((c) and (d)) as responders were incubated with tumor-activated or peripheral-derived $\gamma\delta$ T cells at the indicated ratios in 24-well plates with or without blocking antibodies (2 μg/mL) and in transwell plates (e). On day 3, the proliferation of $CD4^+$ or $CD8^+$ T cells was determined by FCM, wherein CFSE dilution was assessed. Results are representative of 3 independent experiments. $^*P < 0.01$.

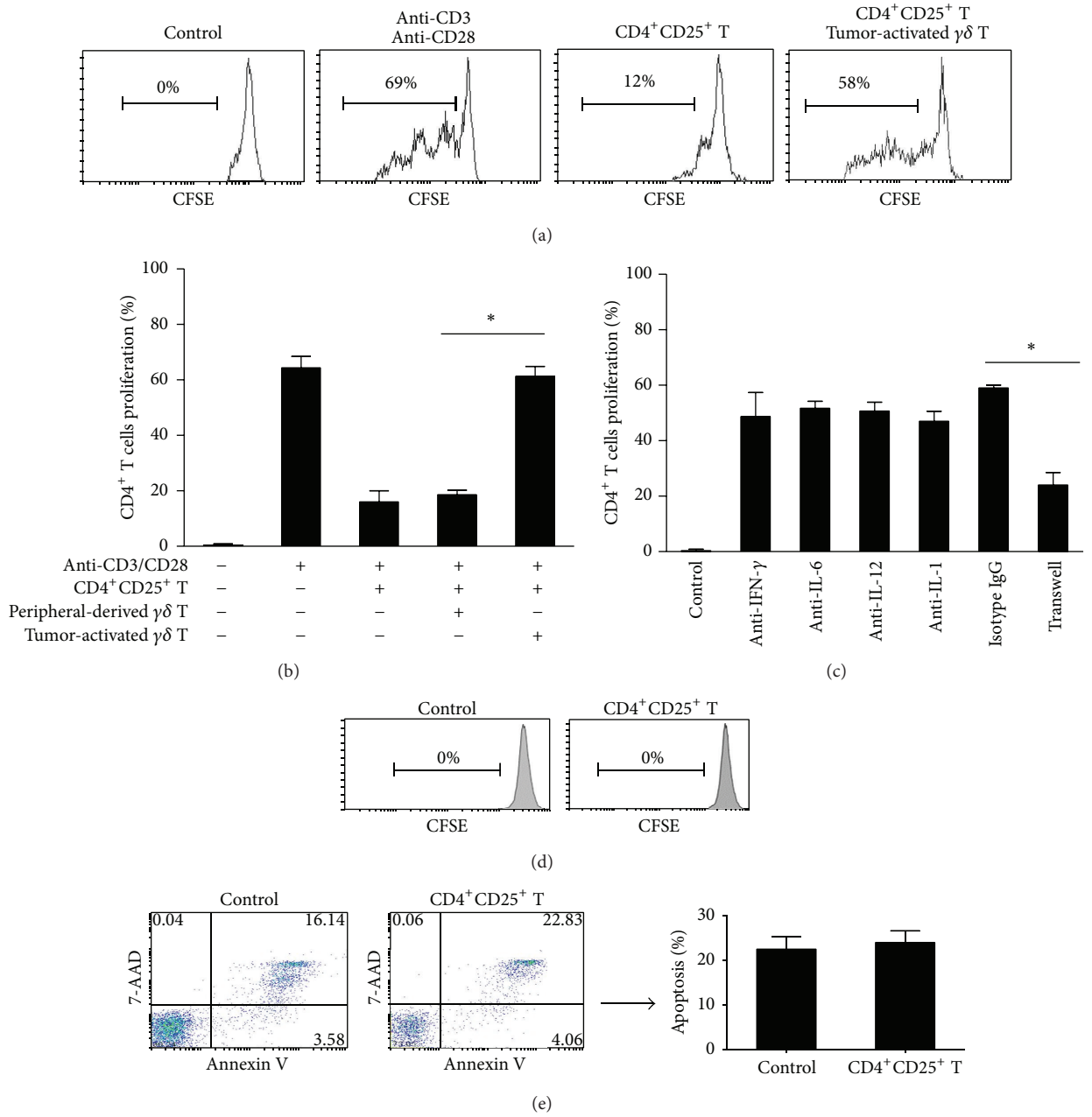

FIGURE 3: Abrogation of immunosuppressive effect of $CD4^+CD25^+$ Treg cells by tumor-activated $\gamma\delta$ T cells. ((a) and (b)) Primary $CD4^+$ T cells were prelabeled with CFSE as responders, and 2×10^5 cells/mL CFSE-prelabeled primary $CD4^+$ T cells were incubated alone or coincubated with autologous $CD4^+CD25^+$ Treg cells at a ratio of 1:1 and/or tumor-activated $\gamma\delta$ T cells at a ratio of 1:1:3 in the presence of anti-CD3 mAb (1 μg/mL) and anti-CD28 mAb (1 μg/mL) with or without blocking antibodies. The same combinations were also incubated in transwell plates (c) as indicated. The proliferation of $CD4^+$ T cells was checked on day 3 by FCM used to assess CFSE dilution. (d) 2×10^5 cells/mL CFSE-prelabeled $CD4^+CD25^+$ Treg cells were incubated alone (control) or coincubated with tumor-activated $\gamma\delta$ T cells at a ratio of 1:1. The proliferation of $CD4^+CD25^+$ Treg cells was checked on day 3 by FCM used to assess CFSE dilution. (e) 2×10^5 cells/mL tumor-activated $\gamma\delta$ T cells were incubated alone (control) or coincubated with $CD4^+CD25^+$ Treg cells at a ratio of 1:1. The apoptosis of $CD4^+CD25^+$ Treg cells was checked on day 2 by FCM. Results are representative of three independent experiments. $^*P < 0.01$.

cancer patients, and it did not change when peripheral-derived $\gamma\delta$ T cells were added to the culture system. However, when tumor-activated $\gamma\delta$ T cells were added, the proliferation of $CD4^+CD25^-$ T cells was significantly increased ($n = 3$, $P < 0.01$), suggesting that tumor-activated $\gamma\delta$ T cells, but not peripheral-derived $\gamma\delta$ T cells, abrogate the immunosuppressive effect induced by $CD4^+CD25^+$ Treg cells. Further, the above-mentioned effects were blocked in a transwell

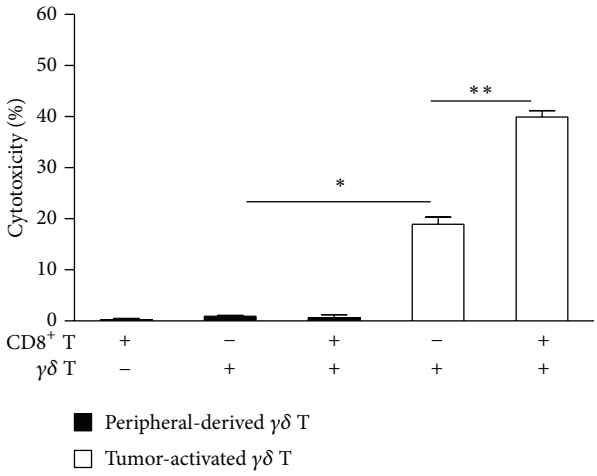

FIGURE 4: Tumor-activated $\gamma\delta$ T cells trigger the cytotoxic effect of autologous $CD8^+$ T cells. Autologous $CD8^+$ T cells (2×10^5 cells/mL) were cocultured with tumor cells in a 24-well plate for 6 h in the absence or presence of peripheral-derived $\gamma\delta$ T cells or tumor-activated $\gamma\delta$ T cells at a ratio of 1 : 1 : 1. The culture supernatants were collected and the cytotoxic activity of effector cells was measured using an LDH assay. Results are expressed as means ± SD and are representative of three independent experiments. $^*P < 0.01$, $^{**}P < 0.05$.

experiment (Figure 3(c); $n = 3$, $P < 0.01$) but not by specific antibodies against cytokines such as IFN-γ, IL-1, IL-6, and IL-12. Further experimental results showed that tumor-activated $\gamma\delta$ T cells neither induced the proliferation of $CD4^+CD25^+$ Treg cells (Figure 3(d); $n = 3$) nor enhanced the apoptosis of $CD4^+CD25^+$ Treg cells (Figure 3(e); $n = 3$), when cocultured with $CD4^+CD25^+$ Treg cells.

3.3. Tumor-Activated γδ T Cells Not Only Directly Kill Tumor Cells but Also Activate the Cytotoxic Effects of Autologous $CD8^+$ T Cells. To determine the ability of tumor-activated $\gamma\delta$ T cells to kill tumor cells and its role in the cytotoxicity of effector $CD8^+$ T cells, we examined the cytolysis of tumor-activated $\gamma\delta$ T cells and primary autologous $CD8^+$ T cells incubated with tumor-activated $\gamma\delta$ T cells. The results showed that tumor cells were not lysed by peripheral-derived $\gamma\delta$ T cells, $CD8^+$ T cells alone, or $CD8^+$ T cells cocultured with peripheral-derived $\gamma\delta$ T cells. Conversely, tumor-activated $\gamma\delta$ T cells could lyse tumor cells (Figure 4; $n = 3$, $P < 0.01$), and cytotoxicity for the tumor cells was increased in the presence of $CD8^+$ T cells ($P < 0.05$), suggesting that tumor-activated $\gamma\delta$ T cells not only directly kill tumor cells but also activate the cytotoxicity of $CD8^+$ T cells.

4. Discussion

In general, APCs such as dendritic cells (DCs) sample Ags from target cells or pathogens by phagocytosis and then present or cross-present processed Ags to MHC class II I molecules, to trigger adaptive immune responses by the release of cytokines, expression of costimulatory molecules, and Ag stimulation [12]. On the one hand, $\gamma\delta$ T cells display characteristics of adaptive immunity, wherein they express a rearranged TCR receptor, generate immunologic memory, and transform into cytotoxic T lymphocytes. On the other, they may also be considered part of the innate immune system, since they respond rapidly to antigenic stimuli, have limited TCR gene usage, and express pattern-recognition receptors [13]. To our knowledge, the present study is the first to identify an APC-like function for $\gamma\delta$ T cells in tumor patients.

Previous studies demonstrated that human $\gamma\delta$ T cells from tonsillar tissue have APC functions, efficiently cross-presenting soluble proteins to effector $CD8^+$ $\alpha\beta$ T cells and inducing effector cells differentiation and activation [1, 11, 21]. However, these results were acquired using $\gamma\delta$ T cells stimulated by the isoprenoid metabolite isopentenyl pyrophosphate (IPP) in vitro, and whether $\gamma\delta$ T cells function like APCs under different disease circumstances, particularly tumors remained largely unknown. In the present study, peripheral-derived $\gamma\delta$ T cells from gastric cancer patients were used. Our findings showed that these peripheral-derived $\gamma\delta$ T cells were activated and proliferated when stimulated by autologous tumor cells in vitro, suggesting that tumor cells were equipped with the signals to activate peripheral-derived $\gamma\delta$ T cells. The underlying mechanism is not clear at present. We previously found that that autologous tumor cells selectively expanded $\gamma\delta$ T cells among $CD4^-CD8^-$ PBMCs from cancer patients and this phenomenon was related to TCR and NKG2D signals.

Accumulating experimental and clinical data indicate that $\gamma\delta$ T cells can recognize aminobisphosphonates and phosphorylated intermediates of the bacterial nonmevalonate isoprenoid pathway, known as phosphoantigens. In addition, high concentrations of IPP, possibly generated because of a dysregulated mevalonate pathway as well as ectopically expressed mitochondrial F1-ATPase/apolipoprotein I complex on malignant cells, can selectively induce $\gamma\delta$ T cell expansion through the TCR pathway [3, 8, 22]. NKG2D recognizes the stress-inducible MHC class I-related chains A and B (MIC A/B) and glycophosphatidylinositol-linked proteins UL16-binding proteins (ULBPs), which are expressed by many tumor cells [23–26]. The engagement of NKG2D provides a costimulatory signal for $\gamma\delta$ T cell activation, allowing for the amplification of TCR-mediated priming upon recognition of ligand(s) on tumor cells.

The tumor-activated $\gamma\delta$ T cells showed APC-like characteristics in terms of phenotype, cytokine profile, and functions. (i) They strongly expressed HLA-DR and the costimulator molecular CD80/CD86 and secreted the proinflammatory cytokines IL-1, IL-6, IL-12, and IFN-γ. (ii) They triggered the proliferation and differentiation of primary $CD4^+$ or $CD8^+$ $\alpha\beta$ T cells and inhibited the function of $CD4^+CD25^+$ Treg cells. (iii) Most importantly, in addition to their direct cytotoxicity, they activated $CD8^+$ $\alpha\beta$ T-mediated cytotoxicity to tumor cells. These phenotypic and functional features are consistent with those found previously [11, 19, 27]. However, our understanding of the APC effects of $\gamma\delta$ T cells on $\alpha\beta$ T cell differentiation is currently rudimentary. Our results showed that peripheral-derived $\gamma\delta$ T cells have no APC-like functions, which exclude their involvement in

the control of $\alpha\beta$ T cell responses in patients with gastric cancer. In contrast, tumor-activated $\gamma\delta$ T cells behave like APCs by the rapid acquisition of APC characteristics in the activation of peripheral-derived $\gamma\delta$ T cells, in a manner reminiscent of mature dendritic cells (DCs). The responses induced by the $\gamma\delta$ T cells were potent, and they induced robust proliferation responses among primary autologous $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, possibly through an unknown antigen-presenting pathway, proinflammatory cytokines, and costimulator molecules. These functions are highly beneficial, and $\gamma\delta$ T cells are a unique and conserved population of lymphocytes that have been the subject of a recent explosion of interest owing to their essential contributions to many types of immune responses and immunopathology [28]. The functional difference between peripheral-derived and tumor-activated $\gamma\delta$ T cells is ascribed to the suppressive state of the immune system in cancer patients.

Considerable attention in immunotherapy research is currently focused on human $\gamma\delta$ T cells because of their functional uniformity [29]. The role of $\gamma\delta$ T cells in oncology is concentrated around their potential applications in cancer treatment, for direct cytolysis. In terms of cellular immunotherapy, it may be important to emphasize that $\alpha\beta$ T cell differentiation induced by $\gamma\delta$ T cells leads to $CD4^+$ T helper cell and effector $CD8^+$ T cell responses. Further, experimental and clinical data indicate that $\gamma\delta$ T cells exhibit a potent HLA-unrestricted lytic activity against various tumor cell lines and display antitumor effects [17, 30, 31]. In addition to TCR-dependent recognition, activation of the killer receptor NKG2D is involved in the cytotoxic activity of $\gamma\delta$ T cells. In general, the cytotoxicity of $\gamma\delta$ T cells to tumor cells involves the TCR and NKG2D receptors and depends on the perforin/granzyme pathway.

Our results also suggested that tumor-activated $\gamma\delta$ T cells directly lyse the target tumor cells and trigger the activation and functioning of $CD4^+$ and $CD8^+$ T cells. Of note, the molecular mechanisms underlying $\gamma\delta$ T cell-mediated activation of conventional $CD4^+$ or $CD8^+$ $\alpha\beta$ T cells are similar to those employed by professional APCs, which involve TCR signals and the costimulatory molecules CD80/CD86. Therefore, a possible mechanism for the APC-like behavior of tumor-activated $\gamma\delta$ T cells is that these cells are loaded with tumor components for a brief period, during which they process and express costimulatory molecules CD80/CD86. The recognition of tumor cells by $\gamma\delta$ T cells does not depend on MHC-mediated Ag presentation, which may represent a key advantage in immunotherapy during advanced stages of cancer [32].

It is well established that the aggregation of $CD4^+CD25^+$ Treg cells in the tumor milieu is involved in the immune escape of tumors and is detrimental for immunotherapy among tumor patients [20, 33, 34]. Further, an interaction between $CD4^+CD25^+$ Treg cells and $\gamma\delta$ T cells has been reported [35]. Recent data have further shown that $\gamma\delta$ T cells from tumor tissues are positively correlated with $Foxp3^+$ suppressive T cells in advanced breast tumors and inversely correlated with relapse-free and overall survival of breast cancer patients [36]. These findings raise interesting questions on whether tumor-activated $\gamma\delta$ T cells are capable of weakening $CD4^+CD25^+$ Treg cell-mediated immunosuppression. Our results demonstrated an additional value to the proposed immunotherapy with $\gamma\delta$ T cells, wherein the immune suppression by $CD4^+CD25^+$ Treg cells can be overcome since it requires cell-cell contact. The underlying mechanism could be that tumor-activated $\gamma\delta$ T cells show enhanced stimulation of $CD4^+$ T cell proliferation, rather than direct suppression by $CD4^+CD25^+$ Treg cells, since our results indicated that tumor-activated $\gamma\delta$ T cells failed to induce proliferation or apoptosis of $CD4^+CD25^+$ Treg cells.

From the results, we speculated that tumor-activated $\gamma\delta$ T cells kill tumor cells and simultaneously present certain Ag(s) from tumor cells. Tumor-antigen signals, together with costimulatory molecules and cytokines, could effectively trigger the activation and functioning of $CD4^+$ and $CD8^+$ T cells. Further investigation of this hypothesis may show that $\gamma\delta$ T cells were compared favorably with professional APCs such as DCs with respect to their advantage of expansion in vitro and direct activation by signals preferentially expressed on tumor cells, such as NKG2D ligands MIC A/B and ULBPs [22, 37, 38], and, thus, these cells may become a continual and renewable source of functional APCs. They may be used to produce tumor vaccines to induce an adaptive immune response against tumors. We believe that $\gamma\delta$ T cells will play a key role in developing cancer immunotherapy strategies.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Chaoming Mao, Xiao Mou, and Yuepeng Zhou contributed equally to this work.

Acknowledgments

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Research Article

Gαs Protein Expression Is an Independent Predictor of Recurrence in Prostate Cancer

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Background. T393C polymorphism in the gene GNAS1, which encodes the G-protein alpha s subunit (Gαs) of heterotrimeric G protein, is significantly associated with the clinical outcome of patients suffering from several cancers. However, studies on the role and protein expression of Gαs subunit in prostate cancer were still unavailable. *Methods*. The immunohistochemical staining was used to assess Gαs expression through tissue microarray procedure of 56 metastatic PCas, 291 localized PCas, and 67 benign hyperplasia (BPH). Gαs expression was semiquantitatively scored and evaluated the correlation with pathologic parameters and biochemical recurrence of prostate-specific antigen (PSA). *Results*. Gαs expression was localized in nuclear and cytoplasm in prostate cancer cells and downregulated in metastatic PCa compared to localized PCa and BPH ($P < 0.001$). Gαs was inversely associated with PSA level and Gleason scores; patients with low expression of Gαs had adverse clincopathological features. In multivariable Cox regression analysis, high Gαs expression and Gleason scores were independent predictors of both PSA progression-free and overall survival. *Conclusions*. Gαs down-expression is associated with adverse pathologic features and clinical PSA biochemical recurrence of prostate cancer. Gαs is an independent predictor to help determine the risk of PSA progression and death.

1. Introduction

Prostate cancer (PCa), the most frequently diagnosed malignancy, has become the second leading cause of cancer-related deaths among men in Western countries [1, 2]. Endocrine therapies which aimed at inhibiting the androgen receptor (AR) function was the mainstay of treatment for advanced prostate cancer based on that the androgen signaling will promote the proliferation of prostate cancer cell. Unfortunately, most of treated patients progressed toward castration-resistant prostate cancer (CRPC) from castration-dependent prostate cancer. And CRPC characterized by aggressive growth and ability to colonize distal organs, which made CRPC still incurable and the median survival time for patients with CRPC was only 12 months [3]. The status of AR was highly predictive of prostate cancer patients that will benefit from endocrine therapy but was not correlated with a better clinical outcome [4, 5]. Prostate-specific antigen (PSA) is a protein produced by the prostate gland cells. The PSA test measures the level of PSA in a man's blood. Although many published studies have assessed the performance of candidate biomarkers in predicting time to relapse of prostate cancer following radical prostatectomy [6, 7], no molecular markers suitable for routine clinical practice that can identify those prostate cancer patients with a high risk of early clinical progression or prostate cancer-specific mortality have been found.

G-proteins are composed of α, β, and γ subunits and α subunit are classified into 4 families: Gαs, Gαi/o, Gαq/11, and Gα12/13. Each of them has multiple members with different expression specificity [8, 9]. Although Gαs is the most extensively characterized and clinically relevant, literature is not unanimous on the role of Gαs in different types of cancers. In lung cancer, Choi et al. found that Gαs could augment cisplatin-induced apoptosis of lung cancer cells through upregulating Bak expression by increasing transcription and by decreasing the rate of protein degradation [10] and the efficacy of radiotherapy of lung cancer may be improved by

modulating Gαs signaling pathway [11]. But in cervical cancer or intrahepatic cholangiocarcinoma (ICC), the situation was just opposite. Cho et al. found Gαs inhibited cisplatin-induced apoptosis by increasing transcription of X-linked inhibitor of apoptosis protein (XIAP) and by decreasing degradation of XIAP protein in HeLa cervical cancer cells [12]. In ICC, Schmitz et al. also found a significant association of both unfavorable disease-specific overall survival and recurrence-free survival with the homozygous TT genotype of the GNAS1 gene which encoded Gαs protein [13, 14]. However, they also reported that T393C polymorphism in the gene GNAS1 was significantly associated with favorable clinical outcome of patients suffering from bladder cancer, chronic lymphocytic leukemia [15], and renal cell carcinoma [16].

The situation was even more complicated in prostate cancer. There were studies reported no association was found between GNAS T393T genotype and prostate cancer [17, 18]. But Liu et al. identified that membrane caveolae-associated Gas was involved in androgen receptor (AR) transactivation by modulating the activities of different PI3K isoforms [19]. More importantly, it had been reported that the expression of Gαs and Gαi decreased 30% to 40% after neoplastic transformation [20]. And the levels of Gαs and Gαi subunits correlated inversely with serum prostate specific antigen in patients with prostate cancer [20], which indicated an important regulatory role of Gαs and Gαi for cell proliferation and neoplastic transformation in human prostate cancer and they may have prognostic value. Therefore, more in-depth investigations are necessary to address this controversy and identify the role of Gαs in prostate cancer.

Thus, we assessed the potential of Gαs as a prognostic marker by determining the level of Gαs protein expression in a series of 347 postradical prostatectomy prostate cancer tissue microarrays (TMA) which include 56 metastatic PCas and 291 localized PCas and 67 benign prostatic hyperplasia (BPH) as controls using immunohistochemistry (IHC). In the present study, we found that expression of Gαs protein was decreased in high grade and metastatic PCas. And low Gαs protein levels were strongly associated with adverse clinicopathologic features and poor clinical outcomes in metastatic and localized PCa patients. Multivariate Cox regression analysis showed that low expression of Gαs was an independent predictor of prostate cancer recurrence and cancer-specific death in metastatic and localized PCa. To the best of our knowledge, this is the first study to identify the independent predictive role of Gαs in patient with prostate cancer.

2. Patients and Methods

2.1. Patient Selection. In order to study Gαs expression in prostate cancer by immunohistochemistry, a total of 347 formalin-fixed, paraffin-embedded prostate tissues between 1994 and 1997 were retrieved from the archives of the First Affiliated Hospital, College of Medicine, and Xi'an Jiao Tong University, and a tissue microarray (TMA) was constructed. The TMA included a series of 56 metastatic PCas and 291 localized PCas. In addition, 67 benign prostatic hyperplasia (BPH) samples were collected as control. This research project was approved by the Ethical Committee of the Xi'an Jiao Tong University, and all the patients had been given their fully written informed consent.

Data were collected on patients with disease baseline and clinicopathologic characteristics as well as 2 treatment outcomes: time to progression and prostate cancer-specific mortality (PCSM). Prostate cancers were graded based on the Gleason system by 2 independent pathologists at the First Affiliated Hospital, College of Medicine, and Xi'an Jiao Tong University in a blind and consecutive manner to ensure adequate diagnosis and grade. The TNM staging system was used to describe the extent of Prostate cancer in patients (based on the AJCC Cancer Staging Manual, Seventh Edition, 2010, Springer, New York, Inc.). TNM stages IIA and IIB were considered TNM stage II.

2.2. Immunohistochemistry (IHC). Paraffin-embedded section of normal and tumor tissue was stained for Gαs expression. Immunohistochemistry for Gαs was performed as previous reported with slight modification. Briefly, slides were deparaffinized in xylene and rehydrated in a graded alcohol series before endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. After nonspecific protein binding was blocked, the primary antibody diluted into recommended concentration for Gαs, which was purchased from Abcam (ab58810), was applied overnight in a humidity chamber at 4°C. Biotinylated secondary antibody was applied for 30 min at room temperature after washing with PBS for 3 times. Visualization was performed using DAB chromogen for 2 to 3 minutes. Negative control was conducted by replacing the primary antibody with preimmune rabbit serum.

2.3. Evaluation of Staining. To evaluate Gαs expression, we used the immunoreactive score (IRS) as previously implemented by Tischler et al. [21], based on the intensity of immune staining and the quantity of stained cells. The intensity of staining was arbitrarily graded as absent (0), weak (1+), moderate (2+), and strong (3+). The percentage of stained cells was use d to quantify the react ion as negative (0% of positive cells), 1+ (<10% positive cells); 2+ (10–50% of positive cells); 3+ (51–80% of positive cells); 4+ (>80% of positive cells). The final value of the analysis of each tissue sample was then expressed as an absolute value through the obtained score by multiplying the two individual scores (i.e., intensity of staining score times the percentage of stained cells score) then generates a final score ranging from − (no expression) to + (weak expression), ++ (moderate expression), or +++ (strong expression). And we identified − and + as negative for Gαs expression and ++ and +++ as positive Gαs expression. Examples of scoring according to staining intensity and the percentage of stained cells are shown in Figure 1.

2.4. Statistical Analysis. SPSS version 13.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. P values < 0.05 were considered significant. Mann-Whitney test was used to calculate the correlation between numerical variables. 2 tests

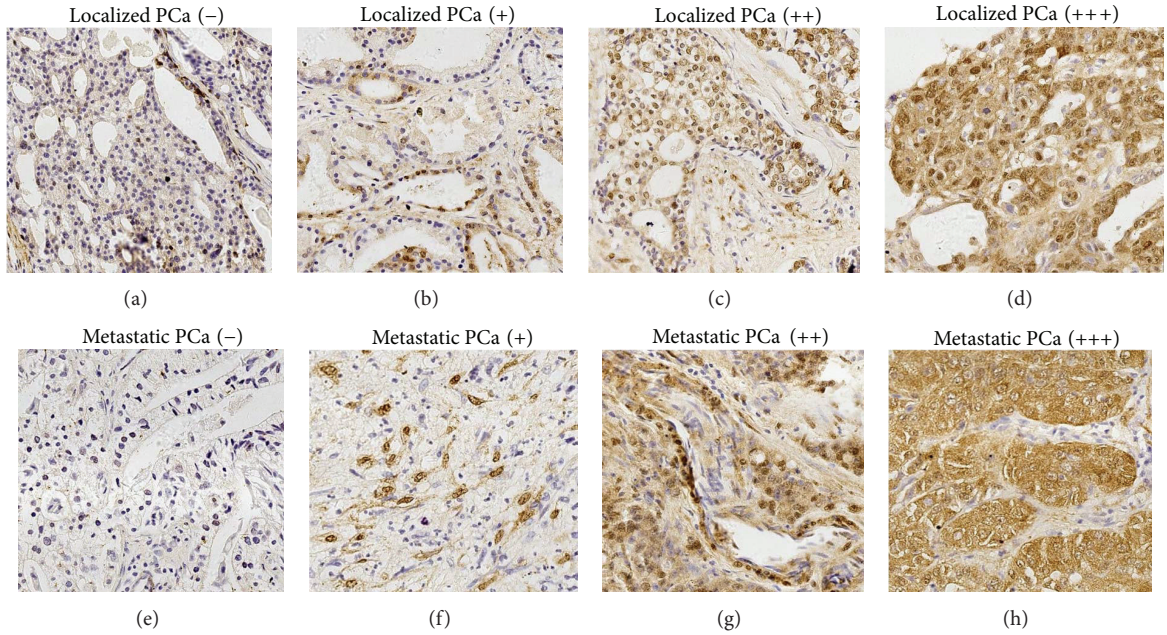

Figure 1: Immunohistochemically stained localized and metastatic PCa tissues from patients. (a) localized PCa tissues without Gαs expression (−); (b) localized PCa tissues with weak Gαs expression (+); (c) localized PCa tissues with moderate Gαs expression (++); (d) localized PCa tissues with strong Gαs expression (+++); (e) metastatic PCa tissues without Gαs expression (−); (f) metastatic PCa tissues with weak Gαs expression (+); (g) metastatic PCa tissues with moderate Gαs expression (++); (h) metastatic PCa tissues with strong Gαs expression (+++). Representative images were taken under a microscope (×20).

were used to evaluate differences in frequency of categorical-variable groups. Spearman's rank correlation was used to analyze the correlation between continuous variables. PSA progression-free and overall survival curves were constructed by the Kaplan-Meier method and compared using the log-rank test. To evaluate the role of prognostic variables, a series of Cox proportional hazards models were fitted to PSA progression-free and overall survival data. Since PSA was a continuous estimate, with the median PSA level for the entire cohort of patients ($n = 347$) as 34.9 ng/mL, we divided the cohort into those with PSA levels ≤35 ng/mL and >35 ng/mL. The following parameters were included: PSA levels (≤35 ng/mL, >35 ng/mL); extraprostatic extension (Yes, No); involvement of surgical margins (No, Yes); involvement of seminal vesicles (No, Yes); involvement of pelvic nodes (N0, N+); Gleason scores (2–6, 7, 8–10).

3. Results

3.1. Histopathologic and Clinical Information. The median Gleason score of all patients was 7 (range: 2–10). 145 patients (41.8%) presented Gleason score of 2–6, 127 (36.6%) patients presented Gleason score of 7, and the remaining 75 cases (21.6%) presented Gleason score between 8 and 10. 49 patients (11.5%) presented TNM stage I; 125 (36.0%) patients presented stage II; 117 (33.7%) presented stage III; and 56 (16.1%) patients presented TNM stage IV. PSA progression was observed in 229 (66.0%) patients at a median interval of 123.5 month (range 7–167). Other clinicopathological features are summarized in Table 2. Moreover, prostate cancer patients who had higher Gleason scores ($P < 0.001$ and $P < 0.001$, resp.), higher TNM stages ($P < 0.001$ and $P < 0.001$, resp.), higher preoperative PSA level ($P < 0.001$ and $P < 0.001$, resp.), positive surgical margin ($P = 0.009$ and $P < 0.001$, resp.), angiolymphatic invasion ($P = 0.004$ and $P = 0.032$, resp.), extraprostatic extension ($P = 0.031$ and $P < 0.001$, resp.), and seminal vesicle invasion ($P = 0.046$ and $P = 0.007$, resp.) present shorter overall survival and PSA progression-free survival (Tables 5 and 6). PSA progression and overall survival time correlated with TNM stage, Gleason score, extraprostatic extension, positive surgical margins, and seminal vesicle invasion demonstrate the representability of study group. The number of patients with positive lymph node involvement ($N = 34$) was too small to find any significant correlation with PSA progression-free survival and overall survival.

3.2. Expression of Gαs in Human Prostate Cancer. To determine the prevalence and clinical significance of Gαs in prostate cancer tissues, we determined the expression of Gαs protein by immunohistochemistry in a retrospective cohort of 347 tumor tissue samples from prostate cancer patients and 67 samples from patients who were diagnosed with benign prostatic hyperplasia (BPH) after tumor resection. Among the 347 patients, 114 patients had not expression of Gαs (−); 73 patients were weak expression (+); 86 patients were moderate expression (++), and 74 patients were strong expression (+++) (as shown in Figure 1). Thus, as we described in the methods section, there were 160 (46.1%) samples positive for Gαs expression and 187 (53.9%) samples negative for Gαs

Table 1: Comparison of Gαs expression among different pathological categories.

Variable	Number	Gαs positive	Gαs negative	χ^2	P
All patients	347	160	187		
Metastatic PCa	56	17	39		
Localized PCa	291	143	148	6.67	0.012*
BPH	67	42	25	12.77	<0.001**

*Significant differences of Gαs expression in metastatic PCa compared to localized PCa.
**Significant differences of Gαs expression in metastatic PCa compared to BPH.

expression in our PCa cohort. We also found that positive ratio of Gαs expression was downregulated in metastatic PCa compared to localized PCa and BPH ($P = 0.012$ and $P < 0.001$, respectively, as shown in Table 1). In patients with BPH, there were 42 (62.7%) samples positive for Gαs expression and 25 (37.3%) samples negative for Gαs expression. In the patients with localized PCa, there were 151 (51.2%) samples positive for Gαs expression and 140 (48.1%) samples negative for Gαs expression. Whereas in the patients with metastatic PCa, there were 9 (16.1%) samples positive for Gαs expression and 47 (83.9%) samples negative for Gαs expression. Gαs antibody mainly showed positive nuclear and cytoplasmic staining in prostate cancer cells. IHC staining for Gαs was sharp and reliable, no background or nonspecific staining was observed.

3.3. Correlation of Gαs with Histopathologic and Clinical Information. Then, we further evaluated the relationship between Gαs expression and clinical features of prostate cancer patients by Pearson chi-square test or Fisher's exact test. We found that the positive ratio of Gαs expression was decreased as the level of Gleason score or preoperative PSA increased. These result showed that there was inverse correlations between Gαs expression and preoperative PSA and Gleason score and TNM stage at diagnosis ($P = 0.030$, $P < 0.001$, and $P < 0.001$, respectively, Table 2). But we found no specific correlation between Gαs expression and the rest of pathological parameters (age; angiolymphatic invasion; extraprostatic extension; positive margin; seminal vesicle invasion; positive lymph node) that we evaluate in the present analysis. In the localized PCa specimens, the expression of Gαs was correlated with preoperative PSA level, Gleason score, and TNM stage ($P = 0.028$, $P = 0.016$, and $P = 0.011$, respectively, Table 3). However, in metastatic PCa specimens, the expression of Gαs was only associated with preoperative PSA level and Gleason score much more significantly ($P < 0.001$, and $P < 0.001$, resp., Table 4).

3.4. Correlation of Gαs Expression with PSA-Free and Overall Survival. In our retrospective cohort with 347 patients, we got the detailed follow-up information of 15 years. At the time of our analysis, 121 patients died and 256 patients progressed. Median time to PSA progression for the whole cohort was 123.5 months (range 7–167 months), while the median time to death was 123.5 months (range 3–179 months). We found that patients with negative expression of Gαs had a higher ratio of PSA progression than those with positive Gαs expression (Table 2). More importantly, negative Gαs expression was associated with PSA progression-free survival and overall

Table 2: Characterization of the cohort of 347 prostate cancer samples.

Variable	Number	Gαs positive	Gαs negative	χ^2	P
All patients	347	160	187		
Age at diagnosis (years)					
≤73	211	96	115	0.081	0.826
>74	136	64	72		
Clinical stage at diagnosis					
I	49	31	18		
II	125	61	64	9.358	<0.001*
III	117	51	66		
IV	56	17	39		
Gleason score at diagnosis					
2–6	145	83	62		
7	127	54	73	15.692	<0.001*
8–10	75	23	52		
Preoperative PSA (ng/mL)					
<35	184	99	85	9.334	0.003*
≥35	163	61	102		
Angiolymphatic invasion					
Presence	114	48	66	1.096	0.350
Absence	233	112	121		
Extraprostatic extension					
Presence	105	53	52	0.993	0.351
Absence	242	135	107		
Positive surgical margin					
Presence	128	60	68	0.048	0.911
Absence	219	100	119		
Seminal vesicle invasion					
Presence	172	78	94	0.079	0.830
Absence	175	82	93		
Positive lymph node					
Presence	34	14	20	0.369	0.590
Absence	313	146	167		
PSA progression					
Presence	213	89	124	4.153	0.047*
Absence	134	71	63		

*Significant differences of Gαs expression among different clinical factors groups in prostate cancer samples.

Table 3: Characterization of the cohort of 291 localized prostate cancer samples.

Variable	Number	Gαs positive	Gαs negative	χ^2	P
Clinical stage at diagnosis					
I	49	31	18		
II	125	61	64	8.972	0.011*
III	117	51	66		
Gleason score at diagnosis					
2–6	141	81	60		
7	112	44	68	8.292	0.016*
8–10	38	18	20		
Preoperative PSA (ng/mL)					
<35	160	86	74	5.336	0.028*
≥35	131	57	84		

*Significant differences of Gαs expression among different clinical factors groups in localized prostate cancer samples.

Table 4: Characterization of the cohort of 56 metastatic prostate cancer samples.

Variable	Number	Gαs positive	Gαs negative	χ^2	P
Gleason score at diagnosis					
2–6	4	2	2		
7	15	10	5	15.049	<0.001*
8–10	37	5	32		
Preoperative PSA (ng/mL)					
<35	24	13	11	11.262	<0.001*
≥35	32	4	28		

*Significant differences of Gαs expression among different clinical factors groups in metastatic prostate cancer samples.

survival. The group of patients with negative expression of Gαs showed significantly shorter overall survival than patients with positive expression of Gαs ($P = 0.001$, Figure 2(a)). These patients also showed a trend for shorter PSA-free survival time ($P < 0.001$, Figure 2(d)). In localized PCa specimens, negative Gαs expression was also associated with better PSA progression-free and overall survival rate ($P < 0.001$, Figure 2(c); $P < 0.001$, Figure 2(f)). In metastatic PCa specimens, a similar trend was found between negative Gαs expression and PSA progression-free/overall survival time ($P = 0.0003$, Figure 2(e); $P = 0.0146$, Figure 2(b)).

As expected, at the univariate level, Gleason scores, TNM stages, preoperative PSA, positive margin, angiolymphatic invasion, extraprostatic extension, and seminal vesicle invasion were associated with PSA progression-free and overall survival. Negative expression of Gαs protein was a prognostic predictor of PSA progression-free and overall survival in PCa patients at univariate level (Tables 5 and 6).

We further conducted a multivariate Cox regression analysis to assess whether Gαs was a prognostic predictor of survival independent of age, Gleason scores, TNM stages, preoperative PSA, positive margin, angiolymphatic invasion, extraprostatic extension, seminal vesicle invasion, and positive lymph node. Multivariate analysis showed that negative Gαs expression was a strong independent predictor of outcome providing survival information (both PSA progression-free or overall survival) above other independent prognostic features (TNM stage, Gleason score), with a hazard ratio of 4.328 and 3.904 and a 95% confidence interval of 1.876–8.432, $P < 0.001$ (negative Gαs group versus positive Gαs group) and 1.278–5.873, $P < 0.001$ (negative Gαs group versus positive Gαs group). In our cohorts, PSA, positive margin, angiolymphatic invasion, extraprostatic extension, and seminal vesicle invasion were not independently associated with outcome at the multivariable level.

4. Discussion

It has been reported that the expression of Gαs correlated inversely with serum prostate specific antigen in patients with prostate cancer and the expression of Gαs decreased 30% to 40% after neoplastic transformation [20]. But there was no study concerning the role of Gαs protein in the prognosis of prostate cancer patients. In the present study, we characterized the expression pattern of Gαs protein in a large number of tissues derived from prostate cancer patients, consisting of localized and metastatic PCa, and assessed the utility of Gαs as a prognostic marker in these patients. In agreement with previous reports, we confirmed that Gαs expression was localized in nuclear and cytoplasm in neoplastic cells. Moreover, we found that expression of Gαs was downregulated in metastatic PCa compared to localized PCa and BPH. And Gαs was inversely associated with PSA level and Gleason scores both in localized and metastatic PCa. At the univariate level, Gαs, Gleason scores, TNM stages, preoperative PSA level, positive margin, angiolymphatic invasion, extraprostatic extension, and seminal vesicle invasion were all significantly associated with PSA progression-free and overall survival. But in multivariable Cox regression analysis, only high Gαs expression and Gleason scores were independent predictors of both PSA progression-free and overall survival. These findings support the potential clinical utility of incorporating Gαs into clinical nomograms to help determine the risk of PSA progression and death.

The prognostic significance of Gαs as biomarker for prostate cancer is likely to its biological functions. Gαs is a member of GTP-binding protein superfamiliy and could independently regulate a variety of effectors including adenylate cyclases, phospholipase Cβ, and ion channels [22, 23]. However, Gαs and T393C polymorphism in the gene GNAS1 which is encoded by Gαs plays distinct roles in different cancers. Gαs could augment cisplatin-induced apoptosis of lung cancer cells [10], but it inhibited cisplatin-induced apoptosis in cervical cancer or intrahepatic cholangiocarcinoma (ICC) [12]. T393C polymorphism in the gene GNAS1 was significantly associated with favorable clinical outcome of

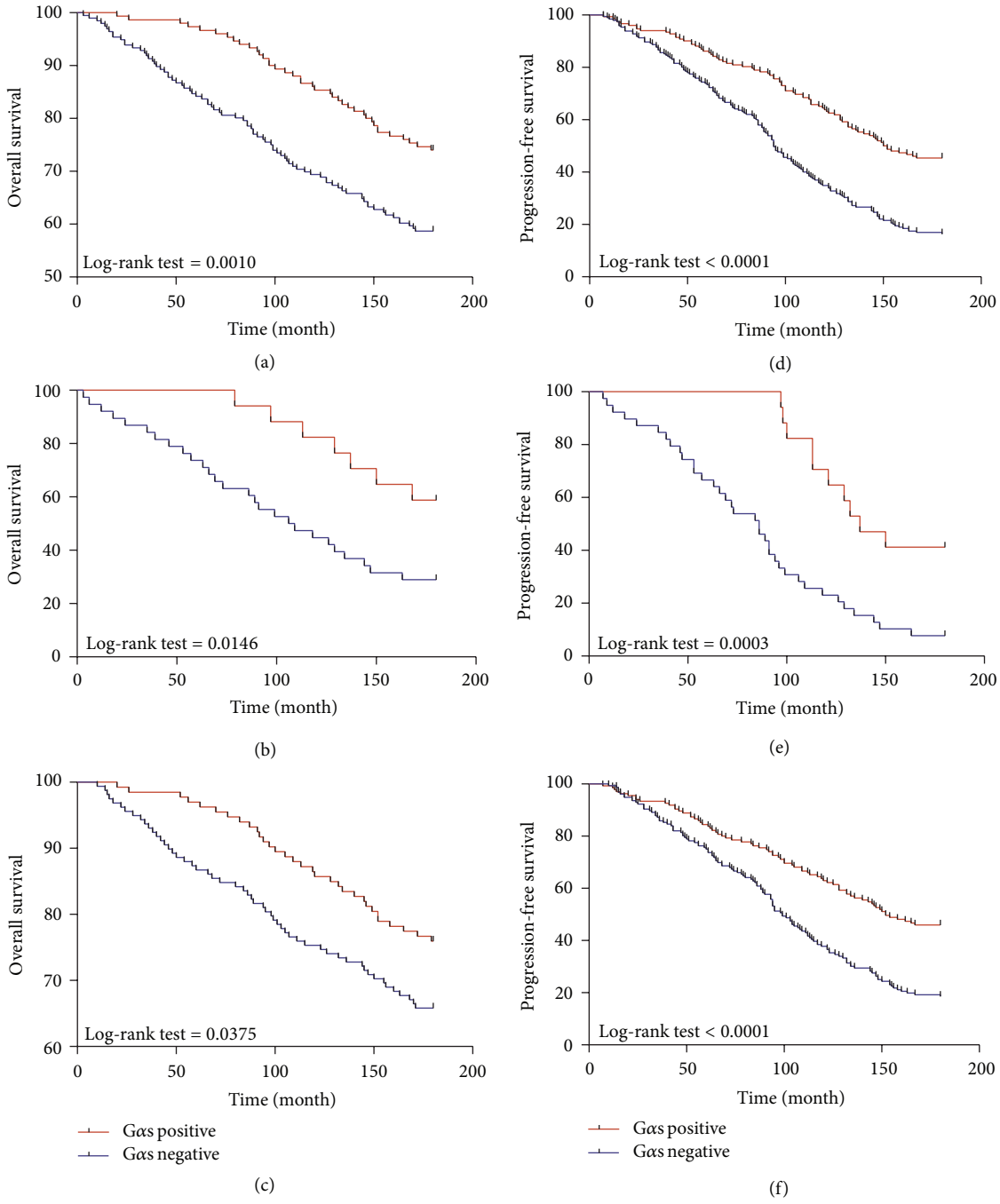

Figure 2: Kaplan-Meier analysis of overall survival (cumulative survival) and PSA progression-free survival of PCa patients relative to Gαs expression. (a) Correlation of Gαs expression with overall survival; (b) in metastatic PCa specimens, the correlation of Gαs expression with overall survival; (c) in localized PCa specimens, the correlation of Gαs expression with overall survival; (d) correlation of Gαs expression with PSA-free survival; (e) in metastatic PCa specimens, the correlation of Gαs expression with PSA-free survival; (f) in localized PCa specimens, the correlation of Gαs expression with PSA-free survival. A statistically significant difference is shown in overall survival and PSA progression-free survival outcome between the different groups of patients, with those having positive expression of Gαs having the better overall survival and PSA progression-free survival.

patients suffering from bladder cancer, chronic lymphocytic leukemia [15], and renal cell carcinoma [16], and significantly associated with unfavorable clinical outcome of patients suffering from ICC and breast cancer [13, 14]. In prostate cancer, previous study reported that low expression level of Gαs was found in T2 stage PCa compared to high levels in normal controls [20]. More importantly, the expression of Gαs was found downregulated in hormone refractory C4-2B and PC3 cell lines compared to hormone sensitive LNCaP and RWPE-1 cell lines. All these studies indicating the

Table 5: Univariate and Multivariate analysis of clinical factors in relation to overall survival.

	Univariate HR (95% CI)	*P*	Multivariate HR (95% CI)	*P*
Negative Gαs	5.832 (3.232–10.763)	<0.001*	3.904 (1.278–5.873)	<0.001*
Age at diagnosis	1.098 (0.921–1.284)	0.495	1.328 (0.493–4.187)	0.276
Clinical stage at diagnosis	2.287 (1.639–3.121)	<0.001*	0.723 (0.298–1.114)	0.294
Gleason score at diagnosis	3.809 (2.778–5.132)	<0.001*	2.153 (1.471–9.357)	0.004*
Preoperative PSA	2.673 (2.007–3.297)	<0.001*	2.158 (0.622–3.192)	0.429
Angiolymphatic invasion	1.224 (1.098–1.989)	0.004*	1.472 (0.897–1.677)	0.172
Extraprostatic extension	1.327 (1.211–2.019)	0.031*	0.819 (0.531–1.396)	0.491
Positive margin	2.127 (1.271–4.918)	0.009*	1.211 (0.682–2.198)	0.514
Seminal vesicle invasion	1.778 (1.281–3.711)	0.046*	1.397 (0.723–2.187)	0.283
Positive lymph node	1.698 (0.831–3.781)	0.064	0.931 (0.871–2.011)	0.592

CI: confidence interval; HR: hazard ratio; rec: recurrence.
*Significant relationships of clinical factors with overall survival.

Table 6: Univariate and Multivariate analysis of clinical factors in relation to PSA progression-free survival.

	Univariate HR (95%)	*P*	Multivariate HR (95%)	*P*
Negative Gαs	5.269 (1.187–7.589)	<0.001*	4.328 (1.876–8.432)	<0.001*
Age at diagnosis	1.132 (0.809–1.727)	0.413	0.743 (0.239–3.158)	0.712
Clinical stage at diagnosis	2.787 (1.131–4.238)	<0.001*	2.135 (0.897–5.328)	0.117
Gleason score at diagnosis	5.821 (3.496–10.825)	<0.001*	3.219 (1.276–8.557)	<0.001
Preoperative PSA	1.784 (1.389–3.476)	<0.001*	0.976 (0.597–2.911)	0.061
Angiolymphatic invasion	1.829 (1.142–2.109)	0.032*	0.734 (0.549–1.291)	0.125
Extraprostatic extension	2.352 (1.399–4.569)	<0.001*	1.892 (0.897–3.219)	0.071
Positive margin	3.404 (1.778–6.091)	<0.001*	2.199 (0.782–3.988)	0.084
Seminal vesicle invasion	3.891 (1.584–5.822)	0.007*	1.329 (0.806–1.986)	0.322
Positive lymph node	2.012 (0.904–4.584)	0.091	1.212 (0.814–1.507)	0.532

CI: confidence interval; HR: hazard ratio; PSA: Prostate-specific antigen.
*Significant relationships of clinical factors with PSA progression-free survival.

functionality and expression of Gαs are selectively modified in human prostate adenocarcinoma and downregulated Gαs levels may play an important regulatory role for cell proliferation and neoplastic transformation in prostate cancer. Thus, we did our efforts to investigate and validate whether Gαs functioned as an efficient prognostic biomarker to predict the outcome of PCa patients. Consistent with previous studies, we found that the expression of Gαs was much lower in metastatic PCa than it is in localized PCa. Furthermore, the patients who had the low expression of Gαs tend to have shorter progression-free survival and overall survival time, nonetheless, metastatic or localized PCa. More importantly, multivariable Cox regression analysis proved that Gαs was an independent predictor of prognosis in prostate cancer.

Though the answer to the discrepancy of Gαs function in different cancers was still unclear, and the role of Gαs in prostate cancer was also in dispute, our results were relatively easy to understand for Gαs had a close relationship with EGFR. EGFR belongs to ErbB oncogene family which also includes ErbB-2, 3, and 4 and is comprehensively expressed in epithelial cells including prostate cancer cells. EGFR are known to regulate cell proliferation, differentiation, angiogenesis, and survival [24]. In prostate cancer, EGFR is elevated along with disease progression. It has been reported that EGFR was highly expressed in DU145 and PC3 cell lines which were hormone-independent human prostate cancer cell lines and responsive to EGF stimulation [25–27]. It was also found that prostate cancer bone metastases express significantly higher level of EGFR [28]. More importantly, EGFR expression increased as prostate cancer progressed from an androgen-dependent to an androgen-independent stage [29]. di Lorenzo et al., found 41%, 76%, and 100% EGFR expression in radical prostate ectomy hormone-sensitive and hormone-refractory metastatic patients in a cohort consisting of 76 patients with androgen-dependent and androgen-independent prostate cancer, respectively [30]. And there was a significant association between EGFR expression and higher Gleason score [31]. Many studies confirmed that overexpression of EGFR contributes significantly to the progression of prostate cancer [31–34].

Zheng et al. reported that overexpression of the stimulatory Gαs promotes ligand-dependent degradation of epidermal growth factor (EGF) receptors and Texas Red EGF, and knock-down of Gαs expression by RNA interference (RNAi) delays receptor degradation [35]. Recently, they demonstrated that EGF-induced, proliferative signaling occurs from

EEA1 endosomes and was regulated by the Gαs through interaction with the signal transducing protein GIV (also known as Girdin). When Gαs or GIV was depleted, activated EGFR and its adaptors accumulate in EEA1 endosomes. Then EGFR signaling was prolonged and EGFR downregulation was delayed, which made cell proliferation greatly enhanced [36]. Basing on our finding that Gαs was downregulated in advanced PCa and the previous studies concerning the function of EGFR in PCa, we hypothesise that downexpression of Gαs inhibit the degradation of EGFR, then androgen receptors which are activated by EGFR were prolonged and cell proliferation increased, eventually causing tumor progression and hormone-resistant in prostate cancer. That maybe the underlying mechanism account for our results in which the expression of Gαs was downregulated in metastatic PCa and inversely associated with PSA level and Gleason scores. But experiments would be desirable to further clarify the relationship between Gαs and EGFR and identify their function in the progression of prostate cancer. However, such functional studies were beyond the scope of this study.

5. Conclusion

In summary, we discovered that Gαs is a promising biomarker of prostate cancer patients. To our knowledge, this is the first study to describe the predictive role of Gαs in prostate cancer. We found that the expression of Gαs was downregulated in metastatic prostate cancer compared to localized prostate cancer. And low expression Gαs was significantly associated with adverse clincopathological features. More important, Gαs was an independent prognostic predictor in prostate cancer. Although our results are promising, Gαs expression needs to be validated in relationship to outcome in the context carefully controlled clinical trials. If confirmed, application of Gαs immunohistochemical analysis should be technically straightforward and feasible. All in all, targeting Gαs could be a promising therapeutic strategy for enhancing the therapy effect of patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lijuan Wang and Guihua Jin contributed equally to this work.

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