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Lead Guest Editor: Ilary Ruscito
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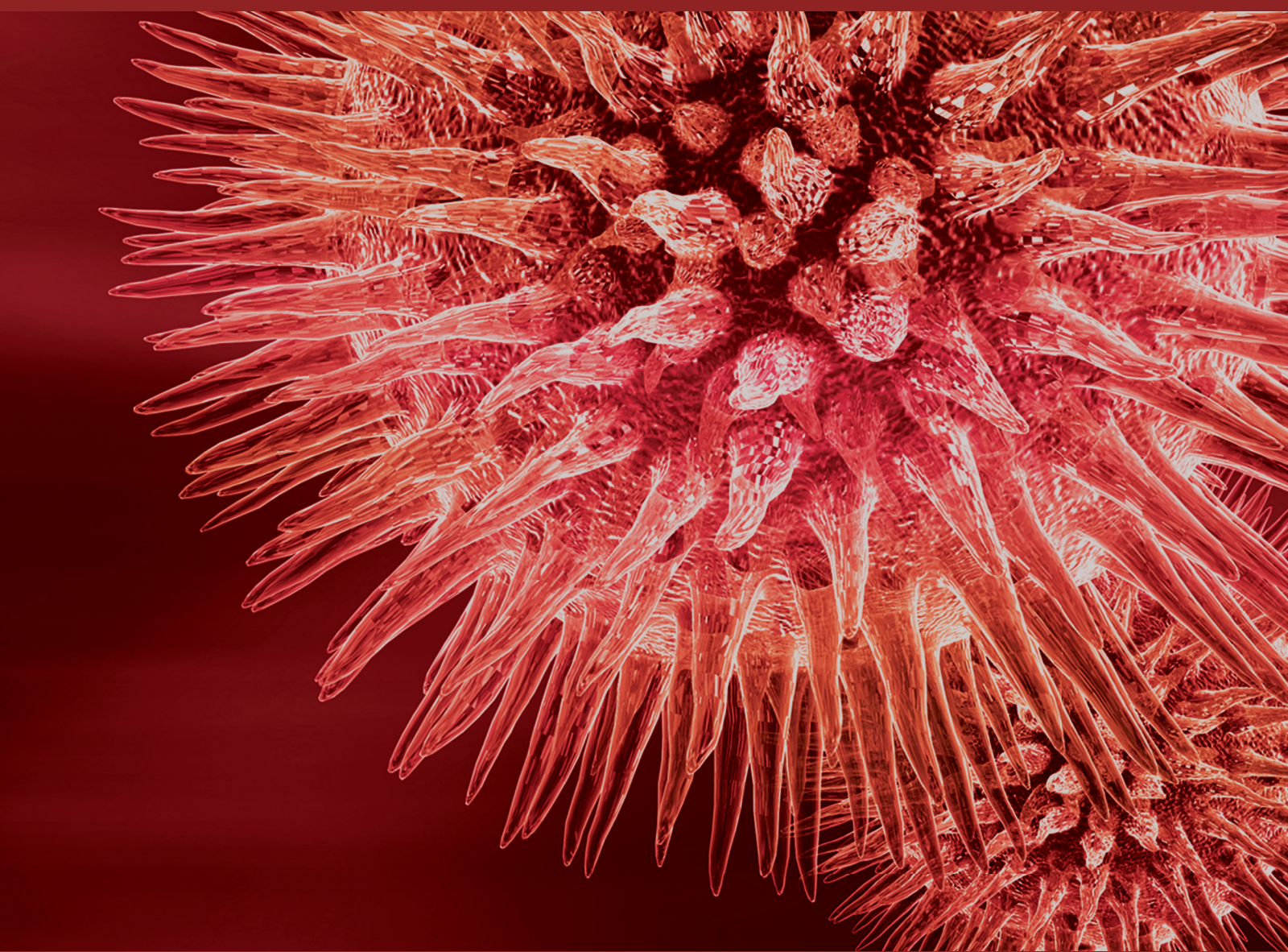

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Hindawi
BioMed Research International
Volume 2018, Article ID 6532019, 2 pages
https://doi.org/10.1155/2018/6532019

Editorial

Immunobiology of Solid Cancers: Cellular and Molecular Pathways as Potential Diagnostic and Therapeutic Targets

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Received 18 January 2018; Accepted 21 January 2018; Published 1 March 2018

In the last four decades, tumor immunology has shed light on identity and functions of cells and molecules involved in tumor rejection through the involvement of the immune system [1]. Several groups of immune cells have been demonstrated to be able to contrast tumor occurrence and tumor progression by killing immunogenic tumor cells, a phenomenon recognized under the definition of "immunosurveillance" [2]. Unfortunately, cancer may evade immunosurveillance and progress through the modifications of its own antigens, which can reduce tumor immunogenicity and/or increase its immunosuppressive action [3]. After years of investigations, harnessing the immune system to attack cancer has recently led scientists to gather enough clinical data to show what a powerful sword immunotherapy can be [4]. Data on unexpected clinical recoveries and long progression-free intervals are increasing regarding patients addressed to immunotherapy treatments [5, 6]. Despite its extraordinary success, only a portion of cancer types and cancer patients benefit from immunotherapy treatments. Understanding the reason why this happens is the big challenge of our time and, in order to answer this question, basic science is crucial: to elucidate how tumor cells and immune cells interact with each other in cancer patients and clarify the mechanisms through which tumor mutational pattern affects the response to therapies is the way to pursue for improving efficacy of current treatments and promoting new anticancer strategies. This special issue was conceived with the aim of collecting new findings in the field of cancer immunology and describing novel biological and molecular evidence on the relationship between cancer and immune system as well as cancer and immunotherapy.

In response to the aim of the special issue, four original research papers and one review article are presented below.

The study reported by B. Cerbelli et al. from Sapienza University of Rome, Italy, showed that immunohistochemical PD-L1 expression in ≥25% of triple negative breast cancer (TNBC) chemo-naïve cells, derived from core biopsies, is an independent predictor for pathological complete response (pCR) after neoadjuvant chemotherapy, thus discussing potentials and limits of PD-L1 future applications as a predictive biomarker for neoadjuvant treatment response in this subset of patients affected by such a clinically aggressive disease.

M. Moschetta et al., at Sarah Cannon Research Institute of London, UK, presented a study assessing the impact of "neutrophil-to-lymphocyte ratio" (NLR) in predicting

PFS among 55 advanced patients enrolled into PD-1/PD-L1 inhibitors phase 1 clinical trials. Results showed a significant longer PFS in patients with a reduction of NLR after two treatment cycles compared to the median baseline NLR, thus advancing the hypothesis that NLR may be a helpful predicting tool in cancer patients treated with anti-PD-1/PD-L1 agents.

An international collaboration between USA (University of Colorado and Yale School of Medicine) and Japan (Kindai University and Chugai Pharmaceutical), coordinated by K. Suda et al., obtained evidence concerning molecular mechanism behind the low expression of PD-1/PD-L1 in NSCLC, associated with reduced efficacy of checkpoint inhibitors (CI) treatments. The study highlighted that EGFR-mutated lung cancer cell lines do not show high PD-L1 expression and, furthermore, after acquisition of resistance to EGFR-TKIs, EGFR phosphorylation affects PD-L1 expression, thus identifying a molecular event able to influence the expression of biomarkers, which regulate patients' access to CI agents.

Apart from its immunomodulatory function, F. Zheng et al., from Huazhong University of Science and Technology, China, identify PD-L1 molecule as a potential biomarker of melanoma cancer stem-like cells, since blocking PD-L1 in melanoma cell lines expressing PD-L1 and ALDH1 impaired tumorsphere formation and induced the apoptosis of tumorsphere cells. These findings raise the need to elucidate the relationship between tumor response to checkpoint inhibitors and clonal evolution of cancer stem cells in the future.

Finally, the review paper by M. Wei et al., Southeast University of China, discusses the role of gastric cancer patients' T cells immunity and disease prognosis, providing a critical synthesis of recent evidence on this still controversial topic.

In conclusion, we find this special issue to be a good opportunity for improving knowledge in the field of cancer immunobiology and immunotherapy, which is a pivotal step to respond adequately to the questions of our time in the battle against cancer.

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Maria Luisa Gasparri
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Hindawi
BioMed Research International
Volume 2018, Article ID 3201940, 6 pages
https://doi.org/10.1155/2018/3201940

Review Article

The Progress of T Cell Immunity Related to Prognosis in Gastric Cancer

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Received 25 August 2017; Accepted 6 December 2017; Published 27 February 2018

Academic Editor: Ilaria G. Zizzari

Gastric cancer is the fifth most common malignancy all over the world, and the factors that can affect progress and prognosis of the gastric cancer patients are various, such as TNM stages, invasive depth, and lymph node metastasis ratio. T cell immunity is important component of human immunity system and immunity responding to tumor and dysfunction or imbalance of T cell immunity will lead to serious outcomes for body. T cell immunity includes many different types of cells, CD4+ T cell, CD8+ T cell, memory cell, and so on, and each of them has special function on antitumor response or tumor immune escape which is revealed in lung cancer, colorectal cancer, breast cancer, ovarian cancer, and so on. But its correlation with gastric cancer is not clear. Our review was preformed to explore the relationship between the progress and prognosis of gastric cancer (GC) and T cell immunity. According to recent researches, T cell immunity may have an important role in the progress and prognosis of GCs, but its function is affected by location, category, related molecule, and interaction between the cells, and some effects still are controversial. More researches are needed to clarify this correlation.

1. Introduction

Gastric cancer is the fifth most common malignancy all over the world after lung, breast, colorectal cancers, and prostate. More than 70% of gastric cancer (677,000 cases) happened at developing countries (456,000 in men, 221,000 in women), and half the total located in Eastern Asia, especially in China [1]. Although the lifestyle and smoking play an important factor, the main risk factor for advanced gastric cancer is infection with the bacterium *Helicobacter pylori* [2]; T cell immunity is a hot topic in recent studies. During the development of cancer, T cells progressively dysfunction and exhaust; however the T cell responses are necessary to control tumors [3]. And they play important roles in several types of cancers like lung cancer [4], colorectal cancer [5], breast cancer [6], and ovarian cancer [7], but the relationship between the T cell immunity and progression and prognosis of GCs is not clear. And there are many subsets of T cells which play different roles in gastric cancer, CD4+ T cell, including regulatory T cells, CD8+ T cell, and CD45RO+ memory T cells [8]. The recent researches are more focused on regulatory T cells.

2. Subsets of T Cell and Molecules Related to Prognosis of Gastric Cancer

T cell immunity is important in tumor response, and there are many subsets of T cells which played different roles in gastric cancer, CD4+ T cell, including regulatory T cells, CD8+ T cell, CD45RO+ memory T cells, and other molecules related to T cell immunity.

2.1. CD4+ T and CD8+ T Lymphocytes. CD4+ T and CD8+ T are two important types of cells in T cell immunity.

CD4+ regulatory T cell is a major cell in self-tolerance and suppresses antitumor immunity [9]. CD4 T cells have effector functions by secreting multiple cytokines or activating other immune cells acting on immunity of tumor [3]. Among CD4+ T cell, Follicular helper T cells (Tfh cells) are special one which are necessary for producing high affinity antibodies. Meanwhile Tfh cells can secrete IL21 and IL4 and show high expression of CXCR5, ICOS, PDCD1 (PD-1), and chemokine CXCL13, which also affect gastric cancer prognosis [10]. Cytotoxic CD8 T lymphocytes are present in tumors and their functions in recognizing tumor epitopes

are nevertheless generally important in antitumor reaction [11]. And CD8 T cells are an important factor on the initial development of tumors, especially in existing tumor, and the presence of CD8 T cells indicates poor prognosis [12].

2.2. Regulatory T Cell. Regulatory T cells (Tregs) are a kind of T lymphocytes with an immunoregulatory capacity, which can inhibit the proliferation and cytokine secretion of effector T lymphocytes. Giving this function, inappropriate production or dysfunction of Tregs could result in severe damage of the host immune system [13]. In recent years, regulatory T cells (Tregs) within tumors, also known as tumor infiltrating Treg cells, have been considered to play a key role in immune evasion [13]. And Tregs are correlated with progression and poor outcomes in gastric cancer ([2]; [14]), but the relation between tumor infiltrating T cells and gastric cancer is unclear.

2.3. Others. In addition, many other related cells and molecules also play a role in prognosis of gastric cancer. Dendritic cells (DC) play the central role in cancer immunosurveillance as the antigen-presenting cells (APC) are involved in the antitumor immune responses [15]. T cell immunoglobulin and mucin domain-3 (Tim-3) is negative regulatory molecules and plays a major role in the tumor immunological tolerance [16]. And B7-H1 (also known as PDL1) is a member of the B7 superfamily [17]. PD-L1 expression has been detected in cancers of the skin [18], lung [19], breast [20], kidney [21], bladder [22], esophagus [23], stomach [24], head, and neck [25], among others. B7-H4 is a coinhibitory molecule which negatively regulates T cell immunity and is rarely expressed in resting antigen-presenting cells (APCs) [26] but is upregulated in a variety of cancer tissues including ovary, kidney, stomach, lung, and pancreas [27–29].

3. The Correlation between T Cells and Gastric Cancer

The factors that can affect progress and prognosis of the gastric cancer patients are various, such as TNM stages [30], invasive depth, lymph node metastasis ratio [31], and tumor immunity. And there are three molecular carcinogenesis mechanisms which may be correlated with GC: chromosomal instability [32], microsatellite instability (MSI) [33], and CpG island methylator phenotype [34]. T cell immunity also plays a role in these mechanisms, and some researches have already suggested that beta-catenin/T cell factor- (TCF-) mediated transcription (canonical Wnt signaling) could result in chromosomal instability (CIN) [35], and MSI gastric cancers possibly express more PD-L1 and have increasing CD8+ T cells before tumor invasive [36].

Nowadays, since the function of T cell immunity in cancer is researched more and more clearly, we found that it can also influence the progress and prognosis of GCs directly or indirectly participating in antitumor responses. For example, DC as the antigen-presenting cells (APC) is involved in the antitumor immune responses, while CD8+ T cell may dissolve and kill tumor cells, and CD4+ cell (including Foxp3+ Tregs) impose restrictions on tumor response.

Except these cells, many molecule such as Th17 [37], CD133 [38], gastrokine 1 [39], angiogenic factor [40], and LKB1 (Sun J et al., 2016) also have possible impact on diagnosis, progress, treatment, and prognosis of gastric cancer.

4. The Correlation and Mechanism between T Cells Immunity and Prognosis of Gastric Cancer

T cell immunity is important in antitumor response and studied in many other cancers. Some studies also show there could be correlation between gastric cancer prognosis and T cells. Haas et al. introduced a phenomenon in their experiment that an increasing stromal FoxP3+ TIL infiltration in tumor issues had a negative correlation with UICC- stage (Pearson's correlation coefficient, $r = -0.40$; $p = 0.001$), number of lymph node metastases ($r = -0.36$; $p = 0.009$), and N category in general ($r = -0.36$; $p = 0.023$). But this relationship could not be seen in other cell types [41]. Cheng et al. showed that Tim-3 was expressed in CD4+ T cells and CD8+ T cells higher in gastric issues and had a meaningful relation with tumor invasion and TNM stage, which could lead to poorer prognosis [16]. And Qing et al. had confirmed that PD-L1 could express more in highly differentiated gastric cancers, and it had an obvious relationship with the depth of invasion (odds ratio [OR] = 3.37; $p = 0.005$), lymph node metastasis (OR = 2.68; $p = 0.020$), tumor differentiation (OR = 3.19; $p = 0.008$), pathological type ($\chi^2 = 8.676$; $p = 0.013$), and survival time (OR = 3.39; $p = 0.003$) [42]. And they proposed that targeting the PD-L1 and APE1 signaling pathways may be a new treatment for gastric cancer, especially deep invasion and lymph node metastasis [42]. Cho et al. indicated that PD-L1 expression was frequently correlated with a lower risk of lymph node metastasis ($p = 0.027$) and lower tumor stages in intestinal type cancer by the Lauren classification [43]. But the mechanism between gastric cancer prognosis and T cells immunity is not very sure.

4.1. CD4+ T Cell and CD8+ T Cell. There are some researches focusing on the relationship between the subsets of CD4+ T cell and the progress and prognosis of gastric cancer. Shen et al. found that CD4+CD25+CD127low/− Tregs are correlated with more advanced stage of gastric cancer through suppressed effector T cell proliferation and express Foxp3 [44]. In another research, Kindlund et al. suggested that CD4+ regulatory T cells can promote tumor growth by inhibiting T cell mediated tumor cell killing, depending on IL-10 and/or TGF-β, but they also showed that CD4+CD25^{High} expresses higher IL-10 [2]. As we all know, *Helicobacter pylori* infection is related to prognosis of GCs. Zhang et al. investigated the potential functions of Follicular helper T cells in the GCs with *Helicobacter pylori* infection. His group found that Th1 and Th17 are the most common subsets of Follicular helper T cells and can be negatively correlated with the disease-free survival of tumor resection [45].

And CD8+ T cells have also been studied. Lu et al. indicated that GC patients with high-density CD8+ had higher overall survival rates than low-density ones by Kaplan-Meier test in MSI-high GCs [11]. But Thompson et al. demonstrated that tumors with high CD8+ T cell density either in intratumor or in stromal had worse progression-free survival (PFS) and OS compared with the lower ones [17]. Tuncel et al. introduced patients with lower numbers of CD8+ T lymphocytes in the tumor, which has a negative correlation with HLA-G and had a poorer prognosis [46].

4.2. Foxp3+ Treg Cell. Although the functions of some immunity cells have been recognized by studies, some controversies are still present.

Foxp3+ Tregs are the most concerned cell and their function is still controversial. Hou et al. showed the level of FoxP3+ Tregs in gastric cancer tissues related to an advanced clinicopathological stage and lymph node metastasis, which indicted poor prognosis [13]. Yuan et al. found that the level of FoxP3 is higher in Tregs and it can inhibit the proliferation of autologous CD4+CD25−T cells in a COX-2-dependent manner to lead to poor prognosis which can be reversed by COX inhibitors [14]. In another article, Tuncel et al. got the similar conclusion that the high numbers of Tregs in the primary tumor, positive regulated by HLA-G, are associated with poor prognosis [46]. But there are some researches having opposite conclusions. Kim et al. showed that GCs with high-density FoxP3+ TILs had significantly higher overall survival rates and low density is closely related to a higher TNM stage, invasion depth, and lymphatic and vascular invasion and proved FoxP3+ T cell density in the intraepithelial cells was an independent predictor for overall survival. But the result of Kim et al. is confined to microsatellite-unstable gastric cancers [47]. Haas et al. suggested that high level of Treg is associated with improved outcome probably via inhibiting local inflammatory process [41]. And Feichtenbeiner et al. found an interesting conclusion that prognostic effect of TILs cells in gastric cancer depends on the distance within cells, and FoxP3+ TILs must be located within 30 and 110 μm far from CD8+ T cells to play its positive impact on prognosis [48]. In addition, Ma et al. investigated the expression of FoxP3 protein in tumor cells and they showed that the high level predicts a good prognosis, whereas high-density Treg is opposite [49].

5. Other Cells and Molecule Related T Cell Immunity in GCs

Except mainly cells in T cell immunity, there are some other cells and molecule also could have their functions in GCs.

Kashimura et al. suggested that the density of CD83+ DCs in negative lymph nodes was an independent prognostic factor by multivariate analysis for patients with metastatic lymph nodes [9]. Gao et al. descripted that the overexpression of B7-H1 in carcinomas has been shown to induce apoptosis in the effector T cells to repress T cell activation and proliferation, which led to lower 5-year OS and DFS [50]. Geng et al. confirmed that lymph node metastasis and B7-H1 overexpression were independent prognostic factors which are negative with gastric cancer through Cox regression multivariate analysis [51]. Shi et al. proposed that soluble B7-H4 (sB7-H4) in circulation is a valuable molecule for predicting the progression and prognosis of GCs and a positive correlation between the two things [52]. And Chen et al. suggested that the expression of T-bet, a key marker for type 1 immune responses, can serve as a prognostic indicator which has negative effect [53]. In a recent study, Kim et al. revealed that decreasing NOVA1 expression in tumor tissue was related to tumor progression and poor prognosis via immune dysfunction of T cells and macrophages [54]. And Th12 [55], Th17 [37, 56], Th1 [57], CX3C chemokines [58], diversity index of mucosal resident T lymphocyte [59], myeloid derived suppressing cells [60], immune activating receptor NKG2D [61], CCR7 [62], and IL-10 [63] are also involved in the progression and prognosis of GCs.

Another interesting thing is the prognosis and progress of the same immunity cells can vary under different locations. Tim-3 is a negative regulatory molecule, only when it overexpresses CD8+ T cell or Tregs can lead to poor prognosis. Another protein is Foxp3+, its higher expression in tumor cells predicts good outcome but in Tregs the function is inverse. It has different prognosis when acting on different cells. Cheng et al. found that CD4+ and CD8+ T cell can be upregulation in GCs by Tim-3, but CD4+ T cell has poorer prognosis. Tim was also an independent factor for GCs, and the lower is the better [16]. Shen et al. revealed that the level of Tim-3 is up in both *H. pylori*-infected asymptomatic and gastric cancer patients, which is on Tregs and CD8+ T cells associated with worse prognosis [64]. And Milašienė et al. observed that higher levels of the absolute number of lymphocyte had a positive effect on overall survival of gastrium in stage III, but there is no effect in stage II [65].

6. Forecast

The subsets of immunity cells have their own special role in response to gastric cancer and lead to different outcomes of patients. But the function is affected by location, category, related molecule, interaction between the cells, and so on. Definite function is still unclear and needs more studies. More studies are needed to investigate the relationship between the T immunity cell and gastric cancer, especially forcing on Foxp3+ Tregs and the influence of location and mutual relations between cells. At the same time, I think that the role of memory T cell is ignored in the progress and prognosis of GCs, and more research is essential.

Because of the T cells exhibiting a possible relationship in the progress and prognosis of GCs, it may provide new theory and way on diagnosis and treatment of gastric cancer. More studies are needed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (no. 81602432) and Program for Jiangsu Provincial Natural Science Foundation of China (BK20140652).

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Hindawi
BioMed Research International
Volume 2017, Article ID 1750925, 7 pages
https://doi.org/10.1155/2017/1750925

Research Article

PD-L1 Expression in TNBC: A Predictive Biomarker of Response to Neoadjuvant Chemotherapy?

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Received 8 August 2017; Accepted 19 November 2017; Published 14 December 2017

Academic Editor: Maria L. Gasparri

Triple negative breast cancer (TNBC) has an aggressive clinical behaviour, with a poorer prognosis compared to other subtypes. Recently, tumor-infiltrating lymphocytes (TILs) have been proposed as a predictive biomarker for a better clinical outcome and pathological response (pR) after neoadjuvant chemotherapy (NACT) in TNBC. These data confirm the role of the immune system in the neoplastic progression and in the response to therapy. We performed a retrospective analysis of 54 pre-NACT biopsies of TNBC and compared both the percentage of stromal TILs and the degree of PD-L1 expression with the extent of pR to standard NACT. A pathological complete response (pCR) was achieved in 35% of cases. Univariate analysis showed (i) a significant association between PD-L1 expression in ≥25% of neoplastic cells and the achievement of a pCR ($p = 0.024$); (ii) a significantly higher frequency of pCR in cases showing ≥50% stromal TILs ($p < 0.001$). However in the multivariate analysis only PD-L1 expression on tumor cells remained significantly associated with pCR (OR = 1,13; 95% CI 1,01–1,27), suggesting that the expression of this biomarker could be associated with a subpopulation of TNBC more likely to respond to chemotherapy. These data need to be confirmed by larger studies.

1. Introduction

Triple negative breast cancer (TNBC) accounts for 10–20% of all breast cancers [1]. It is often associated with high histological grade, presence of lymphocytic infiltration, high rate of distant metastasis, and a poorer prognosis when compared to other breast cancer subtypes. TNBC is generally treated with standard chemotherapy regimens, including both anthracyclines and taxanes, either in the metastatic, adjuvant, or neoadjuvant setting. Neoadjuvant chemotherapy [NACT] is increasingly used in the management of this BC subtype, with pathologic complete response (pCR) rate ranging from 30% to 50% [2–4]. These data point to the need of biomarkers that could be useful to identify the subset of patients more prone to achieve a pCR. In recent reports the presence of tumor-infiltrating lymphocytes (TILs) has been shown to predict the response of TNBC to NACT [5–7]. Moreover, a high number of stromal TILs is predictive

of a more favorable outcome in this BC subset. These data underscore the crucial role of the immune system both in the neoplastic progression and in the response to therapy and support the robustness of biomarkers of tumor-immune system interplay in clinical practice [8]. The interaction between programmed cell death protein 1 (PD-1) and its ligand (PD-L1) represents a mechanism of immune escape and a therapeutic target for poor-prognosis malignancies, such as melanoma and non-small-cell lung cancer (NSCLC) [9]. PD-1 is a transmembrane protein of 40 kDa expressed on CD8+ and CD4+ T cells, natural killer (NK) cells, B cells, activated monocytes, and dendritic cells [10]. It is a negative regulator of the immune system that functions by forming a complex with its ligands (either PDL1 or PDL2). Only limited and contrasting data on the role of PD-L1 in breast cancer have been reported so far. In fact, the expression of this marker has been correlated with either a worst [11] or a better prognosis [12].

Apart from their prognostic or predictive value, the presence of stromal TILs and the expression of PD-L1 are strong markers of immune activation in breast cancer and could be involved in the response to preoperative systemic treatment. In this study we aimed to investigate the role of PD-L1 expression and stromal TILs in predicting the pathological response to NACT in TNBC. We retrospectively analyzed 54 pre-NACT biopsies and compared both the percentage of stromal TILs and the extent of PD-L1 expression on neoplastic and inflammatory cells with the effect of neoadjuvant chemotherapy.

2. Materials and Methods

Between January 2011 and December 2016, 54 consecutive patients with TNBC received standard NACT (4 cycles of doxorubicin + cyclophosphamide Q3W followed by 12 cycles of paclitaxel weekly) at our Institutions. Clinical information, including age, clinical stage at diagnosis, type of surgery, and pathologic response, was extracted from the institutional databases.

2.1. Evaluation of Stromal Tumor-Infiltrating Lymphocytes. Pre-therapy biopsies were retrieved from the Pathology Departments at the Sapienza University Teaching Hospital and the San Giovanni-Addolorata Hospital. Hematoxylin-eosin stained slides were blindly re-evaluated for the presence of stromal tumor-infiltrating lymphocytes (TILs) according to a previously published method [13]. Briefly, TILs were quantified as a percentage of the stromal area of the tumor and expressed as a continuous parameter.

2.2. Evaluation of PD-L1 Expression and Immunophenotyping of the Inflammatory Infiltrate. Serial sections were obtained from each paraffin block for (i) immunohistochemical evaluation of PD-L1 expression on both neoplastic and inflammatory cells and (ii) immunophenotyping of the inflammatory infiltrate. PD-L1 immunostains were performed with one of the antibody clones approved for diagnostic assay (SP142, rabbit IgG, dilution 1 : 200, catalog #M4420; Spring Bioscience, Pleasanton, CA) [14] at 1 : 100 dilution, using an automated immunostainer (Benchmark XT, Ventana Medical System, Tucson, AZ, USA) with the Optiview DAB IHC detection kit (Ventana Medical Systems, Tucson, Arizona, USA) according to manufacturer's instructions. Relevant positive controls (human tonsils and placenta) were used for each run of staining. Negative controls were obtained by omitting the primary antibody. The expression of PD-L1 was evaluated separately on all tumor cells and inflammatory infiltrates. A minimum of 200 neoplastic cells were present in each biopsy sample. A positive stain was defined as the presence of membrane staining, either strong or weak, complete or incomplete, in a percentage of cells $\geq 1\%$, that is, the threshold reported for clinical response to PD-L1 inhibitors in non-small-cell lung carcinoma and has also been reported in breast carcinoma [15, 16]. For each biopsy, both the intensity of membrane staining (scored as 1+ weak, 2+ moderate, and 3+ strong) and the percentage of positive neoplastic cells were recorded, while only the percentage of positive inflammatory cells was evaluated.

Immunophenotyping of the inflammatory infiltrates was carried out with the following antibodies: CD3 for T lymphocytes (Roche, 1 : 100); CD4 (1 : 40) for the helper T subset; CD8 for the cytotoxic T subset (1 : 100); CD20 for B lymphocytes (1 : 200) CD68 for macrophages (1 : 100), and N-CAM (1 : 100) (all from Novocastra, Newcastle, UK). Four images at 20x original magnification (accounting for one mm^2 of tumor field) were acquired from the areas of maximum inflammatory infiltrate by the NIS Elements Viewer mounted on a Zeiss Axioskop 2 microscope. The number of positive cells/mm^2 for each antibody was then manually counted on the acquired images.

2.3. Evaluation of the Pathologic Response to NACT. The degree of pathologic response of each patient to NACT was obtained from the pathology reports. A complete response was defined as the complete disappearance of invasive tumor cells from breast tissue and regional lymph nodes, regardless of the presence of residual ductal carcinoma in situ (ypT0/is, ypN0) [17, 18].

2.4. Statistical Analysis. In the descriptive analysis, quantitative variables were described as mean and range, while qualitative variables were reported as number and percentage. Univariate associations between clinicopathological features and pCR were evaluated using the $\chi 2$ test or Fisher's exact test, when appropriate. To take into account the effects of all variables on pCR, multivariate analysis were performed by a multivariate logistic regression to estimate the adjuster Odds Ratios (ORs). Statistical significance was set at $p < 0.05$. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Clinicopathological features of the 54 patients are detailed in Table 1. Briefly, the mean age at diagnosis was 50 years (range 28–75). In 87% of cases the pre-NACT tumor diameter

Table 1: Clinicopathological features of the study population.

Characteristics	Number of patients (%)
Age (y)	
≤50	30 (55%)
>50	24 (45%)
Pre-NACT tumor size (cT)	
≤2 cm	7 (13%)
>2 cm	47 (87%)
Pre-NACT nodal status (cN)	
Positive	24 (45%)
Negative	30 (55%)
Histotype	
Ductal	51 (94%)
Lobular	1 (2%)
Others	2 (4%)
Nuclear grade	
Grades 1-2	0
Grade 3	54 (100%)
Ki-67	
<50%	14 (26%)
≥50%	40 (74%)
Post-NACT surgery	
Mastectomy	30 (55%)
Segmental mastectomy	24 (45%)
Complete pathological response (pCR) to NACT	19 (35%)

was larger than 2 cm. Axillary node involvement, assessed by echography and confirmed by fine needle aspiration cytology, was present in 24 patients (45%). The most common histologic subtype was ductal carcinoma of no special type (94% of cases). All tumors were of high nuclear grade (G3) with a proliferation index ≥ 50% in the large majority of cases (74%).

After NACT 30 patients (55%) underwent mastectomy and 24 (45%) had conservative breast surgery, A pCR was achieved in 19 patients (35%).

3.1. Tumor-Infiltrating Lymphocytes and Immunophenotype of the Inflammatory Infiltrates in Pre-NACT Biopsies. The results of stromal TILs evaluations are detailed in Table 2. Briefly, stromal TILs were present in 51 pre-NACT biopsies (95%), with percentages ranging from 2 to 80% (Figures 1(a) and 1(b)). Twenty-four cases (45.5%) had 50% or greater stromal TILs (high TILs) and were accordingly classified as lymphocyte predominant breast cancer (LPBC) [13]. Immunophenotyping of the inflammatory infiltrates revealed a predominance of CD3+ T cells in all biopsy samples. In 36 biopsies (70%) the most represented was the CD8+ subset, followed by the CD4+ subset (27%). Intriguingly, NK cells were absent in 96% of biopsies.

3.2. Expression of PD-L1 on Neoplastic and Inflammatory Cells. Membrane staining of neoplastic cells was present in 19 pre-NACT biopsies (35%), with an extent ranging from 1 to 90% (Figures 1(c) and 1(d)). In over 95% of these biopsies (18/19) the percentage of PD-L1 positive tumor cells did not exceed 50%. The membrane stain scored 3+ in 8/19 cases (42%), 2+ in 5/19 (26%), and 1+ in 6/19 (32%). PD-L1 staining of inflammatory cells was present in the majority of biopsies (44 cases, 81%) (Figures 1(e) and 1(f)).

Univariate analysis showed a significant association between the presence of high stromal TILS and the expression of PD-L1 on ≥25% of tumor cells ($p = 0.008$) and ≥10% of inflammatory cells ($p = 0.002$); this association was independent from the prevalent lymphocyte subset (CD8+ or CD4+), and from the CD8/ CD4 ratio.

3.3. Comparison of Histologic and Immunohistochemical Data with the Response to NACT. A pCR was achieved in 19 patients (35%). Univariate analysis (Table 2) showed a significant association between the expression of PD-L1 in ≥25% of neoplastic cells and pCR ($p = 0.02$). The presence of pCR was also significantly more frequent in cases showing features of LPBC (with high TILs) in the pre-NACT biopsies ($p < 0.001$). Moreover, pCR was achieved in 100% of patients showing both high TILs and expression levels of PD-L1 ≥ 25% in neoplastic cells in the pre-NACT biopsies ($p = 0.011$, Table 3).

At multivariate analysis (Table 4), only PD-L1 expression on tumor cells remained significantly associated with pCR ($p = 0.038$) with OR of 1,13 (95% CI 1,01–1,27).

4. Discussion

The immune system is strongly involved both in the tumor surveillance and in the pathogenesis of breast cancer. Moreover, preexisting immunity against tumor cells is a crucial factor that influences the response to chemotherapy. It is now believed that preexisting antitumor immunity is activated or enhanced during the initial cycle of chemotherapy. During the subsequent cycles, together with incoming acquired drug resistance of the tumor cells, the onset of immune resistance mechanisms impairs the efficacy of treatment [19, 20].

Due to the high histological grading and mutational load, along with the activation of large amounts of genes implicated in immune function, TNBC seems to be the subtype more likely associated with immune system involvement. Thus, the identification of novel immunological prognostic and predictive biomarkers would be useful to guide the choice of the most appropriate treatment, as well as the optimal timing of surgery, especially in the neoadjuvant setting.

Either the presence of stromal TILs or the expression of PD-L1 is being actively investigated as prognostic biomarkers in TNBC. This subset of breast cancer has an aggressive clinical and biological behaviour, with higher risk for early recurrences and a poorer prognosis as compared to the other BC subtypes. However, in the neoadjuvant setting, the achievement of a pCR after NACT is associated with long-term survival. In this study, we investigated the hypothesis that the presence of high stromal TILs and the expression

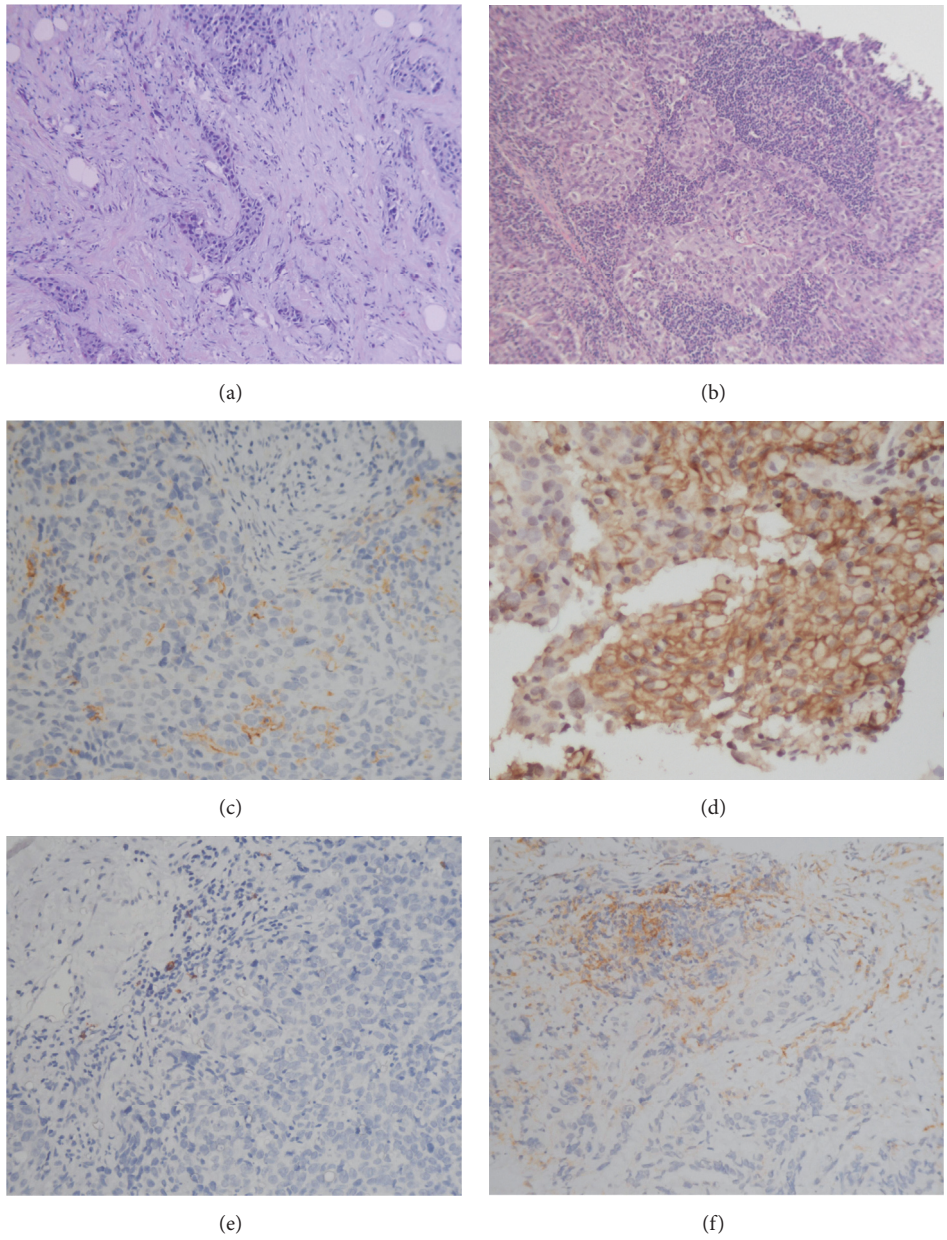

Figure 1: *Evaluation of stromal TILs and PD-L1 expression in TNBC core biopsies*. (a)-(b): low (a) and high (b) level of stromal tumor-infiltrating lymphocytes (haematoxylin and eosin, original magnification ×10). (c)-(d): membranous PD-L1 stain in scattered (c) and diffuse neoplastic cells (d) (PD-L1 immunohistochemical stain, original magnification ×20). (e)-(f): membranous PD-L1 stain in scattered (e) and diffuse inflammatory cells (f) (PD-L1 immunohistochemical stain, original magnification ×20).

of PD-L1, both markers of immune activation in the tumor microenvironment, could be associated with the rate of pCR in TNBC.

We evaluated pre-NACT core biopsies, which proved to be qualitatively and quantitatively adequate for our analysis. In our study population, 35% of patients achieved a pCR, which is in line with recently published literature [21, 22].

On microscopic evaluation of pre-NACT core biopsies, tumor cell expression of PD-L1 was observed in 35% of cases, although at low levels (≥1% <25% in 15/19 biopsies, 79%). Our observation on a pure sample of TNBC confirms the results of Dill et al. [16] who analyzed a large number of BC with various histologic subtypes, showing the highest rate of PD-L1 expression (32%) in TNBC, with only 5% with diffuse expression on tumor cells (>50%).

PD-L1 expression, both on neoplastic and inflammatory cells, was significantly associated with high stromal TILs. Our observation extends the results reported by Mori et al. [23], which showed a significant association between PD-L1 expression on tumor cells and percentage of stromal TILs on surgical breast specimens, and confirms the parallel behaviour of these immune biomarkers in TNBC. The limited

Table 2: Association between stromal TILs, the expression of PD-L1 on tumor cells and inflammatory infiltrate, Ki-67 value, cT, cN, and pCR in the univariate analysis.

	N (%)	pCR	
		N°	*p* value
Stromal TILs			
Absent/low	32 (59%)	*8*	*<0.001*
High	22 (41%)	*11*	
PD-L1 on tumor cells			
0%	35 (65%)	*11*	
≥1–<25%	15 (28%)	*4*	
≥25%	4 (7%)	*4*	*0.024*
PD-L1 on inflammatory cells			
Negative	10 (19%)	*3*	*ns*
Positive	44 (81%)	*15*	
Ki-67			
<50%	14 (26%)	*4*	*ns*
≥50%	40 (74%)	*15*	
cT			
T1	7 (13%)	*3*	*ns*
T2–T4	47 (87%)	*16*	
cN			
Negative	30 (56%)	*13*	*ns*
Positive	24 (44%)	*6*	

Table 3: The achievement of pCR according to levels of both stromal TILs and PD-L1 expression on neoplastic cell membranes (low TILs/low PD-L1; high TILs/low PD-L1; low TILs/high PD-L1; high TILs/high PD-L1).

	PD-L1 on neoplastic cells					
TILs		<25%		≥25%		
	pt	pt	*pCR*	pt	*pCR*	
Low	32	32	25%	0		$p = 0.011$
High	22	18	39%	4	100%	

amount of published reports in pure cohorts of TNBC seems to suggest a favorable prognostic role of PD-L1, despite some discrepancies. Mori et al. [23] demonstrated that the interaction between TILs and PD-L1 correlates with a better clinical outcome. However, when high PD-L1 expression is associated with low levels of stromal TILs the prognosis is poor [24]. In the study of Beckers et al. [25] PD-L1, although associated with a better outcome, failed to show an independent prognostic role in this subset of tumors. These partial discrepancies could be explained by differences in the choice of clinical outcomes, in the methods of evaluation of PD-L1 expression on neoplastic cells (membranous versus cytoplasmic) and the cut-off values adopted, and in the antibodies used and the type of sample evaluated (core biopsies versus surgical samples).

There are only limited data on the predictive value of these two biomarkers in TNBC. We found that in this breast cancer subtype the concomitant expression of stromal TILs and PD-L1 on tumor cells membranes was significantly associated with pCR. According to our results, a cut-off of PD-L1 membrane expression on ≥25% of neoplastic cells in pre-neoadjuvant biopsies predicted pCR for TNBC, regardless of staining intensity. On the contrary, the predictive role of TILs showed only a limited power and no statistical association on multivariate analysis. In light of the preliminary results of the KEYNOTE 173 phase II trial [26], reporting a 90% pCR rate in TNBC treated in this setting with the adjunct of pembrolizumab to standard chemotherapy, we hypothesize that TNBC expressing PD-L1 in less than 25% of tumor cells could represent the subset most likely to benefit from this association.

Immunophenotyping of tumor inflammatory microenvironment revealed an excess of CD8+ with a ratio of CD8/CD4 > 1, in line with previous reports [27, 28], although this observation did not reach statistical significance probably due to our limited sample size. Additionally, we found negligible amounts of NK cells in pre-NACT biopsies, although we cannot exclude the fact that their level could have been increased after the first cycle of chemotherapy due to tumor cells death and the release tumor associated antigens.

In conclusion, we showed that a cut-off value of PD-L1 in ≥25% of tumor cells predicts pCR in TNBC and to our knowledge our study is the first dealing with an exclusive population of TNBC cases. A possible explanation for our observation is that PD-L1 expression could be associated with a subpopulation of TNBC with a more aggressive behaviour,

Table 4: Association between the expression of PD-L1 on neoplastic cells and inflammatory infiltrate, stromal TILs, Ki67, clinical T, clinical N, and pCR in the multivariate analysis.

	N (%)	pCR		
		N (%)	p value	ORR (CI)
Stromal TILs				
Low	32 (60%)	8 (25%)	0.5	1,61 (0,40–6,52)
High	22 (40%)	11 (50%)		
PD-L1 on tumor cells				
0%	35 (65%)	11 (31%)		
1–25%	15 (28%)	4 (27%)	0.038	1,13 (1,01–1,27)
≥25%	4 (7%)	4 (100%)		
PD-L1 on inflammatory cells				
Negative	10 (18%)	3 (30%)	0.058	0,09 (0,01–1,08)
Positive	44 (82%)	15 (34%)		
Ki-67				
<50%	14 (26%)	4 (28%)	0.054	1,05 (1–1,09)
≥50%	40 (74%)	15 (37%)		
Clinical T				
T1	7 (13%)	3 (43%)	0.8	0,8 (0,08–8,09)
T2–T4	47 (87%)	16 (34%)		
Clinical N				
Negative	30 (55%)	13 (43%)	0.27	0,47 (0,12–1,82)
Positive	24 (45%)	6 (25%)		

likely to respond to chemotherapy. Further studies with larger number of cases are warranted to confirm our findings.

Consent

Informed consent was waived from the Ethical Committee.

Disclosure

The preliminary results of this work were presented as a poster at the ESMO 2017 Congress in Madrid, Spain.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank Dr. Elisa Concetta Onesti for her help in the analysis of data and in the preparation of the abstract for ESMO.

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Hindawi
BioMed Research International
Volume 2017, Article ID 1506824, 5 pages
https://doi.org/10.1155/2017/1506824

Research Article

Dynamics of Neutrophils-to-Lymphocyte Ratio Predict Outcomes of PD-1/PD-L1 Blockade

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Received 20 August 2017; Accepted 5 November 2017; Published 28 November 2017

Academic Editor: Ilaria G. Zizzari

Introduction. Baseline neutrophil-to-lymphocyte ratio (NLR) has been repeatedly reported as a significant prognostic factor in advanced cancer patients. We explored whether changes in NLR may predict outcome of advanced cancer patients enrolled into phase 1 trials and treated with PD-1/PD-L1 inhibitors. *Patients and Methods*. Advanced cancer patients enrolled into phase 1 trials between September 2013 and May 2016 and treated with anti-PD-1/PD-L1 agents were included in this retrospective study. NLR was calculated at baseline and after 2 cycles of treatment. Royal Marsden Hospital (RMH) prognostic score and Eastern Cooperative Group (ECOG) performance status (PS) were determined at baseline. Kaplan-Meier estimation and Cox regression analyses were used to assess the impact of NLR dynamics on PFS. *Results*. Among the 55 patients eligible, 26 (47%) were treated with anti-PD-L1 monotherapy, 22 (40%) received single agent anti-PD-1, and 7 (13%) were given a tyrosine kinase inhibitor (TKI) plus a PD-1 inhibitor. Neither ECOG PS nor RMH prognostic score was significantly associated with PFS in our cohort, whereas changes in NLR significantly impacted on PFS. *Conclusion*. Changes in the NLR may be a useful predicting factor in advanced cancer patients treated with anti-PD-1/PD-L1 agents. Further prospective trials are needed to verify these findings.

1. Introduction

Immune checkpoint inhibitors have emerged as potent and effective treatments for various types of haematological and solid malignancies [1]. In particular, blockade of the PD-1/PD-L1 axis can result in dramatic and sustained tumour regression in multiple cancer types [2, 3]. Under normal circumstances, this pathway is necessary to maintain immune homeostasis [4]. When PD-L1 binds to PD-1, an inhibitory signal is transmitted into the T-cell, protecting normal cells from collateral damage. Nevertheless, upregulation of PD-L1 may allow cancer cells to evade immune surveillance [3]. Considering the costs and potential side effects of novel anti-PD-1/PD-L1 agents, it is of vital importance to identify reliable biomarkers to select the most suitable patients for these drugs while sparing nonresponders from toxicity.

PD-L1 expression as determined by immunohistochemistry is considered the most useful biomarker in predicting outcomes of PD-1/PD-L1 blockade [4]. Several studies have investigated the role of PD-L1 expression in tumour and stromal cells as a potential biomarker of response, but the results were somewhat contradictory [4, 5]. Indeed, several factors can limit the reliability of this biomarker, including the use of different monoclonal antibodies for detection of PD-L1, variable procedures for biopsy collection and storage, lack of defined thresholds to describe PD-L1 expression in samples, and intratumour heterogeneity in PD-L1 expression [5] The presence of microsatellite instability, tumour

mutational load, tumour-infiltrating lymphocytes (TILs), myeloid-derived suppressor cells (MDSCs), indoleamine 2,3-dioxygenase, regulatory T cells, and immune specific signatures have been also investigated with promising results [6–8]. Despite the aforementioned methods, there is still a lack of a simple, effective, and definitive biomarker of response to immune checkpoint inhibitors.

Increased neutrophil-to-lymphocyte ratio (NLR) has been reported as an independent poor prognostic indicator in several malignancies and its normalisation following treatment has been found to predict survival in cancer patients considered for early phase clinical trials [9]. Here, we investigated the usefulness of NRL changes in predicting progression-free survival (PFS) in patients undergoing treatment with PD-1/PD-L1 inhibitors within phase 1 clinical trials.

2. Patients and Methods

Data of metastatic cancer patients enrolled in phase 1 trials between September 2013 and May 2016 in our institution were retrospectively reviewed. Patients treated with PD-1/PD-L1 checkpoint-directed therapy were eligible. All the subjects had a histologically confirmed diagnosis of metastatic solid cancer and were intended to receive treatment with an anti-PD-1/PD-L1 agent given as monotherapy or in combination with a tyrosine kinase inhibitor (TKI). Baseline parameters, tumour characteristics, and treatment data were all reviewed and anonymously collected for this study. All the subjects met the standard inclusion criteria for phase 1 trials: Eastern Cooperative Group (ECOG) performance status (PS) 0 or 1; measurable disease based on Response Evaluation Criteria in Solid Tumour (RECIST); adequate bone marrow, liver, and kidney function; life expectancy of at least 3 months. Baseline characteristics recorded in the eligible population included demographic variables, tumour type, anticancer treatment (anti-PD-1 versus anti-PD-L1 versus anti-PD-L1 plus TKI), number of previous lines for metastatic disease, Royal Marsden Hospital (RMH) prognostic score [10], white blood cell (WBC) level, absolute neutrophil count (ANC), absolute lymphocyte count (ALC), and neutrophil-to-lymphocyte ratio (NLR). The RMH prognostic score (range 0–3) was calculated at baseline, taking into account albumin level, lactate dehydrogenase (LDH) level, and number of metastatic sites [10]. The NLR was calculated using the standard formula: NLR = ANC/ALC. NLR was calculated at baseline (cycle 1 day 1), and after 6 weeks (2 cycles) of treatment. Patients were treated until disease progression, death, or unacceptable toxicity. We considered PFS as our main outcome, which was defined as the time from treatment start until progression or death, whichever occurred first.

To investigate the dynamics in NLR between baseline and after 2 cycles of anti-PD-1/PD-L1 therapy, we used a landmark approach by excluding patients who were not able to receive at least 2 cycles of treatment to avoid guarantee time bias. We used multivariate Cox regression analyses with the relative NLR difference as independent and PFS as the dependent variable. To adjust for possible confounding, we introduced the RMH score into the model and additionally added a random effect for tumour entity, in order to account for possible heterogeneity between tumour types. We calculated univariate and multivariate hazard ratios (HR) with accompanied 95% confidence intervals (CI); however, the multivariable analysis is considered as main analysis. To visualize the prognostic effect of the NLR difference, we created Kaplan-Meier plots. All p values are exploratory in nature with a conventional level of significance at 0.05. All analyses were done using the statistical software R (https://www.r-project.org/) and STATA (version 14).

Table 1: Patients' characteristics at baseline. NCSLC = non-small cell lung cancer; ECOG PS = Eastern Cooperative Oncology Group performance status; GI = gastrointestinal; TKI = tyrosine kinase inhibitor; RMH = Royal Marsden Hospital.

Characteristic	n (%)
Sex	
Male	19 (35)
Female	36 (65)
ECOG PS	
0	36 (65)
1	19 (35)
>1	—
Tumour type	
NSCLC	18 (33)
Upper GI cancer	11 (20)
Bladder cancer	8 (15)
Renal cell carcinoma	8 (15)
Breast cancer	7 (13)
Colorectal cancer	2 (4)
Ovarian cancer	1 (2)
Therapy	
Anti-PD-1	22 (40)
Anti-PD-L1	26 (47)
Anti-PD-L1 plus TKI	7 (13)
RMH prognostic score	
0	31 (56)
1	19 (35)
2	3 (5)
3	2 (4)
	Median (range)
Age	61 (40–80)
Number of metastatic sites	2 (1–4)
Number of previous treatment lines	1 (1–6)

3. Results

A total of 67 potentially eligible patients were identified. Of those, 12 subjects received less than 2 cycles and were therefore excluded from the analysis. The characteristics of the included 55 patients are summarised in Table 1. Median age of patients included was 61 years (40 to 80 years). The most represented tumour type was non-small cell lung cancer (NSCLC) with 18 (33%) subjects, followed by upper

Table 2: Distribution of patient population in two groups. Group A: neutrophil-to-lymphocyte ratio (NLR) after 2 cycles ≤ median baseline NLR. Group B: NLR after 2 cycles > median baseline NLR. ECOG PS = Eastern Cooperative Oncology Group performance status; RMH = Royal Marsden Hospital; ANC = absolute neutrophil count; ALC = absolute lymphocyte count; SD = standard deviation; IQR = interquartile range; NLR = neutrophil-to-lymphocyte ratio.

Characteristic	Group A (n = 28)	Group B (n = 27)
Sex n (%)		
Female	11 (39)	8 (30)
Male	17 (61)	19 (70)
ECOG PS n (%)		
0	16 (43)	20 (74)
1	12 (57)	7 (26)
RMH prognostic score n (%)		
0-1	25 (89)	25 (93)
2-3	3 (11)	2 (7)
Intervention n (%)		
Anti-PD-1	14 (50)	8 (30)
Anti-PD-L1	9 (32)	17 (63)
Anti-PD-1 plus TKI	5 (18)	2 (7)
Baseline ANC		
Mean (SD)	5.1 (1.8)	4.1 (1.4)
Baseline ALC		
Mean (SD)	1.3 (0.6)	1.3 (0.5)
Baseline NLR		
Median (IQR)	3.9 (2.7–5.6)	3.0 (2.5–4.8)
ANC after 2 cycles		
Median (IQR)	3.7 (2.8–5.1)	4.5 (3.5–5.7)
ALC after 2 cycles		
Mean (SD)	1.4 (0.5)	1.2 (0.5)
NLR after 2 cycles		
Median (IQR)	2.9 (2.2–3.7)	3.9 (2.8–7)

gastrointestinal (n = 10; 18%), bladder (n = 8; 15%), and breast (n = 7; 13%) carcinomas. Median number of previous lines of treatment for metastatic disease was 1 (range 1–6) while baseline median number of metastatic sites of disease was 2 (range 1–4). RMH prognostic score at baseline was 0 in 31 (56%) subjects and 1 or higher in 24 (64%) subjects. Proportion of patients with ECOG PS 0 or 1 was 36 (65%) and 19 (35%), respectively. In total, 26 (47%) of the patients were treated with anti-PD-L1 monotherapy, 22 (40%) received single agent anti-PD-1, and 7 (13%) were given a TKI in combination with a PD-1 inhibitor.

On univariate analysis, baseline NLR, treatment modality, RMH score, ECOG PS, and number of metastatic sites did not have significant impact on PFS. Baseline median NLR was 3.4 in the overall population. Patient population was divided into 2 distinct groups based on decrease (Group A) or increase (Group B) of NLR in comparison with median NLR after treatment with a PD-1/PD-L1 inhibitor. No substantial differences in distribution were observed between these 2 groups in terms of age, sex, type of treatment, ECOG PS, and RMH prognostic score (Table 2). Increased NLR after 2 cycles of anti-PD-1/PD-L1 therapy had a negative effect on PFS (HR 1.14, 95% CI 1.06–1.23, p = 0.004), (Figure 1). This effect was also observed in our multivariate analysis,

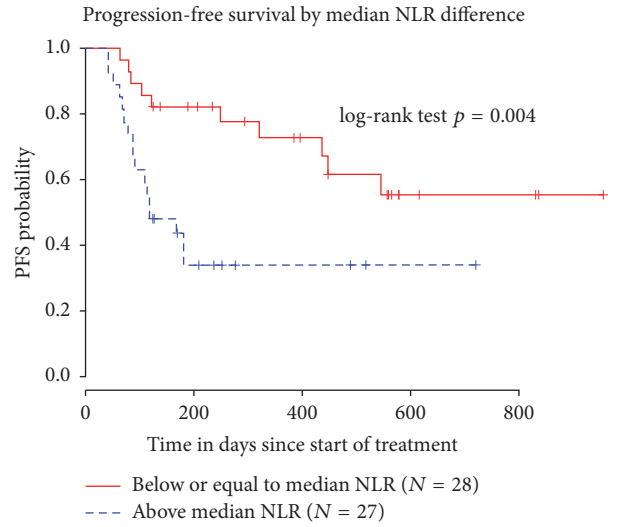

Figure 1: Progression-free survival (PFS) stratified by median differences in neutrophil-to-lymphocyte ratio (NLR) between baseline and after 2 doses of treatment with an anti-PD-1/PD-L1 inhibitor, showing longer PFS in patients with a reduction of NLR compared to the median baseline NLR.

where increased NLR was associated with decreased PFS after adjusting for RMH prognostic score (HR 1.03, 95% CI 1.01–1.04, $p < 0.001$). Baseline median ANC level was significantly higher in Group A than in Group B (p = 0.029). In Group A, a reduction in median ANC was shown after 2 cycles of treatment when compared with baseline ANC level, whereas this was not observed in Group B. After 2 cycles of treatment with anti-PD-1/PD-L1 agent, median ANC was significantly higher in Group B when compared to Group A (p = 0.014). Median ALC did not change significantly after treatment (p = 0.222) and no significant differences were shown between baseline and posttreatment values in both Group A and Group B (p = 0.24) (Table 2).

4. Discussion

Molecular selection in patients undergoing treatment with immune checkpoint inhibitors is an urgent unmet medical need. Ongoing approval of several anti-PD-1/PD-L1 agents and the emergence of safety concerns from immune-related adverse events also highlight the need for biomolecular stratification. Several biomarkers have been investigated, some of which have shown potential usefulness in predicting the activity of these agents. So far, PD-L1 expression in tumour cells remains the most reliable but many technical limitations have been associated with this biomarker [4, 5]. Furthermore, individuals with negative PD-L1 expression can still respond to PD-1/PD-L1 blockade, further questioning the value of PD-L1 expression as a universal biomarker [11]. Therefore, alternative markers of response need to be identified.

Clinicopathologic factors have been extensively investigated in several tumour types and anticancer therapies. Among them, ECOG PS has been repeatedly reported as a strong predictor of survival in multiple settings. The RMH

prognostic score was electively implemented in advanced cancer patients enrolled in phase 1 studies [10]. Unexpectedly, neither ECOG PS nor RMH prognostic score were significantly associated with PFS in our cohort. Our results are not consistent with previous studies showing significant prognostic significance of RMH score and ECOG PS in phase 1 trial patients [10, 12, 13].

Baseline NLR has been reported to predict overall survival in cancer patients undergoing both conventional chemotherapy and targeted treatments, including immune checkpoint inhibitors. In previous reports, a correlation between baseline NLR and survival was shown in kidney cancer and NSCLC treated with IL-2 and nivolumab, respectively [14, 15]. Conversely, in our group of patients, baseline NLR was not found to correlate with PFS, although this result may have been determined by the limited sample size. In our study, we retrospectively analysed phase 1 trial cancer patients with advanced disease who had received at least 2 cycles of treatment with anti-PD-1/PD-L1 agent, to assess the significance of NLR as an independent biomarker in predicting clinical benefit in terms of PFS. Interestingly, a decrease in NLR after 2 cycles of treatment with PD-1/PD-L1 blockade was associated with longer PFS in our cohort. Accordingly, changes in NLR had shown to predict better outcomes in cancer patients undergoing conventional chemotherapy or targeted treatment but had been never investigated in subjects receiving immune checkpoint inhibitors [16–18].

Another important finding in this study was the observation that negative or positive changes in NLR were driven by a decrease in ANC and not by changes in ALC as one would have expected. Though retrospective in nature, our findings on ANC may be interpreted as hypothesis-generating. Despite the fact the T lymphocyte activity is the main target of PD-1/PD-L1 blockade, our results may suggest an important interaction between the neutrophils and tumour microenvironment. We may also speculate that the systemic effect of anti-PD-1/PD-L1 agents involves a crucial effect on circulating myeloid populations included in the ANC as measured by automated full blood cell count analyser. Preclinical evidence shows that MDSCs can impair the efficacy of immunotherapy [19]. Under physiological conditions, there is a low level of MDSCs in the bloodstream while these populations rapidly expand during immunological responses to infections, inflammation, and cancer [20]. MDSCs can adopt multiple mechanisms to induce immunosuppression, including production of arginase 1 and inducible nitric oxide synthase, leading to T-cell inhibition [19, 20]. MDSCs are also known to enhance cancer cell proliferation, confer resistance to anticancer therapies, and promote angiogenesis and metastasis [20]. Concomitant targeting of MDSCs may therefore increase the antitumour activity of PD-1/PD-L1 inhibitors in nonresponders. Moreover, a decreased mobilisation of MDSCs from the bone marrow may represent a systemic effect of anti-PD-1/PD-L1 treatment that needs to be better investigated in preclinical studies. A subsequent decrease of tumour-infiltrating MDSCs may then unleash antitumour activity of TILs and ultimately contribute to the therapeutic effect of anti-PD-1/PD-L1 agents.

Our study has several biases, including its retrospective nature, limited sample size, heterogeneous tumour types, and the choice of PFS as endpoint instead of overall survival. Nevertheless, we showed that NLR, a simple haematological parameter easily obtainable in daily clinical practice, may be used to predict clinical benefit from PD-1/PD-L1 inhibitors. These results are in line with common clinical experience with these agents, where a rapid clinical benefit can be observed despite unusual initial patterns of imaging response. Further studies conducted in larger prospective cohorts of patients undergoing treatment with immune checkpoint inhibitors are needed to confirm the predictive role of NLR in this setting.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Michele Moschetta, Mario Uccello, and Benjamin Kasenda equally contributed to the article.

Acknowledgments

The authors are grateful to the patients and their families for participating in these clinical studies.

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Hindawi
BioMed Research International
Volume 2017, Article ID 1293201, 8 pages
https://doi.org/10.1155/2017/1293201

Research Article

PD-L1 Promotes Self-Renewal and Tumorigenicity of Malignant Melanoma Initiating Cells

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Received 22 April 2017; Accepted 10 September 2017; Published 9 November 2017

Academic Editor: Ilary Ruscito

Recent studies have indicated that therapeutic antibodies targeting PD-L1 show remarkable efficacy in clinical trials in multiple tumors and that a melanoma cell-intrinsic PD-1: PD-L1 axis promotes tumor growth. However, few studies have shown tumor-intrinsic PD-L1 effects in malignant melanoma initiating cells (MMICs). Here, we aim to determine the possible regulatory effects of PD-L1 on MMICs. The ALDEFLUOR kit was used to identify $ALDH^+$ MMICs. Flow cytometry was used to examine the expression of PD-L1 on $ALDH^+$ MMICs. To determine the role of PD-L1 in MMICs self-renewal, we cultured melanoma cells with anti-PD-L1 and measured tumorsphere formation and apoptosis. In addition, the effects of anti-PD-L1 on tumorigenicity and residual $ALDH^+$ MMICs in tumors were evaluated *in vivo*. We demonstrated that melanoma cell-intrinsic PD-L1 was expressed in $ALDH^+$ MMICs. Blocking PD-L1 in melanoma cell lines impaired tumorsphere formation and induced the apoptosis of sphere cells. In addition, blocking PD-L1 inhibited tumor growth *in vivo*. We observed residual $ALDH^+$ MMICs within the tumor. The results showed that blocking PD-L1 also significantly decreased the residual $ALDH^+$ MMICs in the tumors. In conclusion, these results suggest a new mechanism underlying melanoma progression and PD-L1-targeted therapy, which is distinct from the immunomodulatory actions of PD-L1.

1. Introduction

Metastatic melanoma is an extraordinarily challenging cancer, with a 16% 5-year survival rate, and it responds poorly to most standard chemotherapies [1]. It has been established that malignant melanoma initiating cells (MMICs) possess not only the capacity for self-renewal, differentiation, immune evasion, and multidrug resistance, but also potentially vasculogenic mimicry and the ability to transition to migratory and metastasizing derivatives, which are associated with melanoma progression and metastasis [2, 3]. For this reason, melanoma cure is predicated upon effectively targeting and eradicating the MMICs.

Recently, it has been established that programmed death-1 (PD-1) is a prominent checkpoint receptor that, upon binding its ligands PD-L1 or PD-L2, dampens T effector functions by inhibiting signaling downstream of the T cell receptor [4]. PD-L2 is predominantly expressed in APCs, whereas PD-L1 is commonly expressed in various cell types, including tumor cells, immune cells, epithelial cells, and endothelial cells [1, 5]. When PD-1 binds to its ligands in tumors; it leads to T-cell anergy and blocks productive antitumor immune response [6]. The first monoclonal antibody directed at PD-1, Nivolumab, was approved for treating patients with unresectable melanoma in July 2014. The other PD-1 and PD-L1 directed agents are currently in Phase I–III clinical trials in multiple tumor types [7].

In contrast to the immunosuppressive effect of PD-L1, it is also known to contribute to the promotion of tumor cell growth and downregulation of quiescent cells [8, 9]. Furthermore, it has been found that glioma stem cells express lower levels of PD-L1 than differentiated glioma cells do [10]. In head and neck squamous cell carcinoma, PD-L1 is preferentially expressed in $CD44^+$ tumor-initiating cells

[11]. PD-L1 also has suppressive effects on cancer stem cell-related phenotypes of cholangiocarcinoma [12]. These recent data highlight the possible involvement of PD-L1 in the regulation of cancer stem cells in various tumors. However, little research has investigated the role of PD-L1 in MMICs. Here, we report on a study to determine the frequency of PD-L1 expression in MMICs, and the possible regulatory effects of PD-L1 on MMICs.

2. Materials and Methods

2.1. Cells and Cell Culture. B16-F0 and B16-F1 melanoma cell lines were maintained in RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS; ScienceCell), 100 U/ml penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Cells were cultured at 37°C in 95% humidity and 5% CO_2 atmosphere. All cell lines were routinely screened for mycoplasma contamination.

2.2. Flow Cytometry. The ALDEFLUOR kit (StemCell Technologies, British Columbia, Canada) was used to identify the stem/progenitor cells that expressed high levels of the aldehyde dehydrogenase (ALDH) [13]. Briefly, $1 * 10^6$/ml cells were suspended in Aldefluor Assay Buffer (AAB) and incubated with 5 μL ALDH substrate (BAAA) for 45 min. 5 μL diethylaminobenzaldehyde (DEAB) was added to a separate sample containing BAAA for an ALDH-inhibited control. Then, samples were washed and resuspended in AAB. Fluorescence-activated cell gates were established using the inhibited control, DEAB, with the fluorescein isothiocyanate (FITC) channel with excitation and emission wavelengths of approximately 495 nm and 521 nm, respectively. To evaluate the expression of PD-L1 in $ALDH^+$ cells, PD-L1 antibody (10F.9G2, GeneTex) and immunoglobulin G (IgG) isotype-matched control (GeneTex) containing BAAA were added to the cells separately. All samples were incubated for 30 minutes at 4°C. Following incubation, the material was centrifuged, and pellets were resuspended with 500 μl assay buffer prior to data acquisition. Flow cytometry analysis was performed on a BD Biosciences FACSCanto, and data analysis was conducted using CellQuest Pro (B&D Biosciences).

2.3. Tumorsphere Culture. The B16-F0 and B16-F1 melanoma cells were plated as single cells in ultralow attachment six-well plates (Corning, Lowell, MA, USA) and cultured in RPMI 1640 containing 6 mg/mL glucose (Sigma-Aldrich), 1 mg/mL NaHCO3 (Sigma-Aldrich), 5 mM HEPES (Sigma-Aldrich), 4 μg/mL heparin (Sigma-Aldrich), 4 mg/mL bovine serum albumin (Sigma-Aldrich), 20 pg/mL insulin (Sigma-Aldrich), N2 supplement (Invitrogen), supplemented with 10 ng/mL bFGF (Peprotech, Neuilly sur Seine, France), and 20 ng/mL EGF (Peprotech), as previously described [14]. On the second day after seeding, cells were treated with 10 μg anti-PD-L1 (10F.9G2, BioXcell) or control rat immunoglobulin G (IgG). Tumorspheres were observed under microscope 14 days later. Individual spheres with diameters larger than 100 μm from each replicate well were visualized and counted with an inverted microscope.

2.4. Assay for Apoptosis. Cells were double-stained with FITC-annexin V and PI according to the manufacturer's instructions (Annexin-V FITC/propidium iodide (PI) Apoptosis Detection Kit; BD Pharmingen). Analysis was performed by flow cytometry. Early apoptotic cells were stained with Annexin-V alone, whereas necrotic and late apoptotic cells were stained with both Annexin-V and PI.

2.5. Animals and Tumor Model. Adult SPF male C57BL/6 mice were implanted subcutaneously on the right flank with either 5×10^5 B16-F0 or 5×10^5 B16-F1 melanoma cells. Then, 100 μg anti-PD-L1 or control rat IgG was administered intraperitoneally 3, 6, and 9 days following melanoma cell inoculation. All animals were randomly assigned to two groups of 5 mice each. Tumor size was monitored every two days. All surgical procedures and care given to the animals were in accordance with institutional guidelines.

2.6. Statistical Analysis. All data were reported as the mean ± standard error. Statistical analysis was performed using GraphPad Prism 5.0 Software (San Diego). A two-tailed paired t-test was used to determine significant differences. P values < 0.05 were considered statistically significant.

3. Results

3.1. PD-L1 Expression on $ALDH^+$ Melanoma Cells. Previous studies have described the isolation of MMICs from mice using ALDEFLUOR/ALDH as a marker [13, 15]. To determine the expression of PD-L1 in MMICs, we detected $PD\text{-}L1^+/ALDH^+$ subpopulations from these two cell lines. As shown in Figure 1, $ALDH^+$ cells were identified in melanoma cell lines by flow cytometry with the ALDEFLUOR kit. Cells were then incubated for 30 min with mouse monoclonal antibodies specific for PD-L1. The analysis of the percentage of $PD\text{-}L1^+ALDH^+$ cells was gated by $ALDH^+$ cells. We found that approximately 10% to 18% of the cultured murine B16-F0 cells and B16-F1 cells were $ALDH^+$. Approximately, 5% of the $ALDH^+$ cells were $PD\text{-}L1^+/ALDH^+$. These data suggest that PD-L1 may be involved in regulating MMICs.

3.2. PD-L1 Regulated on MMICs Tumorsphere Formation. To determine whether PD-L1 can mediate MMIC self-renewal, we cultured melanoma cell lines with anti-PD-L1. The results showed that anti-PD-L1 significantly inhibited tumorsphere formation in B16-F0 and B16-F1 melanoma cells compared to the control groups (Figure 2). Cancer stem cell-derived spheres were dissociated and passaged; they readily formed secondary spheres [16]. Anti- PD-L1 inhibited secondary tumorsphere generation. Anti-PD-L1 induced a 2-fold inhibition of tumorsphere formation in B16-F0 cells and approximately 1.4-fold inhibition in B16-F1 melanoma cells, in terms of both number and size, compared with control groups.

3.3. PD-L1 Affected the Apoptosis of MMICs Enriched Cells. Tumorsphere formation has been reported as a measure of the presence of MMICs in enriched cell populations. We further explored the effects of anti-PD-L1 on apoptosis in

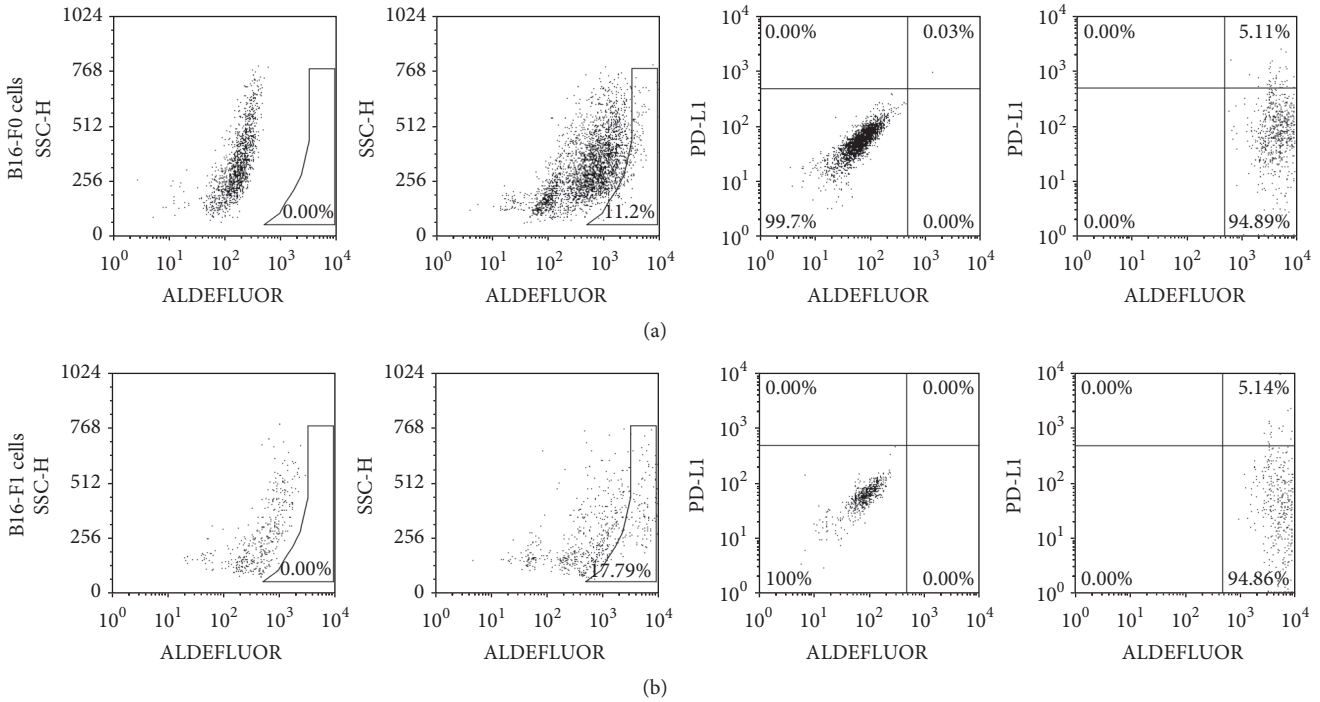

Figure 1: The expression of PD-L1 on MMICs. (a) The left two scatter plots showed the $ALDH^+$ cells identified in the B16-F0 melanoma cells by flow cytometry using the ALDEFLUOR kit. Only $ALDH^+$ cells were gated for analysis of the percentage of $PD\text{-}L1^+ALDH^+$ cells. The right two scatter plots showed the percentage of $PD\text{-}L1^+ALDH^+$ cells in B16-F0 melanoma cells. (b) The expression of PD-L1 in $ALDH^+$ B16-F0 melanoma cells.

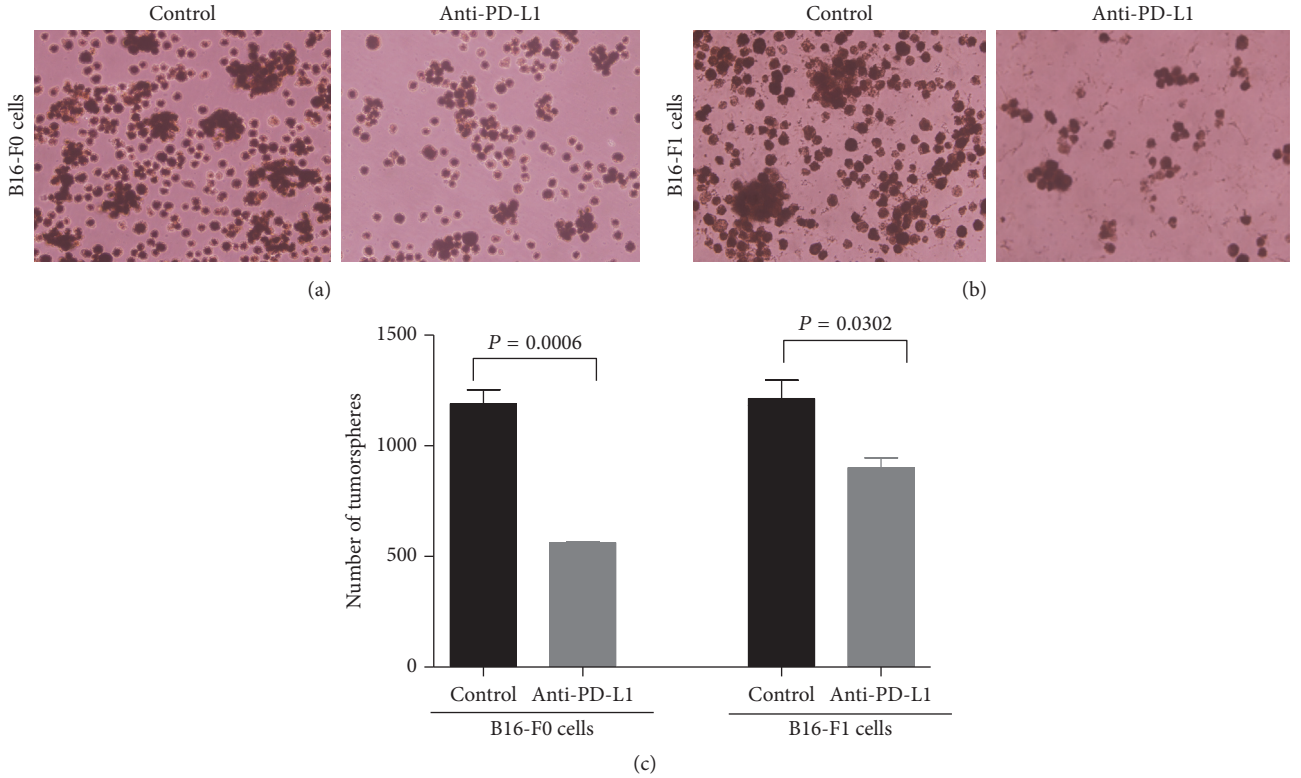

Figure 2: PD-L1 promoted tumorsphere formation. After co-culturing with anti-PD-L1, the sphere formation ability of (a) B16-F0 cells and (b) B16-F1 cells was impaired. (c) The chart showed the number of tumorspheres in each group. Each column represents the mean ± SE of three independent experiments.

melanoma tumorspheres. The data illustrated that anti-PD-L1 induced significant apoptosis in melanoma tumorspheres (Figure 3). Anti-PD-L1 increased the rate of apoptosis by 2-fold in both B16-F0 and B16-F1 tumorspheres. Thus, PD-L1 inhibited apoptosis of MMIC-enriched cells.

3.4. Blockage of PD-L1 Directly Affected MMICs In Vivo. Mice were challenged with melanoma cells (B16-F0 and B16-F1) and treated with 100 μg anti-PD-L1 or control rat IgG 3, 6, and 9 days following melanoma cell injection. Other studies have reported that anti-PD-L1 significantly suppressed tumor growth compared with PBS-injected animals in two animal models. Anti-PD-L1 promoted tumor rejection in 50% of B16-F0 melanoma challenged mice ($P = 0.031$) and 50% of B16-F1 melanoma challenged mice ($P = 0.031$; Figures 4(a) and 4(b)). We observed that anti-PD-L1 decreased residual $ALDH^+$ MSCs within the tumor. As shown in Figures 4(c)–4(e), anti-PD-L1 promoted the rejection of 1.5-fold residual $ALDH^+$ MMICs in the B16-F0 animal model ($P = 0.016$) and 1.4-fold residual $ALDH^+$ MMICs in the B16-F0 animal model ($P = 0.045$). These results suggest that one mechanism for the anti-tumor effects of anti-PD-L1 is related to its ability to suppress the tumorigenicity capacity of MMICs.

4. Discussion

Previous studies have shown that PD-L1 expression is a common phenomenon in immunotherapy-naive melanomas [17–19]. Further studies have indicated that PD-1 expressed by melanoma cells is a tumor growth-promoting mechanism, and PD-1-driven tumorigenesis requires interaction between melanoma-PD-1 and host or melanoma-expressed PD-L1 [18]. Here, we provide several insights into the function of PD-L1 in MMICs, which is separate from its effects on the immune response. Our study found that PD-L1 was expressed in $ALDH^+$ MMICs and induced tumorsphere formation. PD-L1 further inhibited the apoptosis of MMIC-enriched cells. Blockage of PD-L1 directly inhibited tumorigenesis *in vivo* and significantly decreased the residual percentage of MMICs. These results may indicate that melanoma cell-intrinsic PD-L1 promotes self-renewal and the tumorigenic capacity of MMICs.

Traditionally, PD-1 ligands have been expressed in tumor cells, leading to T-cell exhaustion and tumor cell evading the immune response, which was thought to require its receptor interaction [20]. Accordingly, several clinical trials have focused on using PD-L1-blocking antibodies to enhance immunity in cancers [21–23]. However, a recent study found that melanoma-PD-1: host-PD-L1 interactions promoted murine melanoma growth [8]. In melanoma, a subpopulation of cells, namely, MMICs, is capable of not only self-renewal, differentiation, plasticity, immune evasion, and multidrug resistance, but also potentially vasculogenic mimicry, and transitioning to migratory and metastasizing derivatives, which are associated with melanoma progression and metastasis [24]. Thus, we believe that melanoma-PD-L1 may contribute to maintaining the stem cell-like properties of MMICs.

MMICs are known to have high ALDH. Previous studies have successfully used ALDH as a marker to isolate MMICs from mice [13, 15]. Our present flow cytometry results showed that PD-L1 was expressed in $ALDH^+$ MMICs. Glioma stem cells expressed lower levels of the PD-L1 than those of differentiated glioma cells, which contributed to the higher sensitivity of glioma stem cells to the cytotoxicity of the IL-2-activated NK cells [25]. In head and neck squamous cell carcinoma, PD-L1 is preferentially expressed in $CD44^+$ tumor-initiating cells and inhibits IFN-γ secretion by tumor-infiltrating lymphocytes (TILs) incubated with $CD44^+$ cells [26]. These previous studies focused on the expression of PD-L1 in cancer stem cells, which induced immune evasion in cancer. The results presented here demonstrate that anti-PD-L1 inhibited the tumorsphere-forming capacity and induced apoptosis in melanoma cancer stem-like cells. Current phase-I studies targeting PD-L1, BMS-936559, and MPDL3280A have reported significant responses and survival benefits [27–29]. We showed that anti-PD-L1 inhibited tumor growth *in vivo*, which is in agreement with these published studies.

It has been established that the expression of PD-L1 is an indicator of poor prognosis for patients' survival in many cancers, such as pulmonary adenocarcinoma, gastric cancer, colorectal cancer, and esophageal cancer [30–33]. In contrast, studies of the prognostic usage of PD-L1 expression are inconsistent in melanoma [34]. As McLaughlin et al. [35] demonstrated in NSCLC, several factors contributed to false-negative PD-L1 findings, including the fact that tumor samples may be inadequate or not representative of the entire tumor mass, different anti-PD-L1-directed antibodies perform differently, and a quantitative interpretation of immunohistochemical stains has some deficiencies. As described above, PD-L1 expression in melanoma cells showed marked heterogeneity, which may have implications on the study of the prognostic usage of PD-L1 expression analysis. The present experimental data showed that anti-PD-L1 significantly decreased the residual percentage of MMICs, which indicates that the melanoma-PD-L1 pathway may be one of the many mechanisms involved in PD-L1-mediated melanoma progression. According to Tamai et al. [12], PD-L1 can directly affect cancer stem cells, which is distinct from its immunomodulatory action.

Taken together, our results suggest that melanoma-PD-L1 can enhance tumorigenesis by maintaining the stem cell properties in MMICs. Future studies are needed to elucidate the underlying cellular and molecular mechanisms, which will be helpful to maximize its clinical benefits.

Conflicts of Interest

No conflicts of interest exit in the submission of this manuscript.

Acknowledgments

The financial supports of this work are received from the National Nature Sciences Foundation of China (no. 81301954) and Hubei Provincial Health and Family Planning Scientific

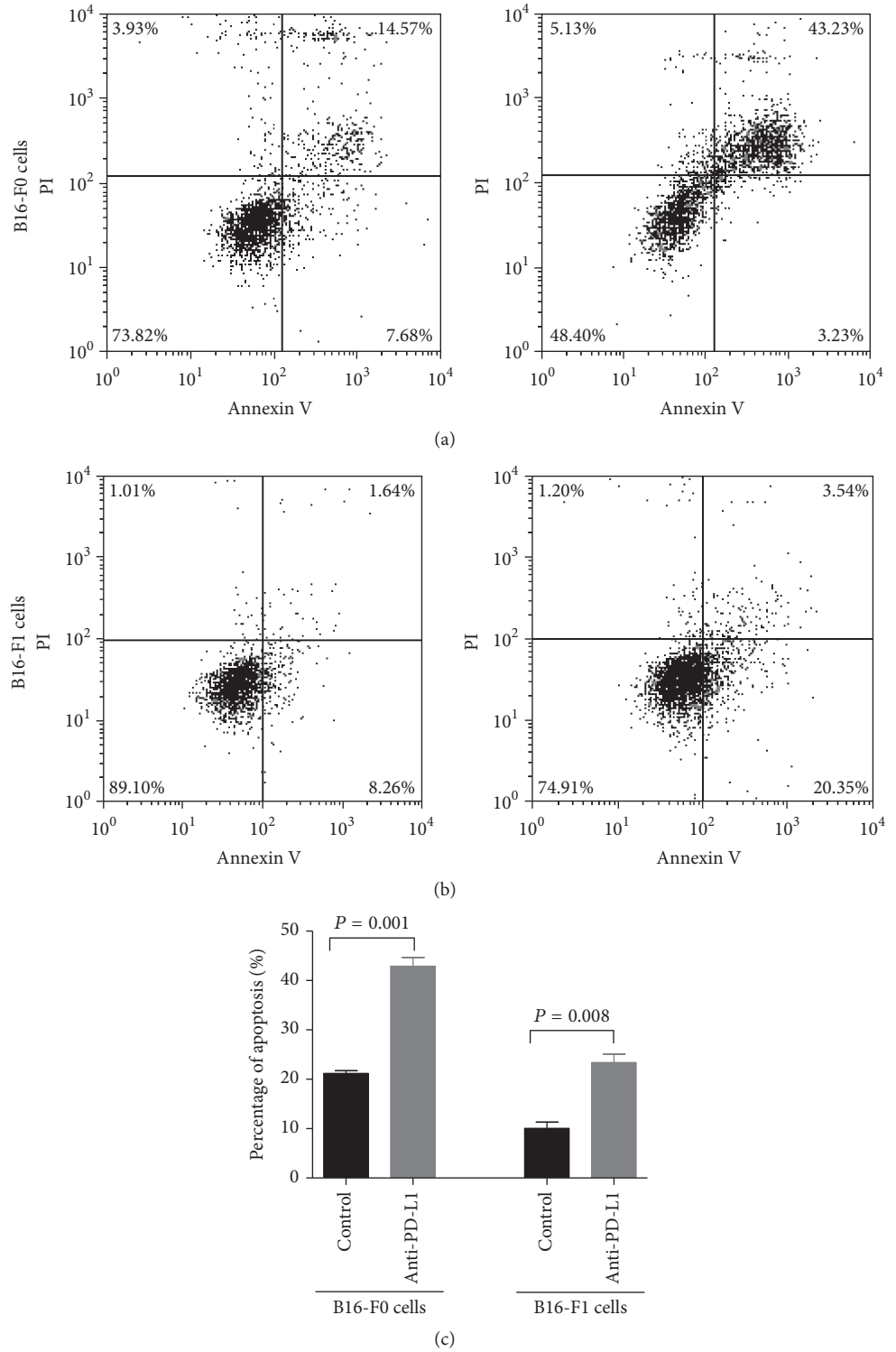

Figure 3: PD-L1 inhibited the apoptosis of sphere cells. After coculturing with anti-PD-L1 for 14 days, tumorspheres were collected and then dissociated into a single cell suspension. The apoptosis rates of (a) B16-F0 spheres and(b) B16-F1 spheres were measured using flow cytometry. (c) The chart shows the apoptosis rate in each group. Each column represents the mean ± SE of three independent experiments.

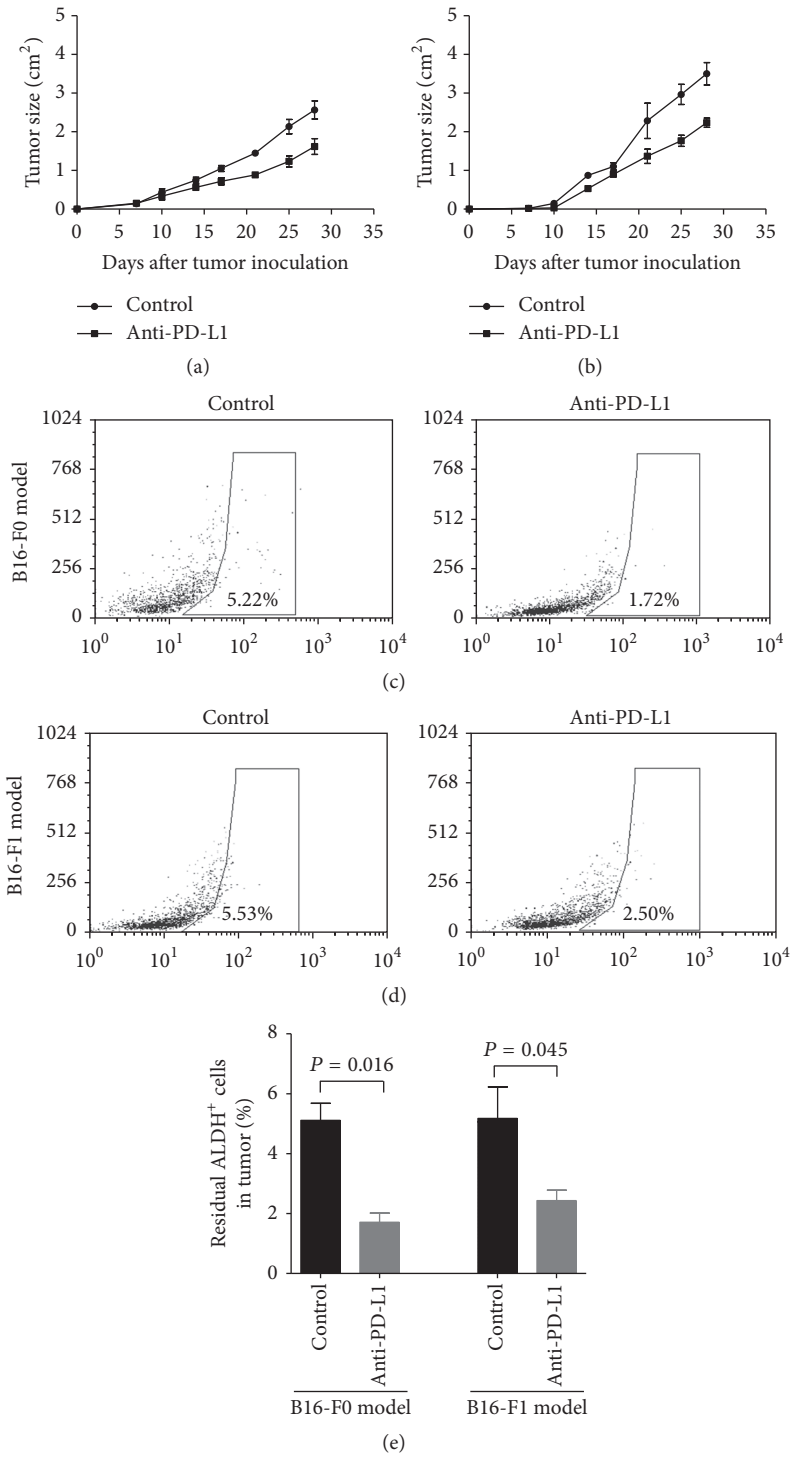

Figure 4: Blockage of PD-L1 affects MMICs *in vivo*. C57BL/6 mice were separately inoculated with (a) B16-F0 cells and(b) B16-F1 cells. Then, mice were administered anti-PD-L1 3, 6, and 9 days after melanoma cell injection. Tumor growth was monitored. The results are shown as the mean ± SE of five mice in each group. (c) Images of tumors from representative animals used in (a) and (b). At the end of the experiment, residual ALDH$^+$ MMICs within the tumor were analyzed by flow cytometry in the (c) B16-F0 and B16-F1 (d) cell models. (e) The data for residual ALDH$^+$ MMICs within two tumor models are shown as the mean ± SE of five mice in each group.

Research project (no. 2015060101010043). We appreciate the English Language Service (Ameican Journal Experts) who edit this manuscript.

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Hindawi
BioMed Research International
Volume 2017, Article ID 7694202, 7 pages
https://doi.org/10.1155/2017/7694202

Research Article

Increased EGFR Phosphorylation Correlates with Higher Programmed Death Ligand-1 Expression: Analysis of TKI-Resistant Lung Cancer Cell Lines

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Received 5 July 2017; Accepted 27 August 2017; Published 8 October 2017

Academic Editor: Ilary Ruscito

Despite the recent development of immunotherapies that target programmed death-1 (PD-1) or programmed death ligand-1 (PD-L1) in non-small cell lung cancer (NSCLC) treatment, these therapies are less effective in NSCLC patients with *epidermal growth factor receptor (EGFR)* mutations. However, the molecular mechanisms underlying this lower efficacy of immunotherapies in *EGFR* mutant lung cancers are still unclear. In this study, we analyzed PD-L1 protein expression in lung cancer cell lines with *EGFR* mutations prior to and after acquisition of resistance to EGFR tyrosine kinase inhibitors (TKIs). We found that parental lung cancer cell lines harboring *EGFR* mutations showed negative (PC9 and H3255 cells) and positive (HCC827 cells) staining for PD-L1 by immunohistochemistry. Comparing PD-L1 expression between EGFR-TKI resistant cell lines and their parental cells, we found that increased phosphorylation of EGFR was related to increased expression of PD-L1. Increased phosphorylation of EGFR was accompanied by the T790M secondary mutation. Acquired resistance cells with *MET* amplification or *EGFR* loss both showed decreased phosphorylation of EGFR and decreased PD-L1 expression. Our results indicate that lung cancer cell lines with *EGFR* mutations (parental cells) do not harbor high PD-L1 protein expression. In addition, EGFR phosphorylation affects PD-L1 expression after acquisition of resistance to EGFR-TKIs.

1. Introduction

Activating mutations in the *epidermal growth factor receptor (EGFR)* gene define one of the most common molecular subtypes of non-small cell lung cancers [1]. EGFR tyrosine kinase inhibitor (TKI) monotherapies (gefitinib, erlotinib, or afatinib) are the first choice for these patients [1]; however, acquisition of resistance to these TKIs is almost inevitable after an average of 1 year [2]. A variety of resistance mechanisms have been identified including T790 M mutation, *MET* or *ERBB2* gene amplification, small cell lung cancer transformation, and epithelial to mesenchymal transition (EMT) [2].

Osimertinib, a 3rd generation EGFR-TKI, is the appropriate second-line drug after acquisition of resistance to gefitinib, erlotinib, or afatinib if a rebiopsied resistant tumor proves the presence of an *EGFR* T790M secondary mutation [3]. However, cytotoxic chemotherapies are still the standard of care as second-line drugs for patients who do not have the *EGFR* T790M mutation [4]. Recent development of immunotherapies that target programmed death ligand-1 (PD-L1) or programmed death-1 (PD-1) has shown dramatic

success in some lung cancer patients [5]. However, these immune-checkpoints inhibitors have shown poorer response rates and outcomes in patients with *EGFR* mutations compared to those with *EGFR* wild-type tumors [6, 7].

PD-L1 protein expression has been pursued as a predictive marker for current immunotherapies. To elucidate the underlying mechanisms of this reduced effectiveness for immunotherapies in lung cancer patients with *EGFR* mutations, we performed the current study to analyze PD-L1 protein expression status, using the FDA approved detection kit system, before and after the acquisition of resistance to EGFR-TKIs in established cell lines harboring *EGFR* mutations.

2. Materials and Methods

2.1. Cell Lines, Reagents, and Generation of In Vitro Resistant Cell Lines. Human lung cancer cell lines used in this study were from the established collections in our labs or as reported in our previous studies [8–10]. PC-9 erlotinib resistant cells were established from PC-9 cells by stepwise exposure to erlotinib from 0.005 μM to 5 μM for about 4 months, and the clone named PC-9 ER clone 5 was isolated with PicoPipet (Nepa Gene, Chiba, Japan). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 1x penicillin/streptomycin solution (Mediatech, Inc., Manassas, VA) at 37°C in a humidified tissue culture incubator with 5% CO_2. All experiments using acquired resistance cells were performed following the removal of drug exposure to avoid the direct effects of drugs on PD-L1 expression. IFN-gamma (Cell Signaling Technology, Dancers, MA) stimulation for 24 hours was performed to mimic an immune cell interaction.

2.2. Immunohistochemistry (IHC) Analysis. Formalin-fixed paraffin-embedded (FFPE) blocks were prepared from cell pellets to perform IHC analysis as previously described [11]. Briefly, cultured cells were gently harvested using Accutase® (Innovative Cell Technologies, Inc., San Diego, CA) and fixed with alcoholic formalin solution for 24 hours. Fixed cells were mixed with melted agarose solution, allowed to solidify, placed in the cassette, and submerged in 70% ethanol. Paraffin-embedding of the agarose cell pellet was performed at our pathology core lab. Antibody against PD-L1 was purchased from Dako (22C3 pharmDx, Dako–Agilent Technologies, Carpinteria, CA). Staining was performed on a Dako Link 48 Auto-Stainer. PD-L1 staining was assessed using the *H*-score assessment.

2.3. Western Blot Analysis. All antibodies were purchased from Cell Signaling Technology (Danvers, MA). Total cell lysates were prepared, and immunoblotting was conducted as described previously [11]. Briefly, cells were cultured until subconfluent, rinsed with phosphate-buffered saline (PBS), lysed in sodium dodecyl sulfate (SDS) sample buffer, and homogenized. The total cell lysate (10 μg) was subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk, membranes were incubated with primary antibodies, washed with PBS, and reacted with secondary antibodies (Cell Signaling Technology), and signals visualized using ECL reagent (Clarity, Bio-Rad, Hercules, CA) and film or detected by an ImageQuant Imager (GE Healthcare Bio-Sciences, Tokyo, Japan).

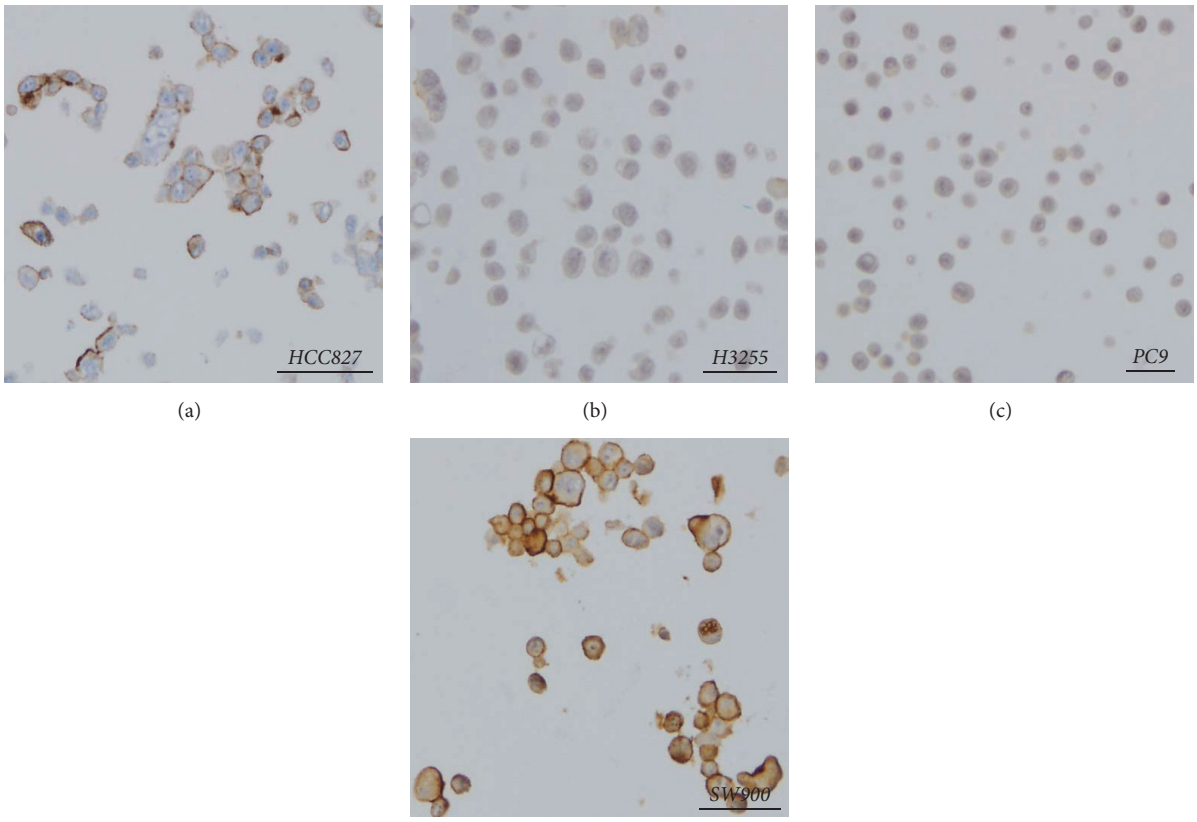
2.4. Flow Cytometry. PC-9 and PC-9 ER clone 5 cells were stained with the mouse monoclonal BV421-conjugated antibody to human PD-L1 (BD Biosciences, San Jose, CA) for flow cytometry analysis with the BD LSRFortessa cell analyzer and the BD FACSDiva software (BD Biosciences).

3. Results

3.1. PD-L1 Expression in Lung Cancer Cell Lines with EGFR Mutation. Initially, we screened for PD-L1 expression in parental lung cancer cell lines by IHC using the Dako 22C3 antibody. The efficacy of the 22C3 antibody was recently demonstrated in clinical trials [12, 13], and the analytical performance seems similar to two other clinically used PD-L1 antibodies (Dako 28-8 and Ventana SP- 263 [14]). As shown in Figure 1, PC-9 cells (del E746_A750) and H3255 cells (L858R) were negative for PD-L1 IHC, and HCC827 cells (del E746_A750) showed positive expression for PD-L1 membrane staining (*H*-score: 145) that is weaker than that observed in other non-small cell lung cancer cell lines such as SW900, a lung squamous cancer cell line (*H*-score: 210). These results indicate that lung cancer cell lines harboring *EGFR* mutation do not have high PD-L1 protein expression prior to EGFR-TKI exposure.

3.2. EGFR Phosphorylation Levels after Acquisition of Resistance to TKIs Is Related to PD-L1 Expression Status. Initial evaluation for PD-L1 protein expression changes focused on comparisons between the parental HCC827 cells and a group of daughter cell lines with acquired resistance to EGFR-TKIs. Each daughter cell line exhibits different type of resistance mechanisms including *MET* gene amplification (HCC827ER), T790M mutation (HCC827EPR), *MET* gene amplification together with T790M mutation (HCC827 CNXR S1), and *MET* gene amplification with *EGFR* loss (HCC827 CNXR S4). As shown in Figures 2(a)–2(e), HCC827 daughter cells that have acquired resistance to EGFR-TKIs demonstrated various PD-L1 expression patterns including slightly decreased PD-L1 expression in HCC827ER and HCC827CNXR S4 cells (*H*-scores: 125 and 120, resp.) compared with parental cells (*H*-score: 145). Meanwhile, both of T790M positive lines (HCC827EPR and HCC827 CNXR S1 cells) had higher PD-L1 expression (*H*-scores: 220 and 190, resp.) compared with parental cells. These results were confirmed using western blot analysis (Figure 2(f)).

Because previous studies have observed a relationship between PD-L1 expression and EGFR activation in lung cancers (but no data regarding EGFR-TKI resistance and PD-L1 expression) [15–18], we compared EGFR phosphorylation status between these cells. We observed that the acquired resistance cells with PD-L1 downregulation have decreased phosphorylation of EGFR (Y992 in HCC827ER cells and



(a) (b) (c) (d)

Figure 1: PD-L1 expression in parental lung cancer cell lines with *EGFR* mutations by IHC (Dako 22C3 antibody). (a) HCC827 showed positive staining (*H*-score: 145). (b) and (c) H3255 and PC-9 cells demonstrate negative staining. (d) SW900 lung squamous cells with positive staining. Images at 20x, captured with a Olympus DP71.

all EGFR phosphorylation sites in HCC827CNXRS4 cells). On the other hand, resistant cells with upregulated PD-L1 have increased phosphorylation of EGFR (Y992 and Y845 in HCC827EPR cells and Y1173 in HCC827CNXRS1 cells).

To generalize our findings in the HCC827 series, we employed two other cell lines (PC-9 and H3255 cells) that developed acquired resistance to EGFR-TKIs via T790 mutation (PC-9ER clone 5, BRC1, 853#10, and H3255XLR80 cells). As shown in Figure 3, three out of four cell lines had higher PD-L1 expression together with increased phosphorylation of EGFR compared to their parental cells. These data also indicated that no single phosphorylation site of EGFR is most responsible for increased PD-L1 expression. Only BRC1 cells showed similar EGFR phosphorylation and PD-L1 expression status compared with parental PC-9 cells.

3.3. Effect of IFN-Gamma Treatment on Parental and Resistant Cells. It has been established that cytokine signaling from the surrounding tumor microenvironment regulates PD-L1 expression in tumor cells [19]. To mimic an immune cell interaction, we treated HCC827 and their resistant daughter cell lines with IFN-gamma and measured PD-L1 expression. We observed that IFN-gamma treatment induced significant amount of PD-L1 protein in all cells examined (Figure 2(g)).

4. Discussion

In this study, we observed that cell lines with acquired EGFR-TKI resistance that harbor increased phosphorylation of EGFR, at any tyrosine residue, showed upregulation of PD-L1 protein expression. This finding is consistent with previous reports, which have observed that EGF stimulation upregulates PD-L1 expression or EGFR inhibition downregulates PD-L1 expression [15–18]. Each phosphorylation site of EGFR provides a binding surface for different substrate proteins, for example, GRB2 adaptor protein – Y1068 [20] or the SH2 domain of PLCgamma – Y992 [21]. However, all major downstream signaling pathways of EGFR, such as AKT serine/threonine kinase (AKT) – mechanistic target of rapamycin (mTOR) pathway [22], Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway [23], or mitogen-activated protein kinase 1 (MAPK1) pathway [17], are reported to induce PD-L1 expression. Therefore, it would be reasonable that increased phosphorylation of any tyrosine residue of the EGFR is correlated with increased expression of PD-L1.

Our results may also provide for a possible explanation for lower efficacy of current immunotherapies in lung cancer patients with an *EGFR* mutation. We observed that PD-L1 protein expression is not high in parental cells with *EGFR*

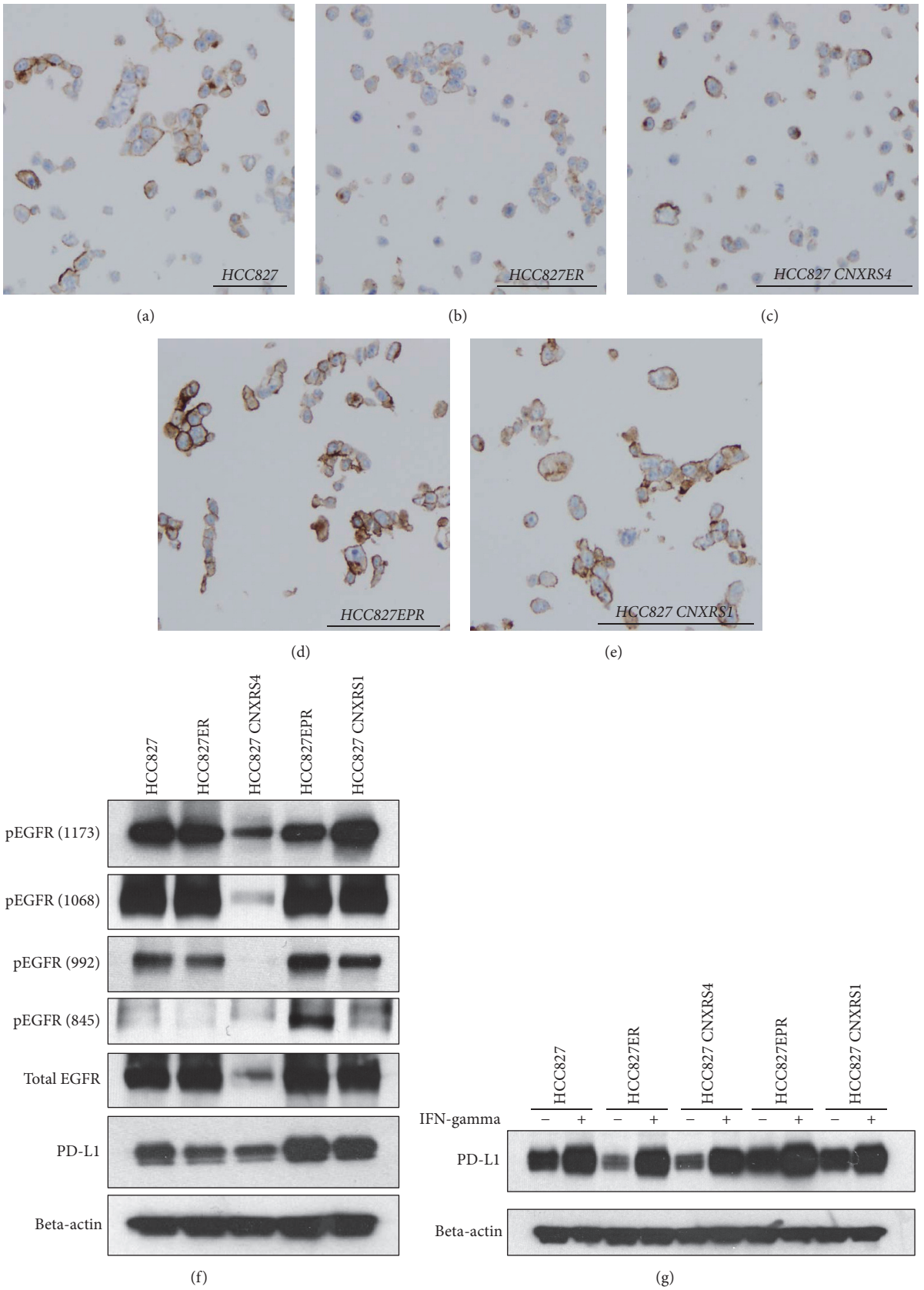


Figure 2: PD-L1 expression in parental HCC827 cells and their EGFR-TKI resistant daughter cells. (a)–(e) IHC staining for PD-L1 (Dako 22C3 antibody) in each cell line, showing decreased PD-L1 expression in HCC827ER and HCC827 CNXRS4 cells and increased PD-L1 expression in HCC827EPR and CNXR S1 cells. (f) Western blot analysis for PD-L1 and phosphorylation of EGFR. Beta-actin was used as loading control. (g) The effect of IFN-gamma exposure (100 U/ml, 24 hrs) for PD-L1 expression in HCC827 and acquired resistance daughter cell lines.

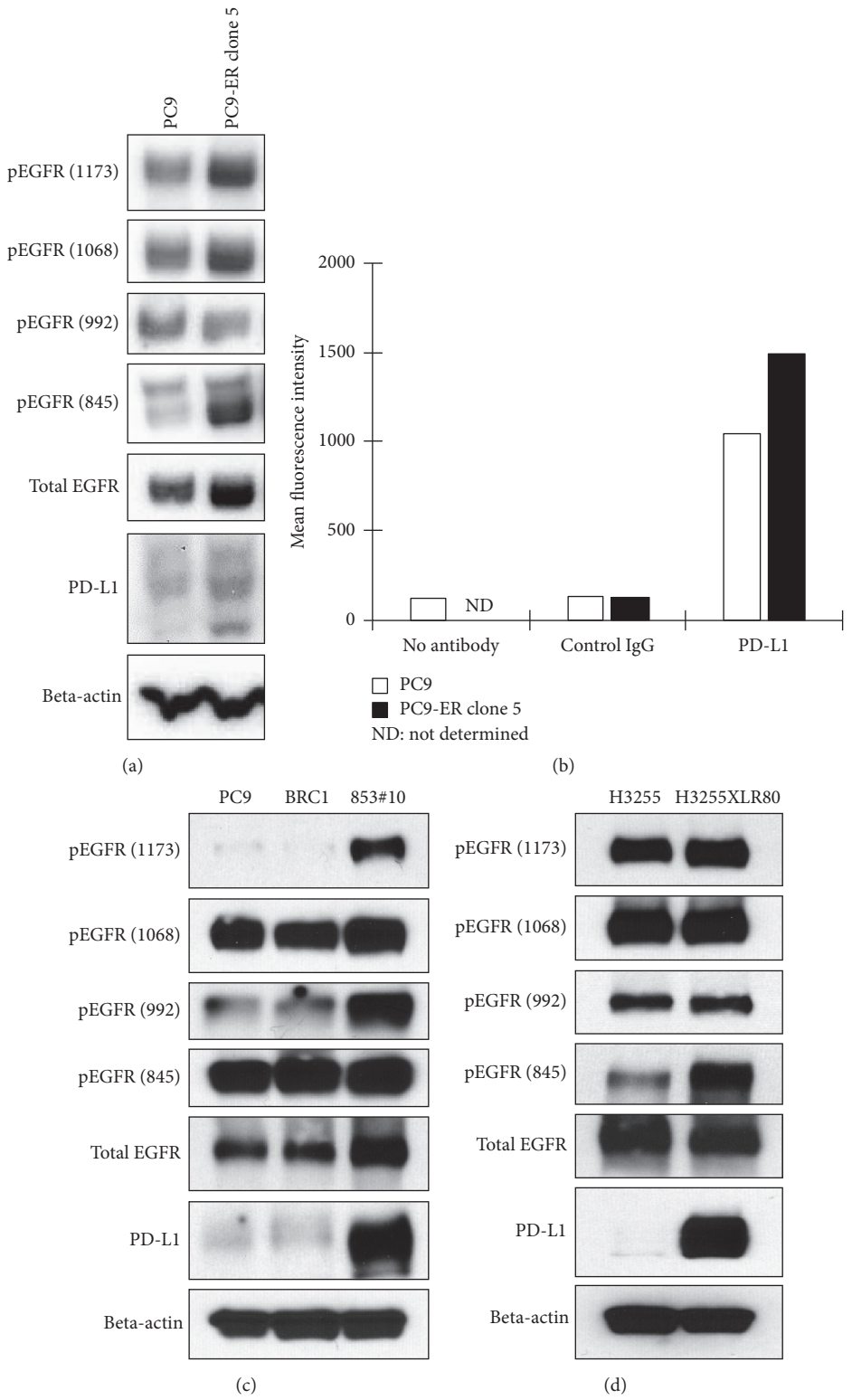


Figure 3: PD-L1 expression and EGFR phosphorylation status in H3255 and PC-9 cells that acquired resistance to EGFR-TKIs. (a) and (b) PC-9 ER clone 5 showed moderate increase of p-EGFRs together with slight increase of PD-L1 expression by western blotting (a) and flowcytometry (b). (c) The other series of PC-9 daughter cell lines resistant to EGFR-TKIs. BRC1 cells showed similar phosphorylation status of EGFR and PD-L1 compared with parental cells, while 853#10 showed dramatic increase of p-EGFR (Y992 and Y1173) and PD-L1. (d) H3255XLR80 also showed dramatic increase of p-EGFR (Y845) and PD-L1.

mutation, and the PD-L1 expression decreased when cells developed resistance to EGFR-TKIs by a non-T790M mediated resistance mechanism. In our previous study, we also found that EMT, another non-T790M mediated resistance mechanism to EGFR-TKIs, decreased PD-L1 expression in lung cancer cells with an *EGFR* mutation [11]. Although acquired resistance cells with increased EGFR phosphorylation (all of them harbored T790M mutation) showed higher PD-L1 expression, osimertinib monotherapy is the current standard treatment for these patients.

Oncogenic signaling within tumor cells and stimuli from the microenvironment both affect PD-L1 expression in tumor cells (constitutive and adaptive PD-L1 expression, resp.). Therefore, our current study looked only at half of the picture (constitutive PD-L1 expression only). However, our results are consistent with clinical findings that showed poorer response rates of PD-1/PD-L1 targeting agents in lung cancer patients with *EGFR* mutations, and may explain at least part of the reasons for the lower efficacy of these agents in these specific patients.

5. Conclusions

In summary, we showed that lung cancer cell lines with *EGFR* mutations do not have high PD-L1 protein expression by an FDA approved PD-L1 test. In addition, we demonstrated that PD-L1 expression changes dramatically after acquisition of resistance to EGFR-TKIs, and that was correlated with phosphorylation status of EGFR. Our data implies possible low PD-L1 expression in TKI-refractory lesions without T790M mutation, and that can be one of the molecular mechanisms that attenuates the efficacy of PD-1/PD-L1 targeting agents in lung cancer patients with *EGFR* mutations.

Conflicts of Interest

The authors declare that they have no conflicts of interest related to this study.

Acknowledgments

The study of PC-9 ER clone 5 was funded by Chugai Pharmaceutical Co., Ltd. The other study was supported by an IASLC Young Investigator Award 2015–2017 to K. Suda.

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