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Role of the Microenvironment in Gastrointestinal Tumors

Lead Guest Editor: Nathaniel Weygant
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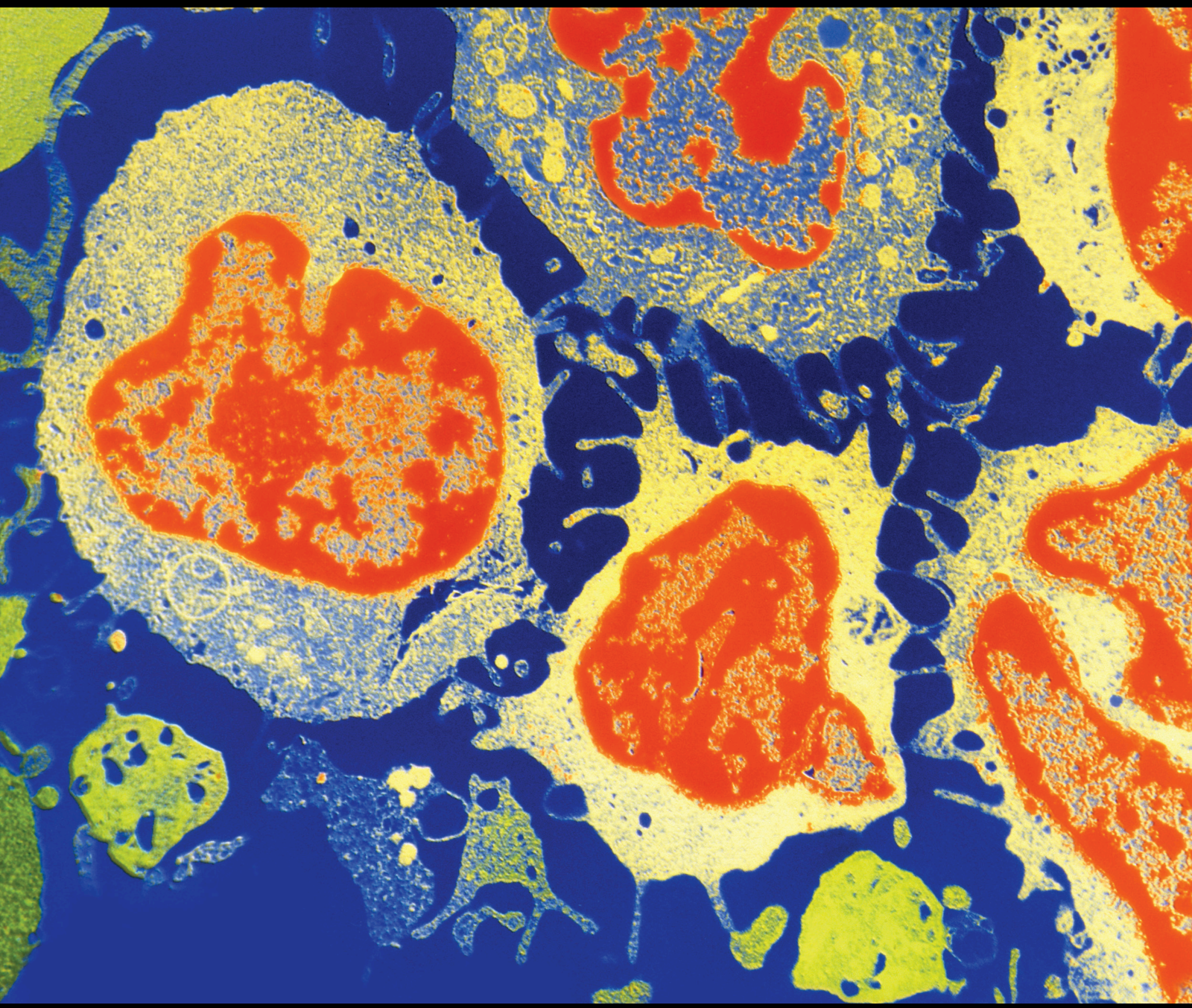

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Editorial Board

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Editorial

Role of the Microenvironment in Gastrointestinal Tumors

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The gastrointestinal (GI) tract maintains a complex environment with diverse epithelial and nonepithelial cell types that regulate discrete and distant processes. Pathogens such as *H. pylori* and inflammatory conditions such as colitis, Barrett's esophagus, and pancreatitis are linked to GI cancers, and recent studies have shown that a targeted mutation in a specific cell type with or without inflammation can be sufficient to initiate cancer. Recent studies using next-generation sequencing to determine molecular subtypes have expanded our understanding of GI cancers, and efforts to go beyond basic pathological assessment including multigene biomarkers, machine learning algorithms, and organoid drug screens are underway with promising results. For therapy, nucleoside analogs, targeted inhibitors, and monoclonal antibodies have demonstrated positive but often limited results in the clinic. New therapies such as anti-PD1 immunotherapy have thus far shown benefit in subsets of patients such as those with microsatellite instability-high tumors, but have not yet delivered the transformational results seen in other cancers. In order to develop effective new therapies for GI cancers and accurately classify patient risk, an improved knowledge of molecular signaling and cell-cell interactions within the tumor microenvironment (TME) is needed. Importantly, in the current clinical context, a more complete understanding of how TME components modulate resistance to therapy and antitumor immunity will be fundamental to improving patient treatment options and outcomes. This special issue seeks to improve understanding of the molecular, cellular, and pathological characteristics of the TME in GI cancers.

In the paper by D. Qu et al., differential activities of cancer stem cell marker doublecortin-like kinase 1's isoforms in pancreatic cancer are described. Importantly, they confirm previous findings that DCLK1 can be coimmunoprecipitated with KRAS and demonstrate that DCLK1 is capable of activating RAS. They support these findings with molecular, bioinformatic, and functional analyses of downstream pathways PI3K/AKT/MTOR and demonstrate the therapeutic use of DCLK1 monoclonal antibody using *in vivo* mouse models. These results further elucidate the functional mechanisms of an important GI CSC marker. C. He et al. also focused on pancreatic cancer and evaluated the effect of irreversible electroporation on immunologic characteristics in patients with locally advanced disease. Their clinical findings demonstrate a prognostic value for CD8+ T cells in this context. They conclude that this may have value as a prognostic tool in pancreatic cancer.

Md. N. Uddin et al., H. Sun et al., and C. Just et al. presented studies concerning the development of prognostic biomarkers for GI cancers. Briefly, Md. N. Uddin et al. used a meta-analysis procedure to develop a colon tumor stroma transcriptional signature. This signature was prognostic in CRC and CD8+ T cells, and prooncogenic signaling pathways were also enriched in colon tumor stroma. H. Sun et al.

investigated the prognostic potential for GLIS2 in gastric cancer. In the context of radiotherapy, low expression of GLIS2 predicted notable radiosensitivity, which might find use in improving the precision of gastric cancer radiotherapy. Whereas Md. N. Uddin et al. and H. Sun et al. utilized meta-analysis and mRNA expression to derive their respective signatures, and C. Just et al. opted to focus on small noncoding miRNAs. They performed a retrospective study of 33 patients receiving neoadjuvant chemotherapy for esophagogastric junction adenocarcinoma using a 96-well array of miRNAs with known malignant roles. They found differential expressions of Let-7f, miR-221, miR-31, miR-191, and miR-194 in this context. These findings could enable improved selection of esophagogastric adenocarcinoma patients for neoadjuvant therapy.

Finally, in a series of review articles, D. Ayers et al., C. Bazzichetto et al., A. Righetti et al., I.-H. Ham et al., E. Pretzsch et al., L. Figueroa-Protti et al., and V. Vautrot et al. cover various topics of emerging importance in the TME. These include cytokine and chemokine signaling, tumor-stromal interactions, specific, mechanisms of metastasis, epigenetic influences, exosomal miRNAs, and immune checkpoint.

Conflicts of Interest

The editors declare they have no relevant conflicts of interest.

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Nathaniel Weygant
Yang Ge
C. Benedikt Westphalen
Wen Wee Ma
Kenneth J. Vega

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Review Article

The Gastrointestinal Tumor Microenvironment: An Updated Biological and Clinical Perspective

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Gastrointestinal cancers are still responsible for high numbers of cancer-related deaths despite advances in therapy. Tumor-associated cells play a key role in tumor biology, by supporting or halting tumor development through the production of extracellular matrix, growth factors, cytokines, and extracellular vesicles. Here, we review the roles of these tumor-associated cells in the initiation, angiogenesis, immune modulation, and resistance to therapy of gastrointestinal cancers. We also discuss novel diagnostic and therapeutic strategies directed at tumor-associated cells and their potential benefits for the survival of these patients.

1. Introduction

Gastrointestinal (GI) cancers represent the most prevalent tumors worldwide and the major cause of death related to cancer. Within this group, we can identify colon, stomach, and liver cancers as the main concerns according to their prevalence (fourth, sixth, and seventh most prevalent, respectively). As a cause of death, stomach (second higher), liver (third higher), and colon cancer (fifth higher) are the main culprits [1]. Nonetheless, this group includes other mention-worthy cancers. Although not within the ten most prevalent, pancreatic ductal adenocarcinoma (PDAC) has one of the worst prognoses and is expected to be one of the major causes of death related to cancer by 2030 [2]. Additionally, the esophagus cancer is highly prevalent in some areas of the globe [1].

The treatment outcomes are also completely different amongst GI cancers. Two of the main reasons concern the timing of diagnosis and the therapeutic approach. As an example, colon and rectum cancers are usually diagnosed at early stages and are treated with surgery (colon cancer [3]), or multimodality treatment including chemoradiotherapy and surgery (rectum cancer [4]) with high rates of success, especially in the latter. Nonetheless, other tumors such as PDAC are usually diagnosed at later stages, when surgery approaches are usually no longer feasible. In these cases, the traditional treatment is based on combined chemotherapy [3–7], with 5 years below 5% [8].

The development of a prominent desmoplastic reaction by both local and distantly recruited stromal cells has been observed in GI cancers. In addition to immune cells, bone marrow- (BM-) derived progenitor cells are recruited to the tumor microenvironment (TMEN) where they differentiate into various stromal cells, such as endothelial cells, pericytes, and fibroblasts [9]. These cells are crucial for both malignization and cancer progression [10] and are frequently associated with poor prognosis [11–14]. Indeed, the interaction of cancer cells and the host microenvironment plays a critical role in strengthening the metastatic proficiency. Thus, a better understanding of the oncological

drivers of these tumors, including their interaction with the microenvironment, is of utmost importance [15, 16]. In this review, we will focus on the role of these tumor-associated cells in the tumorigenesis and progression of GI cancers, as well as on their role in treatment resistance and potential targeted therapeutic approaches.

2. BM-Derived Progenitor Cells

BM-derived cells (BMDCs) are constantly recruited to the TMEN, where they modulate tumor growth and metastasis through the regulation of angiogenesis, inflammation, and immune suppression [17]. Several studies in animal models have implicated BMDCs in the development of carcinomas of the upper GI tract [18, 19], including gastric cancer (GC) [20]. BMDCs were shown to home and repopulate the gastric mucosa in response to *H. pylori* chronic infection, leading to the development of metaplasia, dysplasia, and cancer over time [20]. In another study, BMDCs were found to be about 25% of *H. pylori*-induced dysplastic lesions in a mouse model [21]. Zhang et al. showed that highly metastatic colorectal carcinoma (CRC) cells produce elevated serum levels of OPN, MMP9, S100A8, S100A9, SAA3, and VEGFA. This promoted the setup of hepatic TMENs supportive of metastasis by BMDCs recruitment to this organ [22]. Bone marrow-derived $CD34^+$ $CD31^-$ immature myeloid cells were also found to cluster at the invasion front of CRC in cis-Apc^+/D716 $Smad4^{\pm}$ mutant mice. These immature myeloid cells expressed Metalloproteinases (MMP) 9 and MMP2 and supported tumor invasion at early stages in intestinal adenocarcinomas [23].

Bone marrow-derived mesenchymal stem cells (MSCs) constitute a nonhematopoietic stem cell subpopulation that can populate the TMEN and contribute to tumor growth and progression through paracrine signaling [24]. Data from Beckermann et al. suggested a supportive role of MSCs in angiogenesis [25]. In this study, MSCs display increased vascular endothelial growth factor (VEGF) mRNA expression and protein secretion. They were also found to migrate towards tumor blood vessels of PDAC, *in vitro* and *in vivo*, in response to tumor-secreted growth factors. This is reinforced *in vivo* in an orthotopic mouse model of PDAC, where siRNA directed towards VEGF induces loss of vessel density control by MSCs [25].

Hypoxia can also induce the expression of growth factors that act as chemoattractants for MSCs to the TMEN [26–29]. In fact, the expression of VEGF by MSCs was shown to increase upon stimulation by interferon- (IFN-) γ and tumor necrosis factor- (TNF-) α cytokines through a hypoxia-induced factor- (HIF-) 1α-dependent pathway. This promoted angiogenesis and tumor growth in mice bearing CRC [30].

Some reports also implicate MSCs in tumor progression and metastasis. For instance, S100 Calcium Binding Protein A4 (S100A4) secreted by MSCs isolated from patient-derived hepatocellular carcinoma (HCC) tissues upregulated the expression of miR155 in HCC cells, promoting tumor invasion through the suppressor of cytokine signaling 1- (SOCS1-) MMP9 axis [31]. Exosomes, extracellular vesicles of endosomal origin, can mediate transfer of biomolecules both locally and at distance, playing a key role in the setup of TMENs [32, 33]. For instance, MSCs-derived exosomes supported GC lymph node metastasis and venous invasion by transferring miR-214, miR-221, and miR-222, regulators of the tumor suppressor gene Phosphatase and Tensin Homolog (*PTEN*), to cancer cells [34].

Bone marrow-derived MSCs also have the ability to differentiate into several cell types in the stroma [24], including fibroblasts [35]. Spaeth et al. showed the propensity of MSCs to transition to a tumor-associated fibroblast-like phenotype in ovarian, breast, and PDAC-xenografted tumors. These fibroblast-like cells ultimately contributed to microvascularization and the production of tumor-stimulating paracrine factors [36]. MSCs also favor primary CRC cells metastasis to the liver [37]. Orthotopic transplantation of cancer cells mixed with MSCs (but not cancer cells on their own) into the cecal wall resulted in macroscopic liver metastasis. Interestingly, metastasized colon cancer cells recruited more MSCs to the secondary sites where these were found to differentiate into supporting fibroblast-like cells [37]. Altogether, these results illustrate the role of MSCs in the development of tumor-supporting microenvironments.

Hematopoietic stem cells (HSC) constitute a subpopulation of BM resident cells endowed with long-lived self-renewal and multipotency that sustain the generation of all cells of the blood and immune system. The HSC niche is tightly regulated by osteoclasts and vascular cells within the BM compartment, contributing for the maintenance of a quiescent microenvironment and controlled differentiation [38, 39]. However, tumor-derived soluble factors are able to systemically induce a BM microenvironment switch, from quiescent to protumorigenic and proangiogenic, and stimulate HSC mobilization into the circulation and recruitment to the tumor [39]. These cells can indirectly modulate tumor growth through their ability to differentiate into myofibroblasts and inflammatory cells in the tumor environment.

3. Cancer-Associated Fibroblasts (CAFs)

Fibroblasts are normal components of the connective tissue. These spindle-shaped cells are the main nonepithelial and nonimmune cell elements found in the interstitial space, embedded in physiological extracellular matrix (ECM) [40]. Resident fibroblasts of healthy tissues are considered to be in a resting or quiescent state and are characterized by low metabolic and synthetic activities. In the physiological wound healing process, resting fibroblasts become activated, gaining contractile properties and becoming synthetically dynamic [41, 42]. As the wound closes and evolves into a scar, apoptosis of the activated fibroblasts (myofibroblasts) leads to a significant decrease in their numbers [43]. The inability of myofibroblasts to undergo apoptosis is the driving factor in the development of fibrotic diseases and contributes for the maintenance of other pathological conditions, such as chronic inflammation [42].

In oncologic settings, fibroblasts are frequent components of the TMEN and play an important role at all stages of cancer progression through their phenotypic plasticity and ability to secrete a wide range of signaling molecules.

Next, we emphasize how these cells play a key role in generating tumor-promoting microenvironments in GI cancers.

3.1. Role of CAF in Cancer Initiation and Growth. Multiple studies have highlighted the important role of CAFs in the process of cancer initiation and progression. For example, the abundance of myofibroblasts in CRC-associated stroma is predictive of postsurgery disease recurrence [44]. It has also been suggested that the loss of transforming growth factor- (TGF-) β inhibitory effect leads to the activation of hepatocyte growth factor- (HGF-) mediated cell-cycle regulation and stimulation of epithelial proliferation, promoting invasive squamous cell carcinoma of the forestomach in the $Tgfbr2^{fspKO}$ knockout mice [45]. In addition, the conditional knockout of the TGF-β type II receptor gene in mouse fibroblast-specific protein 1- (FSP1-) positive fibroblasts revealed that TGF-β signaling modulates the proliferation and oncogenic potential of epithelial cells [45].

Recently, it has also been demonstrated that CAFs-secreted HGF regulates liver tumor-initiating cell plasticity through the activation of Tyrosine-Protein Kinase Met/Fos-Related Antigen 1/Hairy and Enhancer of Split-Related Protein 1 (c-Met/FRA1/HEY1) signaling. The activation of this signaling pathway was associated with fibrosis-dependent development in HCC *in vivo* [46]. CAFs-derived HGF was also shown to promote a stemness phenotype in CRC cells [47]. In another study, the deletion of Liver Kinase B1 (Lkb1) gene in stromal fibroblasts resulted in penetrant polyposis in mice, underscoring the involvement of these cells in the tumorigenesis of GI Peutz–Jeghers syndrome. Further analysis revealed that Lkb1 loss induces interleukin-(IL-) 11 expression in gastric fibroblasts and subsequent activation of the Janus Kinase/Signal Transducer and Activator of Transcription 3 (JAK/STAT3) pathway in tumor epithelia, promoting GI tumorigenesis [48].

Emerging data also suggest the switch from normal quiescent fibroblasts into an activated phenotype through epigenetic modifications [49–51]. *Helicobacter pylori* infection, one of the major causes of GC, was shown to induce the secretion of Prostaglandin E2 (PGE2) by gastric epithelial cells. The stromal PGE2 silenced miR-149 through hypermethylation, removing the suppression of its target genes, IL6 and PGE2 receptor 2. This led to elevated IL6 levels that stimulated the stem-like properties of GC cells [49].

3.2. Role of CAF in EMT, Extracellular Matrix Remodeling, and Metastasis. CAFs-mediated signaling also participates in the acquisition and maintenance of cancer cell stemness. One of the common concepts associated with metastasis initiation is epithelial-to-mesenchymal transition (EMT), that is, the process by which cells lose their epithelial characteristics (such as cell-to-cell adhesion and planar and apical polarity) to acquire mesenchymal features (such as motility and invasiveness) [52]. Paracrine signaling through TGF-β between CAFs and cancer cells leads to EMT-driven gain of stemness and metastasis initiation [53, 54]. In fact, targeting CAFs with curcumin reverted the EMT phenotypes of PDAC cells blocking their migration and metastasis [55]. In HCC, myofibroblasts can induce EMT in a TGF-β/platelet-derived growth factor- (PDGF-) dependent manner [56]. Likewise, IL-6 produced by fibroblasts can also activate JAK2/STAT3 signaling in the GC cells promoting their migration and EMT [57]. The miRNA 320a can also affect EMT by decreasing PBX Homebox 3 (PBX3), Extracellular Signal-Regulated Kinase 1 and 2 (ERK1/2) signaling, and N-cadherin expression, and simultaneously increase E-cadherin. When transferred from CAFs to HCC tumor cells via exosomes, this miRNA can inhibit tumor proliferation, migration, invasion, and metastasis. Interestingly, CAF-derived exosomes from HCC patients contain reduced levels of miR-320a, showing how the reduction of an antitumor factor in these vesicles can affect metastasis [58]. Cancer cell-derived exosomes can also reprogram normal adjacent fibroblasts into CAFs. For example, a recent study showed that exosomes derived from early- or late-stage CRC cell lines induce the activation of quiescent fibroblasts into distinct functional subtypes [59]. Specifically, the activation mediated by late-stage cancer exosomes resulted in a proinvasive profile, while fibroblasts activated by early-stage cancer exosomes presented a pro-proliferative and proangiogenic phenotype [59].

Fibroblasts can also remodel the microenvironment and lay the tracks for cancer cell invasion through the surrounding ECM and stromal cell layers [60–62]. For instance, CAF-derived transgelin (TAGLN) induces MMP2 expression and promotes migration and invasion of GC cells [63], while CAF-derived fibroblast activation protein (FAP) remodels the ECM and promotes PDAC cell invasion [64]. In HCC, CAFs secrete different cytokines that activate the hedgehog (C-C motif chemokine 2 (CCL2) and 5 (CCL5) and TGF-β (CCL7 and C-X-C motif ligand (CXCL) 16) pathways in HCC cells, inducing their migration and invasion *in vitro* and metastasis *in vivo* [65]. In addition, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) secreted by cancer-associated MSCs, a subpopulation of CAFs isolated from human PDAC, induced proliferation, invasion, and transendothelial migration of PDAC cells [66].

Colonization is probably the most limiting of all metastasis steps and the microenvironment at the distant sites needs to be favorable for this to happen. Paget's seed and soil theory back in 1889 was the first to suggest that metastasis to a certain organ is not random but depends on interactions with its microenvironment and that cancer cells will seed only in fertile soils [67]. The concept of "pre-metastatic niche" was introduced later in 2001 by Kaplan et al., where they showed that BM-derived cells can form clusters that home to tumor-specific premetastatic sites [67, 68]. In line with this theory, metastatic cancer cells are capable of bringing their own "soil" to the metastatic site in order to facilitate their colonization [69]. A good example is the case of IL11 production by TGF-β-stimulated CAFs, which activate Glycoprotein 130 (GP130)/STAT3 signaling in CRC cells conferring them the survival advantage for efficient organ colonization [70].

3.3. Role of CAF in Angiogenesis. CAFs tailor tumor growth and progression not only by influencing tumor cells but also by indirectly affecting other stromal cells and regulating angiogenesis, inflammation and immune modulation [10]. CAFs are capable of promoting angiogenesis by secreting VEGF and Stromal Cell-Derived Factor 1 (SDF-1) [71, 72]. For example, in a mouse model of GC, α-Smooth Muscle Actin- (α-SMA-) positive fibroblasts were the main producers of VEGF. Activation of these fibroblasts was stimulated by GC cells and shown to increase tube formation by endothelial cells *in vitro* [73]. CAFs are also a source of IL6 in CRC, which in turn can increase VEGF secretion by adjacent fibroblasts and induce tumor angiogenesis in xenografted cancer cells [74]. Pancreatic fibroblasts, also known as pancreatic stellate cells (PSCs), express several proangiogenic regulators such as VEGF receptors, angiopoietin-1, and Tie-2 and produce VEGF in response to hypoxia. Conditioned medium from hypoxia-induced PSCs was able to increase proliferation, migration, and angiogenesis of Human Umbilical Vein Endothelial Cells (HUVECs) both *in vitro* and *in vivo* [75]. In a similar line, hepatic stellate cells (one of the main players in HCC) can produce VEGF and angiopoietin-1 in hypoxic conditions and induce angiogenesis [76, 77].

4. Endothelial and Perivascular Cells

4.1. Role of Endothelial and Perivascular Cells in Tumor Growth. The angiogenic switch is a hallmark of cancer that allows for tumor growth by providing nutrients and oxygen and removing cellular wastes [10]. The establishment of new blood vessels is a crucial step for tumor progression, but the endothelial and perivascular cells that constitute these blood vessels are not just mere bystanders in the game. Endothelial cells (ECs) can promote a cancer stem cell phenotype in human CRC *in vitro* and in cocultured CRC cells ex vivo [78]. Lu et al. showed that CRC stemness is induced through paracrine activation of *Notch* signaling, whereby membrane-bound Jagged-1 on ECs is cleaved by ADAM metalloproteinase domain 17 (ADAM17), releasing a truncated soluble fragment that binds Notch on CRC cells. Importantly, both primary and chemotherapy-naïve liver metastatic CRC liver showed $CD133^+$ epithelial cells located in the proximity to perivascular regions, further supporting an ECs-mediated role in the CRC stem cell phenotype in clinical specimens [78]. A subsequent study found that ECs contributed to chemoresistance in CRC cells via serine/threonine-protein kinase- (AKT-)mediated induction of Nanog Homeobox Retrogene P8 (NANOGP8) [79]. Likewise, in an *in vitro* model of Hepatitis B Virus- (HBV-) induced HCC, increased levels of TGF-β in the conditioned medium of HUVECs boosted the expression of mesenchymal markers, including CD133, and promoted an aggressive phenotype in stimulated Hepatitis B-X Protein- (HBx-) infected hepatoma cells [80]. Immunization of mice with glutaraldehyde-fixed HUVECs resulted in reduced expression of angiogenesis-related antigens (VEGF-2 and vascular endothelial- (VE-) cadherin), suppression of angiogenesis, and smaller esophageal squamous carcinoma (ESC) tumors [81]. This has prompted the evaluation of HUVEC vaccines promoting tumor autoimmune response targeting angiogenesis in pilot trials involving patients with metastatic CRC [82].

Lymphatic endothelial cells (LECs) are also important players in the growth of GI cancers. Expression of Growth Differentiation Factor 11 (GDF11) was increased in CRC patients and positively correlated with tumor grade. GDF11 released in LEC-derived exosomes was also identified as a key modulator of CRC growth *in vitro* and *in vivo* [83]. Moreover, increased proliferation and invasive ability of ESC cells *in vitro* has been demonstrated upon stimulation with conditioned medium from ESC-related lymphatic microvessel endothelial cells. This interaction leads to the upregulation of MMP9 expression and downregulation of Tissue Inhibitor of Metalloproteinases 2 (TIMP-2) expression in poorly differentiated ESC cells and promotes both lymphangiogenesis and growth of these cells *in vivo* [84]. Lymphatic endothelial cells also have an immunoregulatory function in GC by inhibiting the production of IL2, IL10, and IFN-γ cytokines in $CD4^+$ T cells. Coculturing GC cells with both LECs and $CD4^+$ T cells resulted in the upregulation of Programmed Death-Ligand 1 (PD-L1) and Indoleamine 2,3-Dioxygenase (IDO) mRNA expression. This suggests a possible mechanism of cancer immune tolerance and metastatic spread through the lymphatic vessels in GC [85].

Additionally, the development of diffuse-type GC depends on the inflammation mediated by $CXCL12^+$ ECs and C-X-C motif receptor (CXCR) 4^+ gastric innate lymphoid cells (ILCs) that form the perivascular gastric stem cell niche. Endothelial CXCL12 plays a central role recruiting Wnt Family Member 5A- (Wnt5a-) producing $CXCR4^+$ ILCs to the stomach, which in turn activates Ras Homolog Family Member A (RhoA), inhibits anoikis in the E-cadherin-depleted cells and promotes diffuse-type GC growth [86].

4.2. Endothelial and Perivascular Cells Role in Tumor Metastasis. Blood vessels are also a gateway for distant metastasis. The lack of vascular maturation in newborn vessels facilitates cancer cell penetration and promotes distant metastasis. Immunohistochemical analysis of tissue samples from CRC patients revealed a significant correlation between lower microvessel pericyte coverage with increased hematogenous metastasis and poorer survival [87].

The importance of these vascular networks is underscored by the fact that cancer cells undergoing EMT are able to assume the identity and role of pericytes to stabilize the tumor vasculature and improve the vascular support for tumor growth [88]. Shenoy et al. found that the majority of cancer cells undergoing EMT were located preferentially in the perivascular space and were closely associated with ECs and in line with the blood vessels in tumor xenografts. It was further demonstrated that these cells expressed pericyte markers and interacted with ECs, stretching alongside Human Microvascular Endothelial Cells (HMVECs) and exhibiting tight adhesion to EC tubes in a coculture assay *in vitro* [88]. This phenomenon, known as vasculogenic mimicry (VM), whereby cancer cells form *de novo* vascular

networks without the involvement of ECs, represents an alternative paradigm of tumor perfusion in HCC and GC [89–91].

Exosomes are also key mediators in this setting, as they mediate vascular permeability and angiogenesis. Zeng et al. found that CRC-derived exosomal miR-25-3p can be transferred to ECs, where it targets and silences Krüppel-Like Factor 2 (KLF2) and 4 (KLF4). *In vivo*, exosomal miR-25-3p elicited vascular leakiness and promoted CRC metastasis [92]. Exosome-mediated remodeling of the lymphatic network in sentinel lymph nodes was also shown to promote CRC metastasis. The uptake of CT26-derived exosomes by macrophages induced the release of VEGF-C, mediated by exosomal Interferon Regulatory Factor 2 (IRF-2), and promoted lymphangiogenesis in sentinel lymph nodes, which facilitated the development of CRC metastasis [93]. Upregulated secretion of CXCL1 by LECs can also promote migration, invasion, and adhesion of GC cells through the activation of integrin β1- (Focal Adhesion Kinase) FAK-AKT signaling. Activation of the latter induced the expression of MMP2 and MMP9 and increased lymph node metastasis in an animal model of GC [94].

When cancer cells enter the systemic circulation, they get exposed to the harsh circulating conditions. Together with the absence of cell/ECM interaction, apoptosis signaling is triggered and cancer cells undergo rapid anoikis [95]. A recent study in head and neck carcinoma showed that cancer cells bound to Bcl-2 overexpressing ECs (EC-Bcl-2) via E-selectin presented significantly higher anoikis resistance. Additionally, mice coinjected with squamous cell carcinoma cells and EC-Bcl-2 displayed significantly higher lung metastasis [96]. The described chaperoning role of ECs could, in principle, also occur in GI cancers, as increased levels of circulating ECs have also been observed in patients with colon, gastric, and esophagus cancers [97].

5. Tumor-Infiltrated Immune Cells

Infiltrating immune cells from lymphocytic and myeloid origin are also constituents of the TMEN [10]. Lymphocytes are composed by three main lineages that originate from a common precursor identified in the BM: natural killer (NK) cells, T cells, and B cells. T cells are subject to a final lineage decision in the thymus to form mature CD4 (helper) and CD8 (cytotoxic) T cells [98]. NK and CD8 T cells can rapidly degranulate and secrete IFN-γ following antigen receptor triggering, which is particularly important in antitumor responses [99]. T regulatory Cells (TRegs) can contribute to homeostasis by inducing immunotolerance and control of autoimmunity. These cells are derived from the thymus, express CD4, CD25, and Forkhead Box P3 (FOXP3, murine Foxp3) and are capable of inhibiting immune responses mediated by $CD4^+CD25^-$ and $CD8^+$ T cells [100–102].

Myeloid cells comprise polymorphonuclear cells or granulocytes, dendritic cells (DCs), monocytes, and macrophages (extravasated blood monocytes) [103, 104]. Macrophages can display a broad spectrum of activation and polarization states [105, 106]. However, in more general and simplistic models, they are frequently classified as M1, that takes part in type I T helper (Th1) cell responses when stimulated by IFN-γ and is characterized by release of radical oxygen species (ROS), nitric oxide (NO), and proinflammatory cytokines such as TNF-α and IL12; M2, involved in type II T helper (Th2) cell responses and identified by expression of arginase and release of anti-inflammatory cytokines such as IL10 when stimulated by IL4 and IL13 [107].

Myeloid-derived suppressor cells (MDSC) comprise another population of myeloid progenitor and immature myeloid cells endowed with the ability to inhibit T-cell responses [108–110]. DCs were also reported as activators of specific T cells during inflammatory responses and play a central role in protection against infection and malignancy [111, 112]. DCs can also display multiple profiles. Monocyte-derived DCs can perform marked tumor antigen uptake. cDC1s can activate $CD8^+$ T cells, while cDC2s can reprogram protumoral macrophages when injected in mouse models. Importantly, the analysis of tumor biopsies from colorectal cancer patients revealed the presence of all the three abovementioned subsets of tumor-associated DCs [113]. For these reasons, myeloid cells play a pivotal role in the regulation of immune cell responses.

Tumor inflammation has a paradoxical role in promoting tumor growth and progression [10]. Some reports show the association between unresolved infection, chronic inflammation, and tumor initiation. Examples are the relationships between infection by *Helicobater pylori* and GC, chronic pancreatitis and PDAC, and ulcerative colitis and CRC [114–117]. In this section, we describe how immune cells contribute to TMENs in GI cancers.

5.1. Immune Surveillance Evasion. Although increased infiltration of tumors by immune cells has long been thought to be a consequence of failed attempts to eliminate cancer cells, recent studies show that cancer cells can frequently evade immune responses [10]. An important mechanism of immune evasion involves PD-L1/Programmed Death-1 (PD-1) that has been linked to T-cell apoptosis [118]. For example, HCC-derived IL-10 can increase expression of PD-L1 by Kupffer cells, which in turn can decrease the antitumor function and proliferation of $CD8^+PD\text{-}1^+$ cells [119]. In the same line, $PD\text{-}L1^+$ monocytes infiltrates can suppress antitumor T-cell responses and contribute to tumor growth *in vivo* [120]. In both cases, PD-L1 correlated with poor survival in HCC patients and could be targeted by anti-PD-L1 antibodies [119, 120]. CXCL12 produced by FAP^+CAFs has been linked to immune evasion in PDAC, and targeting this cytokine can synergize with anti-PD-L1 immunotherapy in PDAC [121, 122].

In two complementary studies, Wang et al. and Kortylewski et al. demonstrated how the constitutive activation of Stat-3 in cancer and hematopoietic-derived tumor infiltrating cells could inhibit the maturation of DCs, leading to a defective antitumor immune response. In fact, Stat-3 inhibition enhanced the antitumor function of T and NK cells, DCs, and innate immunity against tumors [123, 124]. Furthermore, in a small cohort of patients and healthy control

subjects, Yanagimoto et al. have shown that the numbers and function of DCs were reduced in PDAC patients [125].

PDAC and CRC cells can also evade immune response by expressing an apoptosis-mediating surface antigen FAS (Fas) receptor, which enables these cells to resist Fas-mediated apoptosis and, at the same time, to increase the expression of Fas ligand (FasL), which mediated the killing of T cells in coculture assays [126–128]. The upregulation of FasL was also demonstrated in GC together with downregulation of caspase-3 as an immune escape mechanism [129, 130]. Another strategy by which PDAC cells could escape immune response was through the upregulation of IDO [131], a tryptophan-degrading enzyme that induces an anergic state in T cells through tryptophan starvation [132]. In addition, the presence of Th2 lymphocytes also correlates with reduced PDAC patient survival. Thymic Stromal Lymphopoietin (TSLP), which induces Th2 polarization, was found to be secreted by CAFs after stimulation by TNF-α and IL1β [133].

Immunosuppressive cells such as MDSC and TRegs are elevated in patients with PDAC, esophageal, and GC when compared with controls, being considered an independent prognostic factor for survival in all these three cancers [134]. In fact, by studying a small cohort of patients, Porembka et al. have demonstrated that human PDAC show an increased infiltrate of MDSC when compared with normal pancreatic tissue. Moreover, depletion of these cells in an animal model of PDAC resulted in delayed tumor growth [135]. MDSCs were also increased in HCC patients and could induce the formation of TReg populations, suggesting this as one of the mechanisms responsible for immunosuppression in HCC [136]. Increased populations of TRegs were also found in the blood of patients with gastric and esophageal cancers [137]. In addition, TGF-β1 produced by GC cells was shown to induce TReg development from $CD4^+CD25^-$T cells, and high levels of this factor correlated with elevated TReg numbers in GC patients [138]. Interestingly, Mizukami et al. suggested that the localization pattern rather than the numbers of TRegs might have a higher impact in survival of GC patients. They found that a diffuse pattern of TRegs accounts for poorer survival than peritumoral localization of these cells [139]. TRegs of HCC patients were also capable of impairing the function of $CD8^+$ T cells by decreasing their proliferation, activation, degranulation, and production of enzymes such as granzymes A and B and perforin when stimulated. Not surprisingly, TRegs were associated with higher mortality in these patients [140]. Both CRC- and HCC-associated fibroblasts were also shown to impair NK-cell antitumor cytotoxicity by releasing molecules such as PGE2 [141, 142].

5.2. Immune Cells Role in EMT, Invasion. and Metastasis. In addition to immune surveillance evasion, infiltrated immune cells also promote invasive phenotypes in cancer cells through EMT. For example, PDAC cells increase the conversion of blood monocytes into monocytic MDSC, which in turn act to promote EMT features in these cancer cells [143]. Using *in vitro* coculture studies, Liu et al. have shown that the promotion of the M2 phenotype in macrophages induces the expression of mesenchymal markers in PDAC cell lines *in vitro* and that this effect was dependent on Toll-Like Receptor 4 (TLR4) and IL10 levels [144]. M2 macrophages were also capable of inducing GC invasiveness via activation of the β-catenin pathway [145].

Kim et al. suggested that myofibroblasts can induce the differentiation of myeloid cells into S100A8/9-expressing MDSC and M2 macrophages in CRC by secreting IL-6 and IL-8 [146]. In a mouse model of esophageal cancer, recruitment of MDSC was correlated with IL-6 levels and tumor invasiveness, IL-6 being shown to induce the MDSCs. In fact, IL-6 and MDSC levels predicted prognosis in patients with esophageal cancer [147]. In addition, CAFs and M2 macrophage markers predict clinical outcome in CRC, their expression being inversely correlated with survival [148]. Similarly, CAFs isolated from PDAC patients promoted M2 macrophage polarization that in turn promoted the proliferation of PDAC cells *in vitro* and tumor growth and invasion *in vivo* [149]. Polarized $CD163^+$ (M2) macrophages were also correlated with increased angiogenesis, CXCL12 expression, and tumor progression in GC [150].

The recruitment of immune cells to secondary metastatic sites, and their role in promoting a receptive soil for metastatic growth, has also been the focus of some recent studies. For example, PDAC-derived exosomes containing macrophage migration inhibitory factor- (MIF-) induced TGF-β1 production by Kupffer cells, which induced α-SMA and fibronectin expression by hepatic stellate cells. This supported the influx of BM-derived monocytes, which constituted a liver TMEN supportive of PDAC metastasis [151]. In fact, PDAC liver metastasis depends on the early recruitment of granulin-secreting inflammatory monocytes to this organ. Granulin secretion by metastasis-associated macrophages activates resident hepatic stellate cells into myofibroblasts, which in turn secrete periostin, resulting in a fibrotic microenvironment that sustains metastatic tumor growth [152]. Seubert et al. also demonstrated an increased liver susceptibility towards metastasis through SDF-1-mediated recruitment of neutrophils to the liver. In this study, systemic TIMP-1, which was previously thought to suppress tumor metastasis, was instead driving the increased levels of hepatic neutrophil chemoattractant SDF-1 [153].

6. Stromal Signatures as Prognostic Tools

Based on the significance of stromal cells in tumor growth and progression, much effort has been done on the identification of stromal signatures of cancer prognosis. For example, tumor lymphocyte infiltrates (TIL), such as $CD4^+$/$CD8^+$ T and NK^+ cells, have been generally associated with a good prognosis. On the other hand, infiltration by TRegs, MDSC, M2 macrophages, and CAFs has been seen as a sign of disease progression and poor prognosis, as listed in Table 1.

However, conflicting evidence has shown that increased infiltration of gastric and gastroesophageal tumors by $CD8^+$ T cells was actually associated with a worse prognosis. In fact, patients with high CD8 infiltration also presented PD-L1 expression, which was linked to immune resistance [160].

Table 1: Clinical significance of stromal cells in GI cancers.

Stromal cell	Type of cancer	Levels	Clinical outcome	References
CAFs	GC	High	Metastasis	[154, 155]
PD-L1	PDAC	Expression	Poor prognosis	[156]
PD-L1	GC	Expression	Poor prognosis	[157–159]
PD-L1 and $CD8^+$ T cells	GC/gastroesophageal	High	Lower survival	[160]
PD-L1 and PDL2	Esophageal	Expression	Poor prognosis	[122, 161]
M2 macrophages	PDAC	High	Lymphatic metastasis/poor prognosis	[162]
$CD204^+$ (M2) macrophages	Esophageal	High	Poor DFS	[163]
M2 macrophage and %TRegs	PDAC	High	Lower survival	[155]
CAFs and M2 macrophages	CRC	Expression	Reduced survival	[148]
MDSC	PDAC	High	Progression	[164]
MDSC	GC/PDAC/esophageal	Low	Survival	[134]
Th2	PDAC	Presence	Reduced survival	[149]
TRegs	PDAC	High	Progression/poor prognosis	[165–168]
$TCD3^{low}/TReg^{high}$	CRC	Low/high	Lower survival	[169]
TRegs	CRC	High	Good prognosis	[170, 171, 172]
TRegs	HCC	High	Progression	[140, 173]
$TRegs^{low}/CD8^+$ TIL^{high}	HCC	Low/high	High DFS	[140, 173]
$TRegs^{high}/CD8^+$ TIL^{low}	GC	Ratio high	Lower survival	[174]
TRegs	GC and esophageal	High	Poor survival	[175]
DC and circulating myeloid DC	PDAC	High	Survival	[176]
$CD4^+/CD8^+$ TILs	PDAC	High	Good prognosis after surgery	[177]
$CD4^+/CD8^+$ TILs	Esophageal	High	Good prognosis	[178]
$CD8^+/CD45RO^+$ TILs	CRC	High	Good prognosis	[178, 179]
$CD4^+/CD8^+/CD45RO^+$ TILs	GC	Low	Lymph node metastasis/lower survival	[180]
$CD8^+$ and $FoxP3^+$ TILs	GC (microsatellite unstable)	High	Good prognosis	[181]
M2 macrophages + $CD8^+$ and $FoxP3^+$ TILs	GC (microsatellite unstable)	High	Survival	[182]
M2 macrophages	GC	High	Poor survival and tumor progression	[145, 150]
NK^+ cells	GC	High	Good prognosis	[183]

DFS: disease-free survival.

On the other hand, microsatellite unstable GC patients with high $CD163^+$ (M2) macrophages, $FOXP3^+$, and $CD8^+$ TILs where those with the highest survival advantage [182]. In CRC, as opposite to other cancer types, $FOXP3^+$ TRegs were associated with good prognosis [170, 171]. These are examples on how immune cell signatures are context-dependent and how the complexity of cell interactions and soluble factors released in the TMEN can tip the balance in opposite directions.

Given the diversity of switch mechanisms driving CAFs activation, one can expect to have CAFs with different activated phenotypes in the tumor stroma. Another question is whether all CAFs are in an activated state. Fibroblast plasticity and intratumoral heterogeneity results in an array of CAF signatures associated with different tumor types [184]. Several proteins have been used as markers for the identification of CAFs. Some of the most commonly used biomarkers include PDGFRα/β, α-SMA, and FAP. In addition, FSP1 has been suggested as a marker of fibroblasts in a quiescent state [185, 186]. Other proteins, such as vimentin, desmin, discoidin domain receptor 2 (DDR2), and podoplanin, have also been used in the identification of CAFs [185]. However, it is important to highlight that these proteins are also expressed by other cell types, and the lack of consistent and specific fibroblast molecular markers has been an important limiting factor so far [185]. Opposing actions of CAFs expressing the same protein marker can also be observed in a context-dependent way in TMENs. For example, while in CRC-associated CAFs, podoplanin was correlated with less aggressive tumors and a favorable prognosis [187, 188], its expression by CAFs in lung, breast, esophageal, and PDAC has been associated with an unfavorable prognosis [189–192]. In addition, PDAC patients with fewer myofibroblasts in the tumors had reduced survival, possibly by suppression of the immune surveillance due to increased levels of TRegs [193]. Therefore, one should be cautious when identifying CAFs and extrapolating their role in different tumors based on the analysis of the aforementioned biomarkers. It is increasingly evident that CAFs of tumors from different etiologies present different molecular biomarkers or combination of biomarkers [194].

The full spectrum of this phenotypic diversity and their functional implications in tumor growth, progression, or even therapy resistance mechanisms are yet to be fully understood. However, defining specific tumor-associated immune and CAF signatures might become a valuable prognostic tool and drive the advancement of new therapeutic strategies.

7. Impact of Stroma in Resistance to Therapies

During cancer progression, tumors become more heterogeneous due to a generation of genetically distinct tumor-cell subpopulations and to modifications in TMEN components. In this section, we will describe how tumor heterogeneity, combined with the high plasticity of tumor-associated cells, can influence resistance to therapies in GI cancers.

7.1. Desmoplasia and Tumor Resistance. A desmoplastic reaction, characterized by the formation of a dense fibrosis and increased remodeling and deposition of ECM components, is closely associated to a poor outcome in PDAC and CRC patients [195, 196]. One of the main components of the ECM is hyaluronic acid (HA), which is a high molecular mass polysaccharide. PDAC can express HA into stroma and in peritumoral connective tissue and thus impair vascularity and the delivery of chemotherapeutic drugs into tumors. In fact, gemcitabine-resistant PDAC from patients with resectable tumors showed upregulation in gene pathways related to stroma-ECM receptor interaction, focal adhesion, cell communications, gap junction, and cell adhesion molecules [197]. Enzymatic degradation of HA results in reduction of interstitial flow pressure, reexpands the microvasculature in PDAC [198], and increases the delivery of doxorubicin and gemcitabine in a mouse model of PDAC [199].

The Sonic hedgehog (Shh) pathway also promotes desmoplasia in PDAC, and its inhibition improves delivery of chemotherapy [200]. However, genetic inhibition of the Shh pathway results in more aggressive tumors in a PDAC model [201] and accelerates progression of KRAS-driven PDAC. Inhibiting VEGF receptor (VEGFR) in Shh-deficient mice increased survival and impaired tumor progression [202], suggesting that combinatory approaches could be more effective to overcome tumor resistance.

CAF heterogeneity might be responsible, at least in part, for the protumorigenic and antitumorigenic effects in cancer resistance. For example, PDAC presents a subpopulation with high expression of α-SMA adjacent to neoplastic cells, and another with low expression of α-SMA that locates distantly and secretes inflammatory mediators as IL-6 [203]. Intriguingly, depletion of CAFs based on their α-SMA expression can induce immunodepression and accelerate pancreas cancer progression [193], leading to resistance to chemotherapies. These pieces of evidence indicate that new therapeutic approaches should consider these different subpopulations when looking for effective antitumor therapies directed to CAF.

Chemotherapy can also affect stromal cells, which in turn can promote cancer resistance. A hypoxic TMEN can lead to a metabolic shift based on aerobic glycolysis and lactate production by tumor cells, leading to a low extracellular pH, which is a common feature found in solid tumors. Moreover, chemotherapy-treated CAFs change the expression of metabolic enzymes, leading to increased aerobic glycolysis and autophagy and increased energy production [204]. A recent study showed that drugs targeting mutated K-Ras force cancer cells to get energy thought autophagy in PDAC [205].

Low expression of caveolin-1 in stroma is a marker of autophagy, which occurs via oxidative stress followed by an increase in HIF-1α and NF-kappa B expression [206]. High level of HIF-1α in CAFs is related to an elevated lactate efflux and lower extracellular pH [207]. This acid microenvironment drives EMT, protecting PDAC cells from gemcitabine-induced cell death in a mechanism that involves expression of drug transporters [208]. Moreover, gemcitabine upregulates CXCR4 expression in PDAC cells and promotes their invasiveness through a reactive oxygen species-dependent mechanism [209].

7.2. Soluble Factors and Exosomes Roles in Tumor Resistance. Stromal cells produce soluble factors that play a key role in chemoresistance (Table 2). For example, expression of TGF-β1 by CAFs is frequently present in patients treated with chemoradiotherapy, its inhibition being linked to enhanced chemosensitivity of ESC cells [214]. CAFs can also release IL6, which activates the JAK-1/STAT3 signaling pathway and contributes to chemoresistance of GC cells to 5-fluorouracil (5-FU) [217]. IL-6 secreted by CAFs also plays a role in chemoresistance of ESC cells by upregulating CXCR7. In fact, ESC patients with high expression of CXCR7 and IL-6 presented worse overall survival upon receiving cisplatin after surgery [214].

Tumor-associated macrophages (TAM) release IL6, which activates the IL-6 receptor (IL6R)/STAT3 pathway in CRC cells. STAT3 inhibits the tumor suppressor miR-204-5p, leading to chemoresistance to 5-FU and to oxaliplatin [218]. This suggests that IL6 receptor inhibition in combination with chemotherapy could serve as a suitable strategy to improve chemotherapeutic efficacy through inhibition of the communication between stromal and GC cells [217]. Another example involves cisplatin resistance in GC cells by TAM-derived exosomes containing miR-21 [216]. Exosomal transfer of miR-21 led to downregulation of PTEN and activation of AKT, which resulted in less apoptosis and increased survival in GC cells treated with cisplatin [216].

Moreover, crosstalk between TAM and tumor infiltrating cells through STAT3 can improve chemotherapeutic efficacy by repressing antitumoral $CD8^+$ T-lymphocyte activity [219].

Treatment failure can also result, at least in part, from the increase in exosome release by stromal cells. For instance, gemcitabine treatment increases fibroblast-derived exosomes containing Snail and miR146a, a Snail target, which induce resistance to chemotherapies in PDAC [210] and promote metastasis and chemotherapy resistance by enhancing cell stemness and EMT in CRC cells [220]. Upon exposure to oxaliplatin, CAFs may release exosomes containing long noncoding RNA (lncRNA) H19 to cancer cells, which has competing endogenous RNA potential for miR-141, a tumor suppressor miRNA that targets β-catenin and suppresses the Wnt/β-catenin pathway. In this way, lncRNA H19 promotes stemness of cancer stem cells and oxaliplatin resistance of CRC [211]. Similarly, exosomes secreted by gemcitabine-treated CAFs promote proliferation and

Table 2: Resistance to therapies targeting stromal components.

Drugs	Tumor type	Stromal-derived mediator
Doxorubicin and gemcitabine	PDAC	Hyaluronan [199]
Gemcitabine	PDAC	Sonic hedgehog [200]
Gemcitabine	PDAC	Alpha-SMA [193]
Gemcitabine	PDAC	HIF-alpha [208]
Gemcitabine	PDAC	CXCR4 [209]
Gemcitabine	PDAC	Snail [210]
Oxaliplatin	CRC	LncRNA19 [211]
5-FU	CRC	miR145, miR34-a [212]
Bevacizumab	CRC	VEGF [213]
Cisplatin	ESC	IL6R [214]
5-FU and oxaliplatin	GC	AKT, p38, and survivin [215]
Cisplatin	GC	miR-21 [216]

gemcitabine resistance of PDAC cells by increasing Snail expression [210]. Pancreas-derived mesenchymal stromal cells treated with paclitaxel release exosomes containing paclitaxel which inhibit proliferation of PDAC [221]. Moreover, the intracellular and extracellular expression levels of miR-145 and -34a in CRC cells were associated with 5-FU resistance [212]. The resistance was in part due to the enhanced secretion of these antioncomirs in exosomes produced by resistant CRC cells after 5-FU exposure. This led to decreased intracellular levels of the antioncomirs and sustaining proliferation [212]. 5-FU and oxaliplatin treatment can also induce CAFs to release soluble factors that are taken up by CRC cells, promoting drug resistance through AKT, P38, and survivin translocation [215]. In addition, Snail-expressing fibroblasts can secrete CCL1 and contribute to 5-FU and paclitaxel chemoresistance in CRC [222]. Similarly, increased Snail expression in PDAC cells is correlated with gemcitabine resistance [223].

8. Therapies Targeting Stromal Microenvironment

8.1. Extracellular Matrix Components. In 2003, the first clinical trial of a humanized monoclonal antibody directed to human FAP, sibrotuzumab, was found clinically safe in patients with advanced solid cancers [224]. However, it showed limited clinical response in a phase II trial in patients with CRC [225]. Inspite of promising preclinical findings, therapy strategies targeting CAFs have repeatedly faced obstacles. As we pointed out above, depletion of α-SMA-expressing CAFs can accelerate pancreas cancer progression [193]. Thus, depleting CAFs based on their expression of FAP or α-SMA might not be effective, since other stromal cell types can also express these markers.

Regarding ECM remodeling, Lysyl Oxidase-like 2 (LOXL2) is upregulated in tumor-associated stroma of PDAC, ESC, and HCC [226, 227]. Simtuzumab, an antibody that inhibits LOXL2, blocks the desmoplastic reaction in CRCs in vitro [226]. However, phase II clinical trials of simtuzumab in combination with gemcitabine or FOLFIRI (folinic acid, 5-FU, irinotecan) did not improve the clinical outcome in PDAC or in CRC patients, respectively [228, 229].

Different approaches that inhibit desmoplasia in solid cancers can inhibit tumor growth and improve vascular perfusion and drug delivery. Losartan (an angiotensin I inhibitor) is an antihypertensive drug that reduces collagen and hyaluronan production by CAF through downregulation of the fibrotic signals TGF-β1, Cellular Communication Network Factor 2 (CCN2), and Protein Effector of Transcription 1 (ET-1) [230]. In fact, epidemiological studies demonstrated that gastroesophageal cancer patients presented a moderately reduced cancer-specific mortality amongst users of angiotensin receptor blockers [231]. Based on preclinical studies, a phase II study targeting TGF-β1 by using losartan in combination to FOLFIRINOX (folinic acid, 5-FU, irinotecan, oxaliplatin) in locally advanced PDAC is ongoing with an estimative to be concluded by 2025 (Table 3). Another approach is to inhibit Shh signaling, which drives stromal desmoplasia, by activating the ligand for smoothened (SMO) in CAFs [237]. The SMO inhibitor (IPI-926) reduced the abundance of myofibroblasts in the stroma in PDAC and increased tumor vasculature as well as intratumoral gemcitabine uptake. However, a phase Ib/II clinical trial using IPI-926 in combination with gemcitabine in metastatic PDAC did not show benefits in clinical outcome. Indeed, some patients receiving IPI-926 had a shorter median survival time compared with the placebo group [200].

The tumor stroma can also play an important role in restraining tumor growth, mainly due to the heterogeneous population of fibroblast present in PDAC. Preclinical studies identified a CAF subpopulation expressing high amounts of α-SMA close to tumor cells and CAF subpopulations expressing low α-SMA and secreting IL-6 which could be responsible for the aggressiveness of PDAC [193, 201]. A phase Ib study using enzymatic ablation of hyaluronan by PEGPH20, a PEGylated recombinant hyaluronidase, in combination with gemcitabine showed a potential therapeutic benefit, especially in patients with high expression of HA [232]. In fact, a phase II clinical trial using PEGPH20 in association with gemcitabine and nab-paclitaxel showed improvement in the progression-free survival of PDAC patients [233] and it is now being evaluated in a phase III trial (Table 3). However, PEGPH20 in association with a modified FOLFIRINOX regimen presented high toxicity when compared with FOLFIRINOX alone [238].

Other strategies to inhibit FAP in PDAC showed promising results in preclinical studies [121, 239, 240]. For example, anti-FAP CAR T cells can deplete FAP^+ cells in PDAC and decrease tumor growth through promotion of

Table 3: Drugs targeting stroma components in clinical trials.

Drug and association	Tumor type	Molecular and cellular target	Mechanism	Study phase	ClinicalTrial.gov identifier
Sibrotuzumab	CRC, PDAC	FAP (CAF)	Desmoplasia [224]	II	NCT02198274
Simtuzumab + FOLFIRI	CRC, PDAC	LOXL2	Desmoplasia [229]	II	NCT01479465
Simtuzumab + gemcitabine	CRC, PDAC	LOXL2	Desmoplasia [229]	II	NCT01472198
Losartan + F-NOX	PDAC	TGF-beta1	Fibrosis [230]	II	NCT03563248*
IPI-926 + gemcitabine	PDAC	SMO	Hedgehog pathway inhibition	II	NCT01130142
PEGPH20 + gemcitabine + nab-paclitaxel	PDAC	Hyaluronan	Desmoplasia [232, 233]	III	NCT02715804*
PEGPH20 + FOLFIRINOX	PDAC	Hyaluronan	Desmoplasia [232, 233]	I	NCT01959139*
Pembrolizumab + AMG820	CRC, PDAC	PD-1 (T cells) CSF1R (macrophage)	T-cell apoptosis	II	NCT02713529*
Durvalumab + monalizumab	CRC	PD-1 (T cells) CD94/NGK2a	T-cell apoptosis	I	NCT02671435*
AMG820	CRC, PDAC	CSF1R (macrophage)	M2 polarization [234]	I	NCT01444404
5F9 + cetuximab	CRC	CD47 (macrophage)	Restores macrophage phagocytosis	II	NCT02953782*
Pembrolizumab + cisplatin + 5-fluorouracil	GC, GEJ	PD-1 (T cells)	T-cell apoptosis	III	NCT02494583*
Pembrolizumab + paclitaxel	GC, GEJ	PD-1 (T cells)	T-cell apoptosis	III	NCT02370498*
Ruxolitinib + capecitabine	PDAC	Janus 1 and Janus 2 (pancreatic stellate cells)	JAK-STAT3 pathway inhibition [235]	III	NCT02117479# NCT02119663#
Nivolumab + ipilimumab	Upper GI	PD-1 (T cells) CTLA-4 (T cells)	Block T-cell inhibitory signals and activation of T cells	II	NCT02923934*
Bevacizumab + cisplatin	GC	VEGF-A (endothelial cells)	Angiogenesis [236]	III	NCT00548548
Ramucirumab	Upper GI	Inhibits receptor tyrosine kinase (endothelial cells)	Angiogenesis	II	NCT02241720

GEJ: gastroesophageal junction. *ongoing; #terminated.

antitumor immunity [241]. Recently, a preclinical trial using a DNA vaccine against FAP synergized with anticancer immune therapy targeting Prostate Membrane Antigen (PMSA) in tumor-bearing mice model for prostate cancer [242]. This result suggests that therapies which target both stroma components and tumor cells might be effective for tumors expressing high amounts of FAP, such as CRC and PDAC.

8.2. Immune System. PD-1 is expressed on a large proportion of TILs from many different cancer types, while its ligand, PD-L1, is mainly expressed in antigen-presenting cells and tumor cells [243]. Since tumors can escape the T cell immune response by expressing these molecules, the blockade of this pathway has emerged as a promising anticancer strategy. This approach also showed good results as second and third line of chemotherapy in gastro-esophageal cancer [244, 245]. A clinical study evaluating nivolumab (an antibody against PD-1) monotherapy in heavily pretreated patients with advanced gastric or gastro-esophageal junction cancer showed an increased 12-month overall survival rate compared to the placebo group [244]. In another trial, both objective and complete responses were observed in patients with gastro-esophageal cancer treated with pembrolizumab (an antibody against PD-1) monotherapy, irrespective of PD-L1 tumor expression. Nonetheless, pembrolizumab conferred longer response duration in those patients with PD-L1–positive tumors [245].

CTL4-A (cytotoxic T-lymphocyte antigen 4) signaling diminishes immune response against tumor cells and the use of antibodies against CTL4-A was effective in treating tumors as melanomas [246]. However, clinical trials in PDAC using monotherapies with CTLA-4 or PD-1 inhibitors presented low response rates [247, 248], with the exception of the PDAC patient subpopulation with microsatelite instability [249]. Although the response rates from these studies remain discouraging, they could be improved by combinatory therapies. A phase II study with Nivolumab in association with Ipilimumab (an antibody against CTLA-4) in patients presenting upper GI cancers is ongoing (see Table 3). The first trials using nivolumab and pembrolizumab in HCC were encouraging [250, 251]. However, pembrolizumab as second line of treatment did not meet its coprimary endpoints of overall survival and progression-free survival [252]. A phase III trial with nivolumab in first line treatment is currently underway. Unfortunately, when a better selection of patients based on molecular characteristics from the tumor or on its etiology was performed, the data was inconclusive [250, 251]. Another study showed that a therapy targeting FAP^+ cells that express CXCL12 synergized with anti-PD-L1 immunotherapy in PDAC [121],

and inhibition of its receptor, CXCR4, in sorafenib-treated HCC facilitates anti-PDL-1 immunotherapy [253]. In addition, CXCR4 inhibition increased PD-1 therapy response by inducing mobilization of $CD8^+$ T cells in PDAC [254]. Together, these studies demonstrate important systemic components that might play a role in the clinical outcome and explain, in part, the heterogeneous therapeutic response normally found in these clinical trials.

Natural Killer Cells Antigen 94 (CD94/NGK2a) is the main HLA-E receptor which mediates an inhibitory effect on $CD8^+$ CTL and NK cells, promoting immune evasion in CRC [255]. In fact, increased levels of NGK2a-$CD94^+$ TILs correlate with poor survival in CRC patients [256]. Although metastatic microsatellite-stable CRC patients do not respond to therapies that involve PD-1/PD-L1 blockade [257], a first phase I clinical trial studying an antibody against PD-1 (durvalumab) in combination with an antibody targeting CD94/NGK2a (monalizumab) is ongoing.

Immunotherapy checkpoints have been suggested as a good strategy to impair cancer progression [258], and strategies targeting both the innate and the adaptive immune systems show promising results in CCR [259]. CCL2, which is highly expressed in PDAC, is a chemoattractant for T cells, monocytes, and natural killer cells. CCL2 binds to its receptor, C-C Chemokine Receptor (CCR) 2, which is expressed in monocytes and controls its differentiation into TAMs [260]. CCR2 inhibition in combination with FOLFIRINOX in PDAC has been tried in phase I clinical trial, and the results showed that it was safe and well tolerated [261].

Another mention worthy molecule is CD47, an integrin-associated transmembrane protein. This integrin is overexpressed in solid cancers (e.g. CRC) and is correlated to a poor clinical outcome [262]. Both TAMs and DCs can express the CD47 receptor, signal regulatory protein alpha (SIRPα). The binding of CD47 to SIRPα inhibits phagocytosis of cancer these cells, enabling the tumor to evade immune destruction by first responder cells, such as macrophages [263]. Thus, restoring phagocytosis activity by antigen-presenting cells can enhance antigen priming of T cells. A phase I clinical trial recently described the use of the monoclonal antibody against CD47 Hu5F9-G4 in CRC and PDAC [264]. A Phase II study to evaluate Hu5F9-G4 in combination with cetuximab is ongoing in CRC [265].

As previously mentioned, the M2 macrophages are frequently found in TMEN. Since the intratumoral presence of Macrophage Colony-Stimulating Factor Receptor (CSFR) 1^+ macrophages correlates with the clinical aggressiveness of pancreatic neuroendocrine tumors [266], targeting CSFR1 signaling in TAMs represents an attractive strategy to eliminate these cells and block M2 polarization. A clinical trial using a monoclonal antibody against CSFR1, AMG 820, showed safety and tolerability in patients with advanced solid tumors, including CRC and PDAC [234]. However, since the study did not present significant tumor responses, it was terminated before enrollment into the dose-expansion phase. Preclinical studies have also examined the effects of CSFR1 inhibitors in combination with T-cell target therapies to improve efficacy in PDAC [267]. In fact, a clinical trial using pembrolizumab in combination with AMG 820 is ongoing in PDAC and in CRC, with an estimated date of completion in 2020 (Table 3).

8.3. Angiogenesis. Approaches focused on anti-angiogenesis cancer therapies have been studied in several clinical trials. In GC the results of trials with anti-VEGF were disappointing on the first line treatments (either with bevacizumab or with ramucirumab) [236, 268]. Interestingly, the use of ramucirumab (anti-VEGF2) in association with paclitaxel or in monotherapy showed a significant improvement on the overall survival of gastro-esophageal adenocarcinoma patients and has been approved in this setting [268, 269].

In HCC, the use of tyrosine kinases with antiangiogenic effects were the basis of systemic treatment. Since the first approved drug, sorafenib, several clinical studies showed improvement in clinical outcomes with regorafenib, ramucirumab or cabozantinib, expanding the repertoire of drugs that can be used in this particular disease [220, 270–272].

In CRC, the use of bevacizumab in association FOLFIRI (Folinic Acid, 5-FU, Irinotecan) or FOLFOX (Folinic Acid, 5-FU, oxaliplatin) showed a significant increase in overall survival, being nowadays the standard of care for patients in the metastatic stage of this disease [273]. Nonetheless, the use of bevacizumab as part of the adjuvant chemotherapy treatment in CRC patients was detrimental for survival [274]. Other drugs that change the tumoral angiogenesis, such as the VEGF 1 and 2 inhibitors ziv-aflibercept and ramucirumab, have shown an improvement in overall survival in patients with CRC when in combination with chemotherapy in second line setting after failure of a previous line of chemotherapy [275, 276].

9. Conclusions and Perspectives

Tumor masses are not cancer cells-centered entities that drive malignant progression. Instead, tumor development depends on the complex and intricate tapestry of cell-cell interactions where nontransformed cells of the TMEN play key role in cancer biology. We here summarized how stromal cells can impact tumor growth and progression as well as resistance to antitumor treatment. In fact, we show that most of these cells are important oncogenic drivers, frequently associated with poor prognosis. Therefore, the development of new therapeutic approaches directed to components of the TMEN still has a great unexplored potential. The main challenge on TMEN-directed approaches resides on the complexity of the interactions within the microenvironment, where the same cell type can have opposite effects in tumor growth and progression depending on its cell-to-cell interaction. This is not surprising considering the pleotropic diversity of all the stromal cells described here. Therefore, ideal targeted therapy is unlikely to be solely affecting a single cell type. Instead, the best therapeutic approaches should be those that are capable of tipping the whole balance in favor of tumor inhibition.

Abbreviations

GI: Gastrointestinal
PDAC: Pancreatic ductal adenocarcinoma
BM: Bone marrow
TMEN: Tumor microenvironment
BMDC: Bone marrow-derived cells
GC: Gastric cancer
CRC: Colorectal carcinoma
MMP: Metalloproteinase
MSC: Mesenchymal stem cells
VEGF: Vascular endothelial growth factor
IFN-γ: Interferon gamma
TNF-α: Tumor necrosis factor alpha
HIF-1α: Hypoxia-induced factor 1 alpha
S100A8/9: S100 calcium binding protein A8/A9
HCC: Hepatocellular carcinoma
PTEN: Phosphatase and tensin homolog
HSC: Hematopoietic stem cells
CAF: Cancer-associated fibroblasts
ECM: Extracellular matrix
TGF-β: Transforming growth factor beta
HGF: Hepatocyte growth factor
FSP1: Fibroblast-specific protein 1
Lkb1: Liver kinase B1
IL: Interleukin
JAK: Janus kinase
STAT3: Signal transducer and activator of transcription 3
PGE2: Prostaglandin E2
EMT: Epithelial-to-mesenchymal transition
PDGF: Platelet-derived growth factor
FAP: Fibroblast activation protein
CCL: C-C motif chemokine
CXCL: C-X-C motif ligand
CXCR: C-X-C motif receptor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
SDF-1: Stromal cell-derived factor 1
α-SMA: Alpha smooth muscle actin
HUVEC: Human umbilical vein endothelial cells
ECs: Endothelial cells
AKT: Serine/threonine-protein kinase
ESC: Esophageal squamous carcinoma
LEC: Lymphatic endothelial cells
TIMP: Tissue inhibitor of metalloproteinases
PD-L1: Programmed death-ligand 1
IDO: Indoleamine 2,3-dioxygenase
NK: Natural killer
TRegs: T regulatory cells
FOXP3: Forkhead box P3
DC: Dendritic cells
Th2: Type II helper T cells
MDSC: Myeloid-derived suppressor cells
PD-1: Programmed death-1
Fas: Apoptosis-mediating surface antigen FAS
FasL: Fas ligand
MIF: Migration inhibitory factor
TIL: Tumor lymphocyte infiltrates
HA: Hyaluronic acid
Shh: Sonic hedgehog
5-FU: 5-Fluorouracil
TAM: Tumor-associated macrophages
LOXL2: Lysyl oxidase-like 2
SMO: Smoothened
FOLFIRINOX: Folinic acid, 5-FU, irinotecan, oxaliplatin
CD94/NGK2a: Natural killer cells antigen 94
CCR: C-C chemokine receptor
CSFR: Macrophage colony-stimulating factor receptor.

Conflicts of Interest

The authors declare no conflicts of financial interest.

Authors' Contributions

S. B. and A. G. wrote the sections on BMDCs, CAF, ECs, and immune cells. A. O. and N. C. wrote the sections on stromal signatures of prognostic, tumor resistance, and therapy strategies. B. C. S. conceived and structured the review. All authors wrote and reviewed the manuscript.

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Review Article

Implications for Tumor Microenvironment and Epithelial Crosstalk in the Management of Gastrointestinal Cancers

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Rapid advances in technology are revealing previously unknown organization, cooperation, and limitations within the population of nontumor cells surrounding the tumor epithelium known as the tumor microenvironment (TME). Nowhere are these findings more pertinent than in the gastrointestinal (GI) tract where exquisite cell specialization supports a complex microenvironmental niche characterized by rapid stemness-associated cell turnover, pathogen sensing, epithelial orchestration of immune signaling, and other facets that maintain the complex balance between homeostasis, inflammation, and disease. Here, we summarize and discuss select emerging concepts in the precancerous microenvironment, TME, and tumor epithelial-TME crosstalk as well as their implications for the management of GI cancers.

1. Introduction

1.1. Gastrointestinal Microenvironment. The gastrointestinal (GI) tract is frequently challenged with exposure to bacteria, parasites, viruses, and other pathogens. For tissue to thrive in these chaotic conditions, it is essential to maintain homeostasis in support of pathogen clearance, digestion, absorption, and efficient cell turnover [1, 2]. This necessity has led to unique tissue compartments with specialized cell types in charge of functions that impact both the GI tract and distant organs including the lung, brain, and others [3, 4]. Imbalances in these compartments as well as deleterious hereditary molecular alterations (e.g., loss of the APC tumor suppressor) can lead to inflammatory, precancerous, and cancerous conditions, and an improved understanding of the factors at play may yield new therapeutic strategies against sporadic and inflammation-associated GI cancers [5].

1.2. Inflammation and Injury as a Source of Microenvironmental Instability. Although sporadic and heritable molecular alterations have long been known to be major causes of GI tumorigenesis, recent findings have firmly established inflammation as a hallmark of cancer [6]. Nowhere is inflammatory injury more strongly linked to the development of cancer than within the GI tract where it is implicated in esophageal, gastric, pancreatic, hepatic, intestinal, and other GI cancers. Examples of pathogenic sources of inflammation in these organs include *Helicobacter pylori*, helminths, hepatitis B/C viruses, and various bacterial strains which are able to overpopulate the microbiotic environment under certain conditions [7–10]. Lifestyle factors including smoking, alcohol consumption, processed and red meat consumption, and obesity are also major sources of inflammation which may lead to the expansion of injurious microbiota [11–14].

A number of inflammatory conditions of the GI tract are thought to prime the tissue microenvironment to give rise to tumors. These include gastroesophageal reflux disease, esophagitis, ulcerative colitis, Crohn's disease, gastritis, pancreatitis, hepatitis, nonalcoholic fatty liver disease, and primary biliary cirrhosis. The protumorigenic activity associated with these diseases is likely mediated by their impact on the DNA damage response, immune signaling, and other mechanisms which may be especially enhanced when these conditions are chronic. Indeed, the inflammatory process itself can be thought of as a double-edged sword in terms of cancer because, whereas inflammation is a source of DNA damage which may support tumorigenesis [15], attenuation of inflammatory signaling may support tumor progression as seen in the alternative activation of macrophages [16] (Figure 1).

1.3. Clinical Relevance of GI Inflammation on the Transition to Precancer. Following sustained inflammatory injury in the presence of genetic alterations (e.g., APC mutation), precancerous conditions are often able to take hold in the tissue niche [17]. Examples of this include Barrett's esophagus, intestinal adenomas, pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasia (IPMN), and others [17–21]. Although inflammatory conditions may progress to precancer which ultimately gives rise to cancer, current knowledge suggests that the majority of patients with acute or chronic inflammatory conditions will not experience progression to cancer. As a result, without additional information (e.g., family history of cancer), knowledge of these conditions is often of minimal practical value in cancer diagnosis, prevention, or prognosis. Moreover, even with consistent endoscopic surveillance in patients deemed to be at high risk, cancer may go unnoticed as seen in colitis-associated colorectal cancer (CRC) [17]. Consequently, preventative strategies including enhanced monitoring, biomarkers, and prophylactic drug therapies have become increasingly desirable. In order to effectively develop these strategies, improved knowledge of precancer and TME cell specialization, microbiotic characteristics, intracellular and intercellular signaling, and other characteristics is needed.

2. Targeting Cell Specialization within the Tumor Microenvironment

When viewed as an organ, the tumor can be divided into four major cellular compartments: epithelial, stromal, endothelial, and immune. Among these compartments, the tumor epithelium represents the classical "tumor cell," whereas the stroma, endothelium, and immune compartment comprise the tumor microenvironment (TME). Each of these compartments hosts a variety of cell types with varying functions. The development of single-cell RNA sequencing (scRNA-Seq) technology in recent years has vastly improved the ability to characterize these cells, and their role in cancer initiation and progression is becoming even more apparent [22]. Cancer therapies often target

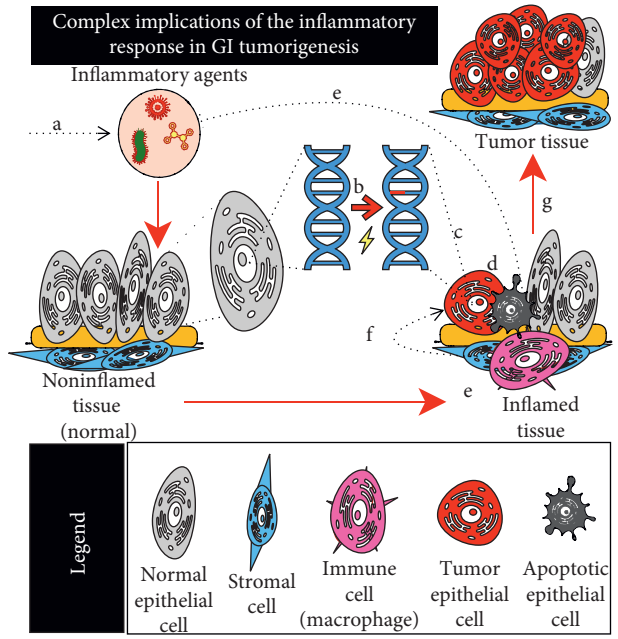

Figure 1: Inflammation and tissue restitution have complex implications for the gastrointestinal microenvironment. (a) The GI epithelium is exposed to a variety of inflammatory agents including bacteria, viruses, parasites, chemicals, and other components which promote injurious shifts in microbial populations and/or directly and (b) induce reactive oxygen and nitrogen species leading to epithelial DNA damage and mutations. Following DNA damage, (c) certain cell types escape DNA repair mechanisms, maintaining these somatic mutations (red), while (d) other cells with effectual DNA repair mechanisms, undergo apoptosis. (e) Macrophages (pink) recruited to the site of injury can engulf pathogens, as well as apoptotic epithelial cell bodies destroyed during inflammation. Macrophage-based detection of signal combinations indicating successful clearance of pathogens (e.g., IL-4 + apoptotic phosphatidyl-serine functional group) can induce (f) macrophage polarization and alternative activation and subsequent anti-inflammatory signaling. (g) Exposure to this signaling may promote tumorigenesis and/or progression in susceptible epithelial cells harboring mutations C or other sources, leading to establishment of tumors and/or metastatic transformation.

specific cell classes in the tumor and TME (Figure 2). However, due to an early focus on tumor epithelia, there has been limited development of TME-targeted agents. The development of effective immunotherapies (e.g., nivolumab and pembrolizumab) demonstrates the potential for expanding the focus to new TME components. Although this approach has not yet yielded extensive benefit in GI malignancies compared to some other cancers, with further knowledge, these advances will expand the therapeutic arsenal.

2.1. Epithelial Cell Types. The majority of cancer research throughout history has focused primarily on the epithelial cell types of the tumor. These cells are often highly proliferative, resistant to apoptosis, and capable of rapidly adapting to insult (e.g., chemotherapy). A variety of studies have shown that tumor epithelial cells are heterogeneous,

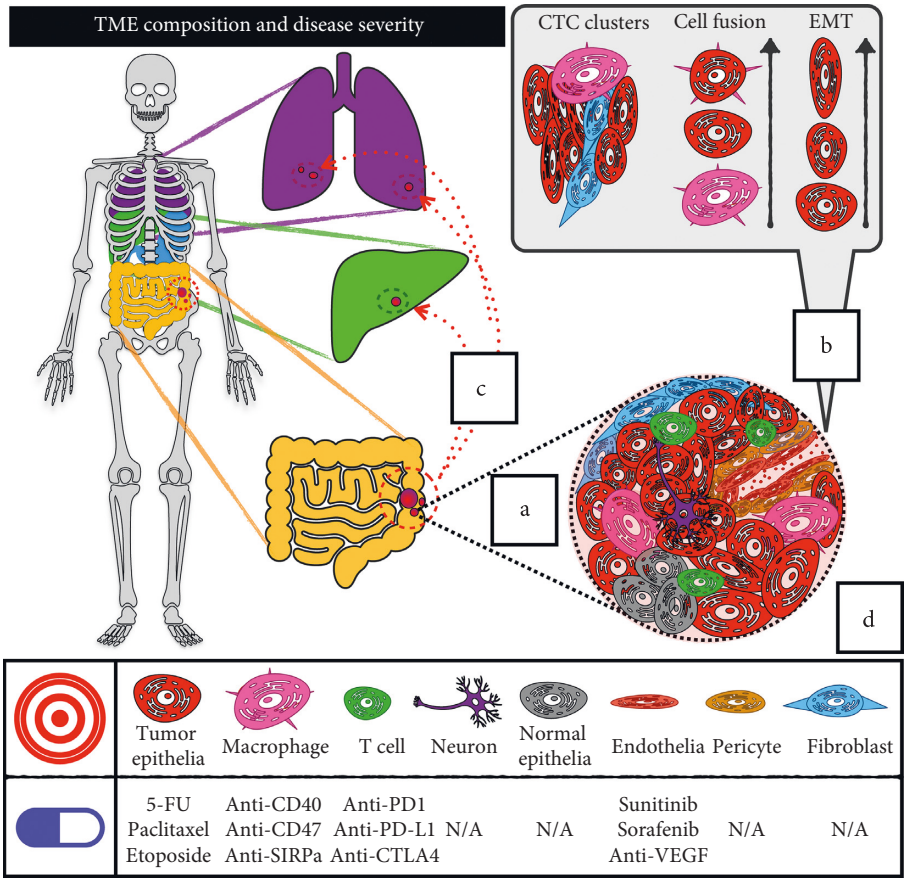

Figure 2: Metastasis and therapeutic efficacy are dictated by complexity within the tumor microenvironment. (a) The TME is comprised of an interacting landscape of unique cell types including tumor epithelia, tumor-associated macrophages, infiltrating T-cells, endothelial cells and pericytes, neurons, and cancer-associated fibroblasts. (b) In metastatic transformation, the TME programs tumor epithelia via EMT, cell fusion, and other processes leading to local invasion and dissemination to distant sites via CTCs and/or CTC clusters. (c) Metastatic dissemination is nonrandom, as in colon cancer where liver metastases are common, although the rules governing this remain to be fully understood. (d) Clinical and developmental drug therapies target or show preference for specific TME components, which factors into their efficacy in various tumor subtypes and combination therapy.

often containing differing mutations, gene and protein expression profiles, and pathway alterations [23]. Evidence supporting various explanations for this heterogeneity has mounted over the years, and its functional importance in resistance and metastasis is evident. Ironically, despite intense basic and clinical research focused on understanding tumor epithelia, research approaches employing relatively homogeneous populations through the use of commercial cell lines, xenografts, and engineered mouse models have led to a situation in which they are some of the most poorly subtyped cells residing within the TME, and a notable lack of markers to identify various subgroups persists.

Radiotherapy and most chemotherapies were developed to target rapidly proliferating tumor epithelia. Examples of these chemotherapies include taxanes (e.g., paclitaxel and docetaxel), nucleoside analogs (e.g., 5-fluorouracil and gemcitabine), platin drugs (e.g., oxaliplatin and cisplatin), and topoisomerase inhibitors (e.g., irinotecan and etoposide). Typically, these therapies demonstrate an ability to interfere in cell replication through one of the three primary mechanisms: induction of DNA damage, interference in microtubule dynamics, or inhibition of DNA synthesis. Many of these antiproliferative therapies have been approved in GI cancers. However, due to adaptive mechanisms within the tumor as well as toxicity to normal cells, they often provide limited benefit. Tumor epithelia may adapt to treatment via quiescence or slower cell division, or through the expression of drug efflux machinery [24]. These characteristics are often linked to epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs), which are capable of recapitulating the tumor with increased metastatic capabilities and drug resistance. Despite these shortcomings, these compounds may increase patient survival, and over the past decade, it has become clear that combinations of these drugs may be more effective than individual agents. This is especially notable in pancreatic cancer where recent studies show that the combination of 5-fluorouracil, oxaliplatin, irinotecan, and leucovorin (FOLFIRINOX) can dramatically improve pancreatic ductal adenocarcinoma (PDAC) survival [25, 26].

2.2. Stromal and Endothelial Cell Types. Stromal cells provide structure to an organ, and the tumor is no exception. Subtypes with known functional importance in the TME

include fibroblasts and pericytes. Cancer-associated fibroblasts (CAFs) surround tumor epithelia providing physical structure, secreting extracellular matrix (ECM), and directing various tumor processes. Additional fibroblasts can be continually recruited from tumor stroma and normal tissue, and CAFs are a primary building block for desmoplasia, which compromises the delivery of conventional and targeted therapies to the tumor [27]. In GI cancers, CAFs are implicated in molecular regulatory processes including cytokine/chemokine secretion, immune checkpoint, tumor growth factor signaling, macrophage polarization, and angiogenesis [28].

Aside from CAFs, mesenchymal pericytes also provide structure within the tumor by maintaining a skeleton for endothelial vessel formation. Interestingly, some findings support the possibility of epithelial-pericyte transition occurring within the TME to support tumor processes such as angiogenesis [29]. Overall, research into cancer-associated pericytes remains limited, but given their role in normal tissue and noncancer diseases, and the importance of angiogenesis in tumorigenesis and progression, a better understanding will likely improve TME targeting strategies [29, 30].

Endothelial cells form blood vessels supported by pericytes and are a key transit point for migrating cells and signaling factors entering and exiting the tumor. Their prevalence is associated with poor outcomes in many cancer types including GI cancers, and high levels of vascularization such as found in clear cell renal carcinoma (ccRCC) and PDAC are associated with potent resistance to chemotherapies [31]. The prominent VEGF pathway is perhaps the best known target within this system [32], but our understanding of tumor-associated endothelia remains limited.

Endothelial cells along with the VEGF, PDGF, and several linked pathways are key components involved in the process of angiogenesis. Angiogenesis pathways interact via a variety of ligand-receptor interactions and are activated by hypoxia-inducible factors. Canonically, hypoxic TMEs induce the expression of transcription factors HIF1A and HIF2A (EPAS1), which in turn upregulate VEGF expression. VEGFs bind to VEGF receptors which regulate endothelial cell viability and migration, recruit immune cells to the tumor, and support lymphangiogenesis [33]. Functionally, angiogenesis within the tumor was recognized early on as a contributor to disease progression and mortality, and vascularization offers additional routes for nutrient uptake supporting growth, as well as dissemination of circulating tumor cells (CTCs). Among GI cancers, this process is especially important in HCC [34], as well as in PDAC where it supports desmoplasia [35], preventing drugs from easily accessing the TME [35]. Based on the importance of this mechanism, a class of angiogenesis inhibitors was developed to target VEGF, PDGF, and related signaling pathways. The most prominent of these inhibitors, sunitinib, has found use in a variety of tumors including PDAC [36]. Other commonly used antiangiogenesis agents include sorafenib, regorafenib, aflibercept, and the anti-VEGF monoclonal antibody (mAB), bevacizumab, which are indicated in some CRCs [33].

2.3. Infiltrating and Auxiliary Immune Cells. Perhaps the most prominent emerging topic in TME research is the immune system. Immune cell subtypes present in tumors include T cells, B cells, natural killer (NK) cells, macrophages, and myeloid-derived suppressor cells (MDSCs) among others. The presence and activation status of these components are key controllers of tumor fate and the response to therapies. A variety of immune subset-specific processes act as levers in this system, including alternative activation of macrophages, presentation of antigens by major histocompatibility complex (MHC), PD1/PD-L1 interaction, and others [37]. Moreover, dated knowledge of immune cell types is slowly being expanded and clarified by scRNA-Seq and other profiling studies demonstrating unique subpopulations of each and expanded hematopoietic and tissue-resident differentiation pathways.

Novel therapies targeting immune-tumor interactions are emerging as the treatment of choice in a variety of cancers. Currently, immunotherapies are more commonly used after conventional first-line therapy but are expected to supplant some of these in the future. Despite the availability of target specific and well-tolerated immunotherapies, and the known importance of the immune system in the GI TME, they have thus far fallen flat relative to the expectations set in clinical trials of some non-GI cancers. However, there are use cases that demonstrate their potential, and effective companion biomarkers and an expanded understanding of the complexities of immune-GI TME interactions may overcome existing challenges to this approach.

Microsatellite instability (MSI) and mismatch repair deficiency (MMRd) may define some immunotherapy-susceptible subsets of GI cancer patients. In MMRd CRCs, PD1-targeted pembrolizumab contributes to significant improvements in progression-free survival through an apparent immune mechanism [38]. In PDAC, similar studies targeting immune checkpoint have demonstrated limited proof of concept for anti-PD1 combination therapies in some patients [39]. Analyses also suggest that advanced gastric cancers may be susceptible to anti-PD1 mAB therapy, which can increase overall survival and reduce adverse events [40]. Taken together, these findings demonstrate the potential for immunotherapies and specifically immune checkpoint inhibitors in the treatment of GI cancer. However, advancing the use of immunotherapies in GI cancer will require combination with chemotherapy, as well as identification of susceptible tumors using specific biomarkers (e.g., PD-1 positive/negative, MSI-H/L, and high/low tumor mutational burdens) and from among the defined tumor subtypes that describe the origin and TME context of disease [41, 42].

3. Cell Interactions, Transformation, and Displacement within the TME

The TME and its associated tumor epithelium is a dynamic compartment driven by intratumoral and extratumoral signaling regulating metabolism, secretory functions, cell populations, and ultimately progression. Exploiting the TME for improved therapies and monitoring will require an

understanding of these specific processes and any signaling crosstalk that may occur between other TME components and tumor epithelia. Cell communication occurs through a variety of autocrine, paracrine, and juxtracrine signaling mechanisms, and signals may be transmitted across long distances (e.g., between discrete organs). Examples of signaling molecules with great import within the TME include cytokines and chemokines, growth factors, and immune-related ligands. Methods for conveying these signals include extracellular vesicles, traditional secretion, and membranous ligand expression. Select signaling pathways and processes of great importance within the GI TME include EMT, CSC-mediated signaling, the formation and dissemination of CTCs, immune-modulatory activities, and potentially organ-organ biological axes.

3.1. Epithelial-Mesenchymal Transition and CSCs within the TME. First identified as an embryonic developmental mechanism, EMT is a process by which tumor epithelia can mimic essential TME cellular components and is now widely considered one of the most important pathways in GI tumor progression. During EMT, stimulation of various signaling pathways leads to the expression of a set of transcription factors (ZEB1/2, SNAI1/2, and TWIST1/2) which remodel tumor epithelial cells to transitional and then mesenchymal cell types characterized by loss of tight junction proteins and expression of mesenchymal markers (e.g., vimentin and fibronectin) [43]. Mesenchymal cells have significantly greater migratory capacity compared to epithelial cells, and evidence suggests EMT is a primary driver of invasion and dissemination leading to metastasis. However, it is less clear how circulating tumor cells (CTCs) that have undergone EMT are able to initiate mesenchymal-epithelial transition (MET) and effectively colonize the distant metastatic site [43]. Moreover, EMT is dynamic within the TME, and transitions can occur in both directions (EMT or MET) in response to environmental stimuli. For example, epithelial cells are often less resistant to chemotherapy agents and EMT may allow them to adapt and escape apoptosis through ABC drug efflux transporter expression and a more mesenchymal-like phenotype. Finally, EMT is inextricably linked to stemness and CSCs, and activation of EMT may potentially induce dedifferentiation of tumor epithelia [43].

Though the existence of CSCs was long hypothesized, the stochastic model of tumorigenesis and progression championed by the Vogelstein group was favored until work by the John Dick lab demonstrated the existence of CSCs definitively by identifying a specific AML subpopulation ($CD34^+/CD38^-$) giving rise to the totality of AML cell types [44]. Like normal stem cells, CSCs are defined by self-renewal and pluripotency and within the TME, they can control tumor progression not only through signaling but also by regulating the overall composition of tumor epithelia through their progenitors. Perhaps the most well-described CSCs in the GI tract are the intestinal crypt base normal stem cells (NSCs) [45] and the terminally differentiated (in normal tissue) intestinal tuft cell [46]. Both of these populations can initiate tumors (cell of origin) in the presence of mutation (e.g., APC loss), while current research suggests that the tuft cell-derived CSC may be more specific to inflammation-associated cancer [47]. However, since tuft cells arise from NSCs, it is probable that these phenomena are inextricably linked and they may need to be studied in tandem. Importantly, the activity of both of these cell types in inflammatory, precancerous, and cancerous conditions may provide an opportunity to understand how different CSC populations interact with immune and other components in the tumor and surrounding tissue.

3.2. CTCs, Dissemination, and Metastatic Colonization and Progression. The importance of the TME to metastatic colonization and progression was first hypothesized in the late 19^{th} century when Stephen Paget proposed what became known as the "seed and soil hypothesis." Through studying many hundreds of cancer patient autopsies, Paget determined that metastasis does not occur by chance. Instead, certain organs provide an optimal environment for this form of cancer progression [48]. Recent findings suggest that the primary tumor may prepare these sites for metastasis at a distance via endocrine signaling, but it must also effectively transform and disseminate cells to these distant sites. Both CSCs and EMT are known to be major factors in transitioning primary tumor cells to CTCs [49], but previous research into CTC biology leaves much to be desired.

Most CTC studies have depended on the use of FACS sorting to identify and characterize these cells. However, FACS techniques have traditionally depended on dissociation to single cells. New findings are demonstrating that CTCs benefit from traveling through the bloodstream in clusters with other CTCs and supportive TME cells including immune cells, fibroblasts, and endothelia [50–52]. However, these CTCs were previously envisioned as a kind of spore for metastasis; these findings suggest that CTC clusters may be more comparable to a metastatic ship containing components and provisions to support the journey from one continent (primary tumor) to another (distant site). Emerging findings also suggest the novel possibility that tumor cell fusion (e.g., macrophage-epithelial fusion) and/or tumor epithelial mimicking of specialized cell functions are involved in the preparation and transport of CTCs to distant sites [53, 54]. However, many pressing questions remain regarding the clonal nature of CTCs and their progeny, the influence of paracrine and endocrine signaling on CTC processes, and the physical properties of CTCs.

3.3. Local and Discrete Immunomodulation in the TME. Infiltrating and auxiliary immune cells regulate cancer through a variety of mechanisms. The most prominent example of this currently is the PD-1/PD-L1 interaction which tumor epithelia and other cells within the TME exploit to activate apoptosis in cytotoxic T cells programmed to destroy them. Modulation of the immune system can be initiated from many different TME components. Secretory products such as cytokines and chemokines are often key regulators of these processes, and cascading events

complicate knowledge of the overall picture. Studies of the inflammatory and precancerous intestinal microenvironment may be one of the best systems to consider when pondering these complex cell and molecular network interactions because of the intestinal epithelium's well-described cellular structure and function.

The intestinal epithelium contains six well-documented cell types: stem cells, antibiotic-producing Paneth cells, hormone-producing enteroendocrine cells, mucin-producing goblet cells, absorptive enterocytes, and sensory tuft cells. Recent studies also provide evidence for functional subgroups of stem cells [55] and tuft cells [56] and an injury-specific reserve stem cell [57]. When challenged by injury, tuft cells sense epithelial damage and respond by secreting IL-25. This IL-25 is detected by innate lymphoid type II cells (ILC2s) through IL-25 receptor (IL17-Rb), which in turn secrete IL-4/13. These secreted interleukins interact with IL-4 receptor on intestinal epithelial stem cells, reprogramming them to produce sensory tuft cells and goblet cells which secrete mucins to protect the intestinal epithelial barrier [58]. As a whole, this system functions in a self-contained fashion to protect and repair the intestinal epithelium, but as a consequence tuft cell hyperplasia occurs, likely increasing the potential for CSC transformation. Given the long-lived nature of some tuft cells [47], this risk may continue long after inflammation subsides, and as tuft cells are also present in GI tumors, this mechanism may be a significant factor in epithelial-immune crosstalk within the TME.

3.4. Evasion of Antitumor Immune Mechanisms within the TME. Several mechanisms allow tumor epithelial cells within the TME to escape detection and eradication by the immune system. Immune cells depend on the presentation of antigens to detect, home to, and neutralize an aberrant cell within the tissue. This process can be subverted by inhibiting antigen presentation machinery directly on tumor epithelial cells [59], deactivating immune antigen-presenting cells (such as dendritic cells), intercepting cytotoxic $CD8^+$ T-cells and natural killer cells, avoiding autophagy from macrophages, activating CAF-based desmoplasia, and other mechanisms [60]. Moreover, the composition of immune cells within the TME is a key facet in tumor progression and response to therapy. This includes patterns of cytotoxic T-cell tumor infiltration, recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs) and *T* regulatory cells (Tregs), and reprogramming of macrophages towards an anti-inflammatory phenotype [60].

Despite a wide variety of innate and adaptive mechanisms by which the immune system maintains surveillance for cancer, tumors are notoriously successful at avoiding immune-based detection. Without expression of major histocompatibility complex (MHC) class I or with damage to associated antigen peptide transport, the immune system's ability to detect aberrant cells, including tumor cells, is highly limited [59, 60]. Essential components in this process include endoplasmic reticulum-based chaperones calnexin and tapasin. These chaperones assist in the transport of peptides and preparation of the trimeric complex of B2-microglobulin, MHC class I heavy chain, and antigen peptide. When presented on the surface of the cell, T-cell receptors (TCRs) detect this complex and perform their associated tasks, and its expression is often associated with improved responses to chemotherapies and immunotherapies, while loss of expression or alterations to antigen presentation machinery can result in resistance to therapy [59].

Various signaling pathways are involved in regulating the tumor immune response and cancer immunosurveillance [60]. Many of these pathways are common to both immune and other TME cells as well as tumor epithelia. Therefore, attempts to target them must take their activity in multiple cellular compartments into consideration. Examples of multicompartment pathways involved in regulating cancer immunosurveillance include MAPK, WNT, PI3K, and STAT3 signaling pathways [60]. For example, IL-6-mediated STAT3 activation is a key driver of M1 (proinflammatory) to M2 (anti-inflammatory) macrophage transition [61] and an active tumor epithelial pathway that directs proliferation and metastasis [62]. Tumor secretory factors driven by molecular signaling pathways often directly regulate TME components. Prominent examples regulating the immune compartment include TGFB, PGE2, and VEGF [60]. Variability in these pathways is one of the reasons that advanced molecular subtyping and personalized therapy is likely to hold great potential in GI cancers.

Individual TME cell types and structural components have varied roles in manipulating cancer immunosurveillance. Mounting evidence suggests that CAFs are able to recruit M2-like macrophages, MDSCs, and Tregs and remodel the TME towards an immunosuppressive and protumorigenic phenotype [63]. Moreover, they may express immune checkpoint markers PD-L1/2 and directly interfere in natural killer cell-mediated cytotoxicity and are in part responsible for defining and altering the properties of extracellular matrix (ECM) [63, 64]. Aside from structural fibers that support the tumor in three-dimensional space, ECM holds a mixture of growth factors, enzymes, and signaling molecules [64, 65] which can regulate the activity of tumor-associated macrophages and entice colonization of the tumor site by endothelial and immune cell subsets [63, 64]. Importantly, it also functions as a track for the migration and invasion of tumor epithelial cells that have undergone EMT as they attempt to disseminate to distant sites [64]. Taken together these concepts demonstrate the importance of the TME to evasion of cancer immunosurveillance.

3.5. TME and Organ-Organ Axes. Recently, functional organ-organ axes (e.g., gut-lung axis and gut-brain axis) have been described in nontumorigenic contexts [3, 4, 66–68], but this concept remains controversial in cancer. Surprising findings in this field demonstrate the importance of the gut microenvironment to cognition and neurological disorders (autism, addiction, and depression) [3, 66], nongut inflammatory conditions (asthma and COPD) [69], and liver diseases (e.g., cirrhosis) [67] among others. The limited

research findings in this area tend to focus more directly on microbiotic populations, but the implications for the GI TME merit interrogation of the contribution of mammalian cells and pathways to these axes and their drivers.

Signaling between the gut and distant organs is hypothesized to occur through pathogenic and/or immune mechanisms. In the pathogenic mechanism, alterations in populations of gut bacteria caused by stimuli such as altered diet, increased stress, or gut disorders can result in secretion of bacterial products and microenvironmental remodeling characterized by altered pH, increased barrier permeability, and the exposure of organs outside of the gut to the byproducts of these alterations [68]. The immune component is thought to be directed by pathogens and, by proxy, the response to their presence occurring through secretion and sensing, priming of progenitor populations for differentiation, and activation of mature populations leading to or orchestrated by molecular and functional alterations in immune cells [70].

Extrapolation of findings concerning inflammatory innate lymphoid type II cells (iILC2s) makes a case for considering the impact of gut-organ axes in the tumor and metastatic microenvironment. Until recently, ILC2s were thought of as resident within each tissue and suspected to originate in the bone marrow. However, iILC2s which arise in the presence of IL-25 or IL-25 stimulating pathogens do not exist in appreciable numbers at homeostasis but become plentiful upon IL-25 stimulation in multiple organs including the gut, lung, and liver. Recent findings trace the origin of these cells to the gut lamina propria, where they are enticed to migrate to distant organs by lipid-mediated chemotaxis [71]. These findings imply that inflammation within the gut can have significant consequences not only to the GI tract and TME but also to non-GI TMEs. Interestingly, activation of ILC2s, which is necessary to protect the gut epithelium during injury, not only regulates the fate of epithelial progeny by stimulating stem cells [58] but also maintains macrophage-dependent immunity [72]. Both of these processes may play an important role in tumor progression and drug resistance, and preclinical evidence indicates that blocking IL-25 signaling through its receptor (IL-17Rb) using a mAB may be an effective therapeutic route in PDAC [73]. However, in another study, direct neutralization of IL-25 with a mAB in a chemically induced inflammatory model of CRC supports tumor progression [74], suggesting that the response to IL-25 therapy would likely be heavily dependent on the characteristics of the patient's TME.

4. Future Directions for GI TME Research and Development

In the immediate term, pressing obstacles to overcoming GI TME protumor mechanisms in the clinic include testing the expanded use of drug combinations like FOLFIRINOX, devising methods to identify patient subsets that may benefit from immunotherapy, and improving management and therapy through precise classification of tumors, development of biomarkers, and integration of computational technologies into clinical workflows. Beyond the immediate term and *in silico* advances such as scRNA-Seq, deep-learning, and advanced imaging technologies, the development of new therapies with increased specificity against traditional biochemical targets, engineered biological therapies like CAR-T and mABs, and others are expected to expand survivability and improve outcomes. Overall, a more holistic understanding of molecular, microenvironmental, environmental, and behavioral contributors to inflammatory damage and cancer in the GI tract is needed to expand translational and clinical applications and prevent and/or delay tumorigenesis and progression.

4.1. Emerging Clinical Importance of the TME. The TME is perhaps the most difficult component for clinicians to monitor in GI cancers. Noninvasive imaging techniques are currently useful to assess macroscopic changes at the organ level but not yet sufficient to identify changes in the TME in most cases. The ability to analyze the TME directly is also compromised by static access to tissue, which can often only be obtained at predetermined times such as following diagnostic biopsy and surgical intervention. Moreover, the TME is not homogeneous, so information gleaned from these specimens may be of limited clinical value. As a result, it will be necessary to develop therapies that target specific TME components and biomarkers that provide a surrogate measure to monitor changes during the course of therapy.

The majority of novel drug classes currently being developed to target the TME falls under the category of immunotherapies. As discussed in the previous paragraph, immune checkpoint inhibitors have shown success in limited subsets of GI tumors, especially CRCs demonstrating MSI or defective mismatch repair [38–40]. High MSI (MSI-H) is characterized by hypermutation linked to MLH1 promoter hypermethylation but can also be induced by hereditary mutations to mismatch repair machinery [75]. Perhaps counterintuitively, the increase in mutations results in enriched neoantigen presentation, making the use of immune checkpoint therapies possible as seen in MSI-H CRC. These therapies work by neutralizing CTLA4 or through blocking the interaction between PD-L1 (CD274), expressed on tumor epithelia and surrounding supportive cells, and PD-1, expressed on infiltrating $CD8^+$ cytotoxic T-cells, a process that is regulated by MHC class I and the components of the neoantigen presentation apparatus. Indeed, ongoing clinical trials are demonstrating promise for these immunotherapeutic agents with or without combination therapy, and the success of PD-1/PD-L1 axis therapies has invigorated the pursuit of targets exploiting other immune-tumor interactions [76]. Prominent emerging targets in this field of development include CD47 [77], CD40 [78, 79], interleukins such as IL-10 and IL-17 [73, 80], inflammatory mediator IDO1 [81], and a variety of engineered viral, vaccine, and cell therapies.

Currently, clinical trials are underway to investigate macrophage targets CD47 and CD40 in GI cancer. CD47 is a highly expressed tumor epithelial extracellular ligand that is detected by the macrophage SIRPα receptor. This "do not eat

me" signal activates phosphatases SHP-1/2 resulting in inhibition of autophagy and ultimately protection of the tumor epithelial cell. CD47 or SIRPα mABs can bind and prevent this interaction, allowing effective autophagy [77]. CD40 expressed on tumor cells can be stimulated by agonist CD40L (CD156) to directly induce apoptotic cell death. In the immune compartment, CD40 activation on dendritic cells leads to the recruitment of tumor-targeting cytotoxic T-cells, and on B-cells, it stimulates endogenous antitumor mAB production. Similarly, in macrophages, CD40 activation leads to cytokine and chemokine secretion, which may have antitumor activity in some contexts. Both CD40 agonist mABs and ligands can be used to simulate this activity [78, 79]. Together, CD40 and CD47 are prime examples of clinical development targets leveraging TME knowledge gleaned from decades of basic laboratory research and are an important step towards phagocytosis modulating anticancer drugs.

Secretory products from the TME are also major drug development targets in oncology. Among these, interleukins are perhaps receiving the most attention at the moment, and evidence for targeting chemokines and their receptors is accumulating. Pegylated IL-10 is one such example (pegilodecakin; AMA0010) that is currently being tested. In PDAC clinical trials, pegilodecakin is able to increase the activation of cytotoxic T cells and improve overall survival when combined with FOLFOX [82]. Further assessments of interleukins in cancer therapy are largely occurring at the preclinical level, with promising findings for targets such as IL-25 [73, 80] as previously discussed. Fortunately, the prior interest in these and related targets for psoriasis, asthma, and inflammation-related disorders has resulted in a variety of existing therapies [83], including mABs with known human safety profiles that could be repurposed for cancer immunotherapy.

Finally, more traditional intracellular biochemical targets with the ability to regulate TME components are being studied. IDO1 is a key enzyme in the conversion of tryptophan to kynurenine. When levels of IDO1 enzyme are high in the TME, cytotoxic T cell and NK cell functions are suppressed, regulatory T cells are activated, and MDSCs expand. Inhibiting the activity of the enzyme can reverse this process and enhance antitumor immunity [81, 84]. Various compounds in this class are being used in clinical trials in liver, pancreatic, and other tumors. Similar to PD1-targeted immunotherapies, IDO1 agents are expected to have therapeutic potential in subsets across the solid tumor spectrum, and companion biomarkers and identification of susceptible tumor subtypes will likely be essential to IDO1-targeted therapy. Additionally, given the mechanism of action, IDO1 inhibitors are also being trialed in combination with immunotherapies such as nivolumab and pembrolizumab [84].

4.2. TME Biomarker Concepts and Personalized Medicine. The effective development of predictive biomarkers utilizing next-generation technologies has only recently become possible. The clinical potential for these markers is demonstrated by the use of the Prosigna (formerly PAM50), Oncotype Dx, and Mammaprint tests in hormone-positive breast cancers. Moreover, the development of comparable GI cancer markers is progressing rapidly as evidenced by the predictive abilities of the consensus molecular subtypes (CMS) developed by the CRC subtyping consortium. These 4 subtypes are characterized by MSI and immune activation (CMS1), canonical signaling through WNT (CMS2), dysregulated metabolism (CMS3), and TGFB and stromal/angiogenic characteristics (CMS4) [42]. These findings not only are improving the understanding of the initiation and progression of CRC but also have practical implications for the treatment and monitoring of patients bearing tumors meeting these biomarker criteria. For instance, standardized CMS subtyping routines provided for RNA-Seq data may be applied to clinical trial data to identify tumor subtypes that are susceptible to therapy. This may lead to approval of drugs that were effective in specific subtypes, but not robust to overcome progression in all 4 CMS subtypes [42]. A handful of clinical trials targeting the CRC CMS4 subtype with novel immunotherapies including anti-PD-1 mAB spartalizumab [85], dual PD-1/TGFB engineered mAB-fusion protein M7824 [86], and a dendritic cell vaccine (AVEVAC) [87] have already begun in the US and EU. Similar subtyping advances in pancreatic [41], gastric [22], and liver cancers leveraging data accumulated from large-scale multicenter projects including the Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC), and others hold similar potential.

Inflammatory biomarkers are another key area of development exploiting the properties of the GI TME. These can be detected by a variety of methods in the tumor tissue, serum, and from other sources. Examples of these include standardized ratios of immune cell types (e.g., neutrophil, lymphocyte, and platelet), levels of circulating cytokines/chemokines, and transcriptome-based subtyping of inflammatory subtypes (e.g., CMS1 CRC (Bailey et al 2016 immunogenic PDAC subtype)) [41, 42, 62, 88, 89]. Gardini et al. identified hepatocellular carcinoma (HCC) patients with increased neutrophil-to-lymphocyte ratio (NLR) or systemic inflammation index (platelet count $\times$ NLR) as prone to disease progression when undergoing sorafenib therapy compared to those with lower ratios. Adjusting for other relevant clinical factors demonstrated the independence of this prognostic measure [89]. Similar findings with NLR in metastatic CRC demonstrate its potential to predict objective response to therapy over multiple courses of treatment (1^{st}–3^{rd} line) [88]. Apart from direct measurements of immune cell types, secreted cytokines and chemokines are potential surrogates for immune activity in the TME including in response to drugs. For example, elevated serum IL-6 levels are indicative of increased risk of HCC and PDAC as well as predictive of PDAC progression [62]. Overall, exploiting objective measures of TME inflammatory characteristics is likely to further personalize therapy in GI cancers.

Concurrent to advances in expanding the use of inflammatory biomarkers and deciphering the underlying molecular characteristics of GI tumors through accumulation of data, improvements to sequencing technology,

machine-learning subdisciplines, and imaging are heralding an era of precision medicine. The use of scRNA-Seq in particular is poised to dimensionally expand knowledge of the GI tumor niche and subtypes and increase the practical value of basic research findings. The two major obstacles to the development and practical use of new and existing next-generation biomarker technologies are limitations in obtaining appropriately sized cohorts with suitable sample quality for analysis and prohibitive financial costs. However, the ability to overcome these obstacles is well within sight, and costs for sequencing are decreasing by the year. Finally, as in previous phases of research and development, these maturing technologies will seed new technologies that will fundamentally shift the approach to biomarkers, such as the recently described Slide-Seq technique which combines standard tissue pathology and scRNA-Seq [90] and other multidimensional techniques.

5. Conclusions

GI cancers are increasingly prevalent worldwide, and the importance of the health of the GI tract in these and non-GI cancers is becoming more apparent. Currently, inflammation is reemerging as a focal point, as the role of the immune system and efficacy of new immunotherapies have taken center stage in oncology. Advances in basic research and technological innovations such as scRNA-Seq, which dimensionally expand our understanding of the TME, are beginning to provide a more holistic concept of GI tumorigenesis and progression. The knowledge gleaned from these advances will support a new generation of therapies and diagnostics, which should enable a breakthrough era of personalized GI cancer management and lead to improved quality of life and survival. In the coming years, basic and clinical researchers should focus on leveraging this growing knowledge to more completely uncover the structure and mechanics of the GI TME and to increase the precision of therapeutic intervention for GI cancer patients.

Conflicts of Interest

The authors declare no conflicts of interest relevant to the contents of this review.

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Review Article

Exosomal miRNA: Small Molecules, Big Impact in Colorectal Cancer

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Colorectal cancer (CRC) is one of the major causes of cancer-related deaths worldwide. Tumor microenvironment (TME) contains many cell types including stromal cells, immune cells, and endothelial cells. The TME modulation explains the heterogeneity of response to therapy observed in patients. In this context, exosomes are emerging as major contributors in cancer biology. Indeed, exosomes are implicated in tumor proliferation, angiogenesis, invasion, and premetastatic niche formation. They contain bioactive molecules such as proteins, lipids, and RNAs. More recently, many studies on exosomes have focused on miRNAs, small noncoding RNA molecules able to influence protein expression. In this review, we describe miRNAs transported by exosomes in the context of CRC and discuss their influence on TME and their potential as circulating biomarkers. This overview underlines emerging roles for exosomal miRNAs in cancer research for the near future.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in men and the third in women in Europe [1]. 772,000 newly diagnosed cases were registered in 2018, and the estimated number of CRC-related deaths is 242,000. Recently, despite the development of therapies revolutionizing cancer treatment like immune checkpoint inhibitors (e.g., anti-PD-1, anti-PD-L1, or anti-CTLA-4 antibodies), clinical prognosis in CRC remains unsatisfactory, with a 5-year survival rate neighboring 13% at the metastatic stage IV of the disease [2]. An area of study carrying hope for future therapies is the understanding of the relationship between patient prognosis and tumor landscape in primary colorectal tumors. Genetic and epigenetic background of the tumor, as well as tumor microenvironment (TME) composition, are the main factors explaining heterogeneity of response to therapy observed in patients. The TME contains many cell types including stromal cells, immune cells, and endothelial cells. The resulting intra- or intertumoral heterogeneity is of prime importance for all aspects of tumor metabolism and explains the differences in tumor abilities to proliferate, invade, and escape therapy [3–6].

In this context, exosomes are emerging as major contributors in cancer biology. Exosomes are lipid-bilayer, cup-shaped nanovesicles (diameter: ~50–150 nm) secreted by cells and originating from the endosomal pathway. Exosome release is a common mechanism, and a broad range of cells secrete exosomes, including tumor cells. As a result, exosomes have been detected in a wide variety of biological fluids (e.g., blood, urine, saliva, malignant ascites, and breast milk) [7, 8]. Cumulative evidence suggests that exosomes

can establish a fertile environment to support tumor proliferation, angiogenesis, invasion, and premetastatic niche formation. Moreover, they may also facilitate tumor growth and metastasis by inhibiting immune surveillance and by increasing chemoresistance via removal of chemotherapeutic drugs. It has been often reported that tumor cells generate more exosomes than normal cells and that circulating exosome levels are increased in the blood of cancer patients when compared to healthy individuals [9–11]. These features make exosomes interesting reservoirs of potential cancer biomarkers such as proteins, lipids, and RNAs. Although there are some CRC tumor markers used worldwide, there is a particular need for new biomarkers due to technical constraints concerning their detection [12]. In this context, exosomes have become in the last few years an important area of research.

Given their role in TME, exosomes have an essential function in cell-to-cell communication, but they also have specific biological functions. The bioactive cargos received by a recipient cell can modify its physiology by tempering with a vast range of processes [13–17]. Exosomes are implicated in tumor cell proliferation [18], increased migration and invasive properties [19–21], resistance to chemotherapy [22], angiogenesis [23], and escape from the immune system [24]. Although miRNA proportion in exosomes may drastically change depending on the physiological context, tissue, or cell type, they often represent one of the predominant RNAs contained in exosomes [25–27]. Exosomes protect miRNAs from degradation, enabling them to be stably expressed in the extracellular space and to be efficiently integrated by specific recipient cells [28]. Consequently, exosomal miRNAs are also deeply implicated in cancer progression. Therefore, modification or inhibition of exosomal miRNAs might be a potential therapeutic strategy in cancer. In this review, we focus on the impact of miRNA on TME in CRC. First, a description of miRNAs and their biogenesis will be presented, followed by a description of exosome biogenesis and composition. We will conclude by a description of the action of exosomal miRNAs in CRC.

2. miRNAs

miRNAs are short single-stranded noncoding RNAs, with a size varying generally between 18 nt and 25 nt (usually 22 nt), that possess the ability to bind complementary target messenger RNAs (mRNAs). miRNAs can induce either translational repression or sometimes degradation of their mRNA targets, thereby constituting a crucial part of post-transcriptional regulation of mRNA expression. Several studies reported the importance of miRNAs in cancer progression, including tumor proliferation, invasion, migration, cell survival, regulation of the immune response, angiogenesis, epithelial-mesenchymal transition (EMT), and cellular stemness [29–35].

In the canonical pathway, miRNAs are at first expressed by the RNA polymerase II as immature stem-loop structure-containing precursors, known as pri-miRNA, of a few hundred to several thousand nucleotides long [36]. However, some pri-miRNAs can be transcribed by RNA polymerase III and some, like miRtrons, are not issued from dedicated transcriptional units but are matured from mRNA introns. A whole cellular machinery is devoted to their processing and nucleocytoplasmic export into functional cytoplasmic miRNAs. First, pri-miRNA precursors are processed into smaller stem-loop pre-miRNAs (approx. 70 nt) by the Microprocessor complex [37]. This complex consists of the Drosha protein, carrying the RNAse activity and DGCR8, that helps determining the proper endonucleolytic cleavage site [38, 39]. Pre-miRNAs are then recognized and exported to the cytoplasm by Exportin-5, where they undergo further endonucleolytic cleavage at the extremities of the stem structure by the RNAse Dicer [40]. The resulting product corresponds to a duplex of 2 complementary miRNAs, the leading strand miRNA or 5p miRNA (formerly at the 5′ extremity of the pre-miRNA) and the passenger strand or 3p or star (∗) miRNA (formerly at the 3′ extremity of the pre-miRNA). This duplex is loaded into a protein complex containing notably Argonaute protein (Ago2), which retains only one of the 2 miRNA strands to form the functionally active RISC complex [41]. The miRNA within RISC complex can recognize and bind to a crucial guide sequence in the target mRNA, located in the vast majority of cases in the 3′-untranslated region (3′-UTR). This sequence, called "seed," corresponds typically to the position 2 to 8 at the 5′ extremity of the miRNA [42]. Mostly, miRNA pairing with its target is rather imperfect and leads to translational repression or destabilization of the mRNA target [43, 44]. Occasionally, complementarity with the mRNA target is almost total, leading to mRNA cleavage and degradation [45]. As of today, there are around 2,000 entries for human miRNAs in the miRBase database (v22.1) (http://www.mirbase.org/index.shtml). Even if the function of most of them is still unknown, miRNAs are predicted to target most existing mRNAs. Over the years, evidence for their involvement in almost all biological processes accumulated, especially concerning their ability to target oncogenic or tumor suppressor genes in multiple cancer-related cellular pathways [46, 47]. miRNAs are present in significant proportions in blood (and several biological fluids such as saliva, urine, and semen), either incorporated in nucleoprotein complexes with Ago2 protein, nucleophosmin1 protein, within high-density lipoproteins (HDL) particles, or finally encapsulated within exosomes or other extracellular vesicles (EVs) [48–50].

3. Exosome Biogenesis and Composition

Exosome biogenesis is initiated by inward membrane invagination of early endosomes to form intracellular multivesicular bodies (MVBs) and then released into the extracellular environment by MVB fusion with the plasma membrane (Figure 1). They differ from other EVs, like ectosomes, which are created by outward budding of the plasma membrane, and apoptotic bodies created during the apoptosis process [51]. Using complex signaling and molecular machineries, like the Endosomal Sorting Complex Required for Transport (ESCRT), newly forming exosomes can incorporate various biologically active molecules. These

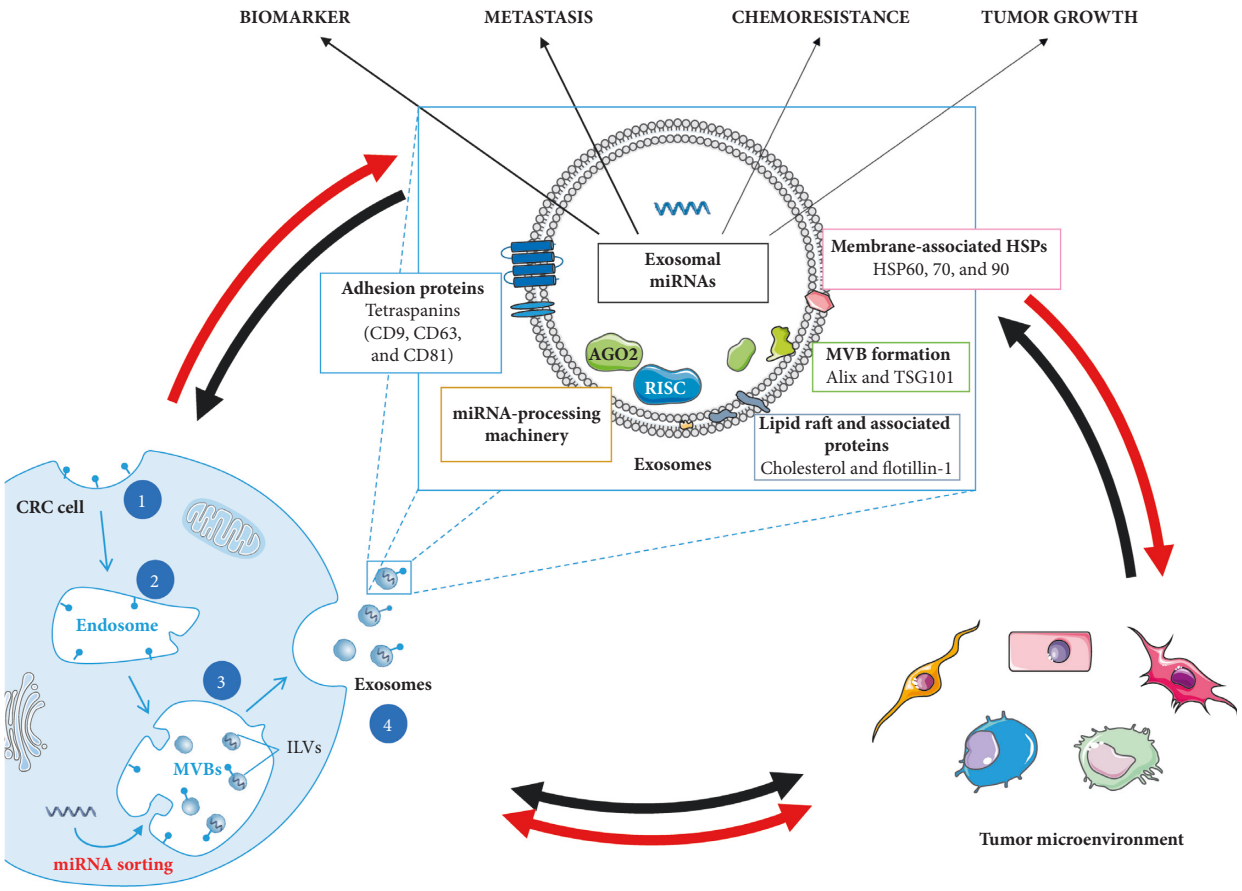

Figure 1: Scheme of exosome biogenesis, composition, and major role in TME modification, in the context of CRC. The biogenesis of exosomes involves 4 different steps: (1) the membrane invagination; (2) endosome formation; (3) generation of the exosome precursors, called intraluminal vesicles (ILVs), by inward budding of endosomes (these accumulations of ILVs are termed as multivesicular bodies (MVBs)); and (4) the fusion of MVBs with the plasma membrane release the ILVs in the extracellular space by exocytosis and become exosomes. Composition: exosomes are composed of different types of enzymes and proteins involved in adhesion, intracellular signaling, immunostimulatory molecules, multivesicular body (MVB) formation, and heat shock proteins (HSPs). Exosomes contain nucleic acids, including miRNA, mRNA, DNA, and small noncoding RNA (snRNA and tRNA). In addition to direct interactions between CRC cells and TME, exosomes, especially exosomal miRNAs, play a key role in the cross talk between cells in TME. CRC cells can release exosomes that will modify TME cells and promote tumor growth, metastasis formation, and chemoresistance. Inversely, stromal cells can also release exosomes that influence tumor cell metabolism. Differential expression of miRNAs within exosomes could also be useful in CRC as biomarker for diagnosis and monitoring.

include different types of nucleic acids and soluble and transmembrane proteins [52, 53]. Among the proteins present in secreted exosomes, some are involved in its biogenesis, like tetraspanins (CD9, CD63, and CD81), Tsg101, and Alix (Figure 1). These proteins are often used as markers, validating exosome enrichment during exosome isolation. Coupled to exosome physical-chemical characteristics (size, density, and buoyancy), they can help discriminating exosomes from other EVs and extracellular particles [54, 55]. Besides, exosome membranes are enriched in lipids (e.g., ceramide, cholesterol, phosphatidylserine, and sphingolipids) and lipid rafts, also playing an important role in their biogenesis and conferring exosomes reinforced rigidity compared to plasma membrane [53]. In particular, ceramide accumulation resulting from conversion of sphingomyelin by sphingomyelinases participates in the formation MVBs [56]. Exosomes also contain proteins that play a functional role in cellular communication, like in antigen presentation. Proteins of the molecular histocompatibility complex (MHC) and various heat shock proteins (Hsp60, Hsp70, and Hsp90) are present in exosomes [57–62]. The incorporation of secreted exosomes into the recipient cell takes place by several mechanisms including macropinocytosis, phagocytosis, endocytosis, or interaction through surface receptors [63, 64].

During their formation, exosomes naturally incorporate cytoplasmic medium. Initially, it was hypothesized to be a nonselective process, resulting in a similar miRNA concentration both in exosomes and parenting cells. Some studies using miRNA for cancer diagnosis or prognosis purposes were implicitly based on the fact that circulating exosomal miRNA levels, especially in body fluids, should reflect accurately the miRNA content of their cells of origin. However, it was rapidly shown in several contexts that the

most expressed endogenous miRNAs in tumor or normal cells were not necessarily the ones predominantly secreted into the extracellular environment [65–67]. It is to note, however, that while some miRNA proportions are very different between the cell and the released EVs, this is not always the case. For example, some miRNAs among the most commonly present in both parent cells and exosomes, and that may be potential CRC diagnostic biomarkers present in tissue, plasma, and serum, are miR-192-5p, miR-10a-5p, and miR-191-5p [68, 69].

4. Exosomal miRNAs in CRC

The way miRNAs are selectively transported into exosomes for secretion (exosomal sorting) is still not completely clear, although several mechanisms have been proposed [70]. In this section, we will address those hypotheses and the role of different types of biomolecules in miRNAs selective transport into exosomes in the context of CRC.

4.1. Role of miRNA Putative Sequence Signals. Several studies suggest the requirement of intrinsic sorting signal sequences in miRNAs, needed for their incorporation into exosomes [71, 72]. One of those sorting mechanisms was described in exosomes from peripheral blood mononuclear cells. It involves recognition of 4-bp RNA motifs, GGAG, by the RNA-binding hnRNPA2B1 protein, provided that it is sufficiently sumoylated [72]. hnRNPC and hnRNPA1, members of the same family of protein, can also bind exosomal miRNAs. Nevertheless, no associated motif has been identified. Another RNA motif, GUUG, was found to be enriched in miRNAs present in exosomes derived from a CRC cell line (SW620) and resembles the GGAG motif recognized by hnRNPA2B1 [73]. This motif was also suggested to be involved in miRNA loading, but it is not known whether it constitutes a specificity of cancer cells or if some RNA-binding proteins, like hnRNPA2B1, intervene in the recognition of this motif.

4.2. Role of Exosome Membrane Lipid Composition. It has been reported that the lipid composition of exosome membranes directly influences exosome biogenesis and composition [53, 56, 74]. This also affects miRNA sorting into exosomes. For instance, the level of neutral sphingomyelinase2 (nSMase2), regulating ceramide synthesis, can influence the quantity of miRNA exported through exosomes [70, 75]. In CRC and hepatocellular carcinoma cell lines, it has been shown that sphingomyelin phosphodiesterase 3 (SMPD3), which also generates ceramide from sphingomyelin, is also involved in miRNA encapsulation [76]. SMPD3 inhibition leads to a decrease in exosomal miRNA levels, while the intracellular miRNA level in CRC cells increases. This influence of SMPD3 was, for example, reported for mir-638, a miRNA also downregulated in exosomes of CRC patients which has been proposed as a biomarker [77, 78].

4.3. Role of Proteins Involved in miRNA Biogenesis and Functions. The miRNA maturation process is connected with miRNA export in exosomes and endosomal trafficking. Knockout of Ago2 leads to the selective decrease of certain miRNA populations in exosomes from several cell lines [79]. In addition, components of the RISC complex can colocalize with MVBs, when MVBs turnover into lysosomes is blocked [80]. In exosomes derived from different cancer cell types, all the essential elements required for pre-miRNAs processing into mature miRNAs, including Dicer and Ago2, are available [10, 81]. When transfected with *C. elegans* pre-miRNA, those exosomes were able to process this pre-miRNA into mature miRNA. This was confirmed to be a Dicer-dependent process. In contrast, miRNA maturation machinery was not detected in exosomes from nontumorigenic cancer cells. CD43, a suspected mediator of active protein transported into exosomes, is enriched in those exosomes. This protein is responsible for the increased level of Dicer, further linking exosome processing with miRNA biogenesis [82, 83]. Probably also related to miRNA biogenesis, it was observed that passenger-strand (3p) miRNAs seem predominant in CRC cell-derived EVs compared to their 5p counterparts [84].

One mechanism highlighted in CRC cells underlines a possible role of the small GTPase KRAS in miRNA sorting. KRAS mutations occur in more than a third of sporadic colorectal cancers, and it has been associated with several other cancers, in particular, regarding tumor aggressiveness [85–87]. Exosomes secreted by KRAS mutant CRC cells can induce growth and migration of wild type (WT) cells [88, 89]. KRAS mutations can influence the recruitment of Ago2, involved in miRNA maturation and secretion, into the nascent exosome [90, 91]. In particular, KRAS mutations affect exosomal encapsulation of several miRNAs implicated in CRC, such as the oncogenic miR-10b, which is selectively retained in WT KRAS-cell exosomes [90]. A higher rate of tumor-suppressor miRNA sequestration and decreased level of oncomiRs were observed in exosomes compared to their parent CRC cells [92]. This process seems to depend on the major vault protein (MVP), a proposed miRNA-binding protein responsible for sorting miRNA to exosomes that is overexpressed in multidrug-resistant cancer cells [93, 94]. Since tumor cells can selectively retain oncomiRs, it was suggested as a phenomenon favoring tumor growth and progression [90, 92]. Moreover, exosome secretion could be used as a way to discard tumor-suppressor miRNAs or other molecules that promote apoptosis, cell cycle arrest, or differentiation, thus also enhancing tumor cell growth and metastasis. This selective secretion was, for example, observed for several tumor-suppressor miRNAs, like miR-23b, miR-224, and miR-921 [95]. In that study, it was shown to be dependent on an important exosome transporter, Rab27, and to significantly affect metastasis and angiogenesis potential of bladder carcinoma cell lines. Because most studies rather focused on how the miRNAs secreted from tumor cells influence their environment, these interesting data need further investigation.

As we will see in the following sections, there are hints that these mechanisms can be disturbed during the tumorigenic process in CRC, explaining the differences systematically observed in miRNA content between exosomes from healthy individuals and CRC patients.

5. Exosomal miRNAs Influence CRC Tumor Microenvironment

Exosomal miRNAs in the tumor microenvironment (TME) have a significant influence on tumor development and progression but are also able to transfer the ability to resist to the anticancer therapy [96–98]. The following section will present the main exosomal miRNAs (exomiRs) proven to be functionally implicated in CRC tumor metabolism. These include miR-21, the miR-200 family, the miR 17~92 cluster, and miR-1246 alongside other relevant miRNAs. Information on expression, role as a biomarker, and function of each miRNA in CRC will be further detailed in the following sections. Available data are summarized in Table 1.

5.1. Exosomal miR-21 and miR-155

5.1.1. Expression and Role as Biomarker. miR-21 was the first shown to be expressed at high levels in the exosomes of 3 different CRC cell lines (HCT-15, SW480, and WiDr) [99]. Interestingly, these tumor-derived exosomes were found to be transferred to normal hepatic and lung cell types, preferred metastasis targets for colon tumors. Later, it was confirmed that miR-21 was overexpressed not only in colon tumor tissue and in liver metastases tissue, but also in plasma exosomes of CRC patients [11, 100]. Exosomal miR-21 expression in plasma has been significantly correlated to its expression on tumor tissue, but also to disease stage, occurrence of liver metastasis, and prognosis. Other studies have reported that this exomiR can be used as a biomarker in CRC [101] but also as a general biomarker of gastrointestinal cancers including esophagus, rectum, and pancreas [102].

miR-21 was systematically found in miRNA populations characterizing circulating exosomes from plasma, feces, and serum in the context of colorectal cancer, as well as in exosomes from different CRC cell lines [84]. It is thereby possible that the circulating biomarker value of miR-21 comes mostly from its presence in exosomes. Nevertheless, it was recently shown that nonvesicular Ago2-associated miR-21 was actively released from HT29 CRC cell lines and that its levels could surpass those of EV-encapsulated miRNA in the absence of chemical lysis [120].

5.1.2. Function in CRC. Exosomal signal of stromal origin, such as exosomes produced by normal fibroblasts (NOFs), can be transferred to CRC cell lines (DLD1 or SW40) and lead to an increased expression of miR-21-5p. This transfer also leads to increased phosphorylation of cell-signaling factors Erk, Akt, and Bad, resulting in an increased resistance to the anticancer drug oxaliplatin (Figure 2(a)).

Overexpression of mir-21 observed in exosomes from CRC tissues leads to a drastic reduction of endothelial progenitors cell (EPC) migration, proliferation, and invasion properties [121]. EPCs are circulating progenitor cells of different types, able to differentiate into functional endothelial cells and to participate in new vessel formation and blood vessel regeneration. This effect on EPCs occurs most likely through direct targeting of interleukin 6 receptor (IL6R) (Figure 2(b)). Since EPCs promote thrombus repair and resolution, it was hypothesized that it led to a higher incidence of deep-vein thrombosis, a prognostic factor in cancer patients.

In the context of CRC, stromal cells themselves can also release miR-21 into the TME, in agreement with previous observations based on stromal microdissections [122]. The altered cancer-associated fibroblasts (CAFs) produce miR-21 rich exosomes, both in regards to intracellular levels but also to the exosome content of NOFs. This increased expression in exosomes is associated with an increase in liver metastasis. These data were confirmed *in vivo* in mouse orthotopic xenografts (Figure 2(c)) [103].

miR-21 is enriched in exosomes produced by M2 macrophages, as is the oncomiR miR-155 [104]. M2 macrophages serves as *in cellulo* model for tumor-associated macrophages (TAMs) present in the TME. These macrophages promote proliferation, invasion, and metastasis of cancer cells, angiogenesis, and immune escape [123]. In CRC cells, both miR-21 and miR-155 are able to target the transcriptional regulator BRG1, resulting in increased migration and invasive behavior (Figure 2(d)). Exosomal miR-21 and miR-155 were thus suggested to be partly responsible for TAM's effects on CRC cells.

5.2. Exosomal miR 17~92 and 25~106b Clusters

5.2.1. Expression and Role as Biomarker. Members of the 17~92 miRNA cluster (miR-17, -18a, -19a, -19b-1, -20a, and -92a-1) were detected in high proportions in exosomes from the LIM1863 CRC cell line, alongside the members of its paralog cluster miR 106b~25 (mir-25b, -93, and -106b) [84]. Interestingly, miR-17, -19a, -20, and -92a are specifically enriched in exosomes as compared to several of their parent CRC cell lines, indicating their potential importance in exosomal communication [106]. miR-19a, -19b, and -92a are also upregulated in serum exosomes of CRC patients compared to those of healthy individuals, which has been linked to liver metastasis recurrence [9]. miR-19a-5p, in particular, was also suggested as a convincing biomarker for CRC severity and lymph node metastasis appearance and prognosis. The upregulation and biomarker value for disease stage of miR-19a-5p, as well as miR-19a-3p and miR-17-5p, were confirmed in serum exosomes of CRC patients [101, 106]. Moreover, miR-17-5p, -18a-5p, -19a/b-3p, -20a-5p, and -92a-1-5p expression is significantly upregulated in exosomes derived from metastatic CRC cell line SW620 compared to exosomes derived from the nonmetastatic SW480 cell line [107].

5.2.2. Function in CRC (miR-25-3p). Concerning the precise role of those two miRNA clusters in exosomal regulation, the main set of evidence comes from miR-25-3p and its action on the formation of premetastatic niche. Serum-derived exosomal miR-25-3p expression has been associated with higher rate of metastases in CRC patients [23]. *In vitro* data suggest that CRC cell-derived exosomes containing miR-25-3p can enter endothelial cells and induce migration, angiogenesis, and vascular permeability. This was confirmed *in*

Table 1: Summary of exosomal miRNA with known functions in CRC. The main source of exosomes in the publications is indicated. Exosomes isolation techniques employed in the literature are indicated with their respective exosome enrichment validation procedures. Without any other mentions, plasma and serum are to be considered from human origin.

miRNA	Exosome source	Isolation technique	Exosome validation technique	Associated function	References
miR-21	(i) Cell supernatant (a) HCT-15, SW480, WiDR (b) CAFs, NOFs (c) Macrophages (ii) Serum (iii) Plasma (whole/mesenteric vs peripheral)	(i) UC (ii) UC/ExoQuick (iii) UC	(i) (a) WB : CsD81, (b) NTA/TEM/WB, (c) TEM/WB (ii) WB : CD81/none (iii) TEM/TEM + WB : Tsg101	Diagnosis biomarker, migration, invasion, liver metastasis, chemoresistance	[11, 99–105]
miR-155	(i) Plasma (whole/mesenteric vs peripheral) (ii) Cell supernatant (macrophages)	UC	(i) TEM/TEM + WB : Tsg101 (ii) NTA + TEM + WB	Migration, invasion	[104, 105]
miR-17~92 and 25~106b clusters	(i) Cell supernatant (SW480, SW620) (ii) Serum	(i) UC + OptiPrep (ii) qEV SEC/ExoQuick/UC	(i) TEM + WB (ii) EM + WB/-/TEM/TEM + WB	Diagnosis biomarker	[9, 23, 101, 106, 107]
miR-25-3p	(i) Cell supernatant (SW480, HCT116) (ii) Serum	UC	TEM + WB	Migration, angiogenesis, vascular permeability, pre-metastatic niches	[23]
miR-200 family	(i) Cell supernatant (a) SW640, SW480 (b) CCL27 (spheroid cultures) (ii) Plasma (mesenteric vs peripheral)	(i) (a) UC, (b) ExoQuick-TC (ii) UC	(i) (a) TEM, (b) None (ii) TEM, WB : Tsg101	EMT reversion marker, preventing permeation, associated with lower survival in exosomes	[98, 108–110]
miR-200b	Cell supernatant (SW480, HCT116)	UC	TEM + NTA	Proliferative activity	[98]
miR-1246	(i) Cell supernatant: (HCT116, HT29, SW480, Colo201, WiDR) (ii) Serum (iii) Plasma	UC	(i) WB : CD81/NTA/TEM (ii) WB : CD81 (iii) NTA + TEM + WB	Diagnosis biomarker, proliferation, migration, angiogenesis, pre-metastatic niches induction, TAM reprogramming	[11, 23, 102, 111]
mir-96 and mir-149	(i) Tissue (ii) Plasma	ExoCapTM + SG + FACS	TEM + WB : CD63	Tumor suppressor	[112, 113]
mir-486-5p	Plasma	Total exosome isolation kit	None	Tumor suppressor	[78, 114]
mir-6869-5p	Serum	Total exosome isolation kit	None	Prognostic biomarker, tumor suppressor	[78, 115]
mir-8073	Cell supernatant-HCT116	UC	None	Tumor suppressor	[116]
mir-193a	(i) Modified CT26 cells xenograft (ii) Serum	(i) UC + SG, pulldown (ii) exoEasy	NTA	Tumor suppressor	[92]
mir-10b	(i) Tissue (ii) Cell supernatant (HCT116)	UC + ExoQuick + exosomes precipitation solution	TEM-IG + WB	Oncogenic, CAF transformation	[117]
mir-142-3p	Cell supernatant (HCT-116, HT-29, SW480, MSCs)	UC	NTA + TEM-IG + WB	Induces stemness	[118]
mir-196b-5p	Serum	—	—	Induces stemness prognostic biomarker	[119]
mir-210	Cell supernatant (HCT-8)	Exosome precipitation solution	TEM	Induces EMT transition	[19]

EMT: epithelial-mesenchymal transition; FACS: fluorescence-activated cell sorting; MSCs: mesenchymal stem cells; OptiPrep: commercial density gradient medium; SG: sucrose gradient; TAM: tumor-associated macrophages; TEM/TEM-IG: electron microscopy (transmission/transmission coupled with immunogold labelling); UC: ultracentrifugation (may include differential centrifugation steps and eventual additional filtering); WB: western blot. Total exosome isolation kit (Invitrogen), ExoQuick (System Biosciences), exoEasy or exoRNeasy (Qiagen), qEV SEC (Izon), ExoCapTM (JSR), and exosome precipitation solution (Macherey-Nagel): commercial exosome purification solution or kits.

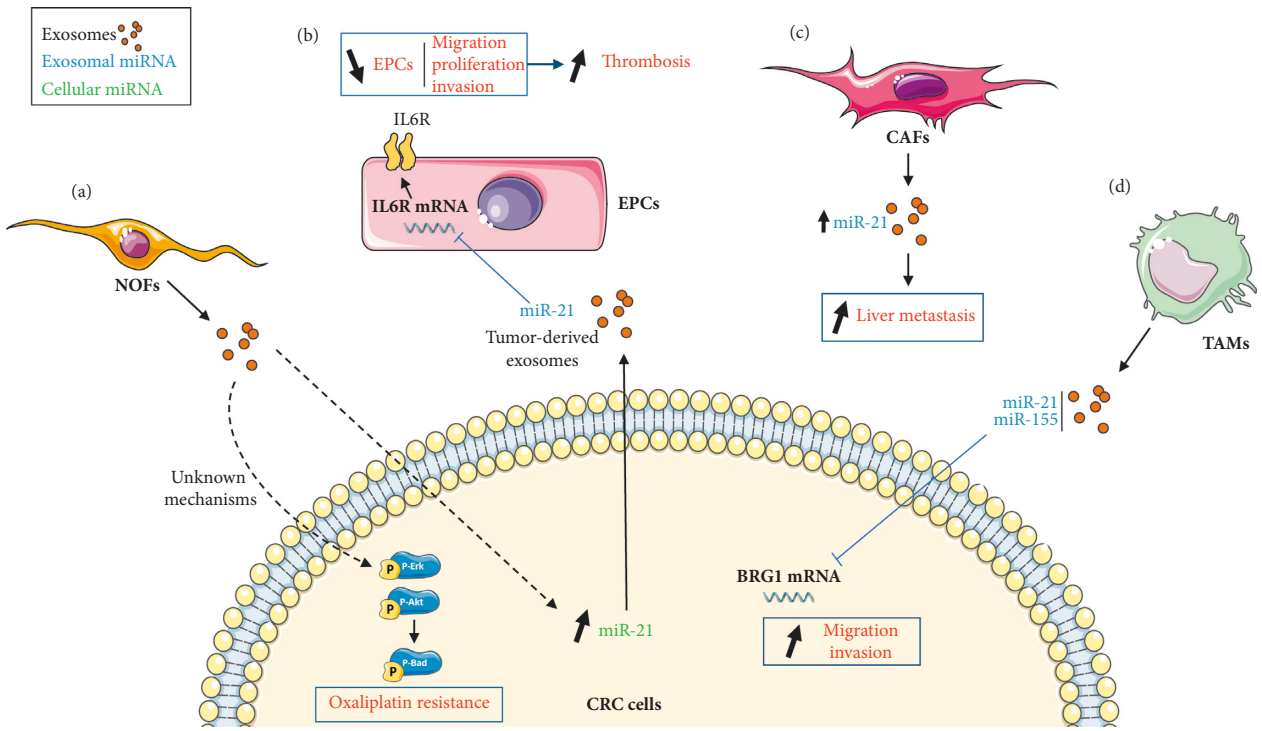

FIGURE 2: Proposed models for the role of exosomal miR-21 in CRC development. (a) Fibroblast-derived exosomes have an effect on CRC cells. The internalization of normal fibroblast- (NOF-) derived exosomes into CRC cells leads to an increase of cellular miR-21 and to the activation of phospho-Erk/Akt pathway, leading to oxaliplatin resistance. (b) CRC cells release miR-21-containing exosomes that are able to inhibit endothelial progenitor cell (EPC) IL6R mRNA transcription, leading to a reduced migration, proliferation, and invasion and favoring thrombosis in CRC. (c) Cancer-associated fibroblasts (CAFs) secrete miR-21-overexpressing exosomes which increase liver metastases. Tumor-associated macrophages (TAMs) also release miR-21-containing exosomes that can negatively regulate BRG1 mRNA in CRC cells and lead to an increased migration and proliferation.

vivo by tail vein injection of exosomes in mice, leading to a higher rate of metastases formation in liver and lungs, in a miR-25-3p-dependent manner. It was suggested to result from miR-25-3p targeting of the transcription factor KLF2. KLF2 negatively regulates expression of angiogenesis factor VEGFR2 and of KLF4, a transcription factor regulating the integrity of endothelial barrier and tight junctions.

5.3. Exosomal miR-200 Family

5.3.1. Expression and Role as Biomarker. Another important family of exosomal miRNAs in CRC is the miR-200 family, which encompasses two miRNA clusters. The first regroups miR-200a, -200b, and miR-429, and the second regroups miR-141 and 200c. Lower expression levels of miR-200c and miR-141 were significantly associated with better survival, in both the tumor draining vein (mesenteric) plasma and the corresponding exosomal fraction [124].

5.3.2. Function in CRC. On one hand, miR-141, -200c, and -429 have a protective effect against tumor progression, but only seemingly active in absence of the epithelial-mesenchymal transition (EMT), a crucial feature of cancer cells acquiring metastatic properties. Indeed, CRC metastatic cells (SW640) treated with the anticancer drug decitabine (DAC) reacquire epithelial characteristics by undergoing EMT reversal (MET). This includes inhibition of their migration and invasion properties. During this phenomenon, exosomal miR-141 and -200c expression increases, while remaining unaffected when DAC has no effect on EMT, like in the corresponding primary tumor cell line (SW480) [108]. This suggests that miR-141 and -200c expression in exosomes is negatively impacted by EMT and positively impacted by the mesenchymal-epithelial transition (MET) (Figure 3(a)).

miR-200c and also miR-141 and miR-429 are expressed in exosomes of naïve CCL27 CRC cell spheroids in 3D culture models [109, 110]. In cells surrounding the tumor, they directly target several members of the ZEB family, which are transcription factors involved in EMT (Figure 3(b)). As a result, miR-200c inhibits EMT in the lymphatic endothelial cells (LECs) co-cultured with CRC spheroids [110], and miR-200c, -141, and -429 inhibit EMT in co-cultured blood endothelial cells (BECs) [109]. Since exosomal expression of those miRNAs is lost in 5-FU (5-fluorouracil) chemoresistant spheroid cultures, surrounding cells engage in EMT transition, visibly weakening the lymphatic (LEC) and blood (BEC) endothelial barriers. By facilitating the crossing of those barriers by CRC cells, this phenomenon could explain increased metastasis occurrence in chemoresistant CRC. Thus, the data suggest that transfer of those miRNAs through exosomes contribute to preventing cell permeation into epithelia and maintaining tissue and organ integrity in normal physiological cell conditions (Figure 3(c)).

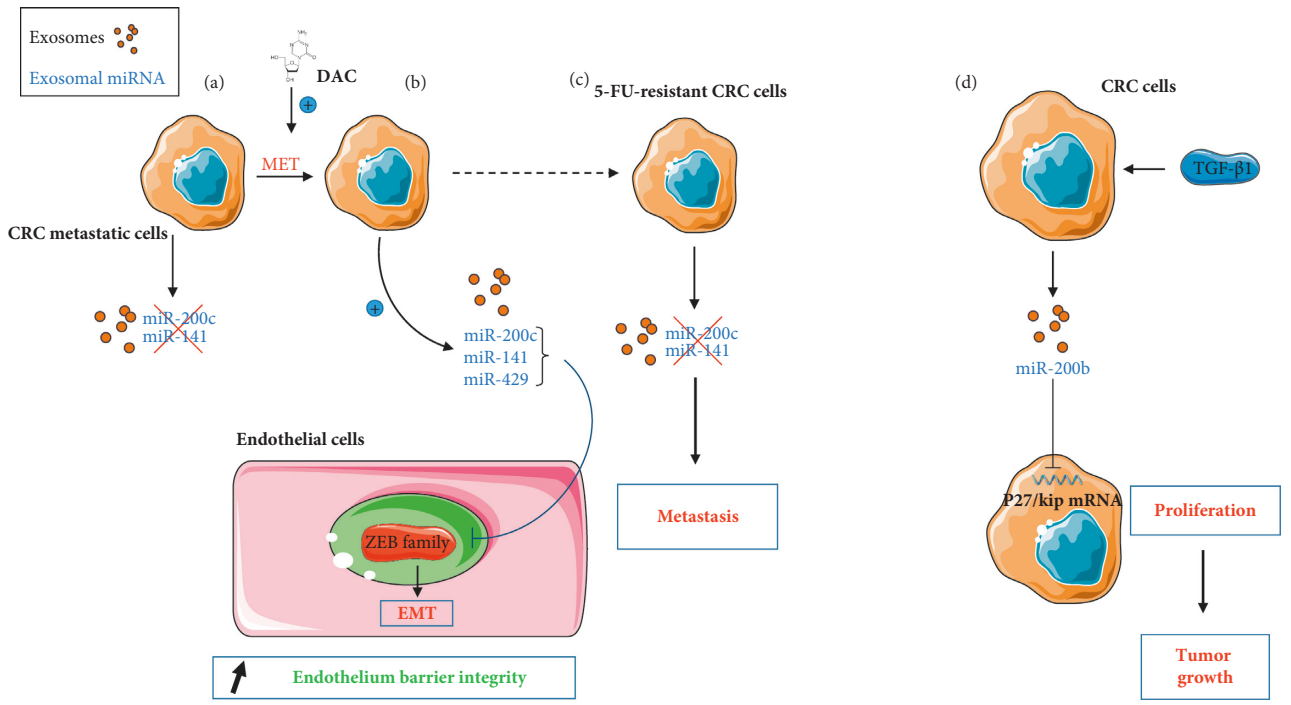

Figure 3: Proposed models for the dual roles of exosomal miR-200 family members on TME in CRC. (a) Upon decitabine (DAC) treatment, CRC cells enter a MET process that stimulates the release of miR-141/miR-200c enriched exosomes. (b) In endothelial cells, exosomal miR-200c, -141, and -429 can also inhibit the expression of transcription factors belonging to the ZEB family, activators of EMT. (c) On the contrary, 5-FU-resistant CRC cells release exosomes without miR-200 family members, favoring EMT. (d) CRC cells exposed to TGF-β1 release miR-200b-enriched exosomes that inhibit p27/kip mRNA, leading to an increased proliferation.

On the other hand, an oncogenic effect of exosomal miR-200b derived from CRC cells (HCT-116 and SW480) was also reported [98]. Exosomal miR-200b level is increased in cells treated with TGF-β1 in a dose-dependent manner and is responsible for the proliferative properties of the resulting exosomes, observed on another CRC cell line. These results were assigned to direct targeting of the antiproliferative cyclin-dependent kinase inhibitor 1B (p27/kip) by miR-200b (Figure 3(d)). The decrease of p27/kip expression was confirmed *in vivo* following miR-200b injection in xenograft of tumor cells. This also led to an increase in tumor size, as expected.

5.4. Exosomal miR-1246

5.4.1. Expression and Role as Biomarker. It was reported that miR-1246 is specifically upregulated in exosomes derived from several CRC cell lines and carcinoma cell lines from the cervix (HeLa), bladder (T24), prostate (PC-3), and liver (HepG2) [11, 23]. In a meta-analysis encompassing literature data from blood, urine, and other bodily fluids, it was the best performing miRNA biomarker for gastrointestinal cancers in terms of specificity and sensitivity [102]. This was in agreement with a previous high-throughput experimental study in serum exosomes, concluding that miR-1246 was the best potential miRNA biomarker for CRC diagnosis in serum together with miR-23a [11].

5.4.2. Function in CRC. Through its action on inflammation, exosomal miR-1246 holds an important role in TME. It was shown that this action was linked to the presence of p53 (*TP53*) mutations in CRC cells. These alterations are one of the most frequent genetic traits of human cancers [111, 125]. The experimental proofs obtained both *in vitro* and *in vivo* allowed to establish a model, in which the presence of *TP53* mutations in CRC cells, specifically resulting in a gain of function (mutp53), led to an increase of miR-1246 levels in exosomes [111]. Exosomal miR-1246 can induce reprogramming of macrophages towards a TAM phenotype, a hallmark of solid tumors associated with poor prognosis (Figure 4). Indeed, those mutp53-reprogrammed TAMs possess an anti-inflammatory cytokine secretion signature (e.g., IL-10, TGF-β, or VEGF). Moreover, their proinflammatory cytokine secretion (e.g., IL-8, IFN-γ, and ICAM-1) is decreased. Mutp53-reprogrammed macrophages also revealed a marked stimulation of extracellular matrix (ECM) degradation activity and enhanced migration and invasion properties. As a consequence, the anti-inflammatory, immunosuppressive, promigration, and proinvasion properties obtained by such macrophages promote tumor growth and metastatic burden to liver and lungs, as confirmed in mice hetero- and orthotopic xenograft models.

Interestingly, a pull-down experiment revealed an association of miR-1246 with hnRNPA2B1, which is suggested to be responsible for miRNA sorting in exosomes in its sumoylated form [72, 111]. The motif recognized by hnRNPA2B1 (GGAG) is carried by miR-1246, and hnRNPA2B1 sumoylation is 3 times higher in mutp53 CRC cells than in the WT CRC cells, suggesting that changes in this mechanism are involved in exosomal miR-1246 oncogenic properties.

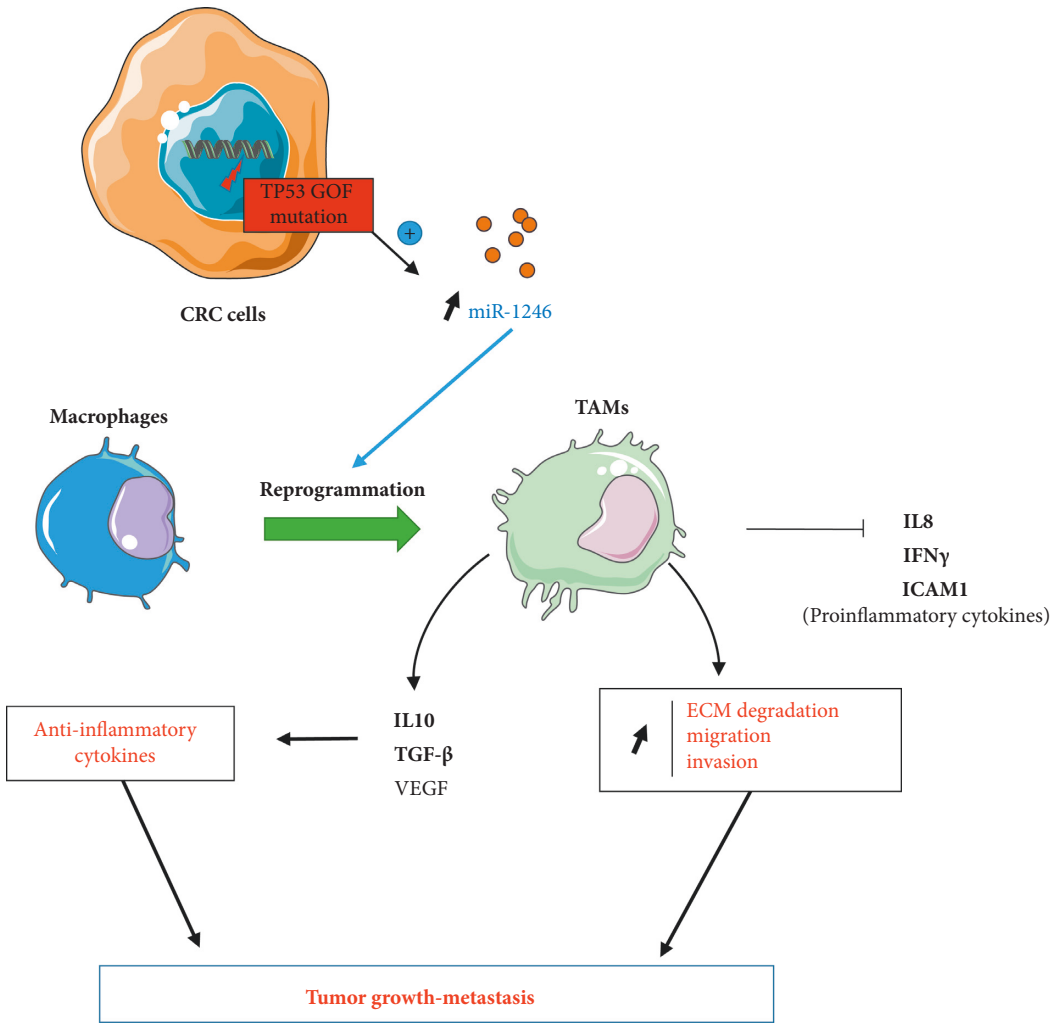

Figure 4: Proposed models for the effects of exosomal miR-1246 on the TME. p53 mutations resulting in gain of function (GOF) in CRC cells induce the release miR-1246-overexpressing exosomes. Exosomal miR-1246 can induce a switch of macrophage phenotype towards a tumor-associated phenotype (TAM), modifying tumor inflammatory state. It leads to a decreased secretion of proinflammatory and increased secretion of anti-inflammatory cytokines. TAMs also present enhanced ECM degradation, migration, and invasion properties. Exosomes are represented as small orange circles.

5.5. miR-149 and miR-96-5p, -486-5p, -6869-5p, -8073, and -193a: Tumor Suppressors. GPC1 (glypican-1) is a member of the heparan sulphate proteo-glycan family and is an important biomarker, found in several types of cancer (breast, pancreatic, and glioma) and involved in angiogenesis and tumor growth [126–129]. It was shown that GPC1 overexpression in CRC cells induces EMT and promotes cell invasion and migration [112]. The miR-149 gene is located within an intron in the *GPC1* gene. miR-149 and miR-96-5p are both able to directly target GPC1 mRNA, resulting in proapoptotic and antiproliferative effects in CRC cells *in vitro* and *in vivo*. Both miRNAs are downregulated in exosomes from CRC tissues or in plasma when compared to those of healthy individuals, while the exosomal GPC1 level is increased [113].

It was shown that exosomal miR-486 was upregulated within plasma exosomes and whole plasma of CRC patients. Therefore, it was suggested as a CRC diagnosis biomarker [114]. However, miR-486-5p possesses a tumor suppressor activity via inhibition of cell proliferation. Indeed, it targets directly PLAGL2, a transcription factor for β-catenin and IGF2 that promotes both proliferation and metastasis and inhibits apoptosis. Nevertheless, in CRC tissues, it has been shown that miR-486-5p expression is inhibited due to a high rate of DNA methylation of its promoter region. The consequent upregulation of PLAGL2/β-catenin/IGF2 pathway leading to proliferation and migration was confirmed in CRC cells.

miR-6869-5p downregulation was also observed in tumor tissues and serum exosomal fractions from CRC patients, and it was proposed as a potential biomarker of CRC prognosis [115]. The tumor-suppressor activity of miR-6869-5p was supported by direct targeting of TLR4, subsequently inhibiting TNF-α and IL-6 production in CRC cells via the TLR4/NF-κB signaling pathway, leading to a decrease in cellular proliferation.

While there is no difference between intra- or extracellular miR-8073 in normal colorectal cells, it is at least 60 times more

expressed in exosomes from CRC cells than in the intracellular extracts. Mizoguchi et al. demonstrated that it can directly target several factors involved in survival, proliferation, and antiapoptosis (FOXM1, MBD3, CCND1, KLK10, and CASP2), resulting in its antiproliferative properties *in vitro* and its effect on tumor growth *in vivo* [116].

Finally, miR-193a was shown to have a tumor-suppressor activity by targeting Caprin1, an upstream activator of the G1/S-specific cyclin-D2 (Ccnd2) and the proto-oncogen transcription factor c-Myc [92]. This causes G1 cell cycle arrest, leading to inhibition of cell proliferation. miR-193a is present at high levels in the exosomal fraction of CRC patients' serum, particularly in advanced stages, with high risks of metastasis. It was also demonstrated that miR-193a sorting into exosomes, which is increased in CRC, was caused by the MVP transporter [92].

5.6. miR-10b: Indirect Oncogenic Activity via CAF Transformation. miR-10b was detected in exosomes derived from multiple types of cancer cells and was particularly enriched in exosomes from CRC cells [90], but also breast cancer [130] and non-small cell lung cancer [131]. It can target directly PIK3CA, thus inhibiting PI3K/Akt/mTor pathway activity, closely associated with the inhibition of cell migration and invasion [117, 132]. Moreover, exosomes derived from CRC cells that contain miR-10b can be transferred to fibroblasts. In the target cells, this results in increased expression of TGF-β and SM α-actin. Expression of those genes are characteristics of myofibroblast-like CAF phenotype [103], and should stimulate tumor cells proliferation. miR-10b was shown to be particularly sensitive to mutation in the exosomal sorting protein KRAS, as KRAS mutations lead to a decreased incorporation of miR-10b in secreted exosomes [90].

5.7. miR-142-3p and 196b-5p: Stemness-Inducing miRNAs. Bone marrow-derived progenitors are an additional important type of stromal cells present in tumors, which are able to release cytokines and exosomes and influence TME. Bone marrow mesenchymal stem cells (BM-MSCs) are indeed able to release exosomes that increase markers of stemness (Oct4, lin28, KLF, Bmi-1, CD44, and SOX2) in CRC cells and their subsequent invasion, adhesion, and drug resistance properties [118]. It has been shown that this effect relies on the influence of miR-142-3p, present in exosomes, which can directly target Numb, an inhibitor of the Notch stem cells pathway [133]. Consequently, exposure to miR-142-3p-containing BM-MSC exosomes results in a boost of tumorigenesis and tumor metastasis, but not tumor weight and size, as shown in orthotopic grafts in mice [118].

miR-196b-5p influences stemness by targeting directly SOCS1 and SOCS3, modulators of stemness pathways, resulting notably in increased STAT3 transcription factor activity in CRC cells and tissues. It increases the production of antiapoptotic factors, like Bcl-2, Bcl-xL, and BIRC, and stem cell factor markers, like NANOG, Bmi-1, OCT4, and SOX2, and increases resistance to the drug 5-fluorouracil [119]. miR-196b-5p was confirmed to be dramatically upregulated in serum exosomes of CRC patients, in a much more distinct manner than in the whole serum, and associated with poor prognosis.

5.8. Lead on miR-210 Importance in EMT. It has been observed that a subpopulation of HCT-8 CRC cells became nonadherent after a few days of culture. Additionally, their proportion increased with culture time, and they developed chemoresistance properties by undergoing EMT [19, 134]. This metastatic-like phenotype can be spontaneously reverted in new cell-free cultures, but not in the presence of other HCT-8 cultured cells. Indeed, the reverse MET phenomenon was inhibited by exosomes produced by cultured cells. As miR-210 is significantly upregulated in HCT-8 exosomes, the authors suggested that it may play a role in MET inhibition [19].

5.9. Other miRNAs. Finally, additional miRNAs found in exosomes were also identified as potential biomarkers in CRC patients. Even if they are not described to play a role in exosomes mode of action, we tried to make a list as exhaustive as possible of the main reported ones in the current state of the art. Data are outlined in Table 2 [105, 135–139].

6. Concluding Remarks

Cell-to-cell transfer of miRNAs by means of exosomes, released by both stromal and tumor cells, plays an important role in tumor progression and metastasis. Several technical obstacles should be overcome to allow improved exosome characterization and further research in particular subjects that remain less covered. Study of miRNA targets and role in CRC provides great hopes for better understanding and characterization of tumor properties, diagnosis and personalized medicine, and innovative therapeutic approaches. These aspects will be briefly discussed in the following sections.

6.1. Limitations regarding Exosome Isolation Methods and Exosome Purity. Exosomes constitute great reservoir of biomarkers since they preserve miRNAs from extracellular environment and have dedicated roles and a specific biology. For example, in one of the first high-throughput characterization of exosomal miRNAs in CRC cells by Ji et al., almost a third of miRNAs from a subpopulation of exosomes were not reported as implicated in colon cancer before [84]. However, exosomal miRNA studies are limited due to a few technical issues. It is currently almost impossible to achieve a very high degree of purity without sacrificing yield when isolating exosomes. There are many approaches to isolate exosomes from the same medium, which are fundamentally different in their principles, resulting in different yields and degree of purity and often used according to the objectives of downstream applications [140].

Unfortunately, it has been shown that the purification method has a great impact on the exosome population

Table 2: Summary of exosomal miRNAs with a potential biomarker role in CRC or whose expression is associated with CRC diagnosis and progression. The main source of exosomes referred in the literature is indicated. Exosome isolation techniques employed in the diverse references are indicated with their respective exosome enrichment validation procedures. Without any other mentions, plasma and serum are to be considered from human origin.

miRNA	Exosome source	Isolation technique	Validation	Associated effect	References
mir-221	Cell supernatant (HT-15, SW480, WiDR)	UC	WB : CD81	Biomarker for tumor size, TNM stage, Dukes stage, lymph node metastasis, recurrence	[99, 135]
mir-215	(i) Cell supernatant (a) HT-15, SW480, WiDR (b) CAFs, NOFs	(i) UC (ii) UC	(i) WB : CD81/ NTA + TEM + WB (ii) NTA + TEM + WB	Upregulation in CAF exosomes	[99, 103]
mir-23a	(i) Serum (ii) Cell supernatant (SW48, SW480, SW620, HCT116, HT29, RKO)	(i) UC (ii) UC	(i) TEM/WB (ii) TEM/WB	Diagnosis biomarker associated with liver metastasis recurrence	[9, 11]
mir-320a, -4476	(i) Tissue (ii) Serum	UC	TEM	Associated with liver metastasis recurrence	[9]
mir-125a	Plasma	ExoQuick	SEM/none	Early tumor stage biomarker	[27, 136]
mir-320c	Plasma	ExoQuick	SEM	Higher levels in CRC patients	[136]
mir-328	Plasma (mesenteric vs. peripheral)	UC	TEM + WB : Tsg101	Liver metastasis biomarker (better performing in mesenteric vein)	[105]
mir-4472-3p	Serum	ExoQuick	TEM + WB	Diagnosis and tumor recurrence biomarker	[137]
mir-6803-5p	Serum	Total exosome isolation kit	None	Diagnosis and prognostic biomarker, associated with stage and lymph node metastasis	[138]
mir-4644	Meta-analysis	—	—	GI cancer diagnosis biomarker	[102]
mir-7641	Cell supernatant (HT-15, SW480, WiDR)	UC + OptiPrep	TEM + WB : Alix, Tsg101	Diagnosis biomarker	[107]
mir-638	Serum	Total exosome isolation kit	None	Biomarker for TNM stages III-IV and liver metastasis	[77, 78]
mir-548c	(i) Serum (ii) Plasma (mesenteric vs peripheral)	(i) Total exosome isolation kit (ii) UC	(i) None (ii) TEM + WB : Tsg101	Diagnosis and prognostic biomarker, associated with faster relapse	[78, 105, 139]
mir-let-7a, -1229, -150, -223	(i) Cell supernatant (HCT116, HT29, RKO, SW48, SW480) (ii) Serum	UC	WB	Potential diagnosis biomarkers	[11]

GI cancers: gastrointestinal cancers; OptiPrep: commercial density gradient medium; TEM/SEM: electron microscopy (transmission/scanning); UC: ultracentrifugation (may include differential centrifugation steps and eventual additional filtering); WB: western blot. Total exosome isolation kit (Invitrogen), ExoQuick (System Biosciences), and qEV SEC (Izon): commercial exosome purification solution or kits.

obtained, including on miRNA content [141, 142]. To help ensure that the obtained isolates are enriched in exosomes, several validation tests also have been proposed. These tests typically include nanoparticle tracking analysis (NTA), exosomes markers detection by western blot, or examination of exosomes by electron microscopy. Studies on the research of biomarkers, mentioned along this review, used different methods of purification, as summarized briefly in Tables 1 and 2. Both the purification method and validation of exosomal enrichment experiments have to be taken into account during result interpretation.

6.2. Nonexosomal vs. Exosomal Extracellular RNAs. Concerning the vesicle-free part of circulating miRNAs secreted by cells by other means, the involved mechanisms are still unclear. Their release could also largely result from cellular lysis. Compared to miRNAs contained in EVs, it is not clear if their role in tumor transformation and progression is negligible or not. To elucidate these roles entirely will remain difficult due to current technical impediments limiting the purity of isolated exosomes and EVs in general [53, 140]. This state of the art was recently backed up by a study in rat serum and plasma, showing that vesicle-free miRNAs are also present in EV fractions after isolation. Moreover, even Ago2-associated part of vesicle-free circulating miRNAs could result from either cellular or exosomal lysis [10, 143].

If the proportion of circulating miRNAs present in exosomes compared to free circulating miRNAs remains elusive, it seems that only a small fraction (down to 10%) of plasma miRNAs are vesicular, whereas in serum or saliva, the majority of miRNA are concentrated in exosomes [48, 49, 144, 145]. In plasma, the fraction of miRNAs present in the vesicle fraction is strongly dependent on the identity of the miRNA considered. Some, like let-7a, were found predominantly enriched in vesicle fractions compared to vesicle-free fractions, while others like miR-16 and miR-92a are preferably associated with circulating Ago2 and seemingly absent from vesicles under physiological conditions [48]. However, it is worth noting that at least in one report (in high-risk colorectal adenomas), 2 serum exosomal miRNAs were considered less efficient biomarkers than their whole serum miRNA counterparts despite their correlated expressions [146]. Although it seems that isolated vesicle-incorporated miRNAs are more stably expressed and constitute more reliable cancer biomarkers than their vesicle-free counterparts [28, 147].

6.3. Exosome Subpopulations in CRC and Their Advantages. Different CRC cell types produce different populations of exosomes. For example, it was shown that CRC cell line exosomes do not contain the same combination of tetraspanin proteins, exosomal markers involved in exosome biogenesis [99]. On the same note, Chen et al. showed that miRNA composition of SW480- and SW620-derived exosomes is significantly different, with more than a third of the miRNAs detected being differentially expressed between the 2 types of exosomes [107]. Moreover, while miRNAs are sorted into exosomes in a differentiation state and cell-type dependent fashion, several types of exosome populations with diverse morphologies have been reported to be secreted by the same cells, in particular in colorectal cancer [148, 149]. The LIM1863 CRC cell line can produce two mutually distinct populations of exosomes, one presenting A33 and the other EpCAM surface proteins, an important cancer-initiating marker in CRC and pancreatic cancer [150, 151]. Their protein and miRNA populations vary significantly between each exosome type and previously determined proteomes of other exosomes, suggesting different effects and/or target recipient cells [84, 150]. Indeed, it was shown that exosomes are tailored to target specific types of recipient cells [152, 153]. This could provide an explanation for the site-specific formation of metastases in colorectal cancer, e.g., the liver, lungs, and lymph nodes. Moreover, their compositions reflect not only this tailoring, but also regulatory events arising in the secreting cell [80]. Exosomes could thus give great advantages for both study of TME and discovery of biomarkers. Indeed, a given exosome population could thereby directly inform us about particular cell types and events they were exposed to, with great specificity. These subpopulations may contain multiple determinants of tumor metastatic potential. The complex interplay between exosome subpopulations, their specific contents, and their potential target cells needs further investigation.

6.4. A Word on Therapeutic Perspectives. Studies on exosomal miRNAs may soon be applied to the clinical setting, as new therapeutic approaches using delivery of miRNA mimics or miRNA antagonist on tumor sites are in development. Several clinical trials concerning the use of miRNAs in the treatment of CRC are currently ongoing and gain more and more interest from biopharmaceutical companies [154]. Furthermore, exosomes themselves constitute a great strategy for the delivery of those new therapeutic actors. Freely circulating miRNAs are rather instable in blood [48] and are also negatively charged, rendering delivery through cell membranes difficult even *in vitro*. Exosomes, on top of their low immunogenicity and cytotoxicity, may enhance therapy deliverability and protect molecules from RNAse activity, making them suitable therapeutic vectors for CRC treatment [155, 156]. Treatment of cells with FF/CAP18 (analog of cathelicidin LL-37), a peptide limiting cancer cell proliferation, induces production of exosomes with antiproliferative properties [157]. This effect is suspected to come from the expression of exosomal miRNAs miR-584-5p, -1202, and -3162-5p. Kyuno et al., have also shown that it is possible to tailor exosomes for therapeutic purposes by transfection with tumor-suppressor miRNAs [158]. The miRNAs in question were miR-342 and -498, which target Claudin7 (cld7) and EpCAM, respectively. Coupled with exosomal expression of ectopic Tspan8, shown to enhance internalization by cancer cells [152], it was sufficient to inhibit tumor growth, motility, and invasion, especially by affecting stemness traits.

To conclude, exosome-carrying miRNAs originating both from the stromal and the tumor cells have a major role in CRC TME. Exosome encapsulation enables miRNA expression in extracellular space and involvement in cell-to-cell communication. Therefore, miRNAs can influence cell inflammatory environment, differentiation status, proliferation, survival, migration, invasion, and angiogenesis properties. Being stably released in the circulatory system, it has been shown at least through venal injection that they can influence distant cell barrier permeability, underlining the role they play in premetastatic niche formation. The delivery of exosome cargo into specific types of target cells may also be one of the mechanisms explaining organ preference of cancer metastasis. For all those reasons, exomiRs constitute a key target for the discovery of biomarkers and new therapeutic approaches in CRC, and an important axis of research in the near future.

Abbreviations

CRC: Colorectal cancer
TME: Tumor microenvironment
mRNA: Messenger RNA
EV: Extracellular vesicle
MVB: Multivesicular body
MVP: Major vault protein
exomiR: Exosomal miRNA
NOF: Normal fibroblast
EPC: Endothelial progenitor cell
CAF: Cancer-associated fibroblast
TAM: Tumor-associated macrophage
EMT: Epithelial-mesenchymal transition
DAC: Decitabine
MET: Mesenchymal-epithelial transition
LEC: Lympho-endothelial cell
BEC: Blood endothelial cell
5-FU: 5-Fluorouracil
BM-MSC: Bone marrow mesenchymal stem cell
MHC: Molecular histocompatibility complex
HSP: Heat shock protein.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Gastric Cancer in the Era of Immune Checkpoint Blockade

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Gastric cancer (GC) is one of the most important malignancies worldwide because of its high incidence and mortality. The very low survival rates are mainly related to late diagnosis and limited treatment options. GC is the final clinical outcome of a stepwise process that starts with a chronic and sustained inflammatory reaction mounted in response to *Helicobacter pylori* infection. The bacterium modulates innate and adaptive immunity presumably as part of the strategies to survive, which favors the creation of an immunosuppressive microenvironment that ultimately facilitates GC progression. T-cell exhaustion, which is characterized by elevated expression of immune checkpoint (IC) proteins, is one of the most salient manifestations of immunosuppressive microenvironments. It has been consistently demonstrated that the tumor-immune microenvironment(TIME)-exhausted phenotype can be reverted by blocking ICs with monoclonal antibodies. Although these therapies are associated with long-lasting response rates, only a subset of patients derive clinical benefit, which varies according to tumor site. The search for biomarkers to predict the response to IC inhibition is a matter of intense investigation as this may contribute to maximize disease control, reduce side effects, and minimize cost. The approval of pembrolizumab for its use in GC has rocketed immuno-oncology research in this cancer type. In this review, we summarize the current knowledge centered around the immune contexture and recent findings in connection with IC inhibition in GC.

1. Introduction

Inflammation is an intrinsic feature of cancer, influencing many processes that take place during tumor development and progression [1–3]. In fact, tumor growth is severely compromised if neoplastic cells are not immersed in an appropriate microenvironment in which neoplastic, immune, and other nonimmune stromal cells coexist [4, 5]. This tumor niche is constantly being reshaped as a result of heterotypic signaling between neoplastic and nonneoplastic cells. Given the relevance of the immune contexture in cancer, we are currently witnessing a change of paradigm in cancer therapy, traditionally focused on cancer cells, with the emergence of therapies centered around the TIME [6, 7]. Immune-checkpoint blockade (ICB) is currently at the lead and profiled as the most promising immunotherapeutic approach in cancer [8–10]; however, despite the very encouraging results in some types of cancer, only a subset of patients obtain clinical benefit from ICB. One of the major challenges is, therefore, the identification of precise and accurate biomarkers to personalize ICB in the clinic. Very likely, predictive biomarkers need to be contextualized to each histology [11].

Infection and chronic inflammation are key players in the pathogenesis of GC. *H. pylori* infection, which is particularly linked to GC of intestinal subtype, the most commonly diagnosed worldwide, triggers chronic and persistent inflammation of the gastric mucosa, characterized by intramural infiltration of inflammatory cells and expression of a vast array of inflammatory mediators [12].

Epstein–Barr virus (EBV) is also associated with the etiology of approximately 10% of the GC cases, especially those located in the proximal stomach [13]. Infiltration of the tumor with CD8+ T cells is a common feature of the EBV+ GC [14]. Environmental and genetic determinants are also implicated in the genesis of this malignancy. Thus, the complex interplay of environment, genetics, infection, and inflammation translates into a very heterogeneous disease at the molecular level [14], which ultimately has an impact in the clinical management of the GC patients.

In 2017, the FDA approved the use of the anti-programmed cell death protein 1 (PD-1), pembrolizumab, in advanced or recurrent GC expressing programmed cell death 1 ligand 1 (PD-L1) [15]. Even before this, various studies had investigated the expression of the PD1/PD-L1 axis in GC, and several found correlation between PD-L1 expression and clinicopathological parameters, including patient survival [16–21]. Interestingly, some reports indicate that *H. pylori* induces the expression of PD-L1 [22–25]. In addition to PD-L1, several other parameters currently suggested as biomarkers of potential clinical relevance for predicting the response to ICB are being studied in GC. In this review, we provide a summary of the current knowledge centered around the immune contexture and the main findings obtained so far in connection to ICB and predictive biomarkers in GC.

2. Epidemiology

GC is one of the most important malignancies worldwide. In 2018, this neoplasm accounted for approximately 1,000,000 new cases and 780,000 deaths globally, which makes it the fifth most commonly diagnosed and the third cause of cancer death [26]. Incidence and mortality rates present substantial variations according to geographic location, with well-defined high- and low-risk areas across the world. More specifically, gastric malignancy is highly incident in Eastern Asia, Eastern Europe, and countries located in the Pacific coast of Latin America; in contrast, incidence rates are generally low in Northern America, Northern Europe, Southern Asia, and Australia [27–29]. Mortality rates also show variations with a very similar geographical pattern [27–29]. Interestingly, incidence and mortality rates are 2-fold higher in men than in women [27].

In the last decades, GC incidence rates are experiencing a steady decline globally [27, 29]. Although the reasons remain obscure, it is speculated that this is at least partially attributed to the concomitant decrease in *H. pylori* prevalence, which is a very well-established factor related to the pathogenesis of GC (discussed below). The decrease, however, is not of the same magnitude in GC of different histological subtypes or anatomical locations [27, 30]. The declining trend is particularly connected to a decrease in the incidence of intestinal subtype, whereas the diffuse subtype remains more or less stable [31, 32]. Similarly, GC of the lower part of the stomach is becoming less common, while the rate of cancer of the gastric cardia is increasing, particularly in high-income countries [27, 30, 32]. Although mortality rates also show a global decline, GC is still one of the most important causes of cancer death [27, 29]. At least in some countries, this downward trend in mortality may be partially connected to the implementation of population-based early-screening programs [33–35]. Nonetheless, the 5-year survival rate remains below 30% in most countries, which is mainly related to the fact that most of the cases are diagnosed at advanced stages, when treatment is likely to fail [36]. Studies in population groups with the same ethnic background but dissimilar access to health care, however, suggest that biological factors could also contribute to explain the mortality and survival of GC [37].

3. Histopathology

GC is classified, among other factors, according to histopathological characteristics and anatomic location. The Lauren histological classification system is probably the most used and categorizes gastric adenocarcinomas into three main histologic types: intestinal, diffuse, and mixed [38]. Importantly, Lauren histological subtypes show substantial differences at the epidemiological, pathological, and molecular levels [38–41]. Anatomical location of the malignant lesions is also an important parameter in the classification of GC. Marked epidemiological and etiological differences have been revealed for malignant tumors located in the distal part of the stomach and those of the most proximal region [42–44].

4. Pathogenesis

The pathogenesis of GC is complex and multifactorial, and differs substantially depending on the histological and anatomical subtype. GC of intestinal subtype, for instance, is the final clinical outcome of a stepwise process known as the Correa Cascade [45]. It starts with *H. pylori* colonization of the normal gastric mucosa, which in conjunction with environmental insults (i.e., diet and lifestyle) triggers a sustained inflammatory reaction resulting in chronic gastritis that, in some patients, may progress to multifocal atrophic gastritis. A subset of them may develop intestinal metaplasia, dysplasia, and ultimately invasive carcinoma [46]. Much less is known about the pathogenesis of the diffuse subtype of GC [47, 48].

Despite the very well-established role of *H. pylori* in gastric carcinogenesis, most of the infected individuals remain asymptomatic or even develop pathologies not related to GC [49]. This feature actually represents one of the most intriguing paradoxes about this bacterial infection. Bacterial strains exhibiting enhanced molecular virulence that ultimately result in stronger inflammatory response are consistently associated with even higher risk of GC [50–52]. Also, a number of polymorphic variants in genes encoding proinflammatory and anti-inflammatory cytokines that play an important role in the immune response triggered by *H. pylori* are also linked to the GC pathogenesis [53–55]. Thus, it is the combination of bacterial, host, and environmental factors what presumably determines the final clinical outcome.

Although the pathogenesis of the malignant lesions arising in the proximal stomach remains very enigmatic

[56], EBV is presumably an important etiological factor for tumors at this particular location, especially those located in the cardia and fundus [13]. EBV-positive tumors constitute around 10% of the cases and, given their very distinctive features, they are actually regarded as a different molecular subtype [13, 14].

5. Tumor-Immune Contexture in Gastric Cancer

Immune contexture is recognized as a crucial determinant of cancer [1, 57]. Infiltrating immune cells including macrophages, neutrophils, dendritic cells, and several lineages of T cells are major constituents of the tumor microenvironment, participating in many processes that take place during cancer initiation and growth [2, 5].

In general, the TIME of overt GC lesions shows an immunosuppressive character (Figure 1(c)). This, however, may vary according to parameters such as tumor histology, anatomical location of the lesion, and molecular subtype, as recently revealed [58]. According to this study, in general, the most prevalent tumor-infiltrating leukocytes were CD8+ T cells, CD68+ macrophages, and CD4+ T cells, representing 15%, 13%, and 11% of all intratumoral cells, respectively. When subdivided according to subtypes, the infiltration with CD8+ T cells, CD4+ T cells, and macrophages was particularly elevated in the EBV+ tumors and the least infiltrated corresponded to the GCs of diffuse subtype. Interestingly, the presence of infiltrating macrophages in GC of intestinal subtype was markedly conspicuous and that of T cells in general was relatively low. Finally, the prevalence of FOXP3+ Tregs in GC was dismal, regardless of the histologic or molecular subtype [58]. Although the latter is probably the most comprehensive, a substantial number of studies have also assessed the composition of the TIME in GC (due to space limitations, we only cite some) [59–69]. Variations in the general prevalence of leukocytes and lymphocytes between studies are expected as a result of the number of patients included, the fact that not all take into account the same clinicopathological and molecular features and the methodological approach used for profiling the immune cell composition and the study design. A general trend, however, in the studies performed so far, is that EBV+ GCs are the most infiltrated tumors, especially by CD8+ T cells. Also, many of the studies have found a significant association between the high number of tumor-infiltrating lymphocytes and improved overall survival, which is particularly robust for the CD8+ T cells [59, 61, 70, 71]. Unlike other types of cancer, there is no current consensus on the morphologic evaluation of tumor-infiltrating lymphocytes in GC, despite some attempts [59]. Therefore, the standardization of a scoring method for quantitation of the tumor-infiltrating lymphocytes in GC lesions is highly needed.

6. Immune Exhaustion

T-cell exhaustion was first defined in chronic infections as the failure of effector T cells to acquire a memory T-cell homeostatic state [72]. During an acute infection, a great portion of activated T cells die after the peak of effector expansion; however, a subset persists losing its effector functions and becoming part of the memory T-cell pool. By contrast, in chronic infections, the ongoing antigen stimulation and persistent inflammation induce a progressive loss of effector T-cell functions, but failing to acquire the antigen-independent memory state [72]. One of the most important features of the T-cell exhaustion phenomenon is the progressive increase in the amount and diversity of inhibitory receptors expressed on T cells, including PD-1, cytotoxic T-lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 protein (LAG-3), T-cell immunoglobulin domain and mucin domain (TIM-3), 2B4, CD160, V-domain Ig suppressor of T-cell activation (VISTA), and T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) [72–74]. Under physiological conditions, these inhibitory receptors, also called ICs, have a crucial role activating negative regulatory pathways in order to prevent autoreactivity and the subsequent immunopathological tissue damage [75]. After T-cell activation, these inhibitory molecules are expressed transiently in functional effector T cells thus maintaining an adequate balance of the immune process [72]. During T-cell exhaustion, however, IC proteins are highly and steadily expressed, and the exhausted phenotype severity depends on the level and number of inhibitory receptors [72].

Although T-cell exhaustion was originally defined in chronic infection, a similar dysfunctional state has been observed in cancer [73]. The role of the immune system in tumor initiation and progression has been widely explored. In fact, the immune-mediated mechanisms play a pivotal role in all stages of tumor biology, regardless of the tissue origin of the tumors. Importantly, the immune system poses a strong selective pressure on the tumor mass that ultimately shapes tumor growth, which has led to the proposal of a cancer-immunoediting process. More specifically, the immune system proceeds sequentially through three distinct phases during tumor development: (1) elimination, in which the innate and adaptive immune systems work together to detect the presence of potentially malignant cells, activate against them, and mediate their destruction; (2) equilibrium, where rare tumor cell variants survive the elimination phase, but the adaptive immune system still prevents their outgrowth and maintains them at bay; (3) escape, in which tumor cells that have acquired the ability to circumvent immune recognition emerge as progressively growing tumors [76]. This last phase can occur through two principal mechanisms: the generation of poorly immunogenic tumor cell variants that are "invisible" to the immune system and/or the establishment of an immunosuppressive state within the tumor microenvironment, which includes the induction of T-cell exhaustion [76]. As a general rule, the inhibitory ligands and receptors that regulate T-cell effector functions in tissues are commonly overexpressed in tumor cells or in nonneoplastic cells in the tumor microenvironment [75].

T-cell exhaustion in cancer and chronic infection share many commonalities, including reduced proinflammatory cytokine production, impaired cytotoxic activity, and elevated levels of multiple inhibitory receptors. Notwithstanding this,

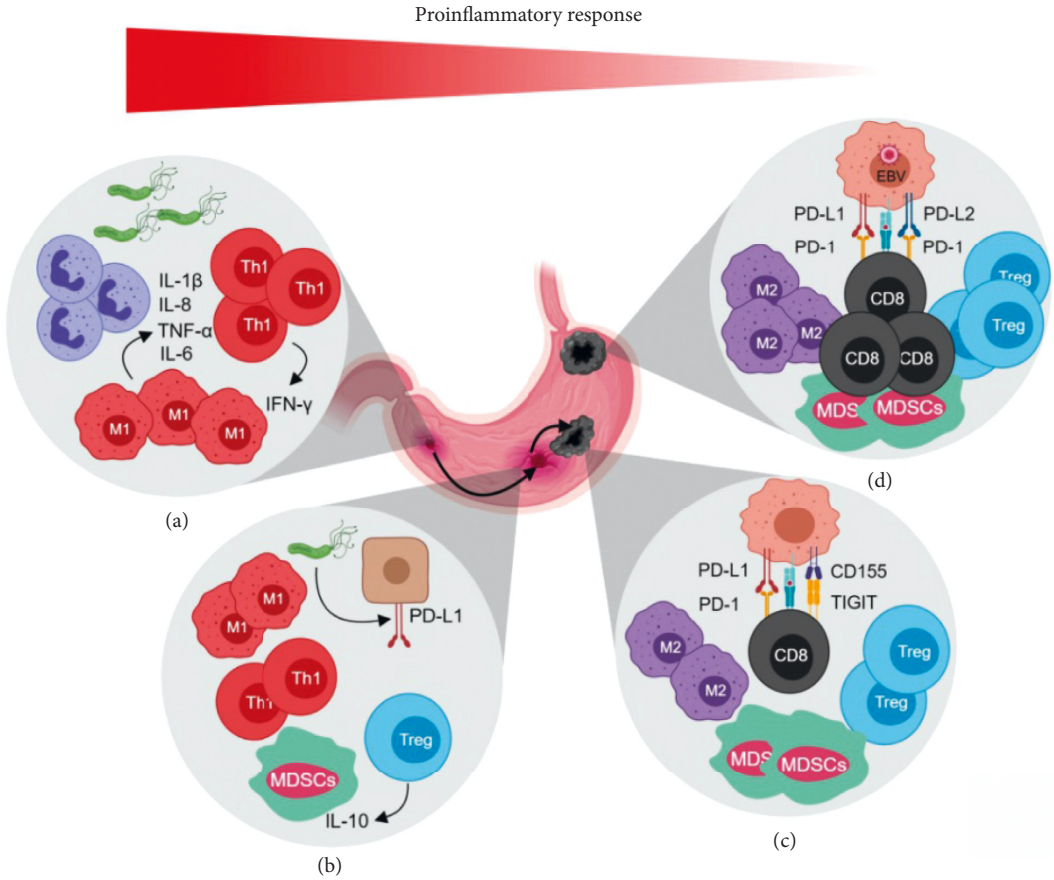

FIGURE 1: Immune contexture in the pathogenesis of gastric cancer. (a) The colonization of *H. pylori* in the normal gastric mucosa triggers an inflammatory response with accumulation of neutrophils and inflammatory macrophages and production of proinflammatory cytokines such as IL-1β, IL-8, IL-6, and TNF-α; leading to Th1 polarization, IFN-γ production, and chronic gastritis. (b) In later stages, *H. pylori* induces the overexpression of PD-L1 in epithelial cells of the gastric mucosa as an immune evasion mechanism, characterized by an increase in regulatory T cells, myeloid-derived suppressor cells (MDSCs), and IL-10 production. (c) Gastric cancer cells express PD-L1 and CD155 which after interacting with PD-1 and TIGIT on the surface of cytotoxic T cells induce T-cell exhaustion and promote the development of an anti-inflammatory tumor microenvironment. (d) Epstein–Barr virus- (EBV-) positive gastric cancer lesions are mainly located in the proximal stomach and are characterized by amplification and, consequently, high PD-L1 and PD-L2 expression with a prominent immune cell infiltration (created with BioRender.com).

differences are also appreciated. In cancer, for instance, priming to tumor antigens is more likely to occur in the absence of inflammation. Consequently, naïve tumor-specific T cells may fail to become properly activated and never differentiate into effector T cells, thus acquiring directly a T-cell exhaustion phenotype [73]. Also, tolerance mechanisms could shape T-cell responses to favor mainly lower-affinity clones [73].

Another important factor influencing intratumoral T-cell activity is the metabolic state of the tumor microenvironment. Effector T cells activate glycolytic pathways for ATP production, even in the presence of oxygen, in a HIF-1α-dependent manner [77, 78]. Glucose metabolism in T cells is promoted by HIF-1α and the AKT/mTOR pathway, which in turn induces the upregulation of glucose transporter GLUT1, providing the T cells enough energy to perform their effector functions [79]. Tumor cells also reprogram their metabolic pathways towards glycolysis, which is mediated by hypoxia and HIF-1α. The fact that proliferating tumor cells increase their glucose uptake limits its availability for T cells as an energy source for their effector functions, affecting the antitumor immune response [80]. Besides changes in the glucose availability in the tumor microenvironment, intrinsic factors in the T cells affect their metabolism. For instance, GC cells express ligands to ICs, such as PD-L1 and CD155, which induce T-cell exhaustion after interacting with PD-1 and TIGIT of the surface of T cells, respectively (Figure 1(c)). PD-1 and TIGIT expression affect T-cell metabolism by inhibiting glycolysis and limiting their effector functions [81, 82]. In fact, downregulation in the expression levels of genes encoding proteins involved in glucose uptake, glucose metabolism, and the AKT/mTOR pathway has been observed in TIGIT+ CD8 T cells from GC patients. Mechanistically, this effect was induced after TIGIT interaction with CD155. Interestingly, the T-cell exhausted phenotype was reversed when the uptake of glucose was increased. Additionally, TIGIT blockade alone or in combination with PD-1

inhibitors improves antitumor immunity in an animal model of GC [82].

As in many other types of cancer, IC overexpression has been described in GC as a mechanism for T-cell exhaustion. Since 2000s, several studies have explored the role of PD-1 and PD-L1 expression in the TIME of GC. More recently, upregulation of other ICs such as CTLA-4, TIM-3, and VISTA has also been reported in human GC [83–85]. Nevertheless, the clinical significance of the differential expression of these immunomodulatory molecules among GC patients has not yet been completely elucidated.

7. Immune-Checkpoint Blockade in Gastric Cancer

The description of the T-cell exhaustion phenomenon in the context of cancer and its role in promoting tumor growth led to a paradigm shift in cancer treatment the past decade. The new vision of tumor therapy has focused in the development of approaches that intend to target or manipulate the immune system in order to reactivate antitumor T-cell functions. One of the most significant breakthroughs so far is the pharmacological blockade of PD-1/PD-L1 and CTLA-4 as novel immunotherapeutic options, which reverses T-cell exhaustion and unleashes strong antitumor immune responses. Importantly, the fact that PD-1/PD-L1 inhibition leads to a reduction in tumor load shows that T-cell exhaustion is not a terminally dysfunctional state and that an active and effective antitumor immune response can be restored [73, 75].

The FDA approval of the IC inhibitors pembrolizumab and nivolumab for the treatment of melanoma in 2014 initiated a new era in the treatment of cancer. Since then, a number of PD-1/PD-L1 and CTLA-4 inhibitors have been approved for the treatment of several cancer types, and many clinical trials are currently running [6]. Specifically for GC, the anti-PD-1, pembrolizumab, was approved by FDA in 2017 for its use in advanced, recurrent GC expressing PD-L1, which was based on the phase II KEYNOTE-059 clinical trial [21]. At present, several clinical trials are evaluating other IC inhibitors, including the anti-PD-1 nivolumab, the anti-PD-L1 avelumab, durvalumab, and atezolizumab, and anti-CTLA4 ipilimumab and tremelimumab. In Supplementary Table 1, we summarize the most representative clinical trials evaluating the safety and efficacy of PD-1/PD-L1 inhibitors in GC. Further details of all ICB clinical trials in GC can be found elsewhere [86, 87]. Of note, the phase II trial ONO-4538 and phase III trial ATTRACTION-2 revealed that nivolumab administration to heavily pretreated GC patients is associated with improved overall survival, compared to patients treated with placebo. These results led to the approval of nivolumab in Japan for the treatment of advanced-stage GC patients progressing after standard systemic cytotoxic therapy, regardless of the PD-L1 status [88]. Some of the current trials in GC are evaluating combinations of PD-1/PD-L1 inhibitors with conventional therapies. The MORPHEUS-GC trial, for example, has eight different study groups that combine IC inhibitors, chemotherapeutic agents, MEK inhibitors, anti-VEGF receptor 2 antibodies, PEGylated recombinant human hyaluronidase, CXCR4 antagonists, and DDP-4 inhibitors [86, 87]. Also, the CIRCUIT trail combines ICB therapy with neoadjuvant short-term-limited local radiotherapy [87]. Combinations of IC inhibitors are also being evaluated in GC. The latter is based on previous studies performed in other cancer types showing that combination of two IC blockers leads to significantly improved response rates. In fact, an ongoing phase I/II trial is analyzing the safety and efficacy of nivolumab plus ipilimumab, compared to nivolumab alone, in patients with chemotherapy refractory GC [86, 87].

Other IC proteins are currently studied in preclinical and clinical settings as potential therapeutic targets in cancer, including LAG-3, TIM-3, and TIGIT [6]. In GC patients, for example, TIM-3 and Gal-9 expressions have been associated with poor patient overall survival, suggesting an important role of these molecules in T-cell exhaustion [89]. Furthermore, the potential of LAG-3 as therapeutic target in GC was recently demonstrated in a mouse model using recombinant soluble LAG-3. More specifically, administration of recombinant soluble LAG-3 reduces tumor growth, enhances the secretion of interferon (IFN)-γ, promotes CD8+ T-cell activation, and increases the survival rate of GC-bearing mice [90]. In this line, the FRACTION-GC trial seeks to further explore the potential of LAG-3 as a novel therapeutic target by including a group of cancer patients who will receive nivolumab plus an anti-LAG-3 antibody [86, 87].

8. Immune-Checkpoint Blockade-Predictive Biomarkers in Gastric Cancer

Cancer patients that respond to ICB generally have long-lasting response rates and manageable safety profile. This, however, is eclipsed by the fact that only a subset of patients derive clinical benefit, which varies according to tumor site. Therefore, the search for biomarkers that can be used in clinical practice to predict the response to ICB is a matter of intense investigation as this may contribute to maximize disease control, reduce side effects, and minimize cost. To date, parameters such as the elevated expression of IC proteins, high mutational load, mismatch repair (MMR) deficiency, microsatellite instability (MSI), high density of infiltrating CD8+ T cells in tumor lesions, and presentation of neoantigens of viral origin are emerging as potential predictive biomarkers [47, 48, 91–96]. Intriguingly, a fraction of patients regarded as potential responders according to these biomarkers do not respond to ICB, which suggest that some parameters of relevance for predicting the response to such agents are still unknown. For instance, studies in melanoma have revealed that the composition and diversity of the gastrointestinal microbiota differ between responders to IC inhibition and nonresponders [97, 98], which is recapitulated in mouse models [99, 100]. An excellent review on ICB predictive biomarkers in cancer has been recently published [101].

A substantial number of studies have characterized the expression pattern of PD-L1 in GC and its correlation with clinicopathological variables. According to these studies,

PD-L1 is expressed in 25 to 65% of GC patients, and it is associated with tumor size, lymph node metastasis, and shorter overall survival [18]. Although it has been widely used as companion test in a large number of clinical studies, its utility as a biomarker has been questioned because not all PD-L1+ patients respond to ICB and, even more intriguingly, some negative patients do respond (Supplementary Table 1). This may be influenced by the lack of a universal cutoff point, differences in the PD-L1 detection assays used, and spatiotemporal intratumor heterogeneity. Many clinical studies using PD-L1 as companion test rely on PD-L1 expression in tumor cells only [8, 9]. More recently, at least in GC, the so-called combined positive score (CPS), which takes into account the PD-L1 positivity on cancer and infiltrating immune cells, has been adopted. This actually showed to be a better scoring method than the percentage of PD-L1+ tumor cells in the KEYNOTE-059 clinical trial with GC patients [70]. Often, clinical trials in GC use a CPS ≥1; nevertheless, it still fails to accurately stratify patients who will benefit from ICB. In the KEYNOTE-061 trial, a CPS ≥10 was evaluated and the overall survival of GC patients treated with pembrolizumab was longer than that of patients under chemotherapy, which could not be recapitulated with a CPS ≥1 (Supplementary Table 1). These results support the notion that the semiquantitative counting of PD-L1 expression needs to be further refined.

MSI is probably one of the most promising predictive biomarkers for ICB. In fact, FDA approved the use of pembrolizumab in patients with unresectable or metastatic solid tumors with MSI or MMR deficiency, regardless of its tissue of origin. This was actually the first time that FDA approved a cancer treatment based on a common biomarker rather than the location of the tumor. Several of the clinical studies performed in GC have added even more evidence that justifies its use as companion test (Supplementary Table 1). Interestingly, in the NCT02589496 trial [102], the only GC patient with high MSI that did not respond to pembrolizumab treatment had a heterogeneous MSI pattern, suggesting that it may be relevant to consider the heterogeneity of the tumors when it comes to assessing the MSI status.

In the clinical study by Kim et al. [102], it was revealed that previously treated metastatic GC patients whose tumors were EBV+ respond particularly well to ICB. It has been demonstrated that EBV+ gastric tumors have very distinctive molecular features, including amplification (also overexpression) of PD-L1 and PD-L2, conspicuous intratumoral or peritumoral immune cell infiltration, especially of CD8+ T cells, and IFN-γ-driven gene expression profile (GEP) (Figure 1(d)) [14, 103, 104]. These results highlight the potential of EBV positivity as the predictor of the response to IC inhibitors in GC; however, more clinical evidence needs to be added to validate its use in clinical settings. Of note, the studies in GC show that MSI and EBV are mutually exclusive biomarkers [102, 103].

Although tumor-infiltrating lymphocyte density, particularly that of infiltrating CD8+ T cells, is strongly associated with ICB response in several cancer types [9, 48], this has not been rigorously evaluated in GC. Some studies in GC, however, have identified immune-related GEPs that correlate with clinical benefit from ICB therapy, especially the IFN-γ-driven and T-cell-inflamed related gene signatures (Supplementary Table 1). Accordingly, the KEYNOTE-059 trial found that all patients with a PD-L1 CPS >20 had high T-cell-inflamed GEP scores. Elevated expression levels of immune-related gene signatures, however, do not necessarily predict ICB response as this may be influenced by other parameters. This is exemplified in the NCT02589496 clinical trial [102], which revealed that gastric tumors with mesenchymal subtype, defined by elevated expression of an epithelial-to-mesenchymal transition (EMT) gene signature, do not respond to ICB despite exhibiting elevated levels of immune signatures. Indeed, the mesenchymal phenotype has been demonstrated to be a negative predictor of response to ICB in other cancer types and a key determinant of poor survival in GC [105, 106]. According to these results, combination of several parameters may be a better strategy in order to accurately predict the response to ICB in GC.

The use in clinical practice of the approach known as liquid biopsy to determine the response to ICB is highly desirable since this is a noninvasive method that enables the constant follow-up of patients undergoing therapy. With liquid biopsy, for example, it is feasible to determine the tumor mutational load through sequencing analysis of blood-derived circulating tumor DNA (ctDNA). The potential of this approach has been demonstrated in a trial of 69 patients representing 23 different cancer types, concluding that the number of mutations detected in ctDNA was positively associated with ICB response [107]. Similar results were found in a study in non-small cell lung cancer patients treated with atezolizumab [108]. In GC, ctDNA sequencing can reproduce tumor tissue exome sequencing and MSI PCR testing to identify patients likely to respond to pembrolizumab. Even more importantly, posttreatment changes in ctDNA predict both ICB response and progression in GC [102]. These findings expand the possibilities of using liquid biopsy in the clinic to perform evaluations of tumor mutational load as the predictive biomarker of ICB response.

9. *Helicobacter pylori* Infection and Its Potential Relevance in the Context of ICB

The inflammatory response mounted against *H. pylori* infection is characterized by a local upregulation in the expression of a vast array of inflammatory mediators and the recruitment of various populations of bone marrow-derived cells to the gastric mucosa, including neutrophils, macrophages, and dendritic, T, and B cells (Figure 1(a)) [109, 110]. *H. pylori* induces the expression of cytokines such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-6, IL-8, IFN-γ, and the cyclooxygenase (COX)-2 enzyme [111–115], as well as the activation of the transcription factor NF-kB [52, 116]. Most of these effector molecules have pleiotropic effects, thus influencing the progression of *H. pylori*-induced carcinogenesis at different levels. One of the best-studied inflammatory mediators in this context is IL-1β, which exerts a proinflammatory function and acts as a strong inhibitor of the gastric acid secretion [112, 117]. The latter creates a less hostile environment for *H. pylori* and other microbial

communities lodged in the stomach. In fact, *H. pylori*-colonized individuals with high-expression polymorphisms in the IL-1β gene cluster, that is, IL-1β and its naturally occurring IL-1 receptor antagonist (IL-1RN), have increased risk for hypochlorhydria, gastric atrophy, and distal GC [53, 118, 119]. IL-1β *per se* is sufficient to induce gastric neoplasia in a mouse model with stomach-specific transgenic overexpression of IL-1β, which is mediated by activation of NF-kB and early recruitment of myeloid-derived suppressor cells (MDSCs) to the stomach [117]. Notwithstanding this, the bacterium generally remains in the stomach of colonized individuals for life, indicating that the immune response is ineffective to clear the infection. In addition, the presence of inflammation for decades supports the notion that the immune response is dysregulated by *H. pylori* [110, 120–122]. The mechanisms by which this bacterium modulates innate and adaptive immunity have been reviewed elsewhere [50, 110].

Both *in vitro* and *in vivo* studies have reported that the expression of some IC proteins in gastric epithelial and activated T cells is upregulated upon *H. pylori* colonization, presumably as part of the strategies to evade and subvert host immune defenses (Figure 1(b)). The upregulation of PD-L1 in gastric epithelial cells following *H. pylori* infection is relatively well documented both *in vivo* and *in vitro* [22–25, 123]. Some of the studies addressing this connection have unraveled aspects underlying the induction and regulation of PD-L1 expression in response to *H. pylori*. For example, it has been demonstrated that the induction of PD-L1 in gastric epithelial cells by *H. pylori* requires its type 4 secretion system (T4SS), whose components activate the p38 MAPK pathway [25]. Importantly, studies in mouse models conclude that the upregulation of PD-L1 results in increased bacterial load, induction of Treg cells in the stomach, and increased IL-10 in serum [25]. Elevated expression of PD-L1 in gastric epithelial cells may induce apoptosis of T cells [24]. Also, the induction of PD-L1 expression in GC cells cocultured with *H. pylori* is inhibited by miR-152 and miR-200b [123]. Interestingly, PD-L1 expression is negatively correlated with miR-152 and miR-200b levels in gastric tumor tissues from human patients [123]. The induction of other IC molecules in the context of *H. pylori* infection has also been reported. For example, a recent immunohistochemistry study found higher levels of Gal-3 in the gastric mucosa of patients with *H. pylori* infection, compared to noninfected subjects [124]. Finally, *H. pylori* stimulation resulted in a significant increase of Tim-3 in an *in vitro* system [125]. Altogether, these observations support the notion that IC protein induction in the context of *H. pylori* infection might contribute to the establishment of a persistent infection, which in turn favors the progression from premalignant lesions to gastric adenocarcinoma through the creation of an immunosuppressive microenvironment.

10. Conclusions

The establishment of a suppressive TIME is a parameter that greatly influences tumor progression. T-cell exhaustion, through the expression of different ICs, is one of the most salient manifestations of the suppressive TIME. The immune-editing process that takes place during GC initiation and progression, from a proinflammatory state induced by *H. pylori* or EBV infection towards a suppressive microenvironment, includes upregulation in the expression of ICs that prevent T-cell-mediated elimination of tumor cells. The fact that this exhausted state can be reverted with the use of monoclonal antibodies has revolutionized cancer treatment. In the context of GC, the recent approval of the anti-PD-1, pembrolizumab, for the treatment of advanced or recurrent GC represents an important achievement since a large number of patients are diagnosed at advanced stages, when the probability of curing the disease is very limited. A major hurdle, however, is the identification of biomarkers that can be used in the clinic to stratify GC patients and personalize ICB therapy. Until now, there are many promising biomarkers that may be helpful as predictive criteria, but none of them seem to be useful by themselves. Instead, clinical trials reflect the requirement of standardizing an algorithm that includes not one but several of these potential biomarkers, such as PD-L1 expression, microsatellite instability (MSI), MMR deficiency, EBV positivity, immune-related GEPs, ctDNA mutational load scores, and mesenchymal subtype.

The induction of ICs expression in response to *H. pylori* infection is a very fascinating finding that may have important implications in gastric carcinogenesis and, therefore, needs to be further explored. To date, very few studies have addressed the molecular mechanisms underlying this relation. A particularly relevant aspect is whether the induction of ICs in the nonneoplastic gastric epithelium colonized with *H. pylori* has an impact in the composition of the microenvironment of manifest GC lesions. More specifically, it is important to know if the expression of ICs in early stages of carcinogenesis favors the creation of a suppressive inflammatory microenvironment, which facilitates the growth and progression of invasive gastric tumors. High expression of ICs and infiltration by effector T cells from very early stages in the sequence of events that culminates with GC could mean a better response to immunotherapy. Ultimately, all this information may also serve as evidence in favor of the use of ICB therapies in early stages of the disease.

Conflicts of Interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of this review.

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Supplementary Materials

The supplementary material submitted along with the manuscripts is a table in which we aim to summarize the most representative clinical trials for immune-checkpoint

blockade therapy in gastric cancer. Most of those studies are ongoing, and a few are already finished. Specifically, in the table, we synthesize the main results obtained in those studies. Of note, in the supplementary table, we only include clinical trials that had a companion test. (*Supplementary Materials*)

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Review Article

Mechanisms of Metastasis in Colorectal Cancer and Metastatic Organotropism: Hematogenous versus Peritoneal Spread

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Metastasis is the major cause of death in patients with colorectal carcinoma (CRC). The most common sites of metastasis are the liver and the peritoneum. Peritoneal carcinomatosis is often considered the end stage of the disease after the tumor has spread to the liver. However, almost half of CRC patients with peritoneal carcinomatosis do not present with liver metastasis. This brings up the question of whether peritoneal spread can still be considered as the end stage of a metastasized CRC or whether it should just be interpreted as a site of metastasis alternative to the liver. This review tries to discuss this question and summarize the current status of literature on potential characteristics in tumor biology in the primary tumor, i.e., factors (transcription factors and direct and indirect E-cadherin repressors) and pathways (WNT, TGF-β, and RAS) modulating EMT, regulation of EMT on a post-transcriptional and posttranslational level (miRNAs), and angiogenesis. In addition to tumor-specific characteristics, factors in the tumor microenvironment, immunological markers, ways of transport of tumor cells, and adhesion molecules appear to differ between hematogenous and peritoneal spread. Factors such as integrins and exosomal integrins, cancer stem cell phenotype, and miRNA expression appear to contribute in determining the metastatic route. We went through each step of the metastasis process comparing hematogenous to peritoneal spread. We identified differences with respect to organotropism, epithelial-mesenchymal transition, angiogenesis and inflammation, and tumor microenvironment which will be further elucidated in this review. A better understanding of the underlying mechanisms and contributing factors of metastasis development in CRC has huge relevance as it is the foundation to help find specific targets for treatment of CRC.

1. Introduction

Of all cancers, colorectal cancer (CRC) is the third most common with metastasis being the major cause of death in the majority of patients [1]. Common sites of distant metastasis are the liver and the peritoneum. About 20% of patients present with synchronous metastases, most commonly in the liver, and up to 60% of patients develop distant metastases within 5 years [2, 3]. Metastases in the peritoneum are found in 25% of patients with an inferior prognosis when compared to other metastatic sites [4]. However, although peritoneal carcinomatosis is considered to be an advanced stage of CRC, in 41–45% of metastasized CRC patients it is the only site of metastatic disease suggesting that peritoneal spread might be a locally advanced form of CRC without other distant metastases [5, 6]. These data point to the complexity of metastatic spread and many attempts have been made to understand the underlying principles of metastasis and organotropism. This review aims to give an overview of the current knowledge of the mechanisms of metastasis and metastatic organotropism in CRC.

2. Organotropism in Epithelial Tumors

Much progress has been made in understanding tumor biology and mechanisms of metastasis but knowledge on the factors influencing the metastatic route of tumor cells,

especially in colorectal cancer, remains poor. Taken from recent findings on other epithelial tumors such as breast cancer and pancreatic cancer, metastatic organotropism is a nonrandom process regulated by different cancer cell intrinsic factors, the tumor microenvironment and the interaction between those cancer cell intrinsic factors and the tumor microenvironment [7]. Having only recently been discovered to play a pivotal role in the metastatic process, there are only very few studies on organotropism in malignant epithelial tumors so far. The next section tries to give an overview of the current knowledge on this hot topic and elaborate on mechanisms of organotropism that have been found to be of high relevance in malignant epithelial tumors.

2.1. Epithelial Characteristics. In pancreatic ductal adenocarcinoma (PDAC), metastatic organotropism to the liver and lung is dependent upon epithelial plasticity mediated by P120 Catenin (P120CTN). P120 Catenin is the binding and stabilizing partner of E-cadherin at the adherens junctions and has been described as a cancer candidate gene [8]. Mono-allelic P120CTN loss accelerates liver metastasis. However, loss of both P120CTN alleles results in the absence of liver metastasis. This might be due to the inability of cancer cells to establish new contacts with neighboring epithelial cells indicating that exhibition of epithelial characteristics is a premise to liver organotropism. Lung organotropism, however, seems to be independent of P120CTN expression and has also been demonstrated in cells with bi-allelic P120CTN loss [9]. The observation that liver metastasis is promoted by maintenance of an epithelial state through factors such as P120CTN and E-cadherin, while repression of E-cadherin is one of the main inductors of epithelial-mesenchymal transition (EMT), is a paradox and underscores the complexity of the metastatic cascade. To summarize, maintenance of epithelial characteristics seems to be a requirement for liver organotropism but negligible in lung organotropism.

2.2. Integrins. Lymphocyte function-associated antigen-1 (LFA-1) is an integrin with adhesive function that immune cells use to invade the liver under inflammatory conditions. Recently though, this integrin has been shown to be also expressed on solid tumors including CRC. A recent study conducted, using an in vitro assay and an in vivo mouse model, could show that decreased expression of the $\beta 2$ subunit of LFA-1, which is required for integrin activation, adhesion, and signaling, correlates with a reduced activation of the liver endothelium and an improved local immune response in the liver. This results in a less tumor-promoting microenvironment. Furthermore, a reduction in early retention of cancer cells in the liver as well as a reduction in metastatic development and tumor size was observed [10]. In summary, the $\beta 2$ subunit of LFA-1 integrin on colorectal tumor cells modulates liver organotropism and a decreased expression is associated with a reduction in liver metastasis.

In breast cancer, apart from cancer subtype and gene signature, metastatic organotropism was found to be regulated by molecular features such as chemokines and integrins. Binding of the chemokine CXCL12 to its receptor CXCR4 is associated with induction of liver metastasis and facilitates extravasation of tumor cells. $\alpha 2\beta 1$ and $\alpha 5\beta 1$ are integrin complexes being expressed on the cell surface where they interact with the liver stroma. Downregulation of these integrins results in reduced liver metastasis by preventing direct interactions of tumor cells with components of the extracellular matrix (ECM). $\alpha 2\beta 1$ and $\alpha 5\beta 1$ expression is upregulated by claudin-2, which is highly expressed in liver metastasis of breast cancer while expression in primary tumors is weak or absent [11].

2.3. Exosomal Integrins. Different exosomal integrin expression patterns have been linked to different routes of metastasis in breast cancer and pancreatic cancer, indicating that exosomal integrins could predict organ-specific metastasis underlying the phenomenon of organotropism [7]. Secreted from tumor cells, exosomal integrins initiate premetastatic niche formation which is defined as a tumor cell-free microenvironment in a putative organ of metastasis. By fusing with the target cells in this microenvironment, induction of inflammation and increment of vascular permeability through Src activation and S100 expression, exosomal integrins prime this microenvironment as a site for seeding of disseminated tumor cells [7]. Integrins $\alpha 6\beta 4$ and $\alpha 6\beta 1$, binding to lung-resident fibroblasts and epithelial cells, are associated with lung metastasis, and integrin $\alpha v\beta 5$, binding to Kupffer cells, is associated with liver metastasis [7].

2.4. Vascularisation of Metastasis. Breast cancer cells metastasizing to the liver show decreased mitochondrial metabolism and increased conversion of pyruvate into lactate. This altered metabolic program is due to expression of pyruvate dehydrogenase kinase-1 (PDK1), a target of HIF1a with VEGF and TWIST as downstream targets, enabling cancer cells to adapt to nutrient insufficiencies and hypoxia in the host stroma [11]. Interestingly, liver metastases emanating from breast cancer are not dependent on hypoxia and increased angiogenesis, while CRC liver metastases depend on these mechanisms [12]. In breast cancer, EGFR, COX2, and MMP-1 and MMP-2 have been shown to promote angiogenesis in lung metastasis. However, in order to overcome nonpermissive signals from resident cells of the lung, lung metastatic cancer cells express Coco and GALNTs [11]. To summarize, liver metastases in breast cancer show a nonangiogenic growth pattern independent of oxygen supply, whereas in CRC a high fraction of angiogenic liver metastases is found.

2.5. Cancer Stem Cell Phenotype. Metastatic spread in CRC has been shown to correlate with different expression of cancer stem cell markers. Liver metastasis in CRC is associated with the expression of cancer stem cell markers CD133, CD44, and β-catenin, whereas in peritoneal carcinomatosis no expression of these stem cell markers can be found. This might indicate that CRC with peritoneal carcinomatosis lack stem cell features needed for dissemination and, bearing the

clinical course of the disease, could possibly be interpreted as an advanced stage of a locally aggressive tumor [13]. Multivariate analysis confirmed that a negative CD133 expression in the primary tumor of colorectal cancer patients with peritoneal carcinomatosis is an independent risk factor for reduced disease-free survival and can predict postoperative recurrence [14]. While the knowledge on stem cell markers associated with peritoneal carcinomatosis is still poor, a few other markers related to liver metastasis have recently been identified. Oct4 gene expression correlates with poor liver metastasis-free survival and Oct4-high cells actively form liver metastasis in vivo [15]. The stem cell markers Notch1 and ALDH1 correlate with lymph node metastasis, advanced stage, and tumor recurrence and represent an independent prognostic factor in colorectal carcinoma [16]. In summary, cancer stem cells seem to have a strong ability for migration and invasion and stem cell markers have been shown to be independent prognostic factors. However, the distribution of cancer cells to different metastatic sites as part of organotropism and the maintenance of biological features is also mediated by the microenvironment and part of a complex bidirectional mechanism [17].

3. Tumor Biology

The process of tumor cells leaving their primary site and forming new colonies in distant tissues is described as invasion-metastasis cascade (Figure 1) [18]. This process consists of five steps: local invasion of tumor cells into surrounding matrix (Step 1), intravasation of tumor cells into circulatory system (Step 2), systemic transportation of tumor cells (Step 3), extravasation of tumor cells into parenchyma of distant tissue sites (Step 4), colonization of distant organs, and establishment of macroscopic tumors (Step 5) [19]. Although the underlying biology of some of these steps is yet to be defined, a few mechanisms have recently emerged and been increasingly recognized to play pivotal roles in the promotion of the invasion-metastasis cascade. However, the mechanisms determining hematogenous versus peritoneal spread are poorly understood.

3.1. Epithelial-Mesenchymal Transition

3.1.1. Transcription Factors. The epithelial-mesenchymal transition (EMT) is a key program that enables stationary epithelial cells to lose their cell-cell adherence and acquire mesenchymal properties that are essential for invasion and metastasis. These include enhanced mobility, invasiveness, increased resistance to apoptosis, and degradation and production of extracellular matrix (ECM) components [19]. The regulation of EMT occurs at different molecular levels (Figure 2).

E-cadherin, a transmembrane protein expressed by epithelial cells, plays an essential role in maintaining epithelial cell polarity and stabilizing cell-cell contacts allowing cells to sustain their epithelial state [20]. Downregulation of E-cadherin expression is associated with lymph node metastasis, advanced stage, poor differentiation, and vascular invasion [21]. A reduction in E-cadherin levels is commonly followed

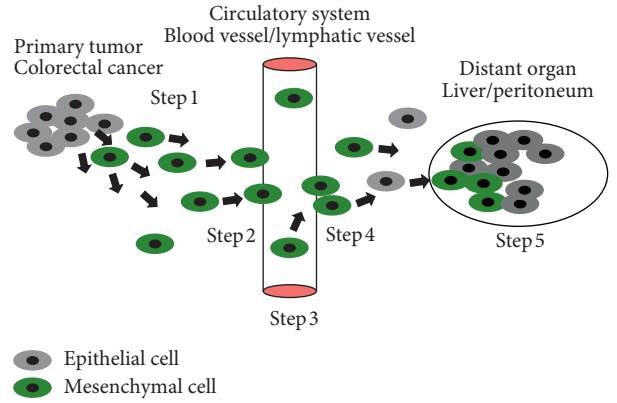

Figure 1: Invasion-metastasis cascade: local invasion of colorectal cancer cells into surrounding matrix (Step 1), intravasation into circulatory system (blood vessels/lymphatic vessels) (Step 2), systemic transportation (Step 3), extravasation (Step 4), colonization of distant organs (liver/peritoneum) (Step 5).

by an upregulation of N-cadherin, an adhesion molecule found in nonepithelial tissues. N-cadherin promotes tumor progression by enhancing fibroblast growth factor receptor (FGFR) signaling and nuclear β-catenin activity [20].

Transcription factors regulating E-cadherin expression in CRC can be divided into two groups: direct and indirect repressors. Direct repressors of E-cadherin bind directly to the E-cadherin promoter and include SNAIL (SNAI1,2), ZEB (ZEB1,2), E12/E47, Brachyury, and AP4 [22–29]. The indirect repressors TWIST (TWIST1,2), FOXC2, TCF4, SOX2, OCT4, Nanog, PROX1, SIX1, PRRX1, HMGA1, and Fra-1 have multiple specific targets and regulate the transcription of E-cadherin at different levels including the activation of direct repressors [30–39].

With respect to the clinical significance in CRC, the repressors AP4, SOX2, and OCT4 have been associated with liver metastasis [29, 32, 33]. Furthermore, an overexpression of the repressor TWIST1, that 85% of CRC patients show a moderate to strong expression of, is associated with nodal invasion, male sex, and poor outcome [40, 41]. Upregulation of SNAI2 significantly correlates with strong vimentin expression, and both SNAI2 and vimentin expression is associated with lymph node metastasis and poor prognosis [42].

Vimentin is an intermediate filament protein that is expressed in mesenchymal and neoplastic cells. Vimentin coexists and interacts with keratin-KRT14 through formation of an intracellular Vim/KRT14 hybrid and can be found in cells at the leading edge of migration. It is hypothesized that this hybrid formation disrupts the rigidity of keratin filaments, thereby promoting cellular migration. Expression of vimentin is regulated by the Vim gene regulating cancer cell migration and invasiveness. It has been shown that knockdown of the Vim gene disrupts keratinocyte colony growth and migration and represses EMT [43].

3.1.2. Pathways. The EMT process is triggered by a multitude of extracellular signals in the tumor microenvironment with subsequent activation of all major cancer cell intrinsic

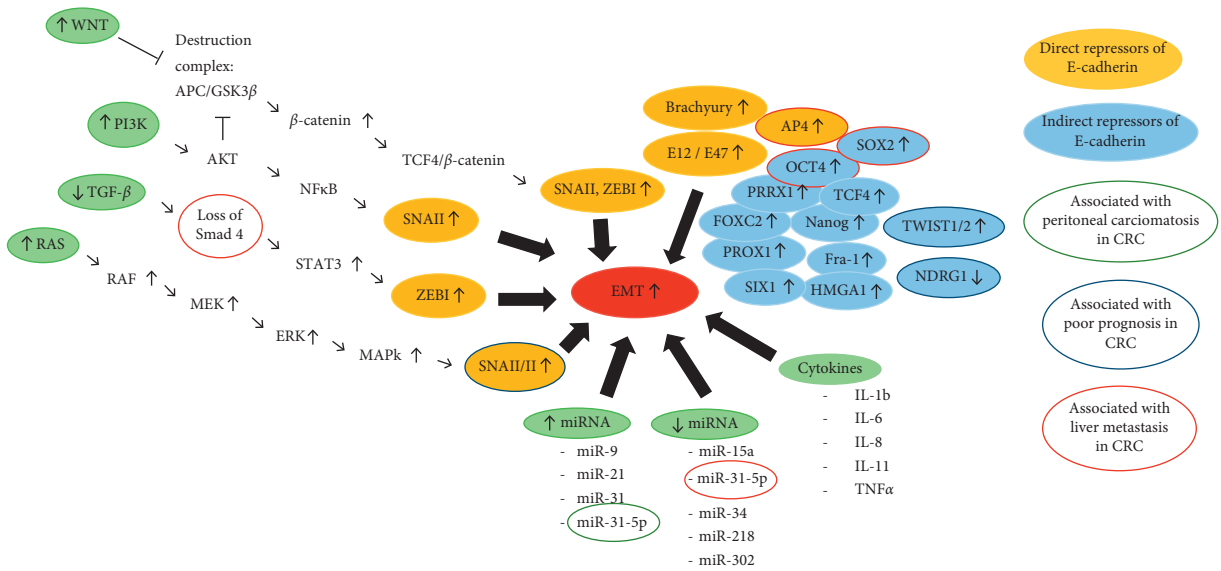

Figure 2: Regulation of EMT at different molecular levels: upregulation of the WNT pathway, PI3K/AKT pathway, and RAS/RAF/MEK/ERK/MAPK pathway and downregulation of the TGF-β/Smad pathway lead to the activation of EMT through downregulation of E-cadherin. E-cadherin repressors can be divided into direct and indirect repressors. EMT can also be regulated on a posttranscriptional and posttranslational level by miRNAs exerting activating and inhibiting functions. As part of tumor cell-tumor microenvironment interactions EMT can also be triggered by a variety of cytokines.

signaling pathways. Considering the current status of literature it seems that all major signaling pathways have some implication in the EMT program of CRC and contribute differently to CRC progression. The following paragraph describes the signaling cascades that are currently considered to contribute significantly to the EMT program in a variety of epithelial tumors including CRC.

Aberrant activation of the canonical WNT pathway leads to the inhibition of the destruction complex that otherwise degrades β-catenin. Subsequently, free cytosolic β-catenin is translocated to the nucleus where it binds to the transcription factor TCF4 inducing WNT target gene transcription including activation of E-cadherin repressors ZEB1 and SNAI1 and upregulation of MT1-MMP9 and LAMC2 that are associated with CRC invasiveness [31, 44]. Inactivating mutations of tumor suppressor genes such as APC and AXIN2 lead to the upregulation of the canonical WNT pathway, thereby promoting EMT [44]. In summary, aberrant activation of the WNT pathway and β-catenin-dependent signaling promote tumor progression and are important EMT regulators in CRC [45].

Inactivation of the TGF-β/Smad signaling pathway leads to tumor progression, and mutations are found in 40–50% of CRC [46, 47]. In the absence of mutations, Smad2 and 3 are activated by TGF-β and transferred to the nucleus with Smad4 regulating transcription [48, 49]. In the presence of mutations, loss of Smad4 that usually suppresses STAT3 activation leads to aberrant activation of STAT3, which is linked to upregulation of ZEB1 expression, reduced E-cadherin, and enhanced N-cadherin and vimentin expression [50, 51]. With respect to clinical relevance in CRC, loss of Smad4 is found in 30% of metastatic CRCs and seems to be a predictor of liver metastasis [52]. Claudin-3 (CLDN3), an integral membrane protein and component of tight junctions, helps cells to remain in their epithelial state and functions as a suppressor of EMT. However, similar to Smad4, loss of CLDN3 expression leads to the induction of EMT through aberrant activation of STAT3. Its loss also leads to upregulation of the WNT pathway and predicts poor patient survival [53]. In conclusion, half of CRC patients present with mutations that lead to an inactivation of the TGF-β/Smad pathway which ultimately leads to the induction of EMT.

N-Myc downstream regulated gene 1 (NDRG1) has been shown to inhibit EMT, migration and invasion through attenuation of the above-mentioned pathways as well as the ErbB signaling pathway, and inhibition of prosurvival autophagic pathways in a variety of cancer cells [54–57]. In CRC patients, NDRG1 expression was found to be an independent prognostic factor for survival and tumor recurrence: CRC patients that are NDRG1 negative face a worse prognosis in cancer-free and overall survival [58, 59].

Another two major signaling pathways regulating EMT are the RAS/RAF/MEK/ERK/MAPK pathway and the PI3K/AKT pathway both triggered by growth factors such as EGF and FGF. Activation of the RAS/RAF/MEK/ERK/MAPK pathway leads to increased expression of SNAI1 and 2. The PI3K/AKT pathway can induce SNAI1 expression through NF-κB or through AKT inhibiting the destruction complex in the WNT pathway which ultimately leads to increased SNAI1 and ZEB1 expression [60, 61]. As described above, activation of SNAI1 and ZEB1 leads to repression of E-cadherin and induction of EMT in CRC. Also involved in these signaling networks, the fibroblast growth factor gene 18 (FGF18) shows elevated expression in CRC and acts as a downstream target of the WNT signaling pathway driving EMT [62].

Finally, we like to add some information on ADAM9 to this synopsis of pathways as this factor has recently

emerged to play a significant role in a multitude of pathways regulating EMT and promoting metastasis in various cancers. ADAM9 is a membrane-anchored metalloprotease and part of the ECM compartment and has been shown to be upregulated in CRC promoting invasion [63, 64]. In CRC liver metastasis, ADAM9, secreted by hepatic stellate cells, binds to CRC cells and promotes carcinoma invasion through tumor-ECM interaction [65]. Studies on HCC cells and lung cancer cells showed that ADAM9, triggered by IL-6 which activates the JNK signaling pathway, induces the expression of the EMT-associated transcription factor SNAIL through NOX1 expression in the cell membrane and ROS production [66, 67]. ADAM9 promotes metastasis through enhanced CDCP1 expression, a promigratory transmembrane protein that is involved in cell-cell interaction and the regulation of anoikis resistance and is overexpressed in metastatic colon cancer [68].

3.1.3. miRNAs. The EMT process can also be regulated on a posttranscriptional and posttranslational level by microRNAs (miRNAs) [19]. Overexpression of miRNA has-miR-31-5p in CRC with peritoneal metastasis inhibits EMT through suppression of c-MET, a kinase mediating EMT. In CRC with hepatic metastasis miRNA has-miR-31-5p is repressed supporting EMT possibly through upregulation of c-MET [69]. miR-200 is involved in the TGF-β pathway and targets ZEB1 as an inhibitor. Loss of tumor suppressor p53 leads to downregulation of miR-200 in CRC and increased expression of ZEB1, thereby promoting EMT [70–73]. Following loss of p53, other miRNAs are downregulated and support EMT in CRC. These include miR-34, miR-302, miR-15a, and miR-218. Downregulation of miR-34 leads to increased expression of SNAI1, cMET, and β-catenin [74]. Decreased miR-302 expression leads to upregulation of transcription factor AP4 and SNAI1 and increased expression of vimentin, all supporting EMT [75]. miR-15a targets AP4 thereby acting similarly to miR-302 [76]. miR-218 when downregulated no longer promotes apoptosis of cancer cells through c-FLIP [77]. miRNAs that when upregulated promote EMT including miR-21, miR-31, and miR-9. miR-21 with TGF-β as an upstream activator downregulates tumor suppressor Pdcd4 and consecutively promotes invasion, intravasation, and metastasis [78]. miR-31, also activated by TGF-β, targets SATB2, a gene that is linked to CRC metastasis [79]. miR-9 directly targets E-cadherin and inhibits its expression [35]. It should be noted that there are many more miRNAs involved in the regulation of EMT, but this review focused on the ones that have been most studied and established to play pivotal roles in the regulation of EMT. In addition, miRNAs have been shown to not only be involved in EMT but likely also in organotropism by targeting and altering the metabolism in the premetastatic niche and regulating cancer stem cell-mediated metastasis. Patients with CRC and peritoneal carcinomatosis show a high expression of miR-31-5p, whereas patients with CRC and liver metastasis only show a low expression of miR-31-5p. This suggests that miRNAs might contribute to defining the site of metastatic spread [69].

3.2. Angiogenesis. Angiogenesis is considered to be a crucial step in tumor growth and establishing a route of transport for metastatic tumor cells. Angiogenesis is not only necessary to sustain tumor growth but also to enable tumor cells enter the vasculature by forming new vessels that connect to the existent circulatory system. This vascular remodeling requires the activation of endothelial cells by proangiogenic factors [63].

The VEGF family members with VEGF A, B, C, D and PIGF are considered the most important inductors of angiogenesis. Other inductors include ANGPT and PLAT [80]. Hypoxia leads to the secretion of VEGF from hypoxic cells by mediation of HIF1a [81, 82]. Other triggers for VEGF expression are EGF, TGF-α and -β, PDGF, ILGF-1, and FGF [83]. Although there are still open questions on how all the VEGF family members are involved in angiogenesis, it has been shown that VEGF A binds to VEGFR2 on endothelial cells promoting migration, survival, and proliferation of those by inducing the MAPK and PI3K pathways. VEGF C acts through VEGFR3 promoting lymphangiogenesis [80].

There is increasing evidence that angiogenesis is not an isolated self-regulated process but a consequence of tumor-microenvironment interactions and is not limited to the generation of new vessels but can also accelerate metastasis.

A recent study highlighted the importance of tumor endothelial cells (TECs) that belong to the group of tumor stromal cells and are a result of tumor cell-microenvironment interactions. The function of TEC is not limited to the formation of new blood vessels to supply the tumor with nutrients and provide a route to disseminate but actually stimulate the tumor to metastasize. When tumor cells enter the circulatory system they physically touch TECs and interact with them through juxtacrine and paracrine signaling. TECs secrete biglycan, a small rich repeat proteoglycan, that stimulates tumor cells to metastasize through activation of NFκB and ERK signaling. Furthermore, TECs are able to downregulate tumor suppressive factors such as Slit2, thereby promoting tumor progression [84].

IL33 is another good example to illustrate the complexity and bidirectional interactions between tumor cells, microenvironment, and angiogenesis. This tumor-derived cytokine induces angiogenesis through recruiting myeloid cells that subsequently secrete VEGF. It also indirectly promotes liver metastasis in CRC by mobilizing macrophages and myeloid cells to remodel the stroma towards a pro-TME rather than changing the invasive or migratory properties and metastatic capabilities of the tumor cells [85]. Various tumor-associated leukocytes including macrophages, neutrophils, myeloid-derived suppressor cells and dendritic cells, mast cells, innate lymphoid cells, $\gamma\delta$T cells, and natural killer cells have been identified to not only lead to an immunosuppressive TME but also contribute to angiogenesis by secretion of VEGF, chemokines, cytokines, proteases, structural proteins, and microvesicles [86].

Other factors that have already been mentioned in terms of their role in the EMT process but also contribute to angiogenesis in various cancers are ADAM9 and FGF18. In lung cancer metastasis, ADAM9 increases the expression of VEGF A, ANGPT2, and PLAT and activates EGFR [67]. FGF18 activates the ERK/MAPK pathway through binding to the

FGFR3 receptor on endothelial cells thereby promoting angiogenesis in ovarian cancer. FGF18 can also activate NF-κB leading to upregulation and secretion of proinflammatory cytokines with subsequent recruitment of tumor macrophages that secrete VEGF A and ECM degrading proteases like MMP9 to promote angiogenesis and creating a protumor microenvironment [87]. Another factor promoting angiogenesis through the ERK pathway is the calcium and integrin binding gene 1 (CIB1) that is upregulated in a variety of cancers, including CRC, often correlating with oncogenic KRas mutations [88]. On phosphorylation mediated by PKD2, CIB1 contributes to angiogenesis by mediating PKD2-induced VEGF production and secretion of tumor cells and VEGFR2 expression [89]. Homeodomain-interacting protein kinase-2 (HIPK2) has been demonstrated to function as a tumor suppressor in various types of cancer and its overexpression leads to the downregulation of VEGF promoter activity. Inhibition of HIPK2 by hypoxia results in induction of EMT and angiogenesis via WNT signaling and increased VEGF promoter activity [90]. A recent study could show that metastatic growth is in fact associated with hypovascularity. Vascularity decreases with increasing metastasis size. The bigger the lesion, the less it is surrounded by vessels, thereby resembling the primary tumor [91]. Although the underlying mechanism is still unknown it has been shown that in pancreatic cancer reduction of fibroblasts leads to increased vessel density pointing to fibroblasts exerting antiangiogenic effects [92].

A recent study could show that under hypoxic conditions CRC cells release exosomes into the tumor microenvironment that promotes angiogenesis. These exosomes, enriched with WNT4, lead to an induction of β-catenin signaling in endothelial cells and stimulate them to proliferate and migrate [93]. Considering the mechanisms of metastasis in CRC it seems that angiogenesis differs between hepatic and peritoneal spread, not in relevance though but in terms of time and order during the metastatic cascade. Liver metastases are considered the result of hematogenous dissemination. However, in order for tumor cells to be transported to the liver they first have to find access to the circulatory system. They also depend on the formation of new small vessels originating around the primary tumor that increase the likelihood of tumor cells entering the blood stream. Once they arrive at the liver, angiogenesis again is required in order for metastases to grow and proliferate [94]. The primary tumor itself can actively support this process by making the liver parenchyma more permissive for the homing and growth of metastasis by recruiting VEGFR-1 expressing haematopoietic progenitor cells that initiate the premetastatic niche [95, 96]. Patients with synchronous liver metastasis and the primary tumor still in situ show a significantly higher VEGFR1 and VEGF A expression in liver parenchyma adjacent to metastases than patients with metachronous liver metastases after resection of the primary tumor which underlines the tumor angiogenesis-promoting abilities of the primary tumor [97]. In contrast, peritoneal carcinomatosis is not considered a result of hematogenous dissemination but the consequence of lymphatic dissemination or tumor cell shedding into the peritoneal cavity with subsequent attachment to distant peritoneum. Hence, angiogenesis seems not to be a key element in the initial steps of the metastatic cascade. However, once the CRC cells arrived in the peritoneum via the mentioned mechanisms, they rely on angiogenesis to sustain proliferation and enable further growth [98].

4. Inflammation and Tumor Microenvironment

The interaction between cancer cells and their microenvironment is considered to be an essential component not only in the early steps of colorectal carcinogenesis but also in tumor progression and development of metastasis. This protumor microenvironment is composed of inflammatory and immune cells involving neutrophils and macrophages, carcinoma-associated fibroblasts (CAFs), environmental conditions such as hypoxia, soluble factors, signaling molecules, and ECM components [82]. Invasion of tumor cells requires the degradation of the basement membrane with subsequent migration of tumor cells through the stroma into neighboring tissues and with respect to liver metastases in CRC invasion into the vasculature requiring angiogenesis.

The process of stromal remodeling is regulated by TGF-β and PDGF, which is secreted by CAFs. Additionally, CAFs produce MMPs, MMP inhibitors, ECM components, cytokines, growth factors, and EMT-promoting factors facilitating not only cancer proliferation and invasion but also metastasis [99].

Through a complex network of soluble factors, macrophages are recruited into the tumor microenvironment (TME) where they exert different functions critically depending on the tumor stage. At early stages, the majority of tumor-associated macrophages (TAMs) are subtype 1 (M1) TAMs that possess proinflammatory characteristics and work to eliminate malignant cells; at later stages macrophages can switch to subtype 2 (M2) TAMS bearing immunosuppressive functions creating a microenvironment permissive of tumor growth. By secretion of ECM-degrading components (e.g., MMP1, 7, 9, 12) TAMs support cancer cells invade the stroma [100]. Furthermore, even proinflammatory M1 macrophages have been shown to promote EMT and activate the β-catenin/TCF4 pathway by releasing proinflammatory factors thereby supporting the metastatic cascade [101].

Besides their role in immune cell recruitment, cytokines can also act to induce EMT in malignant cells. IL-1b promotes EMT through ZEB1 activation, IL-11 via GP130/STAT3 signaling, and IL-6 via STAT3 activation and SNAI expression [102–104]. Downregulation of tumor suppressor p53 by IL-6 results in a reduction of E-cadherin and increased expression of SLUG, further promoting EMT [105]. TGF-β-induced SNAI expression can activate IL8 with subsequent activation of CXCR1 and induction of EMT through PI3K/AKT signaling [106, 107]. TNF-α can induce EMT directly by AKT/GSK-3b-mediated stabilization of SNAI and indirectly by increasing IL8 and CXCR1 [107, 108]. Loss of Smad 4 not only promotes EMT via the signaling pathways described above but also by increasing CCL15 expression leading to the recruitment of CCR(+) myeloid cells. These cells then help CRC cells to invade the stroma and metastasize to the liver by producing MMP9 [109].

In addition to the cancer cell-microenvironment interactions described above recent studies point to several

more specific cells of the microenvironment that play distinct roles in the propagation of peritoneal metastasis. Among them are peritoneal macrophages (PMs), peritoneal mesothelial cells (PMCs), and peritoneal fibroblasts (PFBs). Peritoneal invasion is defined as tumor invasion beyond the peritoneal elastic lamina which creates the invasive tumor microenvironment facilitating tumor progression and metastasis. PMs form the first barrier to invasion by secretion of tumor necrosis factor (TNF). However, as described above, they can also switch to a tumor-promoting M2 phenotype driven by molecules released from CRC cells or by factors found in malignant ascites such as IL-6 and IL-10 [100]. Although the exact function of PMCs in the beginning stages of peritoneal carcinomatosis is not fully understood, PMCs are considered to play a major role in maintaining intraperitoneal homeostasis. Following PM activation, they secrete a variety of mediating molecules to the tumor microenvironment such as cytokines, chemokines, growth factors, ECM components, and adhesion molecules [100]. There is a debate however on PMCs exerting a protective role versus PMCs supporting cancer cell colonization. After tumor cell colonization, PFBs support tumor progression acting similar to CAFs. Recent studies suggest that CAFs might derive from PMCs; however, the mechanisms of this transformation are still unclear [100]. Focus on subpopulations of fibroblasts showed that subperitoneal fibroblasts (SPFs) play an active role in the process of tumor invasion. They show high gene expression of an ECM component and an upregulation of genes associated with cell contraction including α-SMA. Stroma-cell contractile ability and fibrosis with α-SMA expression promote cell migration, invasion, and accelerate metastasis [110]. CD90(+)/CD45(–) cells are a small subpopulation of mesothelilal-like cells (MLCs) found in the peritoneal fluid that also express characteristics of mesenchymal stem cells. In nude mice, intraperitoneal co-injection of MLCs and gastric cancer cells leads to enhanced tumorigenicity and an increased rate of metastatic formation in the peritoneal cavity. Following TGF-β stimulation, MLCs express collagen I, α-SMA, and vimentin and act similar to myofibroblasts. This way they seem to play a supportive role in the development of peritoneal metastasis by creating a permissive tumor microenvironment [111]. Lately focus has also been on peritoneal adipocytes (PAs) which have been found to promote proliferation and invasion of cancer cells through lipid internalization by gastric cancer cells thereby providing nutrients to the malignant compartment. The increased invasiveness is mediated by PI3K/Akt-signaling. Interestingly, only cells from the peritoneum of obese animals could promote tumor growth, underscoring the importance of host factors in the progression of malignant disease [112].

5. Development of Peritoneal Carcinomatosis: An Alternative Concept to the Invasion-Metastasis Cascade

As described above, hepatic spread is the result of the invasion-metastasis cascade, a concept that is applied to explain the mechanism of hematogenous dissemination in epithelial tumors including CRC. However, in the course of this article it becomes clear that in peritoneal carcinomatosis some of the steps simply differ from the proposed concept especially in terms of ways of detachment, dissemination, and attachment of tumor cells. The following paragraph introduces a concept specifically developed to explain the development of peritoneal carcinomatosis as opposed to the invasion-metastasis cascade. Also, differences between hematogenous and peritoneal spread in terms of transport and attachment of tumor cells to distant organ will further be elucidated below.

The development of peritoneal carcinomatosis involves five essential steps (Figure 3): (1) detachment of tumor cells from their primary tumor and gain of motility, (2) anoikis evasion, (3) adherence to peritoneal surface, (4) invasion into peritoneum, and (5) proliferation and formation of peritoneal metastasis [113]. The detachment of tumor cells from their primary tumor can be due to the following mechanisms: spontaneous exfoliation of tumor cells in CRC growing through the serosa (T4 stage), spontaneous tumor cell shedding into the lymphatics around the primary tumor as a result of increased interstitial fluid pressure, surgery-induced tumor spill due to opening of the tumor, transected lymphatics and blood vessels, or postoperative infections due to anastomotic leakage that have been shown to be associated with higher rates of tumor recurrence [98, 101, 113]. On a molecular level, the detachment of tumor cells and subsequent gain of motility is the result of a downregulation of cell-cell adhesion molecules (CAMs) as part of the EMT and changes in the cytoskeleton. Importantly, CAMs include integrins, cadherins, and selectins. Also involved in the EMT process are EGFR, c-MET, and especially in the context of peritoneal carcinomatosis, the EMT trigger TWIST [114].

6. Transport and Attachment of Tumor Cells to Distant Organ

In hematogenous spread platelets and neutrophils help tumor cells that have entered the circulatory system, so called circulating tumor cells (CTCs), avoid elimination by protecting them from shear stress or immune attacks from natural killer cells. TGF-β and platelet-derived growth factor (PDGF) released from platelets inhibit the activity of natural killer cells. By forming protective cloaks combined with fibrinogen, platelets also physically shield cancer cells from natural killer cells. Through TGF-β-induced activation of NF-κB in cancer cells the EMT program is reinforced and promoted so that CTCs will not lose their invasive characteristics needed for extravasation and metastatic colonization by returning to an epithelial state. Extravasation is further supported by ATP secretion from platelets increasing permeability of the vasculature and facilitating the entrance into the tissue [115]. Neutrophils have been shown to support metastatic spread by forming neutrophil extracellular traps that trap tumor cells in the blood stream, this way helping them adhere to endothelial

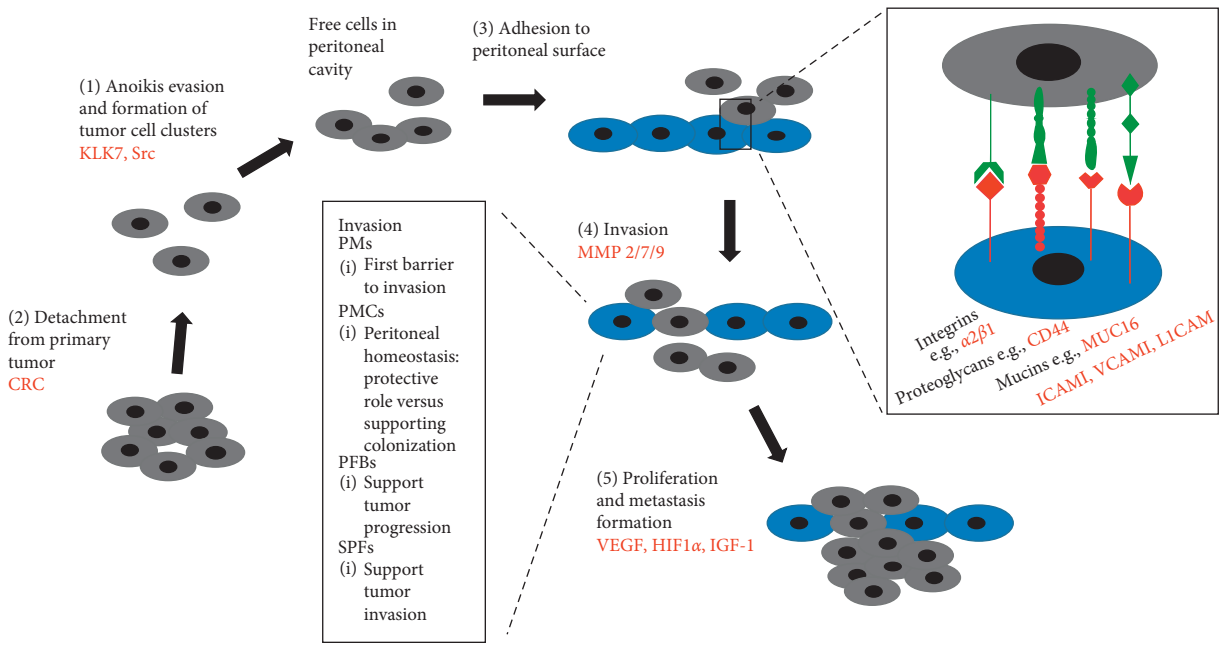

Figure 3: Development of peritoneal carcinomatosis: (1) detachment of colorectal cancer cells from their primary tumor and gain of motility, (2) anoikis evasion, (3) adherence to peritoneal surface, (4) invasion into peritoneum, and (5) proliferation and formation of peritoneal metastasis. Several adhesion molecules have been identified to be crucial in the adhesion process to the peritoneal surface, including integrins, proteoglycans, mucins, and members of the immunoglobulin superfamily. A multitude of cells and factors are involved in the process of invasion including peritoneal macrophages (PMs), peritoneal mesothelial cells (PMCs), peritoneal fibroblasts (PFBs), and subperitoneal fibroblasts (SPFs). Factors that have been identified to play a role in peritoneal carcinomatosis in CRC are highlighted red.

cells, avoid natural killer cell attacks, and extravasate [115]. Due to poor adaptation to the new microenvironment most tumor cells are either eliminated after extravasation or enter a state of dormancy for years. These cells can be activated to grow by prometastatic changes in the microenvironment such as hypoxia, fibrosis, inflammation, and production of ECM components by CAFs which underlines the interdependence of cancer cells and their microenvironment as described above [115]. To evade anoikis in peritoneal carcinomatosis kallikrein-related peptidases (e.g., KLK7) are activated and tumor cells form clusters and continue to proliferate as opposed to the hematogenous dissemination where tumor cells are chemically and mechanically protected by platelets [114]. In addition, studies on CRC cell lines have shown that an upregulation of Src, a tyrosine kinase that plays a major role in cell-matrix and cell-cell contact-mediating adhesions, leads to an increased resistance to anoikis [116].

Recently, focus has been on identifying relevant adhesion molecules in peritoneal spread, and it has been shown that adhesion molecules differ in between hematogenous and peritoneal dissemination suggesting differences in attachment processes. In hematogenous spread, in CRC mainly to the liver, adherence to the endothelium in the hepatic sinusoids is required which involves CD44 binding to hyaluronan and the blood group antigens sLe^a and sLe^x binding to selectins and mucins binding to ECM components [113]. Important adhesion molecules in peritoneal carcinomatosis include integrins (e.g., $\alpha 2\beta 1$) and integrin ligands, proteoglycans (e.g., CD44), members of the immunoglobulin superfamily (e.g., ICAM1, VCAM1, L1CAM), mucins (e.g., MUC16), and the epithelial cell adhesion molecule (EPCAM). There is an ongoing debate on the importance of each of these adhesion molecules with respect to the metastatic site. Data suggest that blood group antigens only play a role in hematogenous spread, whereas L1CAM and proteoglycans only contribute to peritoneal dissemination [113]. Although the exact differences remain unclear there is agreement on the importance of adhesion molecules in metastatic spread especially since free-floating tumor cells in the peritoneal cavity alone do not necessarily lead to peritoneal carcinomatosis [113]. The invasion into the peritoneum requires proteolytic enzymes such as matrix metalloproteases (e.g., MMP2/7/9) secreted by tumor cells or surrounding stromal cells. After invasion, tumor cells not only have to survive in the new environment but also sustain proliferation with IGF-1 and angiogenesis-promoting factors such as HIF1α and VEGF playing pivotal roles. IGF-1 mRNA has been shown to be overexpressed in peritoneal spread in comparison to liver metastases [114].

In search of novel biomarkers, DDR2, a type 1 collagen receptor tyrosine kinase associated with the Src pathway involved in a multitude of processes such as carcinogenesis and adhesion, was identified to be a driver gene of peritoneal carcinomatosis in gastric cancer. In CRC, high DDR2 expression was associated with higher frequencies of T4, lymph node metastasis, peritoneal spread, and poorer prognosis compared to low DDR2 expression, suggesting

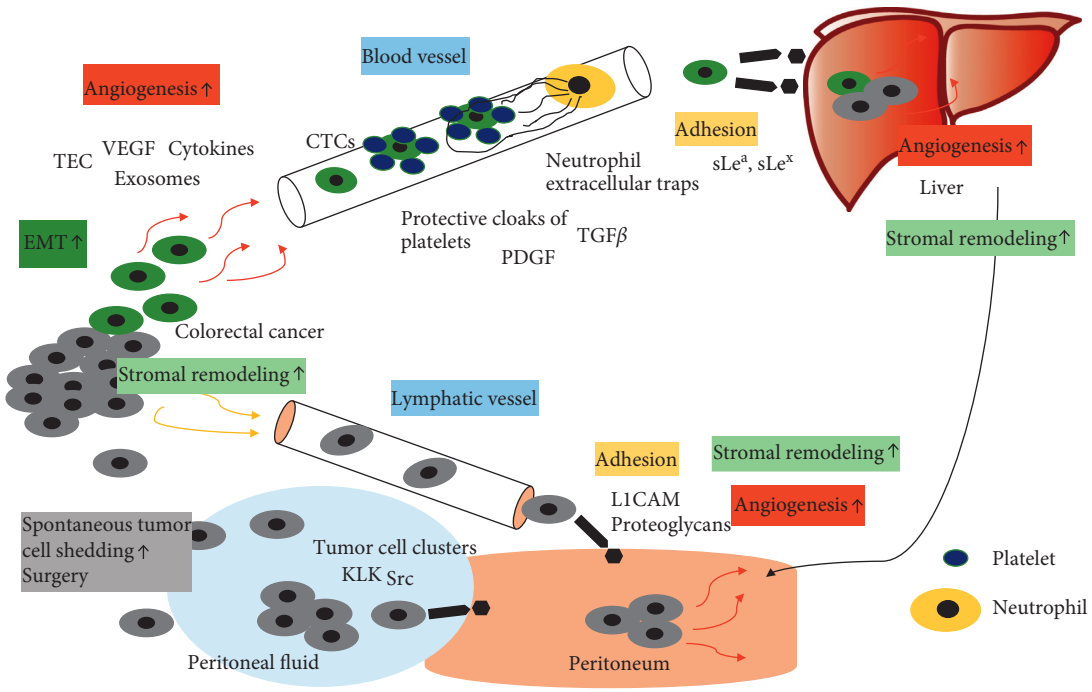

Figure 4: Mechanisms of metastasis in CRC—hematogenous versus peritoneal spread. Hematogenous spread: Tumor cells undergo EMT and leave the primary tumor. Through angiogenesis tumor cells find entrance into the circulatory system. During transport CTCs are protected by platelets and neutrophils from immune attacks and shear stress. Using distinct adhesion molecules CTCs then attach to the liver stroma and form colonies. Peritoneal spread: Tumor cells leave the primary tumor through spontaneous tumor cells shedding, surgical interventions, or exfoliation into the lymphatic system. In the peritoneal fluid, tumor cells form clusters to evade anoikis. Using certain adhesion molecules tumor cells attach to the peritoneum and use angiogenesis to further spread in the peritoneum. Factors that have been identified to play a role in specific parts of this multistep process in CRC are highlighted grey.

that DDR2 expression might be an effective therapeutic target [117].

7. Conclusion

This review aimed to give an overview of the underlying principles of metastatic spread in CRC with respect to liver metastases versus peritoneal metastases (Figure 4). Current data suggest that EMT plays a major role in the beginning stages of tumor spread by enabling mobility and invasiveness. However, it is still unclear whether EMT equally contributes or differs in relevance to local tumor progression and peritoneal spread versus formation of distant metastasis, e.g., hematogenous spread.

Peritoneal carcinomatosis might be an advanced stage of local tumor progression and the result of spontaneous tumor cell shedding into the lymphatic system or exfoliation into the peritoneal fluid, whereas liver metastases are the result of hematogenous dissemination. During systemic transportation to the liver, CTCs are mechanically and chemically protected by platelets and neutrophils that help CTCs avoid shear stress and immune attacks of natural killer cells and support them in adherence to the endothelium and extravasation. During peritoneal spread, tumor cells form clusters to evade anoikis and continue to proliferate. Furthermore, recent studies suggest that adhesion molecules that help cells in the attachment process to their target organ differ significantly between hematogenous and peritoneal metastases. In liver metastases, the blood group antigens sLe^a and sLe^x might play an important role, whereas in peritoneal metastases L1CAM and proteoglycans could be a new focus. Angiogenesis is an important mechanism in CRC to sustain tumor growth. However, it seems that angiogenesis is also an essential step in the early steps of hematogenous metastasis formation by enabling tumor cells to connect to the preexistent vasculature by new vessel formation in the first place. Growing evidence points to the TME playing a crucial role in all stages of tumor development from tumor proliferation to metastasis formation to colonization of the peritoneum or distant organs. Tumor cells, immune cells, soluble factors, and ECM components seem to be all part of an ecosystem provided by the TME and described processes such as EMT or angiogenesis seem to be dependent on bidirectional interactions with the TME. Growing evidence suggests that peritoneal carcinomatosis in CRC is the terminal stage of a locally advanced tumor progress, whereas hepatic metastasis might be a hematogenously spreading CRC that could locally still be controlled. How CRC cells choose their route to disseminate and the underlying mechanisms of organotropism, especially in terms of CRC, have only very recently been started on uncovering. More experimental and clinical studies could contribute immensely to further understand and clarify the underlying principles of mechanisms of metastasis in colorectal cancer and metastatic organotropism. Better understanding of these mechanisms will help provide specific targets for therapeutic interventions in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Overexpression of DCLK1-AL Increases Tumor Cell Invasion, Drug Resistance, and KRAS Activation and Can Be Targeted to Inhibit Tumorigenesis in Pancreatic Cancer

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Oncogenic KRAS mutation plays a key role in pancreatic ductal adenocarcinoma (PDAC) tumorigenesis with nearly 95% of PDAC harboring mutation-activated KRAS, which has been considered an undruggable target. Doublecortin-like kinase 1 (DCLK1) is often overexpressed in pancreatic cancer, and recent studies indicate that DCLK1+ PDAC cells can initiate pancreatic tumorigenesis. In this study, we investigate whether overexpressing DCLK1 activates RAS and promotes tumorigenesis, metastasis, and drug resistance. Human pancreatic cancer cells (AsPC-1 and MiaPaCa-2) were infected with lentivirus and selected to create stable DCLK1 isoform 2 (alpha-long, AL) overexpressing lines. The invasive potential of these cells relative to vector control was compared using Matrigel coated transwell assay. KRAS activation and interaction were determined by a pull-down assay and coimmunoprecipitation. Gemcitabine, mTOR (Everolimus), PI3K (LY-294002), and BCL-2 (ABT-199) inhibitors were used to evaluate drug resistance downstream of KRAS activation. Immunostaining of a PDAC tissue microarray was performed to detect DCLK1 alpha- and beta-long expression. Analysis of gene expression in human PDAC was performed using the TCGA PAAD dataset. The effects of targeting DCLK1 were studied using xenograft and $Pdx1^{Cre}Kras^{G12D}Trp53^{R172H/+}$ (KPC) mouse models. Overexpression of DCLK1-AL drives a more than 2-fold increase in invasion and drug resistance and increased the activation of KRAS. Evidence from TCGA PAAD demonstrated that human PDACs expressing high levels of DCLK1 correlate with activated PI3K/AKT/MTOR-pathway signaling suggesting greater KRAS activity. High DCLK1 expression in normal adjacent tissue of PDAC correlated with poor survival and anti-DCLK1 mAb inhibited pancreatic tumor growth *in vivo* in mouse models.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) has the worst prognosis of any major malignancy with less than an 8% 5-year survival rate and is the third leading cause of cancer-related deaths in the United States [1]. There are four major driver genes for pancreatic cancer: KRAS, CDKN2A, TP53, and SMAD4 [2–4]. KRAS mutations are harbored by 95%

of PDACs and play a key role in PDAC tumorigenesis [5–7]. Active KRAS directs several downstream signaling pathways that play pivotal roles in proliferation, migration, invasion, and survival, which are the most important cellular mechanisms regulating PDAC tumorigenesis and metastasis.

Cells with cancer stem cell-like (CSC) properties have been identified in PDAC. These cells are often resistant to conventional chemotherapy and radiation therapy and as such may explain why current treatments do not cure PDAC or prevent recurrences. Doublecortin-like kinase 1 (DCLK1) is often overexpressed in pancreatic cancer and is coexpressed with other PDAC CSC markers, and recent studies indicate that DCLK1+ PDAC cells can initiate pancreatic tumorigenesis in the presence of mutation and inflammation [8, 9]. Functionally, we have also demonstrated that DCLK1 regulates key oncogenes, pluripotency factors, angiogenic factors, epithelial mesenchymal transition (EMT) related transcription factors, and pancreatic cancer xenograft growth which can be reversed by downregulating DCLK1 or inhibiting its kinase activity [10–13].

Many studies have reported targeting KRAS for PDAC treatment but it remains an undruggable target [4]. DCLK1 is strongly linked to KRAS-mutant cancer, as evidenced by its expression in tumor stem-like cells in multiple KRAS-mutant pancreatic cancer mouse models [14]. Moreover, Westphalen et al. demonstrated that Kras-mutant DCLK1+ tuft cells initiate cancer in the presence of inflammation in support of a CSC role in PDAC and also that DCLK1 forms a complex with KRAS [9]. On the molecular level, recent work using KRAS wild-type colorectal cancer cell line SW48 demonstrated that DCLK1 is transcriptionally induced by knock-in of KRAS G12D, G12V, or G13D, resulting in massive upregulation [15]. When KRAS is targeted with shRNA in these mutant SW48 cells, DCLK1 expression decreases in a dose-dependent fashion [15]. Similarly, when DCLK1 is targeted with siRNA, the expression of KRAS is decreased in a dose-dependent fashion [10, 11, 13]. Here, we report the role of DCLK1 in KRAS-PI3K-MTOR signaling pathway and its implications for chemoresistance and tumor growth. Importantly, our findings demonstrate for the first time that DCLK1 directly activates RAS and that DCLK1-targeted monoclonal antibodies can be used to inhibit PDAC tumor growth in xenografts and the KPC mouse model.

2. Materials and Methods

2.1. Ethics Statement. All animal experiments were performed with the approval and authorization from the Institutional Review Board and the Institutional Animal Care and Use Committee, University of Oklahoma Health Sciences Center and University of Pennsylvania Perelman School of Medicine. Mice were housed under controlled conditions, including a 12-h light-dark cycle, with ad libitum access to food and water.

2.2. Experimental Animals. Athymic nude mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). $Kras^{LSL\text{-}G12D/+}$;$Trp53^{LSL\text{-}R172H/+}$;Pdx1-Cre (KPC) mice have been previously described [16] and were bred and maintained under two pathogen-free facilities at the University of Pennsylvania.

2.3. Analysis of DCLK1 Expression in Various Cancers. For DCLK1 mRNA expression levels in various cancer types, the Cancer Genome Atlas (TCGA) esophageal (ESCA), stomach (STAD), liver (LIHC), pancreas (PAAD), and colorectal (COADREAD) datasets were used. For DCLK1 protein expression levels in various cancer types and in normal tissues, the Human Protein Atlas (THPA) datasets were used [17–19].

2.4. Analysis of TCGA PAAD Data. The standard data run of The Cancer Genome Atlas PAAD dataset was downloaded and sorted for DCLK1 expression. Mann-Whitney U test was used for analysis and comparison of other gene expressions between these two groups (n=45 for each group).

2.5. Clinical Patient Characteristics. Only publicly available, deidentified data were accessed from TCGA for the analysis reported here. Basic characteristics of the PDAC patients used in the survival analysis are provided in Supplementary Tables S1 and S2.

2.6. Immunohistochemical Study of PDAC Tumor Tissue and Normal Adjacent Tissue. A pancreatic adenocarcinoma tissue microarray (US Biomax, HPan-Ade 180 Sur-02) containing 180 microsections including 60 paired tumor and normal adjacent tissues was immunostained with anti-DCLK1 antibody (Abcam, ab31704) following our previously described protocol [20]. Each stained tissue microsection was scored independently by two pathologists and based on percent of tissue demonstrating staining (1 for <10%-4 for > 60%) and staining intensity (1 for lowest intensity, 4 for highest intensity). The resulting scores were multiplied by each other to obtain a composite score.

2.7. Cell Culture and Establishing Stable Cell Lines. Human pancreatic cancer cell lines, AsPC-1 and MIA PaCa-2 (MP2), were obtained from ATCC and grown in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and L-glutamine, without sodium pyruvate (Cellgro) supplemented with 10% fetal bovine serum (Sigma) at 37°C and 5% CO_2. Lentivirus containing human DCLK1-AL cDNA sequence was constructed as described previously [21]. AsPC-1 and MP2 cells were infected with lentivirus to overexpress DCLK1AL-RFP fusion protein (AsPC-DCLK1 and MP2-DCLK1) or red fluorescent protein (AsPC-RFP and MP2-RFP) as control, and selected with puromycin to establish stable cell lines.

2.8. Drug Resistance Assays. Cells (5000 cells per well) were seeded into a 96-well tissue culture plate in triplicate. The cells were cultured in the presence of Gemcitabine (0, 12.5, 25, 50, 100, and 200 nM), everolimus (37.5 μM), ABT-199, or LY-294002 (each at 0, 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50, and 100 μM) with DMSO as a vehicle control. 48 h after treatment, 10 μl of TACS MTT Reagent (RND Systems) was added to each well and cells were incubated at 37°C for 4 h. Once dark crystalline precipitate became visible, 50 μl

of 266 mM NH_4OH in DMSO [22] was added to the wells and placed on a plate shaker at low speed for 1 minute. The plate was measured at OD_{550} using a microplate reader. The OD value of each triplicate was averaged and the results were calculated as a percentage of the DMSO (vehicle) control +/- the standard error of the mean.

2.9. Matrigel Transwell Invasion Assay. Matrigel coated transwell assays (BD Biosciences) were prepared by soaking in serum-free media for 2 h at 37°C in a 24-well plate. MP2-RFP and MP2-DCLK1 cells (5000 cells/well) were seeded into each transwell in serum-free media in triplicate. Cell culture medium containing 10% FBS was added to the bottom of each well as chemoattractant and the cells were incubated for 22 h at 37°C. A cotton swab was used to scrape noninvasive/migratory cells off the top of transwell assays and the remaining cells were fixed with 100% methanol, stained with 0.1% crystal violet, and allowed to dry. After drying all invading cells were counted from each transwell at 4x magnificence.

2.10. In Vitro Spheroid Assay. MP2-RFP and MP2-DCLK1 cells (250 cells/well, n=6 per group) were seeded into an ultra-low attachment 96-well plate in RPMI containing 0.5% FBS and incubated at 37°C under 5% CO_2 for 5 days. Medium without FBS was added on day 3 to prevent evaporation. On day 5, spheroids were manually counted under a light microscope at 10x magnification, and representative images were taken. Spheroids were defined as having at least 10 cells. Efficiency of spheroid formation was calculated by dividing the number of spheroids formed by the number of cells seeded.

2.11. Active RAS Pull-Down Assay. Both AsPC-RFP and AsPC-DCLK1 cells were cultured in serum-free medium overnight, followed by full growth medium (10% FBS) for 15 min in the presence of either DMSO or XMD8-92 (15 μM). Cells were lysed and active RAS were analyzed using the Active Ras Pull-Down and Detection Kit (Thermo Scientific) based on the instruction.

2.12. Coimmunoprecipitation Assay. Both AsPC-RFP and AsPC-DCLK1 cells were lysed with Pierce IP Lysis Buffer (Thermo Fisher Scientific). The cell lysates were used for immunoprecipitation by incubating with anti-RAS antibody for 2h at room temperature, spinning down the precipitates with Protein A conjugated anti-mouse secondary antibody, washing 3 times with Pierce IP washing buffer, and eluting with gel loading buffer. The eluates were separated on a SDS-PAGE and subjected to western blot analysis with anti-DCLK1 antibody (Abcam, ab31704).

2.13. Western Blot Analysis. Total proteins of cell lysates were subjected to Western Blot analysis. The concentration of total proteins was determined by BCA protein assay. Equivalent amounts of total proteins were separated on a SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and probed with the primary antibody. The membrane was then incubated with IRDye 800CW-conjugated secondary antibody. The proteins were detected using Li-Cor Odyssey system.

2.14. Generation of a Human/Mouse Chimeric Antibody. DCLK1-targeted therapeutic monoclonal antibody (CBT-15mAb) and isotype control mAb were supplied in PBS (COARE Biotechnology). In addition, total RNA was isolated from monoclonal hybridoma cells secreting DCLK1 antibody (CBT-15); cDNA was synthesized using a primer downstream of the last variable region for heavy chain (HC) constant and light chain (LC) kappa constant. Each RT-reaction was subject to PCR using degenerate primer sets (USBIO, 11904-10A) to amplify all likely rearrangements. To create the human/mouse IgG chimeric antibody, PCR fragments from the above reaction were inserted into pFUSEss-CHIg-hG1 to express heavy chain and pFUSEss-CLIg-hK to express light chain kappa. Heavy chain was further cloned into pLenti CMV PURO DEST and light chain kappa was further cloned into pLenti CMV BLAST DEST. The expression plasmids constructed above were cotransfected along with packaging plasmids pMD2.G (Addgene), pMDL/RRE g/p (Addgene), and pRSV-Rev (Addgene) into 293T cells. Generation of the concentrated lentivirus was done as described previously [13]. Human 293T cells were infected with both concentrated viruses containing heavy chain and light chain and selected with puromycin and blasticidin (Sigma-Aldrich) to establish stable cell lines. The established cell lines expressing both heavy chain and light chain were expanded into a Bioreactor for production. The conditioned media were collected and purified using a Nab Protein L Spin column (Thermo Fisher Scientific) to produce CBT-15X mAB.

2.15. Xenograft Tumor Study. SW1990 or AsPC-1 pancreatic cancer cells ($0.5x10^6$) in Matrigel were injected into the flanks of 8-week old athymic nude mice (n=6 for CBT-15 vs. isotype control groups and n=7 for CBT-15X vs. isotype control groups for both SW1990 and AsPC-1 cells) and allowed to grow to an average tumor volume of 100 mm^3. Mice with xenografted tumors were injected intraperitoneally (*i.p.*) with CBT-15 mAb, CBT-15X mAb, or isotype control at 25 mg/kg twice per week. Tumor volume measurements were taken every other day using calipers. 30 days from the start of injections mice were killed and tumors excised, measured, and weighed.

2.16. KPC Mice Tumor Study. KPC mice with tumors measuring 50-100 mm^3 were identified using ultrasonography. These mice were injected *i.p.* with CBT-15 mAb or IgG2a isotype control (n=4 for each group) at 25 mg/kg twice per week for four weeks. Tumors were measured by ultrasonography at baseline and once a week after intervention. Mice were killed after four-week treatment.

2.17. Statistical Analysis. All statistical analyses and figures were prepared using R v3.2, GraphPad Prism 6.0, SPSS Statistics 22, and Microsoft Excel. For nonparametric data the Mann-Whitney U test was used, and for parametric data Student's t-Test was used. Kaplan-Meier survival analyses were performed in GraphPad Prism 6.0. Cox regression analyses

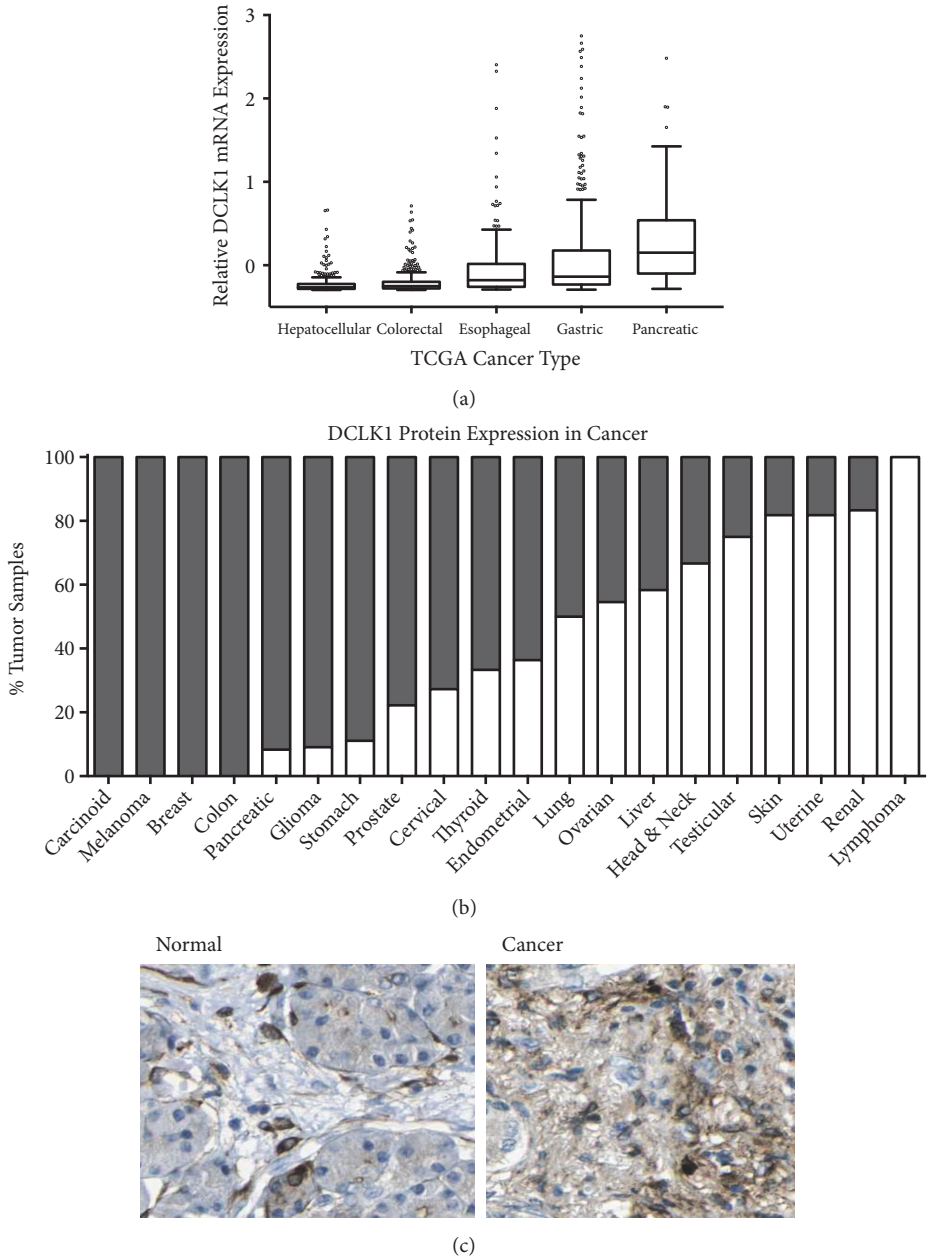
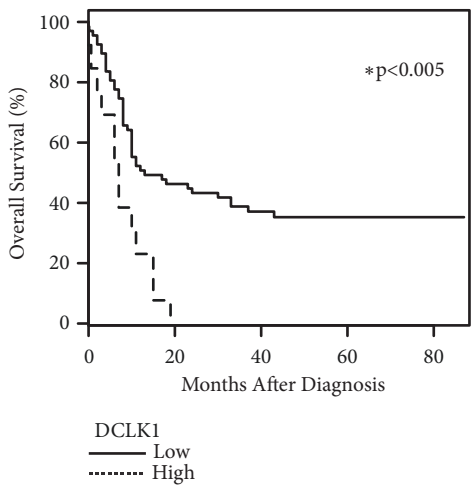


Figure 1: *DCLK1 expression is upregulated in pancreatic cancer and other cancer types*. (a) Relative DCLK1 mRNA expression levels were analyzed using the TCGA esophageal (ESCA), stomach (STAD), liver (LIHC), pancreas (PAAD), and colorectal (COADREAD) datasets. (b) Percentage of DCLK1 protein expression in various tumor tissues was analyzed using the Human Protein Atlas. (c) DCLK1 expression in the normal pancreas and cancer tissues was detected using anti-DCLK1 Ab immunostaining.

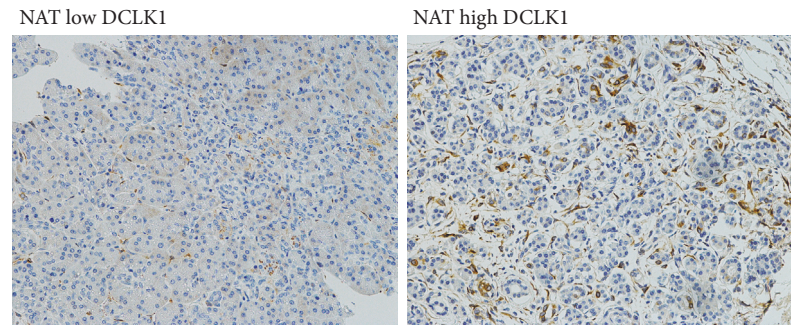
were performed using IBM SPSS Statistics 22. Heatmaps were generated using Genesis. A p-value of less than 0.05 was considered statistically significant for all analyses.

3. Results

3.1. DCLK1 Is Upregulated in Pancreatic and Other Cancer Types. In order to assess DCLK1's gene expression pattern across gastrointestinal cancer types, we analyzed the TCGA esophageal (ESCA), stomach (STAD), liver (LIHC), pancreas (PAAD), and colorectal (COADREAD) datasets and found that pancreatic cancer tissue has the highest DCLK1 mRNA expression levels among the gastrointestinal cancer types (Figure 1(a)). In addition, we analyzed immunohistochemistry staining from the Human Protein Atlas generated using anti-DCLK1 antibody (Abcam 31704) that has been characterized by us and other groups extensively in the past [14, 23–25]. According to the Human Protein Atlas data, 100% of carcinoid, melanoma, colon, and breast and approximately 90% of glioma, pancreatic, and stomach cancer tissue expressed DCLK1. Notable expression (>50%) was also present in prostate, cervical, thyroid, endometrial, and



(a)



(b)

			Fisher's Test	Univariate Cox PH		Multivariate Cox PH	
	N (High)	N (Low)	P	HR (95% CI)	P	HR (95% C.I.)	P
Age (Years)			0.065	1.014(0.991|1.039)	0.239	1.024(0.997|1.052)	0.085
≥65	8	24					
<65	4	41					
Gender (M v. F)			0.999	1.274(0.726|2.234)	0.394	1.047(0.574|1.910)	0.880
Male	7	39					
Female	5	26					
Disease Stage (II v. I)			0.352	**1.979(1.136|3.445)**	**0.014**	**2.354(1.273|4.351)**	**0.006**
Stage II	8	32					
Stage I	4	33					
Disease Grade			0.999	1.743(0.999|3.039)	0.060	**2.127(1.166|3.881)**	**0.014**
>2	1	6					
2	11	59					
DCLK1 (High v. Low)	12	65	N/A	**3.209(1.651|6.240)**	**0.002**	**2.468(1.204|5.058)**	**0.014**

(c)

Figure 2: *High DCLK1 expression in normal adjacent tissue of PDAC correlates with poor survival.* (a) The intensity of DCLK1 expression in PDAC normal adjacent tissue (NAT) was scored based on staining using anti-DCLK1 antibody on a commercially available tissue microarray. Kaplan-Meier analysis of the DCLK1 staining scores demonstrated that patients with high levels of DCLK1 in the NAT had significantly shorter survival time compared to patients with low levels of DCLK1 in the NAT. (b) Representative images of low and high DCLK1 staining in NAT. (c) Multivariate Cox regression analysis of patients included in the TMA.

lung cancer tissue (Figure 1(b)). DCLK1 expression in the normal pancreas is isolated to glandular exocrine cells, while it is overexpressed in both tumor epithelial and stromal cells in the cancer tissue (Figure 1(c)).

3.2. DCLK1 Expression in PDAC Normal Adjacent Tissue Predicts Poor Overall Survival. To further evaluate DCLK1 protein expression in PDAC tumors, we performed immunohistochemistry using anti-DCLK1 antibody on a commercially available tissue microarray with tumor and normal adjacent tissues (NAT) from stages I/II pancreatic cancer patients. We found higher expression of DCLK1 in most of the tumor samples and assessed the effect of DCLK1 expression on patient survival. The expression levels of DCLK1 in the tumor tissues did not predict survival (data not shown). However, patients with high levels of DCLK1 in the NAT had significantly reduced overall survival compared to patients with low levels (median 6-7 months and 12-13 months, resp.) (Figures 2(a) and 2(b)). Controlling for all other factors including age, gender, disease grade, and disease stage using multivariate Cox analysis confirmed this finding (Figure 2(c), p=0.014), suggesting that NAT DCLK1 may be an independent prognostic factor. These findings expand on previous findings demonstrating that tumor DCLK1 predicts survival in PDAC [26] and suggest a potential protumorigenic role for normal DCLK1+ cells adjacent to the tumor.

3.3. PDAC Patients Expressing DCLK1 Demonstrate PI3K/AKT/MTOR Pathway Activation. In order to determine whether DCLK1 expression level correlates with KRAS related pathways in human PDAC patients, we analyzed RNA-Seq expression data from TCGA (PAAD). We grouped patients into DCLK1-low (bottom 25th percentile) and DCLK1-high (top 25th percentile) groups and compared expression of genes downstream of RAS activation. We found that DCLK1-AL and BL are associated with increased EMT based on genetic signature analysis. In addition, DCLK1-high patients have increased expression of PI3K/AKT/MTOR

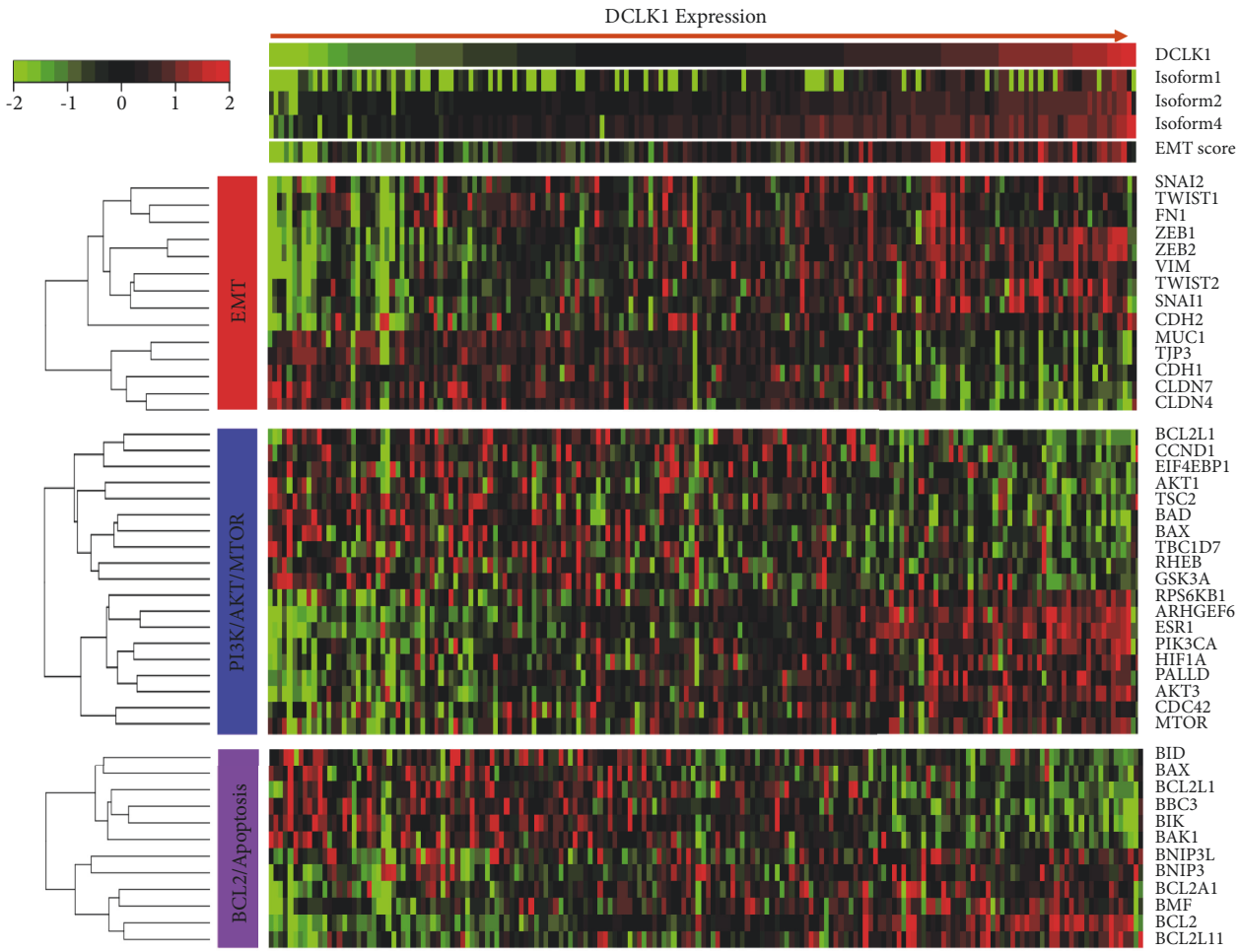


Figure 3: *PDAC patients expressing DCLK1 demonstrate PI3K/AKT/MTOR pathway activation*. RNA-Seq expression data from TCGA PAAD were analyzed. Patients were grouped based on DCLK1 expression and compared expression of genes downstream of RAS activation, grouped into EMT, PI3K/AKT/MTOR, and BCL2/Apoptosis.

and downstream signaling pathways which support stemness, antiapoptosis, and tumorigenesis (Figure 3). Taken together, these findings support a role for DCLK1 in regulating KRAS-mediated pathway activation and confirm recent findings of DCLK1-associated PI3K/MTOR activity [26].

3.4. Overexpression of DCLK1-AL Increases PDAC Invasion, Drug Resistance, and KRAS Activation. To assess the effects of DCLK1-AL on PDAC, we established stable cell lines overexpressing DCLK1-AL-RFP fusion protein in AsPC-1 and MP2 cells using RFP as control (Figure 4(a)). The DCLK1-AL-RFP fusion protein was detected with anti-DCLK1 antibody, while endogenous DCLK1 protein level was barely detectable by western blot in these two lines (Figure 4(a)). To assess the effect of overexpressing DCLK1-AL on pancreatic cancer cell invasion, Matrigel coated invasion assays were performed. Overexpressing DCLK1-AL in MP2 cells increased cell invasion more than 2-fold (Figure 4(b), $p<0.005$) and increased Vimentin expression was also detected in MP2-DCLK1 cells (Figure 4(a)).

Drug resistance is a mechanism by which quiescent tumor stem cells maintain viability while the bulk of the tumor is destroyed by chemotherapies targeting rapidly dividing tumor cells. To assess whether overexpression of DCLK1-AL increases drug resistance, we treated MP2-RFP and MP2-DCLK1 cells with various concentrations of gemcitabine for 48 h and performed an MTT assay. MP2-DCLK1 cells significantly resisted gemcitabine treatment compared to MP2-RFP cells at most doses ($p<0.05$) (Figure 4(c)).

Using a coimmunoprecipitation assay, we found that DCLK1-AL forms a complex with RAS (Figure 4(d)) in DCLK1-AL overexpressing cells consistent with the findings reported by Westphalen et al. [9]. In order to assess whether DCLK1 regulates the activation of RAS, we performed an active RAS pull-down assay to detect the GTP-bound active form of RAS in AsPC-DCLK1 or AsPC-RFP cells following serum starvation and stimulation with FBS-containing media. DCLK1-AL overexpression resulted in an approximately 3-fold increase in active RAS (Figure 4(e)). In order to determine if this activation was regulated by DCLK1 kinase activity, we treated cells with DCLK1 kinase inhibitor

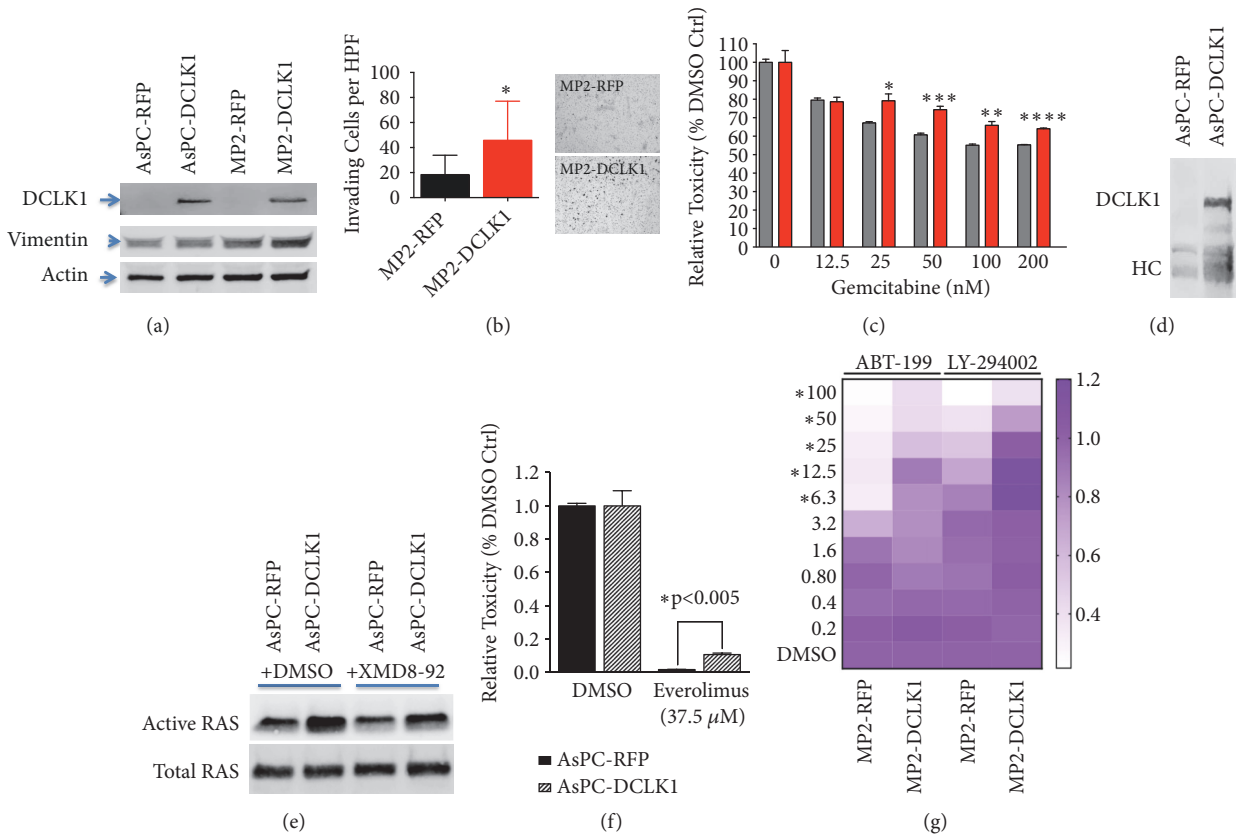


FIGURE 4: *Overexpression of DCLK1-AL in pancreatic cancer cells increases cell invasion, drug resistance, and KRAS activation*. (a) Both AsPC-1 and MP2 cells were infected with lentivirus containing either DCLK1-AL-RFP or RFP cDNA sequence to establish stable cell lines overexpressing DCLK1. (b) Matrigel coated transwell assays were used to study cell invasion activity. (c) Overexpression of DCLK1-AL increases pancreatic cancer cell resistance to Gemcitabine. (d) DCLK1-AL forms a complex with RAS. (e) Overexpression of DCLK1AL increases active RAS in pancreatic cancer cells. (f-g) Overexpression of DCLK1-AL increases pancreatic cancer cell resistance to Everolimus, ABT-199, and LY-294002.

XMD8-92 [20]. Treating AsPC-DCLK1 cells with XMD8-92 (15 μM) for 15 min significantly inhibited the activation of RAS under these conditions. However, XMD8-92 treatment was unable to inhibit RAS-activation in AsPC-RFP cells expressing endogenous levels of DCLK1 (Figure 4(e)). These findings suggest that the use of DCLK1 kinase inhibitors may be beneficial in patients expressing high levels of DCLK1 by impairing RAS activation.

Since high DCLK1 expression in pancreatic cancer patients is correlated with activation of pathways downstream of RAS (PI3K/MTOR) (Figure 3), we also assessed the effect of overexpressing DCLK1-AL on Everolimus (MTOR inhibitor), LY-294002 (PI3K inhibitor), and ABT-199 (BCL-2 inhibitor) treated pancreatic cancer cells. AsPC-DCLK1 cells significantly resisted Everolimus (37.5 μM) compared to control cells ($p< 0.005$) (Figure 4(f)), and MP2-DCLK1 cells significantly resisted both ABT-199 and LY-294002 compared to control cells at most doses ($p<0.05$) (Figure 4(g)). These findings suggest that DCLK1-AL overexpression is an important factor in PDAC drug resistance.

3.5. Anti-DCLK1 Monoclonal Antibodies Inhibit PDAC Tumorigenesis In Vivo. We recently reported that monoclonal antibody CBT-15 targeting DCLK1-AL/BL inhibits renal cancer tumorigenesis *in vivo*. In order to evaluate the effect of targeting DCLK1 in pancreatic cancer tumorigenesis, we utilized a novel mAb (CBT-15G), which differs from CBT-15 which we recently reported in renal cell cancer [21]. To determine its effects on pancreatic cancer tumorigenesis, we established SW1990 pancreatic cancer cells xenografts in athymic nude mice. Upon reaching 100 mm^3 average tumor volume, CBT-15G was delivered *i.p.* biweekly at 25 mg/Kg for 4 weeks and changes in tumor volume were assessed every other day. CBT-15G therapy dramatically reduced SW1990 *in vivo* tumorigenesis over time, which was confirmed by assessing excised tumor volume and weight (Figures 5(a)–5(c)). Following confirmation of CBT-15G's *in vivo* efficacy, the variable region of the mAb was sequenced and a stable 293T cell line secreting the mouse-human chimera version of the mAb (CBT-15X) was generated. Following establishment of the line and purification of secreted CBT-15X, another set of xenografts were prepared as described for the mouse antibody for both SW1990 and AsPC-1 PDAC cell lines. Biweekly *i.p.* CBT-15X therapy also led to a marked, thorough decreased inhibition of *in vivo* tumorigenesis in these xenografts (Figures 5(d)-5(e) and Figure S1).

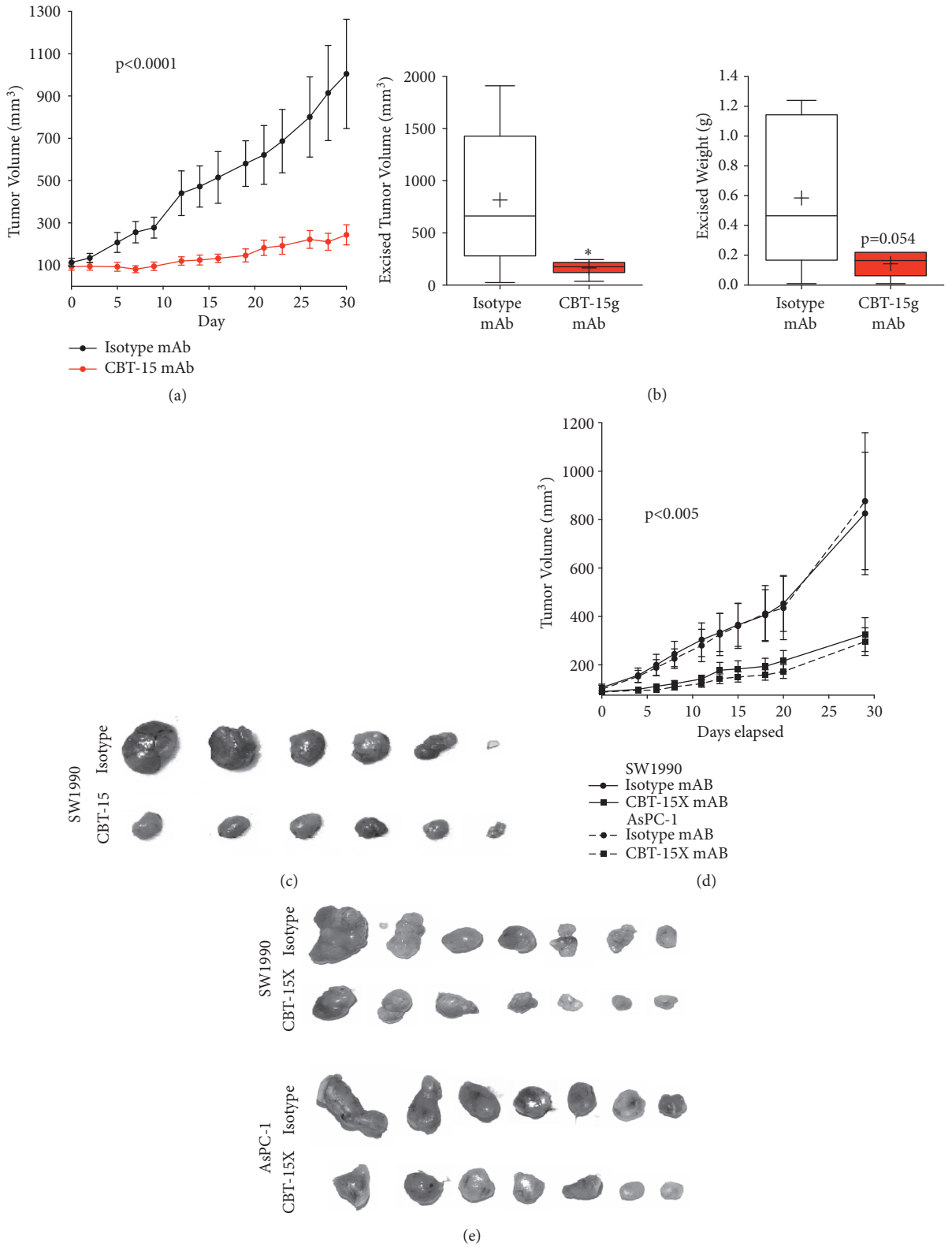


Figure 5: *Anti-DCLK1 mAbs inhibit pancreatic cancer xenograft tumor growth*. (a) Biweekly injection of CBT-15 mAb (*i.p.*) significantly impairs SW1990 pancreatic cells originated tumor xenograft growth ($p<0.0001$) as confirmed by (b) decreased excised tumor volume and (c) decreased excised tumor mass. (d) Biweekly injection of CBT-15X mAb (*i.p.*) significantly impairs SW1990 (solid line with solid squares) and AsPC-1 (dashed line with solid squares) pancreatic cells originated tumor xenograft growth ($p<0.005$) as confirmed by (e) decreased excised tumor mass.

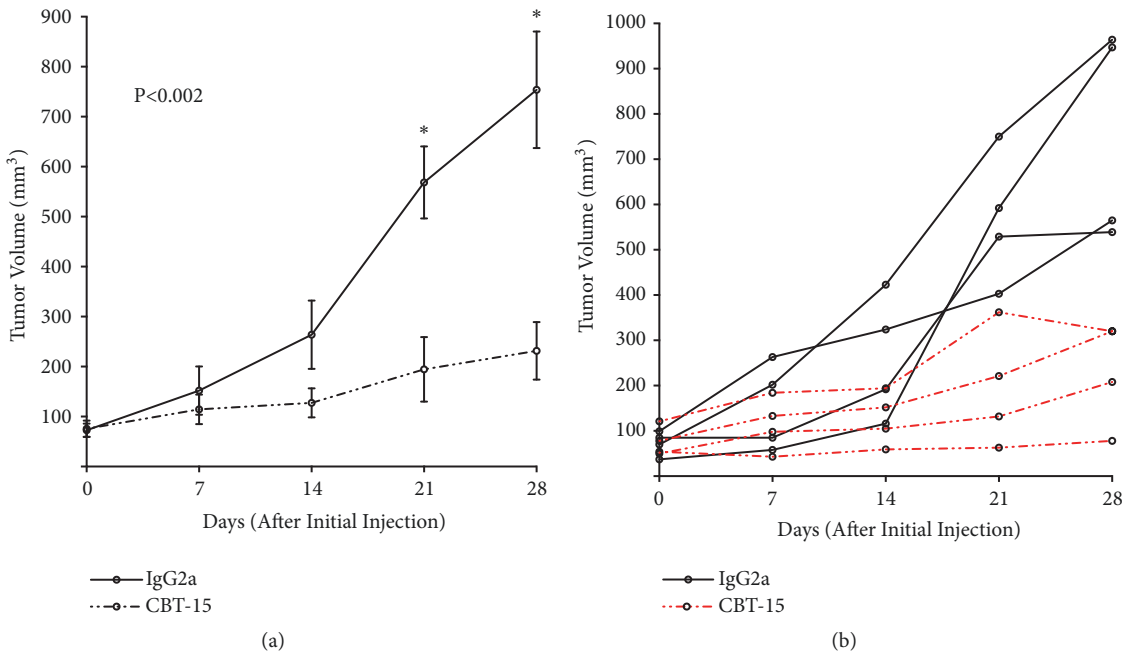


Figure 6: *Anti-DCLK1 mAb inhibits pancreatic tumor growth in KPC mice*. Biweekly injection of CBT-15 mAb (*i.p.*) significantly impairs pancreatic tumor growth ($p<0.002$) in KPC mice (n=4 in each group). (a) Average tumor volumes. (b) Individual tumor volumes.

Although athymic nude mice maintain a partially functional immune system, mAb therapies are best assessed in models with full immune function. Given our recent promising findings in renal cell cancer [21], we converted CBT-15 (an IgA) to both a mouse and fully humanized IgG. To test the activity of this antibody, we delivered it at 25 mg/Kg *i.p.* to KPC mice on a biweekly basis for 4 weeks (16-20 weeks of age). Tumor growth was tracked using ultrasonography. To assess tumor growth accurately, we selected mice with initial tumor sizes <100 mm^3 and only assessed those (n=4 in each group) that survived the duration of the study. The antitumor activity of CBT-15 was clear based on both average and individual differences in tumor growth (Figure 6). In totality these findings provide the first proof of concept for DCLK1-targeted mAb therapy against pancreatic cancer.

4. Discussion

Despite advances in the understanding of pancreatic cancer biology and in surgical and medical therapy in recent years, little impact has been made on the mortality associated with this cancer. Therefore, there is an unmet need to find new therapeutic approaches against PDAC. Zhang et al. reported recently that DCLK1 levels in PDAC tumor tissues predict poor survival [26]; we also found that DCLK1 levels in PDAC NAT can predict poor survival; taken together, these studies suggest that DCLK1 levels could be used as a prognostic biomarker for PDAC.

There are two DCLK1 isoforms transcribed from the α-promoter, isoform 1 (α-short) and isoform 2 (α-long) [13]. It has been reported that overexpressing DCLK1 α-short in pancreatic cancer cells increased cell proliferation, migration, and invasion [27, 28]. In this study, we demonstrated that overexpressing DCLK1 α-long in pancreatic cancer cells also increases these functional properties and drug resistance. In our previous studies of DCLK1 α-long functionality in clear cell renal cancer, we found that its expression strongly supports stemness as determined by 3D spheroid assays, drug resistance assays, and expression of well-described stem cell markers [21]. Similar studies in pancreatic cancer demonstrate comparable results [9, 14, 28]. To assess the potential contribution of stemness to our results in this study, we performed a spheroid assay and found a three-fold increase in spheroid formation efficiency using MP2-DCLK1 cells compared to MP2-RFP cells, suggesting that overexpression of DCLK1-AL increases stemness (Figure S2).

KRAS activating mutations are present in 95% of PDAC tumors, but targeting KRAS directly has been unsuccessful so far and many inhibitors have failed in clinical trials [4]. Here we have confirmed previous studies demonstrating DCLK1 upregulation in PDAC. Importantly, we demonstrate for the first time that its upregulation directly increases the activation of KRAS, suggesting that it is a potential upstream activator. In addition, DCLK1 levels correlate with RAS downstream signaling effectors, PI3K and mTOR in RNA-Seq expression data. These findings offer a potential explanation for previous work showing DCLK1's ability to drive tumor proliferation, migration, and invasion. Functionally, the present study shows that cells overexpressing DCLK1 are resistant to standard doses of the FDA-approved inhibitors against PI3K and mTOR. In fact, approximately 50% more mTOR inhibitor Everolimus and 30% more PI3K inhibitor LY-294002 were required to inhibit cell proliferation. These findings suggest the potential benefits of targeting DCLK1 in these patients as a primary therapy or as a cotreatment with PI3K, MTOR, or EGFR-targeted drugs which have so far demonstrated

insignificant efficacy in trials likely due to the high prevalence of KRAS mutations.

In order to evaluate the effect of targeting DCLK1 *in vivo*, we utilized a novel mAb (CBT-15G) as well as a production-ready version of the mAb that we recently reported against DCLK1 in renal cell cancer [21]. Targeting DCLK1 with these mAbs in xenograft mouse models from $KRAS^{G12D}$ mutant human cell lines AsPC-1 or SW1990 or in the KPC mouse model led to significant inhibition of the tumor growth (Figures 5 and 6). These data demonstrate that DCLK1-targeted mAbs or other targeted therapies may be effective against PDAC.

In summary, the studies reported here illustrate the role of DCLK1 in KRAS activation, PDAC tumor cell invasion, drug resistance, pancreatic tumor growth *in vivo*, and overall patient survival. Analysis of DCLK1 expression across tissue types demonstrates a favorable pattern for targeted cancer therapy. Moreover, it is notable that although DCLK1 is expressed in normal glandular/tuft cells, which play an important role in response to inflammatory injury [29–31], the available data demonstrates that knockdown or knockout of DCLK1 or deletion of DCLK1+ cells [9, 30, 32] does not result in undue toxicity or significantly impacts homeostatic conditions. In combination these findings suggest that targeting DCLK1 may have significant therapeutic potential and a low side-effect profile as a primary therapy or in conjunction with existing drugs.

5. Conclusions

DCLK1 promotes KRAS-driven PI3K/AKT/mTOR signaling in PDAC leading to increased invasive, antiapoptosis, stemness, and tumorigenic properties. DCLK1-targeted therapies may overcome this signaling and improve PDAC outcomes.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Dongfeng Qu and Nathaniel Weygant are equally contributing authors.

Conflicts of Interest

Courtney W. Houchen is a cofounder of COARE Biotechnology Inc. Dongfeng Qu, Nathaniel Weygant, and Randal May have ownership interests in COARE Biotechnology Inc. Other authors have declared that no conflicts of interest exist.

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Supplementary Materials

Figure S1: CBT-15X chimeric mAb inhibits pancreatic cancer xenograft tumor growth. A. Excised tumor volume and tumor mass from SW1990 pancreatic cancer cells originated xenograft. B. Excised tumor volume and tumor mass from AsPC-1 pancreatic cancer cells originated xenograft. Figure S2: overexpression of DCLK1-AL in MP2 cells enhances tumor spheroid formation. A-B. Spheroids formation is significantly enhanced in MP2-DCLK1 cells ($P<0.0001$). C. Representative images display differences between MP2-RFP and MP2-DCLK1 spheroid formation. Table S1: Patient Characteristics. Publicly available, deidentified data were accessed from TCGA, and basic characteristics of the PDAC patients are presented. Table S2: Univariate and Multivariate Analyses. Publicly available, deidentified data were accessed from TCGA for the analysis reported here. *(Supplementary Materials)*

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Research Article

MicroRNAs as Potential Biomarkers for Chemoresistance in Adenocarcinomas of the Esophagogastric Junction

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Concerning adenocarcinomas of the esophagogastric junction, neoadjuvant chemotherapy is regularly implemented, but patients' response varies greatly, with some cases showing no therapeutic effect, being deemed as chemoresistant. Small, noncoding RNAs (miRNAs) have evolved as key players in biological processes, including malignant diseases, often promoting tumor growth and expansion. In addition, specific miRNAs have been implicated in the development of chemoresistance through evasion of apoptosis, cell cycle alterations, and drug target modification. We performed a retrospective study of 33 patients receiving neoadjuvant chemotherapy by measuring their miRNA expression profiles. Histologic tumor regression was evaluated using resection specimens, while miRNA profiles were prepared using preoperative biopsies without prior therapy. A preselected panel of 96 miRNAs, known to be of importance in various malignancies, was used to test for significant differences between responsive (chemosensitive) and nonresponsive (chemoresistant) cases. The cohort consisted of 12 nonresponsive and 21 responsive cases with the following 4 miRNAs differentially expressed between both the groups: hsa-let-7f-5p, hsa-miRNA-221-3p, hsa-miRNA-31-5p, and hsa-miRNA-191-5p. The former 3 showed upregulation in chemoresistant cases, while the latter showed upregulation in chemosensitive cases. In addition, significant correlation between high expression of hsa-miRNA-194-5p and prolonged survival could be demonstrated (p value <0.0001). In conclusion, we identified a panel of 3 miRNAs predicting chemoresistance and a single miRNA contributing to chemosensitivity. These miRNAs might function as prognostic biomarkers and enable clinicians to better predict the effect of one or more reliably select patients benefitting from (neoadjuvant) chemotherapy.

1. Introduction

Since the discovery of microRNAs (miRNAs), which are small, noncoding RNAs with a length of 19–22 nucleotides [1], multiple studies have focused on the importance of their function and participation in human diseases ranging from inflammatory disorders and autoimmune diseases to malignant tumors including melanoma, various epithelial cancers, and hematological malignancies [2]. miRNAs have increasingly been used not only as diagnostic but also as prognostic biomarkers, and several studies have suggested the existence of tissue-specific miRNA signatures which might be used to classify different cancer types [3–5].

Regarding human cancer in general, miRNAs have been found to act not only as oncogenes, promoting tumor growth and dissemination, but also as tumor suppressors, inhibiting tumor cell proliferation and migration and inducing apoptosis [6]. Sometimes, their function varies in a

single organ, showing divergent behavior in specific histologic tumor types (for example, adenocarcinoma vs. squamous cell carcinoma of the esophagus) [7].

Concerning carcinomas of the upper gastrointestinal tract, especially gastric cancer and adenocarcinoma of the esophagogastric junction, a multitude of miRNAs have been identified as useful biomarkers: for example, upregulation of miRNA-17-5p, miRNA-20a, miRNA-106b, miRNA-150, and miRNA-93 has been reported to inhibit apoptosis and to promote cell cycle progression, whereas downregulation of miRNA-29 and miRNA-375 has been shown to increase cell growth and migration [4, 8]. Other commonly dysregulated miRNAs include miRNA-21 and miRNA-19a/b which both promote lymph node and distant metastases as well as invasion of blood vessels when overexpressed [4]. In addition, a seven-miRNA panel consisting of miRNA-10b, miRNA-21, miRNA-223, miRNA-338, let-7a, miRNA-30a-5p, and miRNA-126 has been found to reliably predict survival in gastric cancer patients and is related not only to overall but also relapse free survival [9]. Besides, various miRNAs have been found to be associated with poor survival in both gastric carcinomas and adenocarcinomas of the esophagogastric junction, including miRNA-16, miRNA-21, miRNA-29, miRNA-125b, miRNA-130a, miRNA-141, miRNA-203a, miRNA-222, miRNA-302c, and miRNA-451 [8, 10–15].

Complementing their diagnostic and prognostic significance, miRNAs have also been found to contribute to chemoresistance and/or chemosensitivity via regulation of apoptosis, DNA damage, and repair mechanisms, and epithelial-mesenchymal transition and modulation of drug targets, drug-metabolizing enzymes, and drug efflux transporters [16, 17]. Especially in gastric cancer, the following miRNAs (amongst others) have been found to substantially contribute to chemoresistance: let-7b, miRNA-106a, miRNA-142, miRNA-143, miRNA-21, miRNA-338, miRNA-340, miRNA-497, miRNA-503, and miRNA-582—their target genes including *PTEN* (phosphatase and tension homolog), BCL_2 (B-cell lymphoma 2), and IGF1R (insulin-like growth factor 1 receptor) [18–25]. For esophageal (adeno-) carcinoma, miRNA-141, miRNA-148a, miRNA-200c, miRNA-221, miRNA-27a, and miRNA-296 are said to contribute to chemoresistance [26]. This miR-mediated chemoresistance is mainly directed against commonly used therapeutic agents such as cisplatin, 5-fluorouracil, and vincristine [27].

Following these observations and taking into account that data concerning chemoresistance in carcinomas of the esophagogastric junction are still scarce and many studies have focused on cell lines rather than tissue specimen, our study aimed to further contribute to the knowledge for this entity by comparing miRNA profiles in chemoresistant and chemosensitive cancer tissue.

2. Materials and Methods

2.1. Selection of Cases. Samples from formalin-fixed, paraffin-embedded (FFPE) tissue containing adenocarcinomas of the esophagogastric junction were included in the present study. All patients received neoadjuvant chemotherapy. Only tumor-containing samples of preoperative biopsies (without prior therapy) were used for miRNA analysis, while whole-resection specimens (postchemotherapeutic) were taken to determine the degree of histological regression and thereby treatment response. All cases were collected as part of routine clinical care at the University Hospital of Schleswig-Holstein, Campus Luebeck, during 1997–2013. All analyses performed were in accordance with the Declaration of Helsinki and had been approved by the local Ethics Committee beforehand (reference number 14-242A).

2.2. Histologic Examination. Samples were carefully examined by two researchers (CJ and JK) with a light microscope (Axioskop, Zeiss, Jena, Germany), and histologic tumor types were determined using the current WHO standard [28]. Regression after chemotherapy was determined using haematoxylin- and eosin-(H&E-) stained slides and rated according to the system devised by Becker et al. [29]. Regression grades 1a and 1b were considered as having responded to therapy (responder group), while regression grade 3 was considered nonresponsive (nonresponder group). Cases with regression grade 2 were not included in the study as an assignment to either group could not reliably be undertaken (partial response).

2.3. RNA Isolation and miRNA Profiling. RNA for profiling of miRNA was isolated from FFPE tissue using the RecoverAll™ total nucleic acid isolation kit (Applied Biosystems, Carlsbad, California, USA). RNA concentrations were quantified using the NanoDrop Spectrophotometer (Nanodrop Technologies, Montchanin, New Castle, Delaware, USA). Afterwards, reverse transcription (RT) using amounts of 20 ng of total RNA by applying the miRCURY LNA™ Universal cDNA Synthesis Kit II (Exiqon, Vedbaek, Denmark), containing synthetic RNA Spike Ins, was performed. 5 μl of the RT products was combined with the PCR master mix and nuclease-free water from the miRCURY LNA™ ExiLENT SYBR® Green master mix (Exiqon, Vedbaek, Denmark). After that, 10 μl of the PCR Master mix-cDNA mix was added to each 384-well plate of the miRCURY LNA™ Universal RT miR Ready-to-Use PCR, Cancer focus panel, V4 (Exiqon, Vedbaek, Denmark). Finally, qPCR was performed by using the LightCycler® 480 instrument (Roche molecular systems Inc., Mannheim, Germany). All reactions were carried out according to the manufacturer's instructions. Initial data analysis was executed by using the LightCycler® 480 Software (Roche molecular systems Inc., Mannheim, Germany) to obtain raw Ct values. Ct values were used to determine the amount of miRNA in a sample (both parameters showing an inverse correlation).

2.4. Preprocessing of Data. In order to compensate for variations in quality of extracted RNA, extraction yield, and efficiency of reverse transcription, normalization of data was carried out using GenEx Software Version 6.1 (Trial Version;

MultiD Analyses AB, Munich). The first normalization method used was the Normfinder algorithm which has been described in detail earlier [30]. In our analysis, a panel of 38 miRs from the data set and a single miR (hsa-miRNA-103a-3p) were selected for preprocessing as hsa-miRNA-103a-3p was stably expressed throughout the cohort and has been reported as being highly reliable for normalization of data [31]. Secondly, external controls (so-called Spike Ins—preformulated, commercially available RNAs with defined lengths and binding capacities), which were included in the beginning as potential references, were used to normalize the data [32]. The last normalization method used was the global mean algorithm which—in three steps—reduces nonspecific background noise, calculates the arithmetic mean value for all samples, and then subtracts this value from each individual value [33]. The global mean method is usually applied in cases where large numbers of miRNAs are tested and has been reported to be superior to other normalization methods in this particular setting [34].

2.5. Analysis of Subgroups. To test for significant differences in miRNA expression profiles between responder and nonresponder groups, the Mann–Whitney-*U* test for unpaired samples was applied using SPSS Statistics Version 22 (IBM, Ehringen, Germany). Because exploratory data analysis was performed, adjusting for multiple testing was not required. Afterwards, overlaps of differentially expressed miRNAs between the applied normalization procedures described above were compared.

2.6. Correlation with Clinical Characteristics. To estimate differences in clinical features (age, gender, differentiation grade, nodal status, depth of infiltration, perineural invasion, lymphovascular invasion, and presence of distant metastases) between both groups and between clinical features and miRNA expression levels, the χ^2 test was applied and a p value <0.05 was considered statistically significant.

2.7. Correlation with Overall Survival. To assess the prognostic value of miRNA expression, the median for each analyzed miRNA was calculated. Cases were then dichotomized, either showing an expression level above or below the median as described previously [35]. Overall survival curves were visualized via Kaplan–Meier estimates using SPSS Statistics Version 22 (IBM, Ehringen, Germany). In addition, Cox regression analysis was used to test for independence, taking into consideration gender, depth of infiltration, differentiation grade, nodal status, and presence or absence of distant metastases.

Data were adjusted for multiple testing using the Bonferroni procedure; after that, a p value <0.00052 was considered statistically significant for this test.

3. Results

3.1. Histology. Overall, 24 cases were classified as tubular adenocarcinoma, 3 cases as poorly cohesive carcinoma, 2 cases as mucinous adenocarcinoma, and 4 cases as undifferentiated/unclassifiable according to the current WHO standard [36]. After thorough histologic examination of whole-resection specimens, the cohort consisted of 12 nonresponders (regression grade 3) and 21 responders (regression grades 1a and 1b). Tumor regression was determined as mentioned above; representative examples of regression grading are shown in Figure 1. Further characteristic features of the study cohort are summarized in Table 1.

3.2. Correlation with Clinical Characteristics. Correlation between both groups showed that undifferentiated carcinomas and poorly differentiated carcinomas (differentiation grade 3) were more common in the nonresponder group, while responders showed a higher proportion of tubular adenocarcinomas (p values 0.034 and 0.043, respectively). No differences could be detected concerning gender, depth of infiltration, lymphovascular/perineural invasion, nodal status, or presence of distant metastases (p values between 0.087 and 0.443, as shown in Table 1).

3.3. Therapy Regimen. Neoadjuvant chemotherapy was administered in all cases, and 3 patients received radiation therapy in addition (both belonging to the responder group with irradiation doses of 45 Gy, 59 Gy, and 66 Gy, respectively).

Concerning the nonresponder group, neoadjuvant chemotherapy consisted in most cases of 5-fluorouracil combined with leucovorin, oxaliplatin, and docetaxel (so-called FLOT regimen; 8/12 patients). The remaining patients received either 5-fluorouracil in combination with cisplatin (2 cases) or epirubicin combined with oxaliplatin (2 cases).

In the responder group, a combination of cisplatin and 5-fluorouracil was administered in most cases (12/21 patients). Only 4 patients received treatment using the FLOT regimen, and 2 were treated with 5-fluorouracil in combination with leucovorin and etoposide. The remaining three patients received chemotherapy without 5-fluorouracil containing oxaliplatin, etoposide, and irinotecan.

3.4. miRNA Analysis. Overall, the following 4 miRNAs were differentially expressed between responder and nonresponder groups: hsa-let-7f-5p, hsa-miRNA-191-5p, hsa-miRNA-221-3p, and hsa-miRNA-31-5p.

Concerning the different normalisation methods, only minor variations were detected: applying the Normfinder algorithm, hsa-let-7f-5p, hsa-miRNA-191-5p, and hsa-miRNA-31-5p were differentially expressed (p values 0.03, 0.005, and 0.047). For normalisation with Spike Ins, differences in expression of hsa-miRNA-221-3p and hsa-miRNA-31-5p could be observed (p values 0.022 and 0.02). For normalisation with global mean, hsa-let-7f-5p, hsa-miRNA-191-5p, hsa-miRNA-221-3p, and hsa-miRNA-31-5p were differentially expressed (p values 0.025, 0.014, 0.04, and 0.033, respectively). Finally, for normalisation using

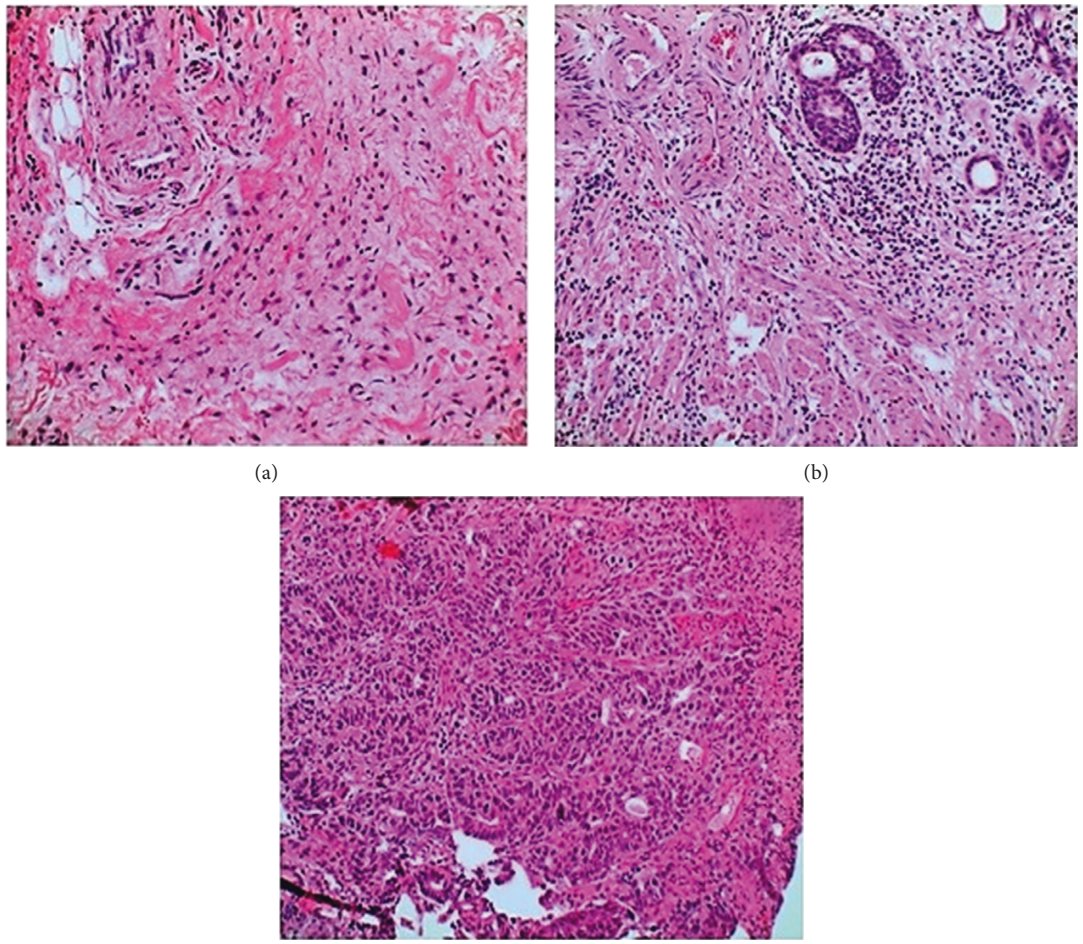

Figure 1: Pictures of histologic tumor regression: (a) regression grade 1a (no vital tumor); (b) regression grade 1b (<10% vital tumor cells); (c) regression grade 3 (>50% vital tumor cells). H&E staining, magnification 200x.

hsa-miRNA-103a-3p, hsa-let-7f-5p and hsa-miRNA-31-5p showed differences in expression (p values 0.038 and 0.036).

Throughout all normalization methods, there was higher expression of hsa-let-7f-5p, hsa-miRNA-221-3p, and hsa-miRNA-31-5p in the nonresponder group, while hsa-miRNA-191-5p showed higher expression in the responder group. The respective miRNA expression patterns (Ct values) are depicted in Figure 2.

3.5. miRNAs and Tumor Differentiation Grade. Correlation between miRNA expression profiles and tumor differentiation grade found that poorer differentiation (G3) was significantly associated with decreased levels of hsa-miRNA-200a-3p and elevated levels of hsa-miRNA-21-5p, hsa-miRNA-222-3p, hsa-miRNA-25-3p, and hsa-let-7d-5p (p values 0.03–0.031).

3.6. miRNAs and Patients' Prognosis. Complete survival data were available for 29 patients with a mean follow-up period of 44.52 months (range 1–100 months). During follow-up, 12 patients (41.38%) died. After adjusting for multiple testing, significant differences in survival according to high or low expression of miRNAs were detected only for hsa-miRNA-194-5p with a p value <0.0001. Survival times in patients with higher expression were nearly three times longer than in those with low expression (97.67 months vs. 32.69 months). Cox regression analysis, however, could not show independence for prediction of patients' survival after taking into consideration gender, depth of infiltration, differentiation grade, nodal status, and presence or absence of distant metastases ($p = 0.462$; hazard ratio 1.469; 95% confidence interval 0.527–4.096). In addition, there was no correlation between expression levels of hsa-miRNA-194-5p and drug response ($p = 0.456$).

Appropriate survival curves and mean survival times including 95% confidence interval are shown in Figure 3 and Table 2.

4. Discussion

The mechanisms underlying chemotherapy and multidrug resistance in human cancer are polymorphic [37]. In this context, miRNAs have been found to regulate apoptosis, DNA repair, and epithelial-mesenchymal transition and

Table 1: Characteristics of adenocarcinoma of the esophagogastric junction according to the responder or nonresponder status.

Characteristics	Responders	Nonresponders	p value
Total n	21	12	
Gender			
Male	18	8	0.198
Female	3	4	
WHO classification			
Tubular	17	7	
Poorly cohesive	2	1	
Mucinous	2	0	**0.034**
Undifferentiated	0	4	
Others			
Differentiation grade			
Well (G1)	0	0	
Moderately (G2)	19	7	**0.043**
Poor (G3)	2	5	
pT (low)			
pT0	2	0	
pT1a	2	0	0.136
pT1b	5	0	
pT2	3	1	
pT (high)			
pT3	8	10	
pT4a	1	1	
pT4b	0	0	
pN			
pN0	12	6	
pN1	3	4	0.203
pN2	6	1	
pN3	0	1	
LVSI			
Present	6	5	0.443
Absent	15	7	
Perineural invasion			
Present	1	3	0.087
Absent	20	9	
Distant metastases			
Present	4	1	0.409
Absent	17	11	

LVSI: lymphovascular space invasion; bold lettering in p values indicates a statistically significant difference.

to modulate drug targets, drug-metabolizing enzymes, or drug efflux transporters [16, 17]. In our study, analyzing adenocarcinomas of the esophagogastric junction, we identified a panel of four miRNAs which were differentially expressed between patients responding or not responding to neoadjuvant chemotherapy. We found higher expression of hsa-let-7f-5p, hsa-miRNA-221-3p, and hsa-miRNA-31-5p in the nonresponder group, while hsa-miRNA-191-5p showed higher expression in the responder group. The molecular mechanisms contributing to chemoresistance or chemosensitivity concerning these four miRNAs are—up to date—not fully understood. Nevertheless, the function and effects of these miRNAs as stated in the literature might give some clues as to what the underlying mechanisms might be: for hsa-let-7f-5p, a proangiogenic effect has been reported; thus, overexpression might contribute to tumor progression via tumor neoangiogenesis [38–40]. For hsa-miRNA-221-3p, cell cycle regulation has been reported as a key mechanism in tumor progression; in addition, in cervical cancer, increased expression levels are associated with epithelial-mesenchymal transition, migration, and invasion by targeting TWIST2 [41, 42]. For human glioblastomas, cervical and colon carcinoma cells downregulation of *PTEN* and activation of Akt and STAT3—mediated by increased levels of hsa-miRNA-221-3p—have been shown as key players in tumor cell survival and radio- and chemoresistance [43–46]. In addition, in hepatocellular carcinoma, upregulation of hsa-miRNA-221-3p decreases the expression of HDAC6, a tumor suppressor, and promotes tumorigenesis [47]. One study focusing solely on esophageal adenocarcinomas showed that the chemoresistance was conveyed through alteration of the Wnt/β-catenin pathway and DKK2, CDH1, CD44, MYC, and ABCG2 expression [26]. Concerning hsa-miRNA-31-5p, only few studies have addressed how increased expression contributes to chemoresistance: in malignant pleural mesothelioma and hepatocellular carcinoma, it promotes chemoresistance by targeting OCT1 and ABCB9 [48, 49]. In ovarian cancer, chemoresistance is increased by modulation of specific calcium-regulated potassium channels [50]. Conversely, an opposite effect has also been reported: overexpression of hsa-miRNA-31-5p decreases levels of stathmin 1, a microtubule-depolymerizing molecule that leads to reduced chemosensitivity in ovarian cancer [51]. In addition, in osteosarcoma, upregulation of hsa-miRNA-31-5p inhibits tumor cell migration and invasion by targeting PI3K3C2A [52]. Regarding hsa-miRNA-191-5p, the few studies conducted so far show that overexpression promotes chemoresistance by modulating p53 and TET1 in cholangiocarcinomas [53]. Furthermore, an association with various estrogen-dependent genes such as ANXA1, PIWIL2, CASP4, ESR1/ESR2, PLAC1, and SOCS2, has been shown in breast cancer—however, up to date, no such data concerning adenocarcinomas of the esophagogastric junction have been published [54].

The miRNA signature we discovered seems to differ from previously published data; other studies found that especially in esophageal carcinoma—in addition to miRNA-221—miRNA-141, miRNA-200c, miRNA-148a, miRNA-296, miRNA-23, miRNA-223, and miRNA-27a are substantially contributing to chemoresistance [26, 55]. Nevertheless, as some studies focus on plasma-circulating miRNAs or cell lines and not exclusively on expression in tumor tissue, results are comparable only to a limited degree [13]. Ours is—to our knowledge—the first study focusing on miRNA expression profiles in adenocarcinomas of the esophagogastric junction and their meaning for therapeutic response based on tissue specimens.

Hsa-let-7f-5p is located on the long arm of chromosome 9 (9q22.3) and shows involvement in immune cell differentiation, angiogenesis, and cellular growth arrest [56–58]. It is commonly affected in multiple human cancers, including melanoma, lung, and head/neck cancer, with downregulation in the majority of cases [59–61]. Nevertheless, upregulation is also encountered, for instance, in papillary, follicular, and

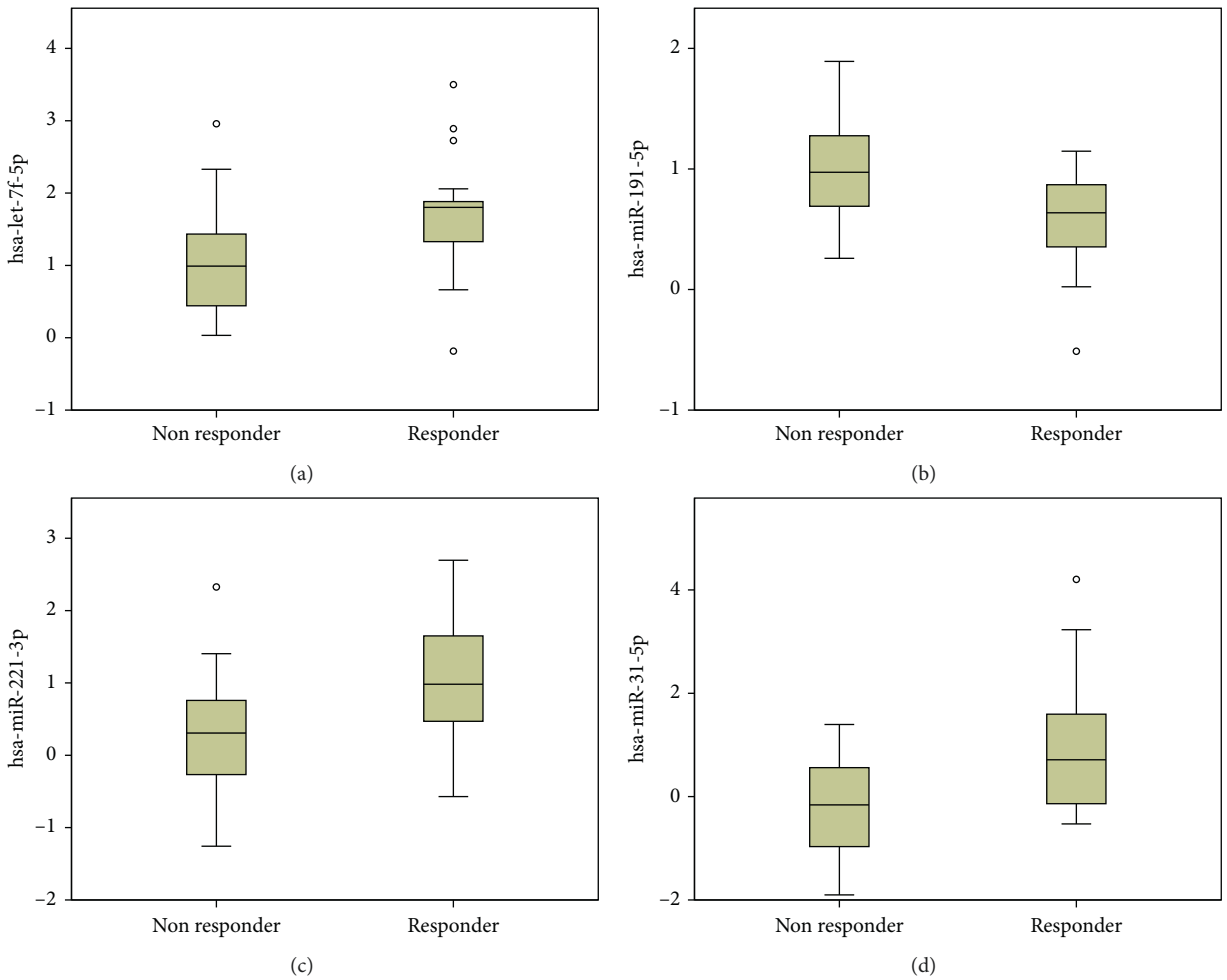

Figure 2: Boxplots and Ct values according to differentially expressed miRNAs in responder and nonresponder groups: (a) Ct values for hsa-let-7f-5p; (b) Ct values for hsa-miRNA-191-5p; (c) Ct values for hsa-miRNA-221-3p; (d) Ct values for hsa-miRNA-31-5p. Lower Ct values indicate higher miR expression, while higher Ct values indicate lower miR levels.

anaplastic thyroid cancer as well as breast cancer and ovarian cancer [62–64]. It has been associated not only with chemosensitivity and treatment response in gastric cancer [65] but also with chemotherapy resistance in breast cancer [37]. In our study, increased expression of hsa-let-7f-5p showed a distinctive association with chemoresistance which seems to be in contrast to previously published data concerning carcinomas of the upper gastrointestinal tract [65]. Still, as data concerning the association of miR expression levels and chemotherapy response are still often controversial and sometimes different functions (either as a tumor suppressor or as an oncogene) have been reported for different histologic tumor types (adenocarcinoma vs. squamous cell carcinoma) in a single organ, our data might only mirror a specific effect for a defined subset of patients [7].

Hsa-miRNA-221-3p is well characterized, and its function and involvement in human cancer has been extensively described. It is commonly known as an onco-miRNA, promoting tumor proliferation, invasion, dissemination, and metastasis [66, 67]. Multiple analyses have focused on cell lines where overexpression is commonly associated with chemoresistance and knockdown restores chemo- and/or radio sensitivity and induces tumor cell apoptosis [26, 68, 69]. In addition, in tissue experiments, hsa-miRNA-221-3p has been shown to promote resistance to a variety of regularly used therapeutic agents including 5-fluorouracil, tyrosine kinase inhibitors, and antiandrogens [37, 70–73].

These findings are difficult to compare with our study population as both responders and nonresponders had been treated using a combination chemotherapy containing 5-fluorouracil in most cases (28/33 cases).

Downregulation of hsa-miRNA-31-5p has been described in a variety of human cancers, for instance, triple-negative breast carcinoma [67] and indicates shortened overall survival in gastric cancer [74] as well as the presence of locally advanced tumor stages, implicating the function as a miRNA with tumor suppressor properties.

Data concerning its association with therapy response are more controversial: while some studies report that overexpression promotes chemoresistance in gastric and ovarian cancer [50, 75] and breast carcinomas [76], others could demonstrate that upregulation in gallbladder carcinomas leads to increased chemosensitivity [77]. In

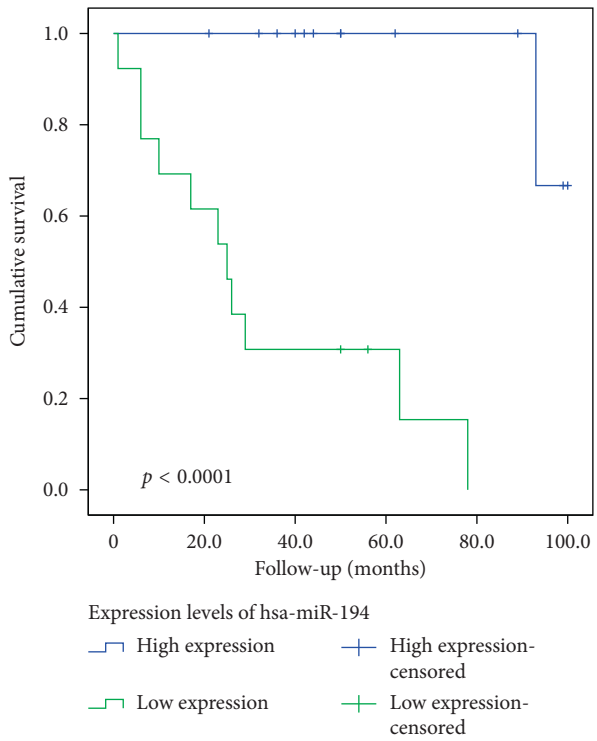

Figure 3: Kaplan–Meier curve showing survival differences according to high or low expression of hsa-miRNA-194-5p with a p value <0.0001. Survival times are given in months.

Table 2: Average survival times according to high or low expression of hsa-miRNA-194-5p.

	Average survival (months)	SD	95% CI	p value
hsa-miRNA-194-5p				
High	97.67	1.91	93.93–101.4	**<0.0001**
Low	32.69	7.89	17.22–48.16	

addition, hsa-miRNA-31-5p expression levels seem to influence the therapy response not only in general but also according to different chemotherapeutic agents: in cell line experiments, it has been demonstrated that downregulation can promote resistance to platinum-based therapies and paclitaxel [78], while upregulation correlates with resistance against 5-fluorouracil [79]. Our study adds to the current understanding as we could demonstrate increased chemotherapy resistance in cases with higher hsa-miRNA-31-5p expression levels. It seems as if the therapeutic response might not only depend on the tumor subtype analyzed but also on the therapy applied and that hsa-miRNA-31-5p might exhibit both oncogenic and tumor suppressive functions according to a specific context.

Throughout the literature, hsa-miRNA-191-5p is described as having oncogenic properties, leading to increased tumor cell proliferation, invasion, and inhibition of apoptosis [80]. This holds true for various epithelial cancer types such as breast, pancreatic, and hepatocellular carcinomas [81] or cholangiocarcinoma. Here, overexpression is additionally associated with decreased overall survival [53]. Concerning its association with chemotherapy outcome, single studies have found hsa-miRNA-191-5p to promote chemoresistance [54], while others could show no influence on chemosensitivity or chemoresistance at all [82]. Overall, literature addressing this particular issue is still very sparse. In contrast to the data described above, in our study, high levels of hsa-miRNA-191-5p showed a clear-cut association with chemosensitive cases responding to neoadjuvant chemotherapy.

It remains to be seen whether these results can be reproduced in larger studies or if a similar effect can be shown in other cancer entities. It may be conceivable that our findings reflect—as it is possibly the case with both hsa-let-7f-5p and hsa-miRNA-31-5p—an effect which is only discernible in a defined subset of tumors or specific tumor entities.

Due to the small case number in our study and the often limited availability of both pretherapeutic biopsies and resection specimen in a single institution, we additionally consulted a web database (GEO database) to supplement our data. Here, we could find only one additional study analyzing chemotherapy response in a very small cohort (8 cases) of upper gastrointestinal carcinomas, namely, stomach cancer, proposing a miRNA signature used for predicting chemotherapy outcome [65]. This signature, however, differed from our findings, showing an association of chemoresistance and high expression of let-7g, miR-342, miR-16, miR-181, miR-1, and miR-34. Whether this might be due to small case numbers in both studies or whether this reflects differences between two cancer entities with a fundamentally different pathophysiology remains to be seen. Another study analyzed esophageal adenocarcinomas (14 cases) and reported that miRNA-221 contributes to chemoresistance via the Wnt/β-catenin pathway, supporting our findings [26].

In addition, we correlated the miRNA expression with patients' overall survival and could demonstrate that overall survival was significantly correlated with expression levels of hsa-miRNA-194-5p (p value <0.0001) although independence could not be demonstrated applying Cox regression analysis ($p = 0.462$; hazard ratio 1.469; 95% confidence interval 0.527–4.096). In accordance with our results, where higher levels of hsa-miRNA-194-5p corresponded to prolonged survival (97.67 months vs. 32.69 months), overexpression has been reported to inhibit cell proliferation and to act as a tumor suppressor in laryngeal SCC, prostate cancer, melanoma, bladder cancer, NSCLC, and clear cell renal carcinoma [83–87]. In addition, overexpression has been found to inhibit growth and proliferation in gastric cancer cell lines and esophageal squamous cell carcinoma [88, 89]. Other miRNAs which are commonly linked to prolonged or shortened overall survival in gastric or esophageal carcinomas—for instance, miRNA-16, miRNA-21, miRNA-29, miRNA-125b, miRNA-130a, miRNA-141, miRNA-203a, miRNA-222, miRNA-302c, and miRNA-451—could not be reproduced in our study [4, 8, 9, 11, 14]. This might well be due to a smaller number of patients included in our study which limits the statistical reliability as well as the fact that, after adjusting for multiple testing, only a p value <0.00052 was considered statistically

significant which excluded a few otherwise statistically significant miRNAs.

5. Conclusion

Our results imply that miRNAs might play an important role in the evolution of chemoresistance and/or chemosensitivity in adenocarcinomas of the esophagogastric junction. Nevertheless, as—due to the restricted availability of both pre- and posttherapeutic tissue samples in a single institution—the number of patients in our study was limited, statistical results should be interpreted with caution. In addition, only tumor tissue was compared without establishing baseline levels of miRNA expression in nonneoplastic mucosa.

Regardless of the abovementioned limitations, our results contribute to other studies postulating that miRNAs might be a pretherapeutic means to predict therapy response in the future and better stratify patients who benefit from neoadjuvant therapy or who might not benefit at all and therefore could be spared of adverse effects of noneffective treatment strategies.

It remains to be seen if the miRNA signature we established for adenocarcinomas of the esophagogastric junction can be reproduced in future studies or for different tumor entities.

Abbreviations

Ct: Cycle threshold
hsa: *Homo sapiens*
miRNA: MicroRNA.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Part of this study has been presented as a scientific poster at the ASCP Annual Meeting 2017 (Chicago, Illinois).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Christina Just and Juliana Knief contributed equally to this work.

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Research Article

Low Expression of GLIS2 Gene Might Associate with Radiosensitivity of Gastric Cancer

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Human gene GLIS family zinc finger 2 (GLIS2) is a member of GLI-similar zinc finger protein family. Previous studies indicated GLIS2 gene involved in tumorigenesis mechanisms. However, the association between GLIS2 expression and radiosensitivity of gastric cancer has not been well understood. In this study, we used the gastric cancer database in TCGA, and significant association was observed between the low expression of GLIS2 and radiosensitivity of patients with gastric cancer. The adjusted HR values for radiotherapy were 0.162(0.035-0.756) and 0.089(0.014-0.564), with p values 0.021 and 0.010, respectively, in training and testing data, for these patients with low expression of GLIS2, while for patients with high expression of GLIS2, there was no significant survival difference between radiotherapy and nonradiotherapy groups. The adjusted HR were 0.676(0.288-1.586) and 0.508(0.178-1.450), with p values 0.368 and 0.206 in training and testing data, respectively. Further study showed that, for low expression patients, radiotherapy did not significantly increase new tumor event rate and disease progression rate, which partially supported our assumption. These results suggested that low expression of GLIS2 might significantly associate with the radiosensitivity of patients with gastric cancer. The GLIS2 gene might be a potential effective molecular marker of gastric cancer for precise radiotherapy.

1. Introduction

Gastric cancer accounts for a large proportion of cancer death worldwide. GLOBOCAN2018 data showed that over 1000,000 people were newly diagnosed with gastric cancer in 2018 [1], nearly 783,000 people died. According to Chinese cancer statistics in 2015 [2], gastric cancer is one of the top four common malignant tumors in China, with the second highest morbidity and mortality. Clinically, the treatment of gastric cancer mainly focuses on surgery, radiotherapy, chemotherapy, immunotherapy, and combined treatment of traditional Chinese medicine as adjuvant treatment methods. Based on extensive application of radiotherapy in clinical practice worldwide, many researchers have paid great attention to how to utilize radiotherapy to improve the life quality of patients with gastric cancer in different directions. Chung et al. did retrospective analysis on the efficacy of treatment of gastric cancer by volumetric-modulated arc therapy (VMAT) and found that VMAT could effectively reduce the treatment time and the irradiation dose at the liver and kidney to alleviate the toxicity of radiotherapy [3]. Some clinical trials had focused on providing optimal radiotherapy strategies for patients with different stages of gastric cancer [4–7]. However, they did not reach consistent results.

Due to diverse sensitivity to radiotherapy in individuals, clinical doctors would like to screen radiosensitive

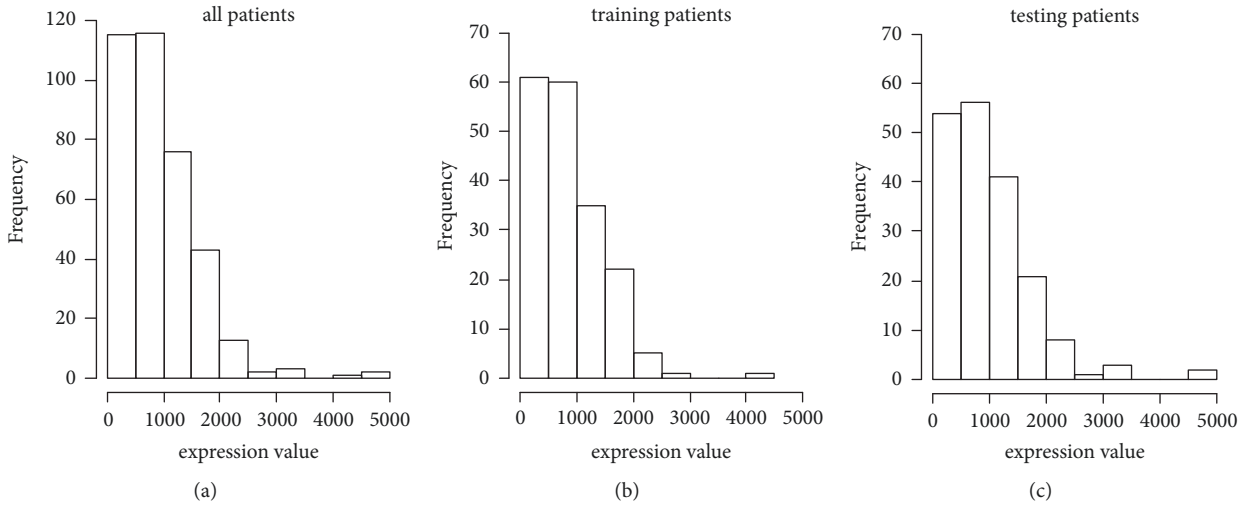

Figure 1: Expression distribution of GLIS2 gene of patients with gastric cancer. (a) Expression distribution of GLIS2 gene in all data. (b) Expression distribution of GLIS2 gene in training data. (c) Expression distribution of GLIS2 gene in testing data.

patients who can obtain higher survival benefits. Based on the genome sequence technology, researchers could find potential biomarkers to predict radiosensitive patients, then oncologists and surgeons could adjust their treatment strategy to reduce adverse reactions and improve radiotherapy efficacy [8]. Therefore, it is particularly imperative to find gene biomarkers that can accurately and sensitively predict whether patients with gastric cancer are sensitive to radiotherapy, so as to provide evidence to formulate targeted radiotherapy regimens.

GLIS2 gene is a dual-function transcriptional regulator; its regulation would play an important role during embryonic development [9]. GLIS2 gene could regulate self-renewal capacity in hematopoietic progenitor cells and promote differentiation of megakaryocytes [10, 11], which was identified as one of several genes required for optimal repopulation [12]. Nevertheless, overexpression of human GLIS2 had a negative effect on reprogramming [13, 14], leading to a decreased number of ESC-like colonies, denoting that GLIS2 gene might be associated with cancers. Moreover, expression levels of GLIS2 gene make crucial contribution to maintaining normal renal structure and function [15]; previous studies reported that loss of GLIS2 could lead to increased renal cell apoptosis and fibrosis in human and mice [16]. Mutant mice lacking GLIS2 function showed anterior bowel defects, including esophageal and tracheal stenosis, as well as pulmonary hypoplasia and pulmonary function defects [17].

Recent study had shown that overexpression of GLIS2 had significant association with chemoresistance of gastric cancer [18]. Relationship between the expression of GLIS2 and the sensitivity of radiotherapy for patients suffering from gastric cancer has not been well studied. We assumed the expression level of GLIS2 associated with radiosensitivity of patients. Sensitive patients could obtain survival benefits after radiotherapy. To verify this hypothesis, we analyzed the relationship between GLIS2 expression and radiotherapy sensitivity based on gastric cancer data from TCGA, to provide references for clinical treatment of gastric cancer.

2. Data Sources and Methods

2.1. Data Sources. In the present study, we analyzed normalized mRNA sequencing data of GLIS2 of the patients with gastric cancer. The data was downloaded in December 2016 from TCGA (The Cancer Genome Atlas, http://cancergenome.nih.gov/) [19, 20], by using TCGA assembler [21]. To clean data, we combined the clinical survival information from several raw data files and eliminated the data of patients with no survival time or survival outcome to obtain effective patient survival information. Then we screened the clinical factors needed among the available data and combined them to obtain a complete clinical data file. In addition, we kept the patients with clear information on radiotherapy. Furthermore, we deleted repeated expression information of normal tissue and combined the mRNA sequencing data of GLIS2 with the clinical data obtained in previous steps to obtain the data used for the present study, which contained 371 patients.

2.2. Analysis Method. In the present study, radiotherapy sensitivity was defined as the improved survival benefits of the patients receiving radiotherapy, compared with the patients who did not receive radiotherapy [22, 23]. The gene that can predict individual radiosensitivity could be a potential biomarker for radiosensitivity prediction. In order to validate the hypothesis of this study, we randomly split the data into training data and testing data. Firstly, we generated random number between 0 and 1 for all patients, by using the R function runif(). Then, we picked up a half of patients with small random number, as training data. The rest patients were treated as testing data. The same analysis was performed for both training and testing data. Since the expression level of GLIS2 gene showed skewness distribution in the training data (Figure 1), the median values in the training data were defined as the critical threshold of high and low expression. Then, univariate and multivariate Cox regression analysis were performed for patients with high

Table 1: Associations of clinical indicators and GLIS2 expression level with total survival in training data.

	Univariate analysis		Multivariate analysis	
	HR	P values	HR	P values
Radiotherapy				
Yes	0.458(0.247-0.849)	0.013	0.453(0.226-0.909)	0.026
No	1.000		1.000	
Gender				
Male	1.422(0.886-2.283)	0.145	1.645(0.994-2.724)	0.053
Female	1.000		1.000	
Age				
>60	1.498(0.894-2.509)	0.125	1.569(0.895-2.751)	0.116
≤60	1.000		1.000	
Histologic type				
NOS	0.956(0.556-1.641)	0.868	0.969(0.550-1.706)	0.912
DT/MT/SRT	0.774(0.405-1.480)	0.438	0.724(0.356-1.473)	0.373
PT/TT	1.000		1.000	
T Stage				
T3/T4	1.891(1.088-3.285)	0.024	1.642(0.870-3.098)	0.126
T1/T2	1.000		1.000	
N Stage				
N1/N2/N3	1.837(1.086-3.105)	0.023	1.433(0.666-3.080)	0.357
N0	1.000		1.000	
M Stage				
M1	3.178(1.616-6.250)	0.001	3.305(1.435-7.614)	0.005
M0	1.000		1.000	
Pathological stage				
III/IV	1.810(1.129-2.902)	0.014	1.268(0.609-2.639)	0.526
I/II	1.000		1.000	
Targeted therapy				
Yes	0.753(0.481-1.176)	0.212	1.010(0.352-2.898)	0.986
No	1.000		1.000	
Chemotherapy				
Yes	0.771(0.497-1.196)	0.246	0.792(0.291-2.160)	0.649
No	1.000		1.000	
GLIS2 expression				
High	1.232(0.794-1.910)	0.352	1.353(0.854-2.144)	0.197
Low	1.000		1.000	

Note: HR: hazard ratio; NOS: not otherwise specified; DT: diffuse type; MT: mucinous type; SRT: signet ring type; PT: papillary type; TT: tubular type.

and low expression. Supplemental Table S1 showed the basic patient characteristics.

In the present study, survival analysis model of R packages survival was adopted for analysis, and survival curves were plotted by R packages rms. P value of 0.05 was taken as the criterion of significance. Missing values were multiple imputed by R package mice [24].

3. Results

3.1. Correlation Analysis of GLIS2 Expression Level and Clinical Indicators with Survival. In this study, the overall survival was the main observation outcome. The Cox proportional hazard model was used to evaluate the association between GLIS2 expression level and clinical factors with survival. Tables 1 and 2 illustrated the analysis results of training and testing data, respectively. The analysis results showed that radiotherapy could improve the overall survival of patients. However, in both datasets, there were no significant associations between expression level of GLIS2 and overall survival.

These results showed radiation therapy was significantly associated with overall survival. However, we argued that not all patients benefitted from radiation therapy. More accurate radiotherapy can be achieved if sensitive patients are effectively screened. In other words, poorer sensitive patients could be protected from noneffective radiotherapy and the adverse reactions.

Table 2: Associations of clinical indicators and GLIS2 expression level with total survival in testing data.

	Univariate analysis		Multivariate analysis	
	HR	P values	HR	P values
Radiotherapy				
Yes	0.334(0.161-0.690)	0.003	0.309(0.128-0.744)	0.009
No	1.000		1.000	
Gender				
Male	1.100(0.668-1.813)	0.708	1.193(0.711-2.003)	0.504
Female	1.000		1.000	
Age				
>60	1.309(0.793-2.160)	0.293	1.346(0.794-2.282)	0.270
≤60	1.000		1.000	
Histologic type				
NOS	1.521(0.811-2.853)	0.191	1.843(0.962-3.534)	0.065
DT/MT/SRT	1.065(0.520-2.180)	0.863	1.570(0.739-3.333)	0.240
PT/TT	1.000		1.000	
T Stage				
T3/T4	1.700(0.909-3.183)	0.097	0.994(0.472-2.091)	0.986
T1/T2	1.000		1.000	
N Stage				
N1/N2/N3	2.227(1.169-4.240)	0.015	2.280(0.995-5.228)	0.051
N0	1.000		1.000	
M Stage				
M1	1.167(0.559-2.439)	0.681	1.162(0.536-2.520)	0.704
M0	1.000		1.000	
Pathological stage				
III/IV	1.952(1.162-3.279)	0.012	1.703(0.831-3.490)	0.146
I/II	1.000		1.000	
Targeted therapy				
Yes	0.580(0.356-0.946)	0.029	0.997(0.340-2.924)	0.996
No	1.000		1.000	
Chemotherapy				
Yes	0.608(0.376-0.982)	0.042	0.679(0.249-1.856)	0.451
No	1.000		1.000	
GLIS2 expression				
High	1.401(0.869-2.260)	0.166	1.184(0.725-1.936)	0.500
Low	1.000		1.000	

Note: abbreviations were the same with Table 1.

3.2. Relationship between GLIS2 Expression Levels and Clinical Indicators. We analyzed the relationship between expression levels of GLIS2 and clinical factors by using the chi-square test. The analysis results in Table 3 showed that there were no significant associations between the expressions of GLIS2 and any clinical indicator.

3.3. Relationship between Radiotherapy and Survival in High and Low Expression Groups. The main idea of this study was to discuss whether the patients with low expression of GLIS2 were sensitive to radiotherapy. In order to obtain reliable results, we split the data to two part, training data and testing data, and performed survival analysis respectively. Table 4 demonstrated that, in the training and testing data, for low expression subgroup, there were significant associations between radiotherapy and overall survival. The similar results could be found between univariate and multivariate analysis. The adjusted HR for radiotherapy vs nonradiotherapy were 0.162(0.035-0.756) and 0.089(0.014-0.564) in training and testing data, respectively. For the patients with high expression in training and testing data, radiotherapy could not significantly improve the overall survival, with the adjusted HR 0.676(0.288-1.586) and 0.508(0.178-1.450), respectively.

Figure 2 illustrated the survival curves of radiotherapy and nonradiotherapy groups based on different expression levels of GLIS2 gene in the training data and testing data. In the low expression group, the survival time of the patients receiving radiotherapy was significantly prolonged, shown in

Table 3: Relationship between expression levels of GLIS2 and clinical indicators.

	Training data(n=185)				Testing data(n=186)			
	High	Low	χ^2	P values	High	Low	χ^2	P values
Gender			0.264	0.608			0.978	0.323
Male	56	61			71	53		
Female	36	32			30	32		
Age			0.000	1.000			2.287	0.131
>60	64	63			59	59		
≤60	28	28			42	25		
Histologic type			5.681	0.058			7.173	0.028
NOS	44	54			50	42		
MT/DT/SRT	30	16			33	17		
PT/TT	18	22			16	26		
T Stage			1.518	0.218			0.080	0.777
T3/T4	69	59			77	63		
T1/T2	23	31			23	22		
N Stage			0.000	1.000			0.243	0.622
N1/N2/N3	64	63			73	57		
N0	28	29			28	27		
M Stage			1.278	0.258			0.330	0.565
M1	4	9			12	7		
M0	88	84			89	78		
Pathological Stage			0.019	0.891			0.013	0.909
III/IV	48	44			51	42		
I/II	42	42			46	41		

Note: abbreviations were the same with Table 1.

Table 4: Association analysis between radiotherapy and survival under different expressions of GLIS2.

Data	GLIS2 expression	Unadjusted(RT vs NRT)		Adjusted(RT vs NRT)	
		HR	P values	HR	P values
Training	High (n=92)	0.694(0.332-1.452)	0.332	0.676(0.288-1.586)	0.368
	Low (n=93)	0.165(0.040-0.686)	0.013	0.162(0.035-0.756)	0.021
Testing	High (n=101)	0.677(0.294-1.558)	0.359	0.508(0.178-1.450)	0.206
	Low (n=85)	0.116(0.027-0.509)	0.004	0.089(0.014-0.564)	0.010
All Data	High (n=193)	0.694(0.401-1.200)	0.191	0.673(0.360-1.257)	0.214
	Low (n=178)	0.145(0.053-0.401)	<0.001	0.170(0.055-0.521)	0.002

Note: adjusted factors: gender, age, histologic type, TNM stage, pathological stage, chemotherapy, and targeted therapy.

Figures 2(b) and 2(d). In the high expression group, Figures 2(a) and 2(c), radiotherapy had no significant effect on the overall survival.

We further performed survival analysis by combined training and testing data (Table 4). The same conclusion could be reached. The unadjusted and adjusted HR were 0.694(0.401-1.200) and 0.673(0.360-1.257) for high expression subgroup, respectively. However, for low expression group, the unadjusted and adjusted HR were 0.145(0.053-0.401) and 0.170(0.055-0.521), respectively. For low expression group, radiotherapy exhibited significant clinical efficacy.

Survival curves for all high and low expression patients were shown in Figures 3(a) and 3(b), respectively. Figure 3(c) contained the survival curves for patients who received radiotherapy. It can be seen that the survival rate of patients with low expression was significantly prolonged after receiving radiotherapy.

In summary, in the high expression group, patients who received radiotherapy achieved no significant survival benefits than those who were not treated by radiotherapy, while in the low expression group, the survival rate of patients was significantly improved if they received radiotherapy. The results suggested that the low expression of GLIS2 gene might effectively indicate the radiosensitivity of patients. These patients would obtain significant survival benefits from radiotherapy.

3.4. Associations among GLIS2 Expressions and Clinical Assessment Factors after Adjuvant Therapy. Figure 4 showed the associations among the expression levels of GLIS2 and

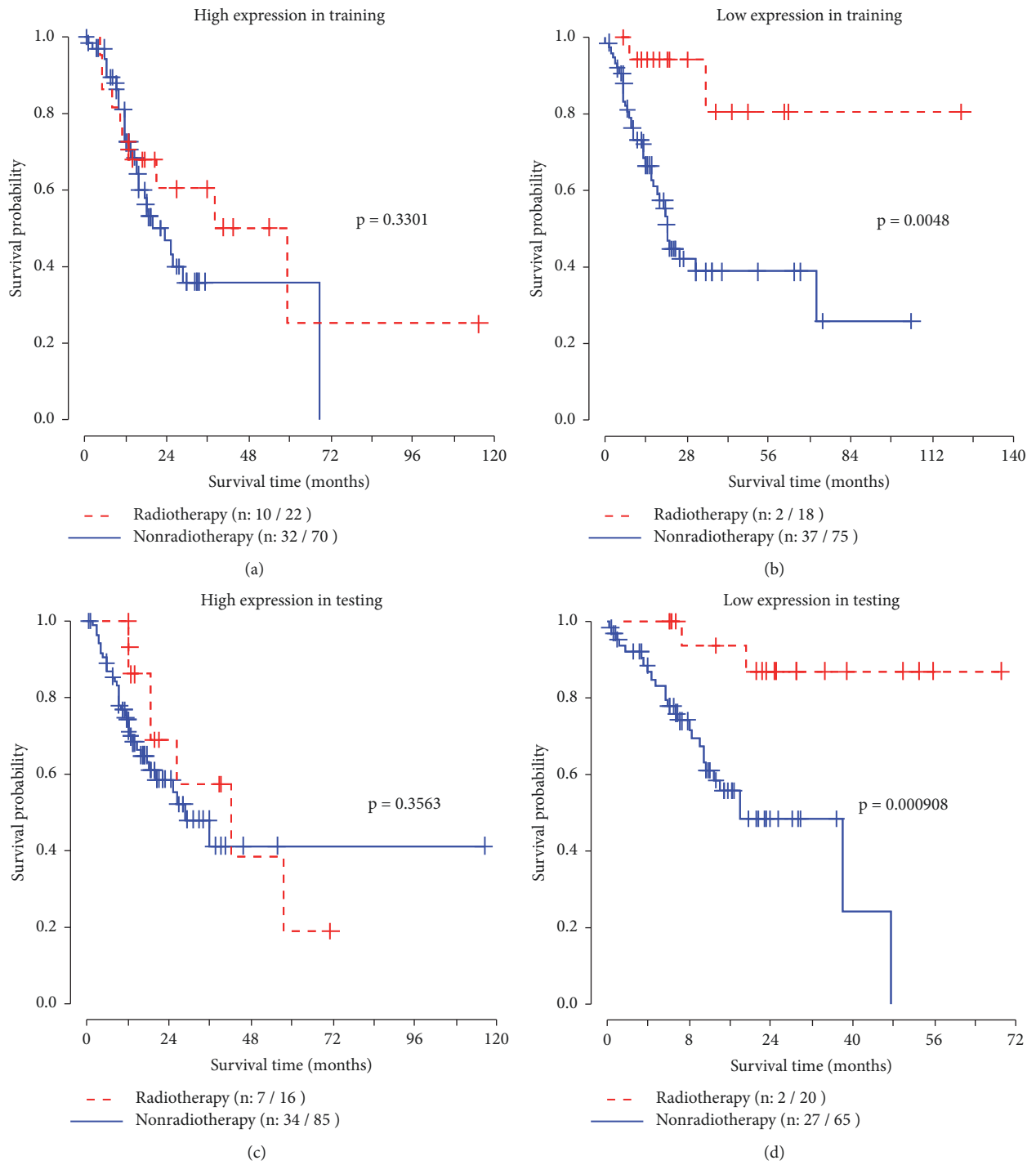

Figure 2: Survival curves under different expression levels of GLIS2 in training and testing data. Log-rank test was used to estimate the P values.

two clinical assessment indexes: the new tumor event and progressive disease. There was a significant difference in new tumor event rate between high and low expression group patients under radiotherapy. The low expression group hold lower new tumor event rate 22.2%, which was a half of high expression group (48.6%). Disease progression rate was also approximately significant lower (13.9%) in low expression group, compared with high expression group (32.3%), for patients who received radiotherapy. The results in Figure 4 suggested that, for low expression patients, radiotherapy did not increase new tumor event rate and disease progression rate and even decreased the two rates. These results partially supported that patients with low expression of GLIS2 might be sensitive to radiotherapy.

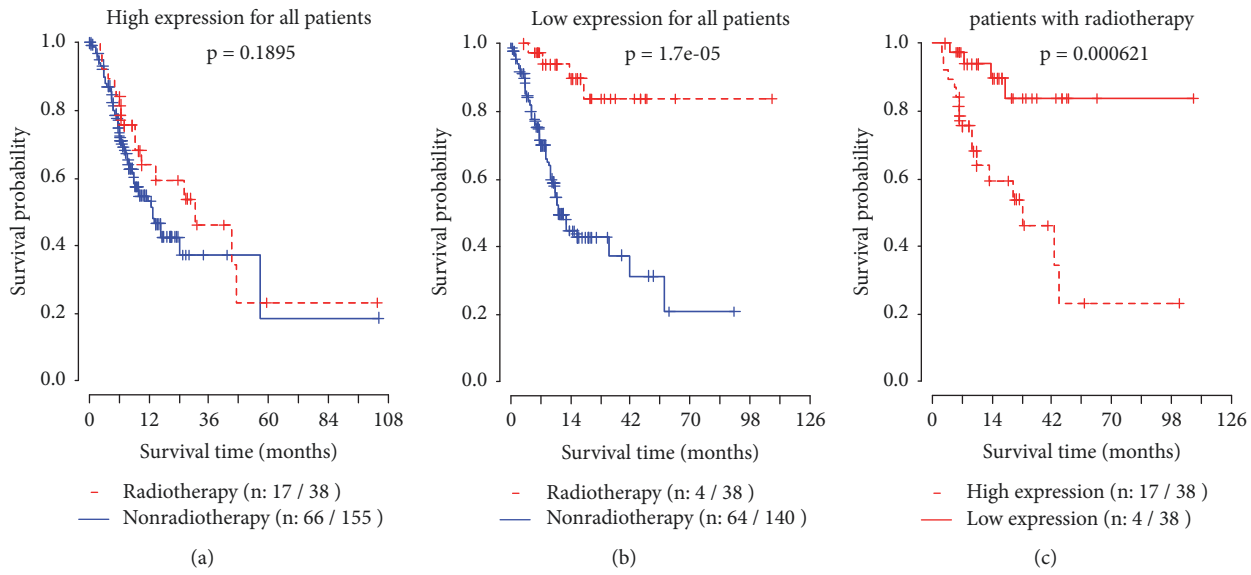

Figure 3: Survival curves under different expression levels of GLIS2 for all patients.

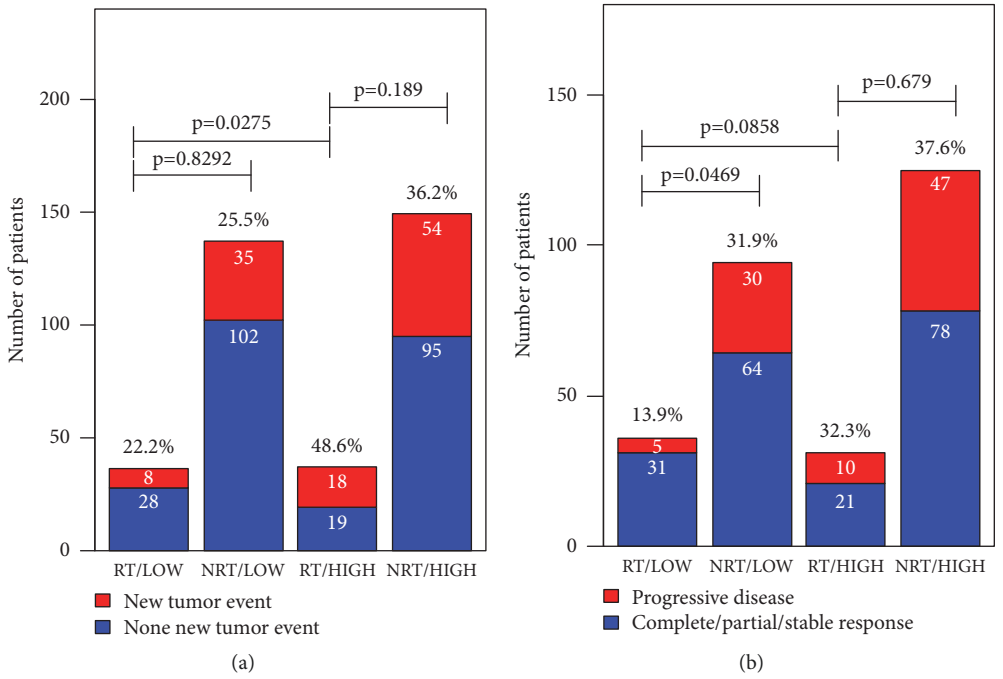

Figure 4: Associations among GLIS2 expressions and clinical assessment factors. Chi-square test was used for comparisons of rates of different groups. RT: radiotherapy; NRT: nonradiotherapy; HIGH: high expression of GLIS2 gene; LOW: low expression of GLIS2 gene.

4. Discussion

Radiotherapy is an essential part of adjuvant treatment to cancers, whereas it is also a double-edged sword. It not only kills tumor cells, but also promotes radioimmunity, induces distant metastasis, and damages normal tissues [25]. Studies had reported that increased irradiation had toxic effects on the skin and other normal tissues [26]. Therefore, improving the efficacy of radiotherapy and reducing toxicity had attracted worldwide attention. According to variations of radiosensitivity in individuals, dividing patients with gastric cancer and giving radiation treatment to patients with significant radiotherapy sensitivity would make radiotherapy more accurate, while eliminating the adverse reactions of patients who are not sensitive to radiotherapy after radiotherapy [27]. It can be seen that finding appropriate biomarkers to distinguish sensitive populations is of great importance for clinical treatment. However, effective molecular markers had not been found in gastric cancer so far.

We made use of public data from TCGA in the present study. Due to the difficulties of collecting clinical samples and the large number of potential genes available for external validation, the development of a reliable diagnostic classifier using early nonrandomized phase II data is often not feasible. To overcome these difficulties, we performed an internal validation procedure that randomly divided the data into two parts and analyzed them separately. We found significant associations between expression levels of GLIS2 gene and survival outcome. Low expression of GLIS2 gene might indicate radiosensitivity of patients. Table S2 illustrated the research results of data. Radiotherapy did have significant effect on improving total survival time of patients suffering from gastric cancer. However, we argued that, in clinical practice, not all patients, some patients with gastric cancer will benefit from radiotherapy. The need for radiation depends on what type of surgery, whether the cancer has spread to somewhere else of body, and in some cases, the age or other clinical factors. If clinical doctors can predict radiosensitivity of patients, they could evaluate sensitive patients more effectively and perform accurate radiotherapy.

In our analysis, we chose median as cutoff to divide high and low expression group. We also performed analysis on other cutoffs, as shown in Figure S1. The results suggested that, when the cutoff larger than median was selected to divide high and low expression group, for patients with high expression, there was no significant survival difference between radiotherapy and nonradiotherapy, while for patients with low expression, patients who received radiotherapy had better survival than who did not receive radiotherapy. We also found that if we selected other cutoffs lower than median, like 10% to 40% quantiles, for these patients with high expression, the HR of radiotherapy were statistically significant, which may be caused by including too many relative lower expression patients. These results were consistent with our conclusion, that low expression patients could be predicted as radiosensitive patients.

It is known that radiotherapy type in gastric cancer includes preoperative, postoperative, and palliative therapy. Preoperative radiotherapy is mainly used in patients with locally advanced gastric cancer to reduce tumor burden and control tumor progression for surgery. TCGA did not provide clear information about radiotherapy type. But, if preoperative therapy was used, the expression data of cancer tissue would be not useful.

Postoperative radiotherapy was the main direction of our research, which is usually combined with chemotherapy to treat patients with resectable gastric cancer as adjuvant therapy. Macdonald et al. conducted Gastrointestinal Cancer Intergroup phase III Trial (INT 0116) and found that postoperative chemoradiotherapy (CRT) could significantly improve the survival rate of patients after radical gastrectomy, though lack of strict trial control [28]. Furthermore, the result of phase III trial led by Lee et al. suggested that additional postoperative radiotherapy to surgery and postoperative chemotherapy could effectively reduce the local recurrence rate and improve the progression-free survival time of patients with positive pathologic lymph nodes [29, 30].

Palliative radiotherapy is mainly applied to the treatment of patients with advanced gastric cancer, focusing on reducing bleeding, pain and other symptoms to improve the quality of life of patients. For M1 stage patients, palliative radiotherapy might be used to improve survival of these patients. We further performed analysis on M0 stage patients and removed M1 stage patients. The results (Table S3) were consistent with our previous results in Table 4. We treated the M stage of patients as a covariate in our analysis.

The mechanisms of the association between GLIS2 and radiotherapy are still not clear. According to the report published by Masetti et al. [31], CBFA2T3-GLIS2 is the most frequent chimeric oncogene identified in non-Down's syndrome acute megakaryocytic leukemia (non-DS-AMKL). It regulated molecules involved in the Hedgehog pathway and Wingless/Integrated (WNT)/β-catenin pathways, such as GATA3, HHIP and β-catenin. GATA3 was demonstrated to interact with HIF-1α to enhance cancer cell invasiveness [32], and inhibition of HHIP promoter methylation suppressed human gastric cancer cell proliferation and migration [33], which would affect the treatment and prognosis of patients with gastric cancer. β-catenin regulated cell adhesion to impair DNA repair [34], leading to increased DNA damage and sensitivity of treatment for cancers [35, 36]. GLIS2 could also regulate the interaction between β-catenin and T-cell factor/Lymphoid enhancer factor (TCF/LEF) to affect the activation of cyclin D1, which may have association with poor tumor differentiation and prognosis in gastric cancer [37]. These findings suggested that GLIS2 might be an important gene associated with tumor DNA repair and tumor cell cycle. The changes of tumor DNA repair and tumor cell cycle also associated with another important clinical treatment, the radiotherapy. Therefore, GLIS2 may also involve in molecular response under adjuvant radiotherapy. Mechanisms of GLIS2 and radiosensitivity of gastric cancer require further study.

In the present study, expression levels of GLIS2 gene did not associate with overall survival. However, in the subgroup analysis, we came to conclude that gastric cancer patients with low expression of GLIS2 were supposed to possess high radiosensitivity, while patients with high expression of GLIS2 gene were not sensitive to radiotherapy. Population selectivity of radiotherapy has certain guiding value for treatment of gastric cancer.

In clinical work, it was found that the degree of tumor retraction was significantly different after radiotherapy, mainly because of the large individual differences in radiosensitivity. DNA is the main target of ionizing radiation. Cancer risk is usually associated with changes in DNA repair, cell cycle, or apoptotic pathways [38], which plays important roles in radiosensitivity. Gene mutations, polymorphisms, and epigenetic modifications related to DNA repair function can make radiosensitivity variant [39]. In radiotherapy, the survival time varies greatly due to sensitivity of radiotherapy. If cancer patients can be predicted to exhibit radiotherapy sensitivity or resistance, oncologists and surgeons can then alter the treatment to reduce adverse reactions or improve the efficacy of radiotherapy. CBFA2T3-GLIS2 is an important prognostic factor for patients with non-DS-AMKL [10, 40, 41]. Studies had linked the function of GLIS2 to autosomal

recessive kidney disease and found that GLIS2 was the most common genetic cause of end-stage renal failure [42]. These studies had demonstrated that the GLIS2 gene had important links to cancers. In our research, we concluded that GLIS2 gene might be an effective molecular marker which was independent of tumor clinical indicators and an indicator of prognostic assessment indexes, which was consistent with the conclusion of previous study [18].

Studies on the association between radiosensitivity of gastric cancer and GLIS2 expression had not been reported before. In this study, internal validation strategy was used to make up for the small sample size and the defects of data only from TCGA. The relationship between GLIS2 and radiosensitivity of gastric cancer was explored, which provided a new reference for clinical improvement of the therapeutic effect on gastric cancer, and an important clue for basic research on radiosensitivity of gastric cancer. Furthermore, we should mention that there were some limitations of this study. We used the data only from TCGA. Sample size was small. In addition, there was no external validation study on our results, like real data from clinical study. Although the limitations existed, the present study still provided a potential helpful clue for further study.

Data Availability

The datasets used in the present study are available from The Cancer Genome Atlas database (http://cancergenomec.nih.gov/).

Disclosure

The funding body did not play any roles in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Haitong Sun and Jincheng Gu contributed equally to this work.

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Supplementary Materials

Table S1: basic patient characteristics. Table S2: associations of clinical indicators and GLIS2 expression level with total survival in all data. Table S3: association analysis between radiotherapy and survival under different expressions of GLIS2 for M0 stage patients. Figure S1: the HR values of radiotherapy along with different cutoffs. We chose different quantiles as cutoff to divide high and low expression group for further survival analysis. *(Supplementary Materials)*

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Review Article

Role of Cancer-Associated Fibroblast in Gastric Cancer Progression and Resistance to Treatments

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Although the survival of gastric cancer (GC) patients has gradually improved, the outcomes of advanced GC patients remain unsatisfactory despite standard treatment with conventional chemotherapy or targeted agents. Several studies have shown that cancer-associated fibroblasts (CAFs), a major component of tumor stroma in GC, may have significant roles in GC progression and resistance to treatments. CAFs are a major source of various secreted molecules in the tumor microenvironment, which stimulate cancer cells and other noncancerous components of GC. Surprisingly, these factors could be involved in gastric carcinogenesis. Cytokines, including interleukin-6 and interleukin-11, or growth factors, such as fibroblast growth factor produced from CAFs, can directly activate GC cells and consequently lead to the development of an aggressive phenotype. Galectin-1 or hepatocyte growth factor can be involved in CAF-derived neovascularization in GC. In addition, recent studies showed that CAFs can affect tumor immunity through M2 polarization of tumor-associated macrophages. Finally, the current study aimed to introduce several inhibitory agents and evaluate their suppressive effects on CAFs in patients with GC progression. However, further studies are required to evaluate their safety and select appropriate patients for application in clinical settings.

1. Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and is a major cause of cancer-related mortality [1]. The standard treatment for GC without distant metastasis is resection of the stomach and proper lymph node dissection, and postoperative systemic chemotherapies are recommended for stage II or III [2, 3]. Despite standard treatment for patients with resectable GC, patients with advanced stage GC still show poor prognosis; therefore, the 5-year overall survival rate of patients with stage III was about 20%–40% [4–6]. Meanwhile, the therapeutic option for GC patients with distant metastasis (stage IV) or patients with recurrence after resection is systemic chemotherapy with multidrug regimens, but the outcomes are poor with a reported mean survival time of about 10 months or less [7–10]. Recently, a variety of molecularly targeted agents has been proposed to enhance the survival rate. However, most clinical trials either have not shown a survival benefit, except for trastuzumab and ramucirumab as combined agents with palliative chemotherapy, or are still ongoing [11]. The limited benefit of treatments for GC is increasingly attributed to the tumor stroma, including extracellular matrix (ECM), fibroblasts, immune cells, and microvasculature, as it is well known that GC has a profuse, noncancerous proportion that contributes to GC progression [12–14].

Among these various components in the tumor stroma, cancer-associated fibroblasts (CAFs) have been suggested to play a key role in tumor development [15]. CAFs remarkably influence the tumor microenvironment (TME) via the secretion of cytokines, chemokines, and growth factors [16, 17]. Those secreted proteins enhance cellular migration, alter the metabolism of epithelial tumor cells [18, 19], control the metabolic flexibility of cancer cells [20], and play a significant

role in the development of resistance to therapeutic agents [21, 22]. The function of CAFs in cancer progression has been explored in various *in vitro* experimental models using CAFs isolated from primary human solid tumor tissues [23]. Usually, the cancer cells were cocultured with CAFs, which have shown increased migration, invasion, and survival ability, and tumorigenesis of cancer cells was compared with those cocultured with normal fibroblasts [23–25]. Not only *in vitro* but also *in vivo* experiments have confirmed that CAFs advance tumor growth and promote metastasis of cancer cells when coinjected into murine xenograft models [23, 26]. In spite of these results, the unique makers of CAFs and main contributors according to the type of cancer have not been clarified. There have been several well-established indicators of CAFs, such as platelet-derived growth factor α (PDGRFα), PDGFRβ, and alpha-smooth muscle actin (α-SMA) [15]. However, these markers are typically expressed only in a fraction of fibroblasts within the tumor and are not specific to CAFs. In addition, CAF-derived contributors involved in cancer progression could differ depending on the type of cancer, because they have different carcinogenesis and progression mechanism. Therefore, the mechanisms of communication between CAFs and specific types of cancers need to be investigated.

Based on those characteristics of CAFs in solid cancers, previous studies have shown that the histologic accumulation of CAF in various types of cancers (colon, esophagus, breast, and liver cancer) could be a poor prognostic maker [27–30]. In GC, type IV of traditional Borrmann's classification has a profuse fibrotic stroma showing poor prognosis due to high recurrence in the peritoneum [31–33]. Those clinical studies have implied that CAFs accumulated in GC tissues might enhance the progression and metastasis of GC. In addition, the high throughput gene expression profiling in GC tissues revealed that the tumors with a high expression of "stroma signature" genes contained a high proportion of fibrotic stroma including CAFs and could be a surrogate marker for predicting the prognosis of GC patients [34]. Our previous study also investigated if the accumulation of fibroblast in a specific subtype of GC, signet ring cell carcinoma, was related to the clinical outcomes [35]. In this study, a higher proportion of CAFs, which was evaluated by immunohistochemical staining for α-smooth muscle actin and Masson's trichrome staining for stromal collagen, was significantly related to poor prognosis than a lower proportion of CAFs. Taken together, it would be easily assumed that CAFs have a big impact on the GC progression, due to their direct effects on cancer cells or indirect effects on other ecosystems within malignant tumors. The former could induce the stemness or metastatic potency of cancer cells through paracrine or direct contact, while the latter could control noncancerous microenvironments such as angiogenesis or tumor immunity.

To explore the function of CAFs in malignant tumors through experimental models, CAF should be steadily isolated from bulky tumors. To date, several methods to isolate fibroblasts from GC tissues have been established and isolated fibroblasts were confirmed by expression of specific markers [35–38]. For example, the fresh tissues harvested from patients were immediately moved into a clean bench and were cut into small pieces in a culture dish. After mincing with scalpels, a coverslip was placed over the tissue forming a sandwich. Fibroblasts usually outgrew in a monolayer and were subsequently collected [35]. However, because those cells were not immortalized, most researchers used the fibroblasts with low passage number for subsequent experiments.

We aimed to provide an update on the mechanism of CAF-induced GC progression in the view of tumorigenesis, invasion and metastasis, angiogenesis, and tumor immunity. We also aimed to introduce the potential therapeutic strategies that can target the effect of CAFs on the GC cells.

2. Origin of CAFs Accumulated in GC

Although CAFs are the predominant cell type within the tumor stroma of various solid cancers, the origin of CAFs is not fully understood. Previous studies have suggested some candidates for the origin of CAFs such as fibroblast in normal tissues [63], specific cells around vessels such as pericytes and vascular smooth muscle cells [64], endothelial cells [65], and bone marrow-derived stem cells [66]. In GC, a pericyte was suggested as one of the origins of CAFs [67]. Here, GC cells could secrete exosomes, which could induce the transition of pericytes into CAFs, but this experimental result was not proven in GC patients' samples. Bone marrow-derived stem cells have also been proposed as the origin of CAFs in GC [68]. This result was proven in the tissues harvested from patients with secondary GC who have previously undergone bone marrow transplantation for various hematologic diseases. Another study insisted that CAFs could be induced from normal resident fibroblasts of the stomach by stimulation of TGF-β derived from the scirrhous GC cells [69].

However, in other solid tumors such as those of the head and neck, breast, and pancreas, recent studies show that CAFs contained in one tumor were heterogeneous, presenting different gene expression patterns and a variety of functions [21, 70, 71]. These results imply that CAFs in GC may also include various subtypes that originated from multiple sources, and it would be important to determine which subtype has a crucial role in GC progression. To the best of our knowledge, no study has evaluated the heterogeneity of CAFs in GC; hence, it should be investigated in future studies.

3. Role of CAF in Gastric Carcinogenesis

Gastric carcinogenesis is a very complicated process. Because high-throughput genetic profiling in GC tissues did not reveal driver mutation during gastric carcinogenesis [72], the role of environmental factors such as infection and food could be emphasized [73]. Most studies have proposed that those factors could enhance precancerous inflammation in the gastric mucosa, which can lead to GC [74, 75].

Although the role of CAF during gastric carcinogenesis has been rarely reported, several candidates derived from

fibroblasts have been suggested as the contributors to the occurrence of chemically induced GC in murine models [76, 77]. In one of those models, GC developed in Lewis rats provided with drinking water with N-methyl-N'-nitro-N-nitrosoguanidine [77]. During carcinogenesis in this rat model, SPARC-stained fibroblasts appeared in the interstitial portion of early initiation stage of stomach tumors in the test rat; however, this was not observed in the control rat. These results suggested that SPARC-expressing fibroblasts probably contributed to GC development. Hiroto K et al. studied the role of CAFs on carcinogenesis using N-methyl-N-nitrosourea- (MNU-) induced gastric tumorigenesis mouse model [76]. In this study, compared with normal gastric tissues, IL-6 expression in GC was significantly increased, and IL-6 knockout mouse had a lower incidence of MNU-induced GC than wild-type mice. These results imply that IL-6 induced from CAFs has an important role during gastric carcinogenesis.

4. Role of CAF in GC Invasion and Metastasis

The invasion and metastasis of cancer cells have long been the causes of death and great challenges for GC patients even after undergoing complex clinical treatments [78]. The poor prognosis and low survival rate of GC patients are mainly due to metastasis [45], and almost 60% of GC deaths are due to peritoneal recurrence [50]. However, the specific mechanisms of GC metastasis have not been clarified.

The epithelial-mesenchymal transition (EMT) is a biological process by which epithelial cells lose their cell polarity and cell-cell adhesion, gain migratory and invasive capacity, and become resistant to apoptosis. Moreover, the EMT increases the production of components of ECM and gains the invasive properties to become mesenchymal stem cells, which play an important role in the initiation of metastasis during cancer progression [47]. In GC, high-throughput molecular analysis revealed that the expression of EMT gene signature in primary GC was significantly related to poor prognosis [79]. As described earlier, because CAFs were known to advance tumor cell metastasis and invasion by overexpression of a variety of factors that can enhance EMT phenomenon [39], the exploration of communication mechanism between GC cells and CAFs could be crucial in the field of GC metastasis research.

One study described that the effect of CAFs on increased migration of GC cells was more significant than normal tissue-associated fibroblasts. This study suggested that microRNA-106b is a CAF-specific maker and has a crucial role in the reinforcement of phosphatase and tensin (PTEN) signaling in GC cells [40]. While this study did not indicate the exact communicators between GC cells and CAFs, Wu X et al. [41] showed that GC-derived CAFs secrete significant quantities of IL-6, which can induce EMT phenomenon and increase migration of GC cells through activation of Janus kinase 2/signal transducers and activators of transcription (JAK2/STAT3) pathway in GC cells. In addition, they showed that deprivation of IL-6 by inhibiting the JAK/STAT3 pathway with a specific inhibitor markedly diminishes these phenotypes in GC cells induced by CAFs. Another secreted factor such as fibroblast growth factor 9 (FGF-9) could be suggested as a communicator between GC cells and CAFs [42]. This study showed that the CAFs isolated from GC tissues could secrete FGF-9 into the extracellular area under the regulation of miR-214 and the secreted FGF-9 could induce EMT in GC cells. CAF-derived exosomes could be a key player in the communication between GC cells cultured from scirrhous type GC, which is a subtype of GC with abundant fibrotic stroma [37] because exosomes are cell-derived vesicles containing functional biomolecules that can be transferred to recipient cells [43]. In particular, CD9 is a specific marker of exosomes that originated from CAFs, and CD9 exosomes from CAFs could increase the migration and invasion ability of GC cells.

Taken together, the evidence suggests that CAFs may play a pivotal role in the migration and invasion of GC cells. In addition, other factors such as stromal derived factor 1 (SDR1), CXCL12, and interleukin 11 have been suggested as CAF-derived inducers for migration and invasion of GC [36, 38, 44, 46, 48, 49, 80, 81], and their mechanisms are listed in Table 1. The mechanisms associated with CAF-induced motility of GC cells could be a novel target in the treatment of GC.

5. Role of CAF in Angiogenesis of GC

Pathological angiogenesis is a hallmark of cancer [82]. Growth, invasion, and metastasis of malignant tumors depend on neovascularization that is controlled by proangiogenic and antiangiogenic elements [83, 84]. Past studies have shown the positive correlation between the expression of factors related to tumor angiogenesis and poor clinical outcomes of GC patients [85, 86]. Moreover, antiangiogenic agent, a monoclonal antibody VEGFR2 antagonist, is one of the few targeted agents showing clinical benefit in metastatic GC patients [87]. A plethora of factors have been proposed as contributors to angiogenesis, but major factors should be clarified before administering novel targeted agents to block GC angiogenesis.

Increasing evidence has shown that chemokine secretion by CAFs may support the recruitment of bone marrow-derived angiogenic cells [58]. CAFs may be a major source of angiogenic factors [88]. In GC, galectin-1 [89] and hepatocyte growth factor (HGF) [90] have been proposed as CAF-derived secretory proteins, which contribute to GC angiogenesis. Galectin expression in CAFs was positively related to increased expression of endothelial cell marker, CD31 [89]. Ding X et al. [90] discovered that the phosphorylation of Akt and ERK1/2 was increased in GC cells treated with HGF and cocultured with CAFs. Both Akt inhibitors and ERK1/2 inhibitors reduced the angiogenic and vasculogenic abilities of HGF. However, these results have been confirmed using an *in vitro* angiogenesis assay (tube formation assay). To elucidate the correlation between CAFs and GC angiogenesis, the inhibitory effects of CAF-derived proteins on GC

Table 1: Mechanisms involved in CAF-induced migration or invasion in gastric cancer.

Author	Year	CAFs derived contributor	Regulation of contributor in CAFs	Receptor in cancer cell	Activated signaling pathway in cancer cell	Experimental models	Reference
Yu B et al.	2013	Transgelin (TAGLN)			Metrix metalloproteinase-2 (MMP-2)	Transwell migration Invasion assay Xenograft model	[39]
He XJ et al.	2014	Galectin-1 (Gal-1)		Integrin receptor	Gal-1/Integrin β1 axis	Transwell migration Invasion assay	[40]
Kasashima H et al.	2014	Lysyl oxidase-like 2 (LOXL2)		Integrin receptor	Src/focal adhesion kinase (FAK) pathway	Wound healing assay Invasion assay	[41]
Izumi D et al.	2016	CXCL12		CXCR4	Integrin β1/FAK pathway	Invasion assay Real-time imaging	[42]
Qiao J et al.	2016	Stromal cell derived factor 1 (SDF1; CXCL12)	Serum response factor (SRF)	CXCR4	SDF1-CXCR4 axis	Transwell migration Wound healing assay Invasion assay Xenograft model	[43]
Wang X et al.	2017	Lumican			Integrin β1/FAK pathway	Transwell migration Invasion assay xenograft model	[44]
Wu X et al.	2017	Interleukin-6 (IL-6)		Cell-surface type I cytokine receptor complex	JAK2/STAT3 pathway	Transwell migration Peritoneal xenograft	[45]
Ding X et al.	2018	HGF/IL-6		c-MET/IL-6R	JAK2/STAT3/twist1 pathway	Transwell migration Invasion assay Xenograft model	[46]
Miki Y et al.	2018	CD9-positive exosomes			MMP-2	Wound healing assay Transwell migration	[47]
Suzuki M et al.	2018	TGF-β1				Invasion assay Wound healing assay	[48]
Wang X et al.	2018	IL-11			JAK/STAT3 and MAPK/ERK pathway	Transwell migration Invasion assay	[49]
Wang R et al.	2019	Fibroblast growth factor 9 (FGF9)	Downregulation of miR-214			Transwell migration	[50]

angiogenesis should be investigated in GC animal models for clinical application.

6. Role of CAF in GC Resistance to Chemotherapy

Chemotherapies for GC have shown some clinical effects; however, some patients still show progression and recurrence after chemotherapy in clinical settings, and there are many obstacles to overcome this issue. One of the commonly reported reasons for failed chemotherapy in clinics is the occurrence of drug resistance. Unfortunately, the underlying mechanism of multidrug resistance in GC remains unclear.

Previous studies evaluating chemotherapy resistance have focused on the tumor microenvironment. In particular, cancer cell–ECM interactions, CAF–ECM adhesion, and cytokine or chemokine-mediated signaling pathways have been considered as TME-related resistance to chemotherapy [91, 92]; CAFs may have a major role in those mechanisms. CAFs have been confirmed to regulate chemoresistance by secreting cytokines, including stromal cell-derived factor-1α, IL-6, and IL-7 [93–95], and may also increase intratumoral interstitial fluid pressure, thus indirectly inhibiting the uptake of anticancer drugs [96]. However, the role of CAF in chemoresistance has not been clarified.

Only one study was published describing that CAFs secreting IL-11 could contribute to resistance to combined chemotherapy regimens in GC cells by activating gp130/JAK/STAT3/Bcl signaling pathway [97]. Recently, our group investigated the GC CAF-specific secretory protein involved in chemoresistance [98]. Through the analysis of transcriptome between fibroblasts from paired normal gastric and GC tissues, IL-6 was suggested as a CAF-specific cytokine. In addition, transcriptome data and immunohistochemical staining for GC tissues revealed that IL-6 was usually expressed in the fibrotic stromal cell. CAF-derived IL-6 could induce resistance to 5-FU or cisplatin in various experimental models, such as *in vitro* and *in vivo* xenograft, and tocilizumab, a monoclonal antibody that inhibits the binding of IL-6 to its receptor, effectively suppressed the development of drug resistance. If those results were applied in the clinical setting, it could have prevented the occurrence of chemoresistance in GC patients.

7. Role of CAF in Tumor Immunity of GC

The Cancer Genome Atlas (TCGA) project for GC revealed four molecular subtypes [72]; among them, Epstein–Barr virus- (EBV-) positive and microsatellite instability subtype was associated with high-density tumor-infiltrating lymphocytes and showed a better prognosis compared with other subtypes [99]. Some previous studies have reported that infiltrating immune cells had an effective host immune response against GC cells [100, 101]. Taken together, the tumor escape from immune response could deteriorate the outcome of GC patients; therefore, this mechanism could be a good target to improve the patients' prognosis. However, the exact mechanisms involved remain unknown.

CAFs produce a plethora of cytokines and chemokines potentially contributing to tumor immunity at various stages of cancer progression. The direct or indirect effects of IL-6, IL-8, IL-10, TGF-β, C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 9 (CXCL9), and CXCL10, but not limited to those, on tumor immunity in patients with oral, breast, and pancreatic cancer have been investigated [102–104]. The role of CAFs in the regulation of tumor immunity is seldom reported in GC. However, recent studies show that CAFs were deeply involved in M2 polarization of macrophage suppressing immune clearance [105, 106]. CAFs could induce M2 polarization in tumor-associated macrophage (TAM); it has been well reported that the accumulation of M2 macrophage was significantly related to the poor survival of GC patients [107] and M2 macrophages directly induced invasion and metastasis of GC cells or indirectly reduced immune response within GC tumors. The proportion of CAF in the deep portion of the primary GC is higher than that in the superficial layer, which positively correlates with the increased number of M2 macrophages [105]. Other studies reported that neurooncological ventral antigen 1 (NOVA1), a marker of activated CAFs, was suppressed in GC microenvironment including CAFs, and NOVA1 suppression was significantly correlated with immune dysfunction such as an accumulation of M2 macrophage [106]. Although several secretory proteins such as macrophage colony-stimulating factor (M-CSF) [108], interleukin 33 (IL-33) [109], CCL2, and interleukin 6 (IL-6) [110] have been suggested as stimulators derived from CAFs for M2 macrophage in esophageal and pancreatic cancers, it has never been reported in GC and should be discovered to be applied for clinical setting in the future.

8. CAF-Targeting Agents

As the role of CAFs in the progression of solid tumors becomes clearer, several therapeutic approaches to inhibit the function of CAFs have been suggested as novel agents.

Some of those CAF-targeting agents have been already applied in clinical settings in patients with various malignant or nonmalignant diseases, but they were not used as CAF inhibitors. Nilotinib is an inhibitor of the c-KIT receptor and is effective in the treatment of chronic myeloid leukemia, melanoma, and gastrointestinal stromal tumors [111–113]. Aside from c-KIT receptors, nilotinib also inhibits other receptor tyrosine kinases such as platelet-derived growth factor receptors (PDGF-Rα and PDGF-Rβ) or discoidin domain receptors (DDR1 or DDR2) [52, 114]. A previous study reported that PDGF-R was expressed in CAFs, not in cancer cells [60]. As activated PDGF-R signaling pathway in tumor stroma can increase the proliferation of cancer cells [57] and stimulate GC angiogenesis [61], nilotinib could be used as a potential inhibitor for GCs with a profuse fibrotic stroma [51]. Tocilizumab has been clinically used in several patients with rheumatic disease as an inhibitor

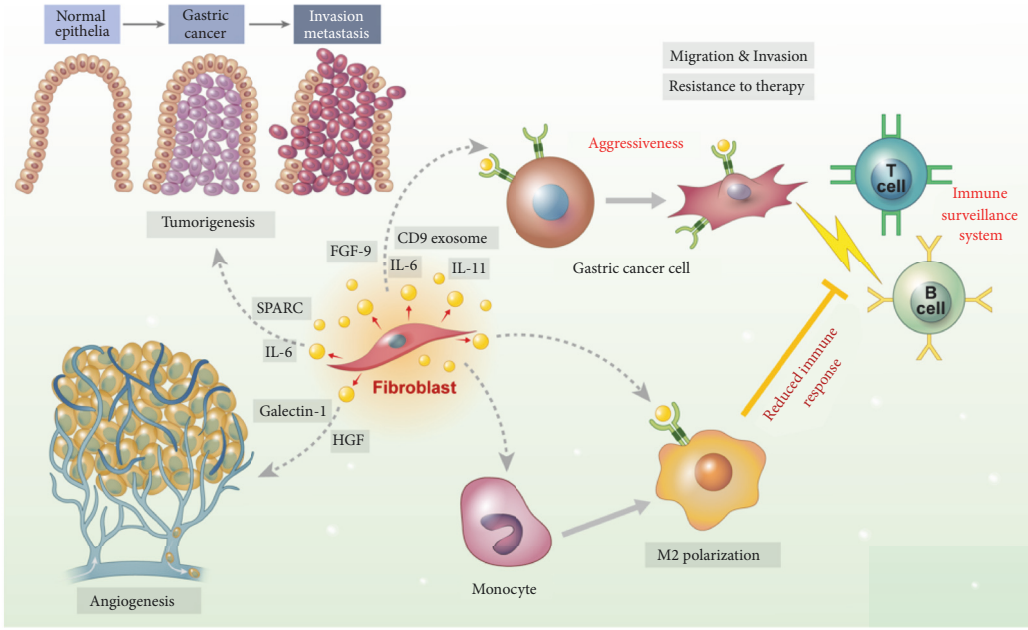

Figure 1: Schematic image for role of cancer-associated fibroblasts in tumor microenvironment of gastric cancer.

of a pleiotropic cytokine such as IL-6. This drug has been proposed as a potential inhibitor of GC cells and CAFs. The *in silico* analysis using TCGA database of GCs revealed that low expression of both *IL-6* and *IL-6R* genes was significantly related to improved survival of GC patients and the *in vivo* experiments described that tocilizumab could efficiently reduce tumor growth in xenograft models of GC cells mixed with CAFs [53].

Several natural products have been proposed as suppressors of CAF activity. Astragaloside IV, the main component of nontoxic Chinese herb, inhibited cellular migration by reducing the ability of CAFs to promote GC cell migration and invasion through regulation of microRNA such as miR-214 in the nontoxic low dose [54]. Paeoniflorin, the principal bioactive component of Radix Paeoniae Rubra, inhibited the secretion of IL-6 from CAFs and consequently inhibited the migration- and invasion-promoting capacities of GC CAFs [55].

Other agents have been evaluated to determine their efficacy in suppressing the migration- and invasion-promoting capacities of GC CAFs through various preclinical models [46, 56, 59, 62, 115–118]; these agents are listed in Table 2. However, future studies are required to determine the toxic effects and indications of those agents.

9. Conclusion and Future Perspectives

Studies have shown that the CAFs are an important component in the TME of GC, and previous studies revealed the potential effects of CAFs including carcinogenesis, metastasis, invasion, angiogenesis, resistance to therapy, and tumor immunity in various GC models (Figure 1). However, the inhibitory mechanism of CAFs on GC cells as well as TME has not been applied in GC treatment. Moreover, the specific markers and origin of CAFs remain controversial. Recent advanced technologies for single-cell transcriptome profiling have uncovered spatial, functional, and genomic heterogeneity of cancer cells and associated host cells in TME [119]. The single-cell RNA-sequencing for lung [120], pancreas [121], and colorectal cancer [122] revealed that CAFs in solid tumors have molecular and functional intra- and interheterogeneity and suggested specific CAF subpopulations as targets for cancer treatment. However, to the best of our knowledge, there has been no report that studies CAFs heterogeneity through the single-cell molecular profiling in GCs. Considering the functional role of CAFs in GCs, further studies evaluating CAF heterogeneity are warranted to determine the critical CAF subtype that expresses specific targets for GC treatment.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Table 2: Agents for inhibition of communication between CAFs and GC cells.

Author	Year	Agents	Origin	Cell lines	Target molecules	Tools	Outcomes	Reference
Yashiro M et al.	2003	Tranilast	Chemical	OCUM-2D	Matrix metalloproteinse-2 Transforming growth factor-beta 1	Invasion assay	Invasiveness	[51]
Onoyama M et al.	2013	Nilotinib with Everolimus	Chemical	TMK-1 MKN-1 KKLS	PDGF-R tyrosine kinase and Mammalian target of rapamycin	Xenograft	Tumor growth Angiogenesis Metastasis Stromal reaction	[52]
Hara M et al.	2016	Itraconazole	Chemical	HT-29 (colon) MKN-28 MKN-45	Mitogen-activated protein kinase Protein S6	Flow cytometry Angiogenesis assay Xenograft	Angiogenesis Tumor growth	[53]
Izumi D et al.	2016	AF-310-NA AMD3100 PF-573,228	Antibody Chemical Chemical	AGS KATOIII	CXCL12 CXCR4 Focal adhesion kinase	Real-time imaging Invasion assay	Migration Invasiveness	[42]
Jin H et al.	2017	7rh	Chemical	MKN-45 MKN-74	Discoidin domain receptor 1	Spheroid culture Xenograft	Tumorigenesis	[54]
Pang T et al.	2017	Fiber-modified hexon-chimeric oncolytic adenovirus	Adenovirus	MKN-45	Fibroblast activation protein (for CAF-specific infection)	Cell viability assay Xenograft	Tumor growth	[55]
Wang Z et al.	2017	Triptonide	Natural products (*Tripterygium wilfordii*)	BGC-823	microRNA-149 ↑ microRNA-301a ↓	Colony formation assay Wound healing assay Invasion assay	Tumorigenesis Migration Invasiveness	[56]
Wang ZF et al.	2017	Astragaloside IV	Natural products (*Astragali Radix*)	BGC-823	microRNA-214 ↑ microRNA-301a ↓	Proliferation assay Wound healing assay Invasion assay	Proliferation Migration Invasiveness	[57]
Ding X et al.	2018	LY294002 U0126	Chemical		AKT ERK 1/2	Angiogenesis assay	Angiogenesis	[58]
Dong R et al.	2018	Polyphyllin I	Chemical	MKN-45	Fibroblast activation protein alpha Hepatocyte growth factor	Proliferation assay Xenograft	Proliferation Tumor growth	[59]
Karakasheva TA et al.	2018	Tocilizumab	Antibody	ESCC EAC	IL-6 receptor alpha	3D culture 3D organotypic culture Xenograft	Tumorigenesis	[60]
Wang ZF et al.	2018	Paeoniflorin	Natural products (*Radix Paeoniae Rubra*)	AGS	microRNA-149 Interleukin 6	Wound healing assay Invasion assay	Migration Invasiveness	[61]
Chen G et al.	2019	Metformin	Chemical		Calmodulin-like protein 3	Proliferation assay	Tumor promotion	[62]

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Review Article

CXCL12 and Its Isoforms: Different Roles in Pancreatic Cancer?

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CXCL12 is a chemokine that acts through CXCR4 and ACKR3 receptors and plays a physiological role in embryogenesis and haematopoiesis. It has an important role also in tumor development, since it is released by stromal cells of tumor microenvironment and alters the behavior of cancer cells. Many studies investigated the roles of CXCL12 in order to understand if it has an anti- or protumor role. In particular, it seems to promote tumor invasion, proliferation, angiogenesis, epithelial to mesenchymal transition (EMT), and metastasis in pancreatic cancer. Nevertheless, some evidence shows opposite functions; therefore research on CXCL12 is still ongoing. These discrepancies could be due to the presence of at least six CXCL12 splicing isoforms, each with different roles. Interestingly, three out of six variants have the highest levels of expression in the pancreas. Here, we report the current knowledge about the functions of this chemokine and then focus on pancreatic cancer. Moreover, we discuss the methods applied in recent studies in order to understand if they took into account the existence of the CXCL12 isoforms.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal gastrointestinal tumors; indeed, it is characterized by poor prognosis and the survival rate is only 8%. Because of asymptomatic clinical course, patients at the moment of diagnosis already present advanced or spread diseased stage. In particular, more than 80% of patients have unresectable carcinomas at the moment of diagnosis. The mutation of oncogene K-Ras, an earliest genetic event, is the first factor which promotes the progression of ductal epithelial cells from a normal state to a neoplastic intraepithelial conformation (pancreatic intraepithelial neoplasia, PanIN) [1–3]. As a consequence of mutations in this protein, several downstream processes are activated, such as proliferation, metabolic reprogramming, antiapoptosis, evasion of the immune response, and remodeling of the microenvironment. PDAC cancer cells are composed of different subpopulations, such as epithelial cancer cells, $CD24^+/CD44^+$ cancer stem cells, $CD133^+$ cancer stem cells, and mesenchymal cancer cells; furthermore, each cell population is genetically heterogeneous. However, to understand the tumor onset, behavior, and drug resistance, it is important to study also the stromal component of PDAC (cancer microenvironment). This component consists of several cell types: cancer-associated fibroblasts (CAFs), T cells, pancreatic stellate cells (PSCs), macrophages, endothelial cells, and others [4, 5].

Cancer cells and surrounding microenvironment exchange signals with each other by releasing signaling molecules [6]. In particular, PSCs produce extracellular matrix molecules (i.e., laminin, fibronectin, and periostin) [7–10] while macrophages release matrix metalloproteinases (e.g., MMP-9) [11]. Both tumor and stromal cells release growth factors, including FGF, EGF, VEGF, HGF, and TGF-β and the inflammatory messenger IL-6. Instead, COX-2 is released by macrophages [11–13]. The chemokines CXCL12, CCL2, and CCL22 are produced by CAFs and macrophages. In particular, CXCL12 is mainly released by CAFs, which represent the 50% of tumor stroma [8]. All these released factors promote the activation of numerous signaling pathways, crucially linked to PDAC development [14]. Among inflammatory cytokines, the CXCL12 chemokine plays an important role in tumor-stroma communication. In PDAC microenvironment, the CAFs activation is induced by molecules released by cancer cells, in particular, IL-6, TGF-β, IL10, PDGF, and FGF [12, 13]. The activated CAFs release

the CXCL12 chemokine, which binds to its two receptors, CXCR4 and ACKR3, highly expressed on the cancer cells surface [8, 15, 16]. This binding allows the activation of numerous signaling pathways in pancreatic cancer cells, such as phospholipase C, MAPK, and PI3K-Akt-mTOR, as well as JAK/STAT pathways. The activation of one or more of these pathways supports the tumor growth and invasion, promotes resistance to drug therapy, provides possible niches for the metastasis development, and protects tumor cells from the host's immune system [17, 18].

Regarding the latter, it is known that CXCL12 exerts a predominant immunosuppression effect by sequestering $CD8^+$ T cells and preventing them from attacking the cancer cells [19]. Moreover, by depleting fibroblast activation protein (FAP)-expressing CAFs, it was possible to attain immune control of the PDAC development and to restore the antitumor effects of anti-CTLA-4 and anti-PD-L1 immune checkpoint antagonists [20–22].

2. Chemokines

Chemokines are a family of low molecular weight proteins (8-14 kDa) involved in the immune system's response. They activate leukocytes and direct their migration from the circulation to an inflammation site. Among chemokines there is high sequence homology (about 20-50%); indeed their tertiary structure stability is due to conserved amino acids, like cysteines, that form the characteristic chemokine "Greek key" (three antiparallel β-pleated sheets are overlaid by a C-terminal α-helix) holding protein structure through two disulphide bonds [23] (Figure 1). Chemokines are synthesized from a propeptide, consisting of different amino acids number based on the protein, which is subsequently removed during the cell secretion of the mature protein. Most chemokines are equipped with four residues of cysteine, two of which are located at the N-terminal end, one in the middle, and one near the C-terminal end. The portion preceding the first β-sheet consists of about 20 residues at the N-terminal end, the motif with the conserved cysteine (CC, CXC, CX3C, or C), an "N-loop", and a single 3_{10} helix. The first disulphide bond occurs between the first conserved cysteine residue and the 30s loop, which is located between the first and the second β-sheet while the second disulphide bond is between the second conserved cysteine residue and the 40s loop, located in the third β-sheet. These disulphide bonds give the structure to the chemokines and the ability to integrate with the receptor [24, 25].

About 45-50 chemokines, all structurally similar, have been identified in humans. They are classified into 4 subfamilies based on the localization of the cysteine residue in the NH2-terminal region. The class of CXC chemokines (α-chemokine) comprises chemokines with two cysteine residues separated by one amino acid. In mammals, 17 different chemokine CXCs were identified; these, in turn, can be divided into two categories: the "ELR-positive" chemokines, with the specific amino acid motif (the ELR motif Glu-Leu-Arg), immediately before the first cysteine, and the "ELR-negative" chemokines which do not present this sequence. In neutrophils, for the interaction between ligand and receptor, this three-amino-acid sequence is very important and is highly conserved in all members of the CXC chemokine family. The subfamily of CC chemokines (β-chemokine) is characterized by the presence of two adjacent conserved cysteine residues; they are also called the CC chemokine ligands (CCLs). Twenty-seven members of this group have been identified. Most of them can contain four cysteine residues; others can also have six cysteines. CC chemokines trigger the movement of monocytes, natural killer cells, and dendritic cells. In the class of C chemokines (γ-chemokines) only two chemokines have been described: XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β). They are different from all other chemokines because they have only one NH2-terminal cysteine residue. The CX3C chemokine (δ-chemokine) subfamily presents two cysteine residues at the NH2-terminal that are separated by three amino acids. Only one chemokine has been discovered, that is, fractalkine (or CX3CL1). It is both released by and bound to the cell that expresses it and acts as both a chemoattractant and a cell adhesion molecule [24–30].

2.1. Chemokine Functional Roles. Chemokines and their receptors play important physiological roles in the human organism. Through a concentration gradient, they act as chemoattractant factor driving the cellular migration. Based on their constitutive or inducible production, they are classified into homeostatic and inflammatory chemokines, respectively. The homeostatic chemokines are produced in the thymus and lymphoid tissues and do not need to be stimulated by external stimuli. Some chemokines control the immune surveillance process directing the leukocyte homing; others play a role in embryogenesis, haematopoiesis, and neurogenesis and promote angiogenesis [31]. Homeostatic chemokines are CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12, and CXCL13. The inflammatory chemokines are released by many different cell types and drive the cells of both adaptive and innate immune system. Inflammatory chemokines are produced in high concentrations during infection or injury and they act as a chemoattractant for leukocytes, recruiting monocytes, neutrophils, and other effector cells from the blood to sites of infection or tissue damage. Their production is stimulated by proinflammatory cytokines like interleukin-1. Typical inflammatory chemokines include CCL2, CCL3, and CCL5; CXCL1, CXCL2, and CXCL8 [29].

2.2. Chemokine Receptors. Chemokine receptors are mainly anchored on the leukocyte surface and, based on their mechanism of action, are divided into two groups: G-protein-coupled receptors (GPCRs), which activate signaling through G proteins, and atypical receptors, acting through the binding with β-arrestin [24]. About 18 chemokine receptors have been identified and are characterized by 7-transmembrane domains. They are classified into the CXCR, CCR, CR, and CX3CR groups based on the respective chemokine family they can bind. However, the interaction between a chemokine and its receptor is not completely exclusive; indeed, each receptor can recognize more than one chemokine type, and a chemokine can bind multiple receptors [29].

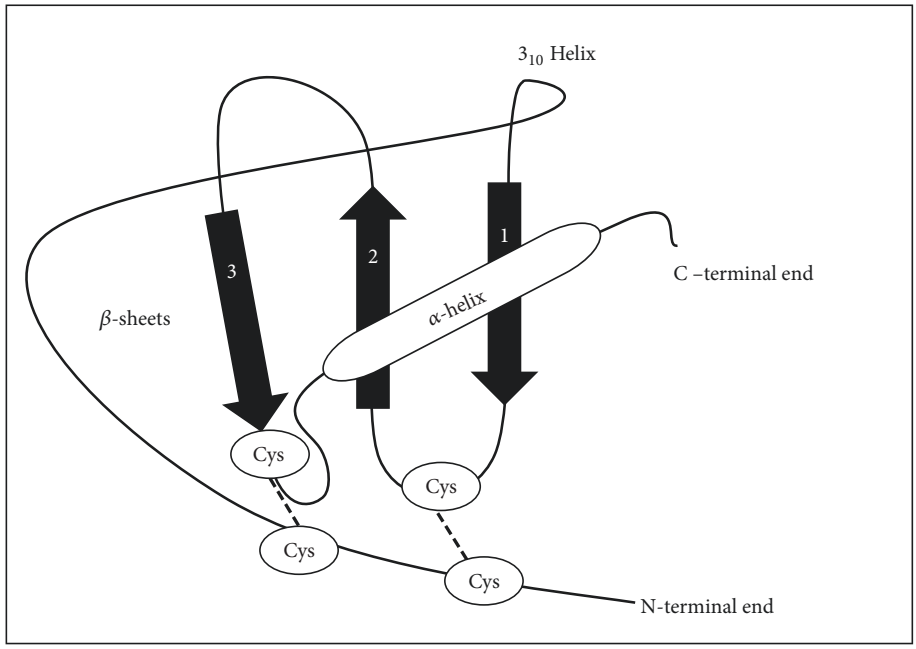

Figure 1: CXC chemokine structure. Chemokine presents three antiparallel β-sheets and a C-terminal α-helix; this tertiary structure is owing to the presence, starting from N-terminal region, of an "N-loop", a single 3_{10} helix, and then the 30s and 40s loops. The two cysteine residues at the N-terminal end allow the formation of disulphide bonds which are fundamental to the chemokine structure and receptor interaction.

Approximately 350 hydrophilic and hydrophobic amino acids constitute these receptors. Going from extracellular to intracellular environment, there are (i) a short N-terminal region that allows the specificity of ligand binding, (ii) seven-transmembrane domains that lead to the formation of 3 extracellular and 3 intracellular loops, and (iii) a C-terminal region suitable for receptor regulation and for G proteins binding that triggers the intracellular signaling after receptor activation [32]. In addition to the C-terminal portion, G proteins can also bind to the receptor through the third intracellular loop [32]. The interaction between chemokines and their receptors induces a receptor conformational change, with consequent signal transduction and, in the end, the cellular responses [24], such as chemotaxis, immune cell migration, and inflammation but also tumor initiation, promotion, and progression [29].

3. CXCL12

CXCL12 (C-X-C motif chemokine ligand 12) is one of the most studied chemokine proteins. It has a key role in both physiological and pathological conditions. Originally, CXCL12 was identified as a pre-B cell growth factor (PBGF), which plays an important role in homeostatic processes. Subsequently, it has actually been discovered that PBGF is constitutively expressed by bone marrow stromal cells, so it has been called factor 1 derived from stromal cells (SDF1) [27, 31]. This homeostatic chemokine plays a constitutive role in physiological processes such as embryogenesis, haematopoiesis, angiogenesis [31, 33, 34], development of cardiovascular and nervous systems [35], regulation of different cell functions like differentiation, distribution, activation, immune synapse formation, effector function, proliferation, and survival in the immune system [33]. In contrast to all these physiological functions, it also plays an inflammatory role in many diseases. It is involved in different pathological processes, such as HIV infection, neovascularization, chronic inflammatory disorders, tumor growth, distant metastasis formation, and chemoresistance [28, 31, 33, 36–38]. In cancer, the binding between CXCL12 and its receptors causes different pathways activation, that, through cancer cells, migration, angiogenesis, and epithelial to mesenchymal transition (EMT) [39], are involved in tumor initiation and progression [36, 40].

Contrarily to common CXC chemokines, whose genes are found on chromosome 4q21, the gene that encodes the protein CXCL12 is found on chromosome 10q11 [28]. Although seven different splicing variants of CXCL12 chemokine have been discovered (α, β, γ, δ, ε, θ, and the predicted isoform iso7), the functional roles of each one are still unknown. Until now, most studies have focused mainly only on three isoforms, i.e., α, β, and γ. Out of four exons in total, the first three are shared with all other splicing variants of CXCL12, but each one differs from the others in the terminal region.

In order to be biologically active, CXCL12, initially secreted as propeptide, is subjected to the proteolytic removal of 21 amino acids present at NH2 terminal end (Figure 2). This monomeric mature form, which has undergone cutting, is unstable at blood level and tends to glycosaminoglycans (GAGs) binding, escaping proteolytic degradation [41, 42]. Three parallel β-strands and an overlying α-helix constitute the ordered structure present among the disordered N- and C-terminal ends. In the mature form of CXCL12, the first 8 amino acids (AA) at the N-terminal play an important role in the interaction with the receptor; in particular, the

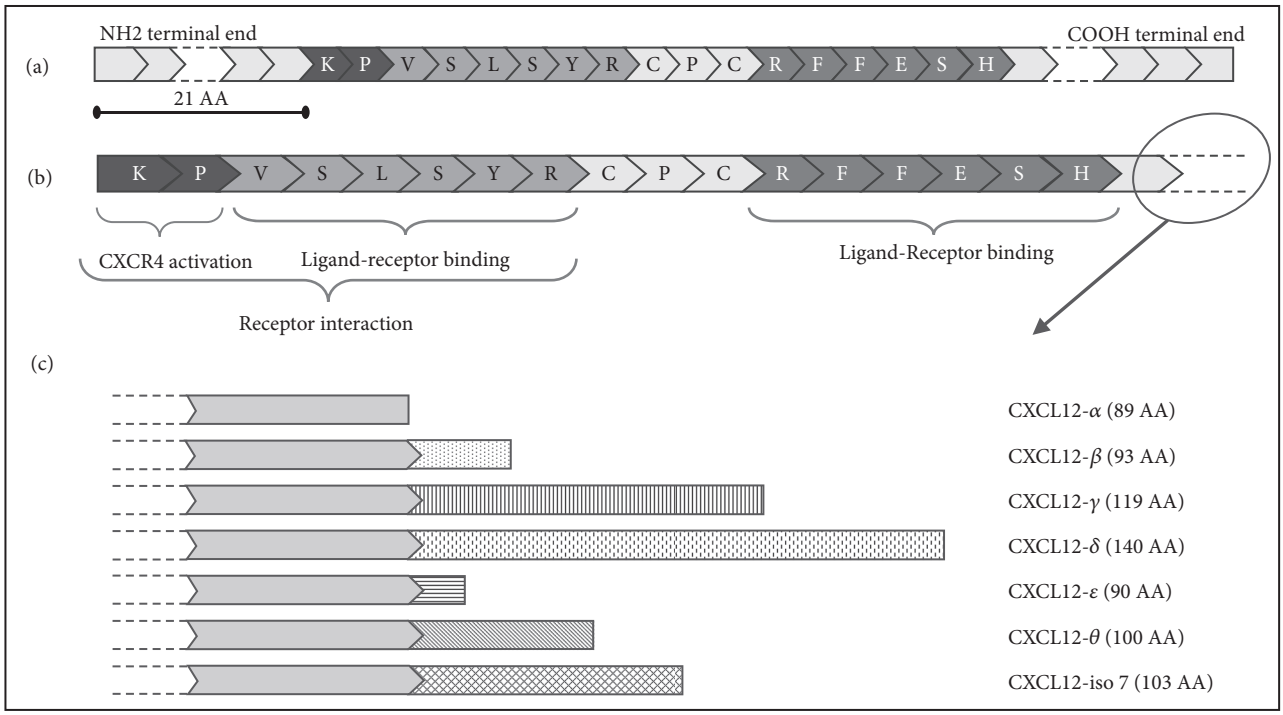

Figure 2: CXCL12 sequence. (a) The CXCL12 immature form, the propeptide, which includes the 21 amino acids at the N-terminal end, that will be removed. (b) The mature CXCL12 form has undergone a proteolytic cut of 21 amino acids at the N-terminal end. The first 8 amino acids of the mature CXCL12 allow the receptor interaction; in particular, the first two, lysine and proline, activate the CXCR4 receptor while the other six are used for the receptor binding. Also, the "RFFESH" sequence allows the ligand-receptor binding. (c) Representations of all CXCL12 isoforms are reported. They all have the same starting sequence, but each one differs from the others in the terminal region length.

first two AAs (Lys-1 and Pro-2) activate CXCR4, while the other six allow ligand-receptor binding [28, 37]. Another element exists in the CXCL12-CXCR4 interaction: the RFFESH sequence. This motif is involved in ligand-receptor binding and, thanks to the structural changes, it allows the N-terminal AA activation of the receptor (Figure 2) [27]. In the stabilization of the receptor binding (between the organized region and disorganized C-terminal one), glycosaminoglycans (GAGs) such as heparin and heparan sulphate play an important role. Thanks to their negative charge, these long polymers of disaccharide units form an extracellular matrix that attracts positively charged chemokines towards itself. This chemokine-GAG interaction is essential for the chemokine gradient generation. The binding to the GAGs protects CXCL12 from NH2-terminal truncation and inactivation [27, 28, 37].

3.1. Expression. CXCL12 is expressed in various human tissues (liver, pancreas, spleen, and heart) by different cells, like stromal cells, fibroblasts, and epithelial and dendritic cells [36, 43–45]. Only blood cells do not seem to express CXCL12. Furthermore, CXCL12 can be secreted in tumor microenvironment by carcinoma-associated fibroblasts and mesenchymal cancer stem cells [37, 46]. The expression and activity of CXCL12 are regulated by three different factors: hypoxia, ACKR3 scavenging, and posttranslational modifications. Hypoxia is a characteristic component of inflammatory stages, that, through its tissue mediator HIF-1 (Hypoxia-Inducible Factor-1), has been shown to induce CXCL12 expression and secretion by fibroblastic and endothelial cells. Thanks to the presence of a HIF-Response Element (HRE) on the CXCL12 promoter, HIF-1 is able to regulate the promoter's transcription activity [47, 48]. The second regulatory factor involves the atypical receptor ACKR3. It can act as a scavenger, influences the chemokines gradient, and decreases inflammation. Indeed, it sequesters and internalizes CXCL12 to allow the activation of downstream pathways or the lysosome ligand degradation [49]. Regarding the posttranslational modifications, which alter the function of CXCL12, they involve both chemical and enzymatic modifications, including NH2-terminal truncation, COOH-terminal truncation, citrullination, and nitration. The NH2-terminal truncation is implemented by serine proteases, in particular, the transmembrane serine protease dipeptidyl peptidases IV (DDP4) and the intracellular serine protease dipeptidyl peptidases VIII (DPP8). DPP4, also called CD26, splits proteins containing an Ala or Pro residuals in the penultimate position of their amino acid sequence. The DPP4 cleavage of CXCL12 induces a loss of calcium-dependent signaling and chemotaxis. These changes lead to a decrease in the binding ability of GAGs (especially heparin) and CXCR4. Moreover, DPP4-truncated CXCL12 is inactivated but still able to bind the CXCR4 receptor, so it acts as an antagonist [31, 50]. Being an intracellular protease, DPP8,

differently from DPP4, can cleave CXCL12 only after ligand internalization or DPP8 release after cell lysis.

Regarding the COOH-terminal truncation, the enzymes involved in this modification are the secreted carboxypeptidase N, the plasma membrane carboxypeptidase M, and the lysosomal Cathepsin X. Due to the presence of the lysine at the C-terminal end, the carboxypeptidase M and N degrade only the α-isoform [37, 42]. CXCL12 degradation occurs in both blood and tissues and does not inactivate CXCL12 but halves its activity and receptor binding affinity. This causes lower receptor binding capability, chemotaxis, cell proliferation, GAGs binding, and a greater predisposition to NH2-terminal truncation. Also, Cathepsin X decreases CXCL12 activity.

The enzyme implicated in the citrullination is the peptidylarginine deiminase (PAD) and involves the hydrolysis of the imine group of Arg into the ketone group, resulting in citrulline (Cit) formation. This switch from Arg to Cit causes an alteration of protein structure and subsequent modification of its interaction with other proteins. The effects on CXCL12 activity are inhibition of receptor binding, signal transduction, and chemotaxis. The CXCL12 citrullination has a greater impact on decreasing CXCR4 activity than ACKR3.

The nitration process takes place by the chemical factor peroxynitrite and leads to the nitration of the residues of Tyr and Trp with the formation of nitrotyrosine. Tyrosine nitration can either increase or decrease protein activity or have no effect on it. The CXCL12 nitration can be induced chemically or it can occur naturally. This nitration, assessed by in vitro studies with lymphocyte and monocyte, results in reduced intracellular calcium mobilization, IP3 accumulation, and ERK1/2 phosphorylation, with the consequent decrease in chemotaxis. It also reduces cellular signaling and migration [27, 31].

Various cell and tissue types secrete CXCL12 whose expression is regulated by enzymatic or lysosomal degradation. CXCL12 has a rather short half-life in the bloodstream, about 30 minutes, due to processes such as degradation by proteases, binding to GAGs, and following sequestration. The above-mentioned processes permit gradient formation in various compartments [34, 42, 46]. It has been demonstrated that enzymes responsible for proteolytic degradation are metalloproteinases (DDP4 and MMP2) and leukocyte elastase (Cathepsin G). Still, there are no data about their roles in physiological in vivo processes [43, 51–54]. Nevertheless, at the cellular level, CXCL12 is digested by lysosome after being internalized by ACKR3 receptor [55, 56].

3.2. Dimerization of CXCL12 Receptors. CXCR4 and ACKR3 can form homo or heterodimers on the cell membrane. Depending on whether CXCL12 binds to its monomeric or dimeric (homo-ether dimer) receptors, different signaling pathways may be activated [33]. In particular, (i) the CXCR4 monomer signaling is preferably mediated by the G proteins which activate the MAPK/PI3K/Akt pathways; (ii) the CXCR4 homodimerization, induced by CXCL12, involves the activation of the JAK/STAT pathway, but it is not known if G proteins or β-arrestin are involved; (iii) the ACKR3 monomer acts as a CXCL12 scavenger and activates ERK 1/2 signaling via β-arrestin; (iv) the CXCR4/ACKR3 heterodimer formation shifts the signaling from G proteins to β-arrestin. By GRK-dependent phosphorylation, β-arrestin signaling may cause the CXCR4 internalization, the CXCL12 scavenging, and/or the activation of the ERK1/2 pathway resulting in cell survival increase.

CXCR4 is internalized and degraded only after CXCL12 binding; instead, ACKR3 is continuously internalized independently of the ligand binding [41, 55] and it is not degraded [41].

3.3. CXCL12 Interactions. Through the interactions with two different CXC chemokine bind receptors, CXCR4 (CD184) and ACKR3 (CXCR7), and through the glycosaminoglycan (GAGs) binding, the chemokine CXCL12 plays a role in physiological and pathological conditions.

3.3.1. CXCR4. CXCR4, also known as cluster of differentiation 184 (CD184), is characterized by seven-transmembrane domains, usually categorized as G-protein-coupled receptor (GPCR). Originally, CXCR4 was identified as leukocyte-derived seven-transmembrane domain receptor (LESTR) for its role as a cofactor in human immunodeficiency virus (HIV) cell entry. Subsequently, its role as the receptor of CXCL12 ligand was discovered [27]. Regarding the extracellular structure, CXCR4 has a different organization compared to normal GPCR receptors. Indeed, this receptor, at the end of helix VII, has two helical turns longer than the other receptors. This extension allows the formation of a disulphide bond between Cys274 and Cys2, and together with the extracellular loop 2 (ECL2, Cys186) and the extracellular end of alpha helix III (Cys109) is essential for binding with CXCL12 [28, 34]. In the last ten years, the interest in this receptor is exponentially increased because many cell types express it, including the cancer cells. In particular, it is expressed in leukocytes, lymphocytes, epithelial and hematopoietic progenitor stem cells, cells of lymphoid organs like the bone marrow, thymus and lymph nodes, lung, small intestine, and nonhematopoietic cells, such as endothelial, epithelial, and stromal cells (fibroblasts). The gene encoding this receptor is located on chromosome 2. CXCR4 has two alternative isoforms: CXCR4-A and CXCR4-B. The CXCR4-B isoform is more expressed and undergoes a splicing process, while the CXCR4-A isoform does not undergo splicing, differs in 5 amino acids, and is four amino acids longer at the NH2 terminal. Functionally, they are both active [27, 31]. Regarding the regulation of CXCR4 expression, it is known that the Nuclear Respiratory Factor 1 (NRF-1) and HIF-1 increase its transcription levels, while the transcriptional suppressor Ying Yang 1 (YY1) inhibits its expression. Numerous molecules induce transcription of CXCR4, such as growth factors and cytokines (BFGF, VEGF, interleukin, and TGF-β), as well as second messengers: calcium and cyclic AMP [28, 34, 57].

3.3.2. CXCL12/CXCR4 Pathway. The CXCR4-mediated signaling occurs mainly by G proteins binding. The CXCL12 binding to CXCR4 extracellular portions induces a conformational change of the receptor tertiary structure that triggers the heterotrimeric G-protein ($G_{\alpha\beta\gamma}$) activation (linked to

the DRYLAIV sequence present in the second intracellular loop of CXCR4) and, in turn, by converting GDP in GTP dissociates into α-subunit (G_α) and $\beta\gamma$-complex ($G_{\beta\gamma}$). After CXCL12/CXCR4 binding, the receptor is internalized and degraded by lysosomes [41]. The α subunits can be $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$. Depending on subunit binding, CXCR4 can activate different signaling pathways, such as phospholipase C, MAPK, and PI3K-Akt-mTOR. The activation of one or more of these pathways results in cellular migration, proliferation, activation of adhesion molecules, and chemotaxis. In tumor, it is correlated with progression, survival, angiogenesis, and metastasis development [27, 34, 38].

CXCR4 also seems to trigger signaling through JAK2/3 binding; in particular, some authors think that JAK2/3 cause intracellular calcium mobilization and chemotaxis, through the kinases transphosphorylation and subsequent CXCR4 phosphorylation, resulting in STAT 1/2/3/5 recruitment and activation. Other authors, based on models of JAK-deficient cell lines, claim that CXCR4 does not use JAK/STAT to activate the downstream pathways [27, 34]. Finally, this receptor can also bind to β-arrestin and induce activation of the p38 MAPK pathway [27, 31].

3.3.3. ACKR3. The second receptor for CXCL12 is the "atypical" chemokine receptor ACKR3, so called because of its different transduction activation method. Indeed, the activation of ACKR3 excludes the common G-protein signaling and occurs through β-arrestin binding. In the past, this receptor was named RDC1; subsequently, the orthologue GNR1 cloned from leukemic pre-B cells was wrongly identified as a polypeptide intestinal receptor. A few years later, RDC1 was classified as calcitonin gene-related peptide (CGRP). Consequently, with the discovery of binding to CXCL12, RDC1 was renamed as CXCR7 and then ACKR3 [58, 59].

Compared to the CXCR4 receptor, CXCL12 appears to bind to the ACKR3 receptor with a 10 times greater binding affinity (apparent $K_D = 0.4 \pm 0.1$ nM for ACKR3, apparent $K_D = 3.6 \pm 1.6$ nM for CXCR4) [60, 61]. In addition to CXCL12, ACKR3 can also recognize the chemokine CXCL11. In human, ACKR3 gene is located on chromosome 2 and it includes a different amino acid sequence (DRYLSIT) from the usual sequence of chemokine receptors (DRYLAIV). ACKR3 is expressed by different cell types, such as hematopoietic cells, activated and vascular endothelial cells, fetal hepatocytes, placenta cells, neuronal progenitor cells, and endothelial cells of tumor tissues. In particular, this receptor is highly expressed on the cell surface of T lymphocytes, during chemotaxis processes mediated by CXCL12. Its expression is associated with the ability of B cells to differentiate into plasma cells following activation [27, 28, 31]. This mechanism translates into several biological aspects such as regulation of the immune response and migration of T cells, stem cells, and neural progenitor cells.

There are contrasting opinions about the specific role of ACKR3 [33]. While some authors think that this receptor has a protumor role, since it induces cell migration and proliferation [55], others recognize an antitumor role (decoy role) of ACKR3 since it can prevent CXCR4-ligand binding by scavenging CXCL12 [56].

3.3.4. CXCL12/ACKR3 Pathway. There is not much data on the CXCL12/ACKR3 pathway. It would seem that by β-arrestin binding, ACKR3 can induce the activation of Akt and its MAP kinases ERK1 and ERK2. Despite the lack of calcium mobilization, following the receptor activation, the β-arrestin pathway is activated and CXCL12 scavenger is obtained. Moreover, the signaling mediated by ACKR3, in addition to the CXCL12 sequestration from the microenvironment, promotes cell migration, survival, and adhesion [27, 28, 31, 58, 59]. Unlike CXCR4, this receptor, following its CXCL12-binding internalization, is not degraded but it is recycled on the plasma membrane [41].

3.3.5. GAGs. The CXCL12 chemokine carries out its functions not only by binding to chemokine receptors but also by interacting with glycosaminoglycan (GAGs), such as heparin and heparan sulphate, which either are attached to the proteins of the cell surface or form the extracellular matrix itself. The negative charges of GAGs interact with the positive charges of the CXCL12 allowing the chemokine gradient formation [27, 31, 34].

4. CXCL12 Isoforms

Until now, six isoforms of CXCL12 have been discovered: α-isoform, β-isoform, γ-isoform, δ-isoform, ε-isoform, and θ-isoform; and the isoform-7 still remains predicted. The CXCL12 is the only CXC chemokine with alternative splicing variants and it is also the only one to be regulated and processed more at the posttranslational level than by transcriptional mechanisms. All seven isoforms share the first 3 exons (1-68 AA) and differ in length for the last exon. The amino acid sequence of this exon confers the specific length of each isoform: 89 AAs for α-isoform, 93 AAs for β-isoform, 119 AAs for γ-isoform, 140 AAs for δ-isoform, 90 AAs for ε-isoform, and 100 AAs for θ- isoform [27, 59] (Table 1, Figures 2 and 3).

Our protein alignment (Figure 3) shows that α, β, and ε isoforms differ from each other in a few AAs. Γ and δ isoforms are the longest; instead, isoform 7 is quite different from all the others.

It is known that the α, β, and γ isoforms differ in binding affinity with the extracellular matrix (ECM); in particular, γ-isoform has a greater affinity than β-isoform, which, in turn, is greater than α-isoform. Due to the binding with the extracellular matrix, CXCL12 is protected from cell degradation process and consequently has a slower tissues release. This mechanism leads to the formation of specific gradients based on the different affinities of isoforms ECM binding [37, 62].

Each variant seems to have different expression and function [59], but we do not know with certainty their specific involvement in physiological processes [43, 63]. They also appear to be involved in tumor development processes such as apoptosis, metabolism, and development of metastases (Figure 4) [33].

4.1. CXCL12 α-Isoform. Among the variants of CXCL12, the α-isoform is the most studied. This variant is not present in

Figure 3: Protein multialignment of all CXCL12 isoforms.

Table 1: CXCL12 sequence information. The gene CXCL12 (alias SDF1, NCBI Gene ID: 6387) produces 7 splicing isoforms. Data about different CXCL12 sequence variants are reported. Note that, according to NCBI Ref Seq, the transcript variant 5 corresponds to protein isoform 7.

ISOFORM	UNIPROT ID	LENGTH (WEIGHT)	REFSEQ ID
α	P48061-2	89AA (10,103 KDa)	NP_954637.1, NM_199168.3
β	P48061-1	93AA (10,666 KDa)	NP_000600.1, NM_000609.6
γ	P48061-3	119AA (13,705 KDa)	NP_001029058.1, NM_001033886.2
δ	P48061-4	140AA (15,495 KDa)	NP_001171605.1, NM_001178134.1
ε	P48061-5	90AA (10,192 KDa)	/
θ	P48061-6	100AA (11,395 KDa)	/
7	P48061-7	103AA (11,004 KDa)	NP_001264919.1, NM_001277990.1

the blood due to enzymatic degradation; instead, it is highly expressed in tissues, more in adult than in fetal ones [27, 37]. In particular, it is expressed in bone marrow, pancreas, liver, lungs, spleen, heart, lymph nodes, and thymus and has also been found in skin, small intestine, and neurons [34, 38]. Its amino terminal region makes it a specific ligand for CXCR4 and ACKR3 for the promotion of angiogenesis [28]. CXCL12-α can induce, in vitro, an increase in the survival rate of hematopoietic progenitor cells. Depending on the tissue expressing it, this isoform is able to manage hematopoietic stem cells in the bone marrow, to guide germinal cells during development, and to induce neurostimulation of the central nervous system. In breast cancer, low expression of CXCL12-α corresponded with worse metastasis-free survival [46].

4.2. CXCL12 β-Isoform. The β-isoform, despite functional similarities with the α-isoform, is particularly correlated with the vascular system. Thanks to differences in its fourth exon, this isoform is not degraded by blood carboxypeptidase N. Indeed, the sequence of this variant includes five additional residues at the C-terminal region, which contain one motif for HS (heparan sulphate) binding [64]. Like the α-isoform, it acts as a specific ligand for CXCR4 and ACKR3. It is highly expressed in vascularized organs like spleen, liver, bone marrow, pancreas, and kidney [37], in the endothelial cells of brain microvessels [38] and also in fetal tissues like liver and lung [27]. Unlike CXCL12-α, CXCL12-β promotes angiogenesis, as observed in vitro by the endothelial tube formation assay [65]. In bladder cancer, the mRNA levels of CXCL12-β are associated with poor prognosis and are potential predictor of metastasis and future recurrence [66]. In breast cancer, low levels of CXCL12-β correlated with worse metastasis-free survival and recurrence-free survival [46].

4.3. CXCL12 γ-Isoform. Thanks to its C-terminal end binding site, this isoform is the variant with the highest GAGs binding affinity and it is able to escape inactivation by proteolytic enzymes. CXCL12-γ is not expressed in fetal tissues but highly

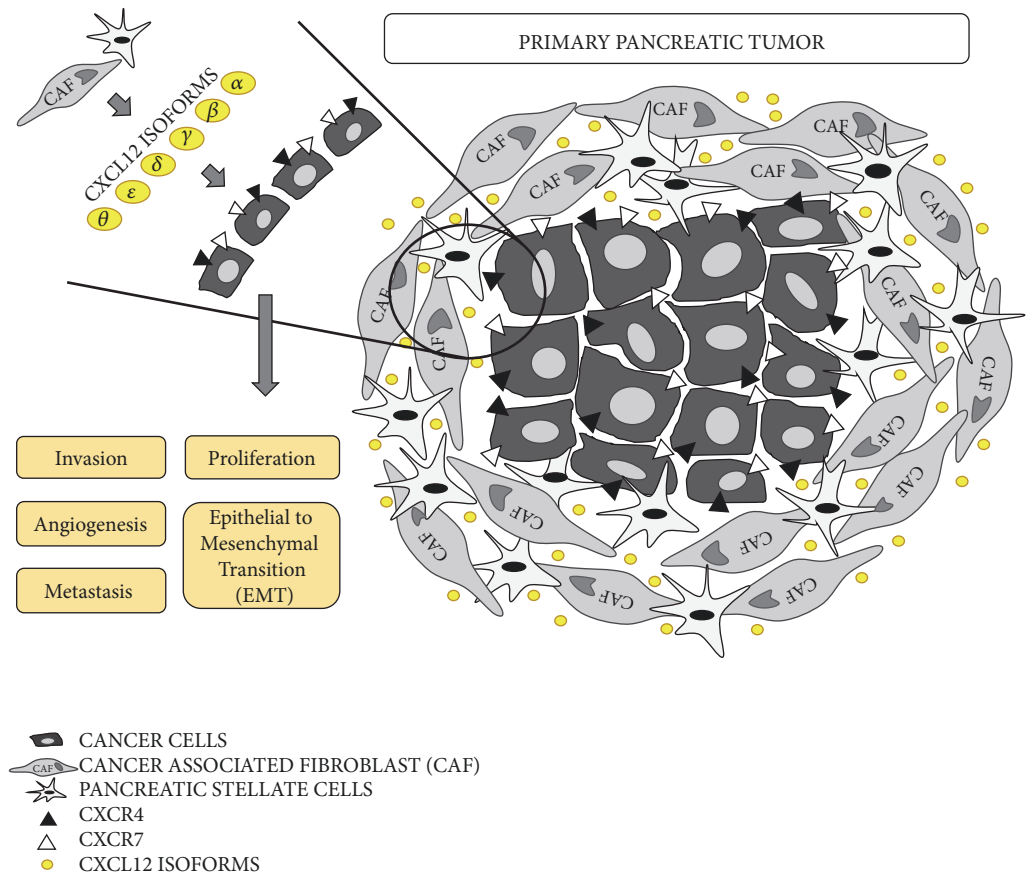

Figure 4: Primary pancreatic tumor microenvironment. PDAC stromal cells, like cancer-associated fibroblasts (CAFs) and cancer stellate cells, release the different CXCL12 isoforms in the tumor microenvironment. Cancer cells, which expressed the CXCL12-receptor on their surface, can bind the ligand. This ligand-receptor binding can cause tumor invasion, cellular proliferation, angiogenesis, epithelial to mesenchymal transition (EMT), and metastasis.

expressed and active in less vascularized organs, like heart and brain [27, 37, 59]. Once secreted, it binds immediately to the cell surface, reducing its presence as a free form [28]. Functionally, it is able to induce a weak in vitro chemotaxis, while in vivo it is the most active isoform which stimulates chemotaxis [27]. CXCL12-γ, thanks to its stable binding interaction, is characterized by a greater long-term effect than the α-isoform; indeed, in mice injected with both isoforms, it produces an inflammatory reaction 5 times longer [37]. As demonstrated in vitro by the endothelial tube formation assay, CXCL12-γ drives angiogenesis similarly to CXCL12-β [65]. In colorectal cancer, CXCL12-γ is positively associated with tumor size [44]. In prostate cancer, CXCL12-γ plays a key role in induction of cancer stem cell and neuroendocrine phenotypes, which are known to promote tumor growth, metastasis, chemoresistance, and the progression to the metastatic castration resistant prostate cancer [67]. In breast cancer, higher levels of CXCL12-γ correlate with improved metastasis-free survival and recurrence-free survival [46]. Unlike the other isoforms, CXCL12-γ significantly increased the breast cancer metastatic tropism for bone marrow [65].

4.4. The Other CXCL12 Isoforms. For about ten years, three additional isoforms of CXCL12 have been identified but their specific role has not yet been established. The highest levels of expression of each variant were found in the pancreas [27, 63]. The δ-isoform is also expressed in the liver, spleen, and lungs [37], while the isoforms ε and θ have also been found in the heart, kidneys, and liver [63]. In breast cancer, higher expression of CXCL12-δ correlates with better overall survival [46].

5. CXCL12 and Pancreatic Cancer

Numerous studies are trying to shed light upon the tumor roles of CXCL12, such as its effects at cellular levels and interactions with CXCR4 and ACKR3 receptors [68, 69]. The CXCL12/CXCR4 axis seems to play an important role in the processes of invasion, proliferation, migration, metastasis, and angiogenesis in pancreatic cancer (Figure 4) [17, 18].

Indeed, both pancreatic cancer cells and tissues highly expressed CXCR4 and ACKR3 receptors on their surface, which are activated by CAFs-released CXCL12 chemokine [70, 71]. In particular, according to immunohistochemistry data, 56.7% of pancreatic cancer tissues, 50.0% of para-cancerous tissues, and 53.3% of pancreas surrounding lymph nodes express CXCR4 compared to 18,3% of the normal pancreatic tissues [72]. Another study reported a positive

CXCR4 expression in 80% of cancerous tissues, 70% of para-cancerous tissues, and 73.3% of lymph nodes compared to 26.7% of the normal pancreata [68]. Moreover, 73% of human pancreatic cancer tissues express both CXCR4 and ACKR3 [73]. Interestingly, pancreatic stellate cells isolated from pancreatic cancer tissues do not express CXCR4 [74, 75].

Regarding CXCL12 expression in normal pancreas, only ductal epithelial cells expressed CXCL12, whereas acinar and endocrine cells do not express it [71, 76]. In pancreatic cancer, the CXCL12-α isoform was moderately or strongly expressed at the protein level in primary pancreatic stellate cells isolated from PDAC tissues [74]. Moreover, ELISA assay revealed release of CXCL12-α from fibroblasts but not from pancreatic cancer cells [17]. However, in this paper, the "α-isoform" term is reported only in the title and keywords, but since the product identifier of the used R&D ELISA kit is not reported and R&D ELISA kits exist for total CXCL12 or for both α and β simultaneously, probably referring to the "α-isoform" is inaccurate.

In pancreatic cancer tissues, the expression of CXCL12-α isoform, assessed by western blot, was higher than adjacent tissues [77]. In addition, as seen by immunohistochemistry, 45.3% of PDAC tissues expressed CXCL12 protein and it was correlated with histological grades of disease severity [68]. On the contrary, there is other evidence that CXCL12 protein was frequently expressed in normal tissues (56.7%), in para-cancerous diseases tissues (46.7%), and in pancreas surrounding lymph nodes (50%); instead, only 13.3% of tumor tissues expressed it. However, it is not specified to which isoforms authors refer in these papers [78]. Regarding the last two works, we assume that data referred to the total CXCL12 but we cannot be sure since the product identifiers of the used antibodies are not reported in the Methods section.

Also in pancreatic cancer it has been shown that the interaction between tumor and surrounding stroma, mediated by the CXCL12/CXCR4 axis, influences the tumor growth and its aggressiveness. In particular, by administering the culture medium of pancreatic stellate cells (PSCs) to AsPC-1, BxPC3, and SW1990 cancer cells, an increase in proliferation, migration, and invasion due to activation of CXCL12-α/CXCR4 axis was observed [75]. In another study, it was reported that pancreatic stellate cells, which produced CXCL12-α chemokine, cause an increase in tumor growth [74].

Interestingly, chemoresistance was observed in pancreatic cancer cells treated with CXCL12-α and, subsequently, with mTOR-targeted therapies or gemcitabine. For example, it was shown that the activation of CXCR4 by CXCL12-α isoform in the HS766T cell line promotes chemoresistance to mTOR inhibitor temsirolimus [79]. Additionally, PSCs produced CXCL12-α which inhibited gemcitabine mediated apoptosis of pancreatic cancer cells through an IL-6 autocrine loop [74]. Furthermore, an interesting work showed that CXCL12 RNA expression and protein secretion levels were increased when fibroblasts were cocultured with gemcitabine-treated pancreatic cancer cells resistant to gemcitabine. On the other hand, gemcitabine exposure of these cancer cells induced the increase of CXCR4 protein expression. The strengthening of the CXCL12/CXCR4 axis caused enhanced invasive behavior and in vivo tumorigenicity [80]. Unfortunately, it is not clear which isoform the authors investigated, since the RNA expression data were obtained using a TaqMan probe which covers all CXCL12 isoforms. Regarding immunohistochemistry assays, the product identifier of used antibody is not reported. Probably, they investigated the CXCL12 α-isoform, but the "anti-SDF-1α" word appears only in the Methods section.

In pancreatic cancer, it has been demonstrated that the chemokine CXCL12 could play both protumor and antitumor roles. Several in vitro studies have investigated the CXCL12 roles by administering it to PDAC cell lines. The treatment of Panc-1 and SW-1990 cell lines with CXCL12-α isoform showed the upregulation of the matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9). Mechanistically, MMP-2 upregulation was partially mediated by p38 in Panc-1 cells [77]. The observed expression modification of MMP protein family members was also studied in MiaPaCa2 cell lines. The administration of CXCL12 to this cell line promoted proliferation and invasion through the expression of MMP-2, MMP-9, and uPA (urokinase Plasminogen Activator) proteins [70]. However, in this paper, it is not clear which isoform was used since the catalogue number of the CXCL12 recombinant protein is not reported and, at present, the company that supplied this product sells both α and β isoforms. In vitro studies went further to explore other potential pathways activated upon CXCL12 treatments. In particular, it was shown that CXCL12 administration on Panc-1 cells increased ERK and Akt phosphorylation, enhancing cell proliferation [81]. The treatment with CXCL12 of MiaPaCa2, HPAF, and ASPC1 cell lines also caused Akt and ERK activation and the consequent phosphorylation and destabilization of the NF-κB inhibitory protein, IκB-α. This caused the nuclear accumulation of NF-κB, its binding to SHH (Sonic Hedgehog Homolog) gene promoter, and the consequent expression and release of SHH [82]. Unfortunately, it is not declared which CXCL12 isoform was used in the last two papers. However, these mechanisms are worth further investigating. Indeed, SHH has an important role in the tumor microenvironment as shown in hTERT-HPNE cells, a normal pancreatic cell line, where this ligand induced desmoplasia [83].

Interestingly, the administration of CXCL12 in pancreatic cancer cells showed also the activation of signaling pathways mediated by ACKR3, which, therefore, has not only a scavenger role for CXCL12. Indeed, ACKR3 stimulation by CXCL12 in Panc-1 cell lines leads to ERK1/2 phosphorylation through β-arrestin-2 without increasing K-Ras activity unlike CXCR4 stimulation [73]. In this experiment, the catalogue number of CXCL12 protein is not reported, so it is not clear which isoform was used.

In PDAC, other molecules connected to the CXCL12/CXCR4/ACKR3 axis have been discovered. For example, in murine models, it was shown that the loss of the tumor suppressor KLF10 (Kruppel-like factor 10) induced metastases in PDAC mouse models through the activation of CXCL12/CXCR4 pathway [84]. In this work, both an antibody detecting all CXCL12 isoforms and the Mouse Cytokine Array able to detect only the α-isoform were used. Moreover, in stellate cells obtained from primary

pancreatic cancer tissues, the protein Galectin-1 is involved in stimulating the production of CXCL12 through the activation of NF-κB [16]. In this study, different techniques have been applied to investigate different CXCL12 isoforms. In particular, a specific ELISA kit was used for the CXCL12 α-isoform; an antibody recognizing all isoforms was used for immunohistochemically staining; RT-qPCR primers detecting both the α and δ isoforms were used for CXCL12 mRNA quantification.

A recent study proposed an additional mechanism for PDAC neural invasion mediated by CXCL12 [71]. It was already known that cancer cells express chemokine receptors and therefore they are attracted by the chemokines released from the nerves. Through the nerves, cancer cells disseminate and give rise to metastases. Recently, it was shown that precancerous cells release chemokines in order to attract Schwann cells (SC) from the nerve and induce tumor dissemination in early carcinogenesis. In particular, in vitro and in vivo studies in murine models have shown that PDAC cells, by the release of CXCL12-α, attract SC cells and determine a decrease in pain sensation given by alteration of SC, spinal astrocytes, and microglia molecular pain pathways [71].

Most of the studies have assigned a protumoral role to CXCL12 in several cancer types, but a study demonstrated that CXCL12 could play also an antitumor role in pancreatic cancer. Indeed, the stable CXCL12 gene reexpression in MiaPaCa2 cancer cells, which is usually epigenetically silenced, caused a significant decrease in tumor growth and migration. In particular, the cell cycle was arrested, and the migration and liver metastases development were reduced. These factors led to tumor growth decrease, both in vivo and in vitro, and to a survival rate increase [76]. Unfortunately, it is unclear whether all the CXCL12 isoforms or only some of them play this antitumor role. Indeed, according to the manufacturer, the antibody used for sandwich ELISA recognizes both CXCL12 α and β isoforms, but probably its cross-reactivity with the other isoforms was not assessed.

Overall, very little is known about the functional differences among the CXCL12 isoforms, especially in PDAC. The dissection of their specific functions would allow a deeper understanding of the PDAC carcinogenesis and progression mechanisms. Moreover, CXCL12 isoforms could represent new prognostic and predictive biomarkers for several cancers. For example, they could be dosed for a better patient staging, prognosis, and prediction of the metastatic potential and tropism. Regarding the latter, it is known that CXCL12-γ levels are associated with metastatic tropism for bone marrow [65]. In preclinical studies and clinical trials, the inhibition of the CXCR4 receptor by, for example, the CXCR4 antagonists AMD3100 and TN14003 and the CXCL12 analogue CTCE-9908, showed promising antitumor effects in different cancers, including pancreatic cancer [40, 85]. Besides anti-CXCL12 antibodies, the only molecule neutralizing all isoforms of CXCL12 is the L-RNA aptamer NOX-A12 [85], which is currently under evaluation in clinical trials as anticancer agent in chronic lymphoblastic leukemia, multiple myeloma, and metastatic colorectal and pancreatic cancer with liver metastasis (source: Clinicaltrials.gov). However, since some molecular differences among CXCL12 isoforms exist, once the specific functions of each isoform in tumor development and progression are clarified, it will be necessary to design CXCL12 isoform-specific therapies.

6. Conclusions

In conclusion, data show that CXCL12/CXCR4/ACKR3 axis is involved in keeping the communication between pancreatic cancer and its microenvironment. Most of the results attribute a protumor role to CXCL12, but it could also have an opposite role. Actually, there are six CXCL12 isoforms, and a seventh predicted variant has been identified, so it is possible that they have different effects. Although many studies have investigated the role of this chemokine, they have not clarified the functions of each isoform in pancreatic cancer yet. This is, in part, because it is not always clear which isoform was investigated and, in part, because the recombinant δ, ε, θ isoforms are still not available. Therefore, further studies would be useful to evaluate the specific role of each variant related to pancreatic cancer. The knowledge of these mechanisms could suggest novel strategies to treat PDAC; indeed, it could emerge that some CXCL12 isoforms should be blocked or administered. However, future studies are necessary to establish the optimal stage for this intervention.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Immunomodulatory Effect after Irreversible Electroporation in Patients with Locally Advanced Pancreatic Cancer

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Purpose. Irreversible electroporation (IRE) has been demonstrated to be a safe and effective method for locally advanced pancreatic cancer (LAPC). The aim of this study was to evaluate the immunomodulatory effect after IRE and to evaluate the prognostic value of variations of the immune parameters in LAPC patients after IRE. *Methods.* Peripheral blood samples of 34 patients were obtained preoperatively and on the third day (D3) and seventh day (D7) after IRE, respectively. The phenotypes of lymphocytes were analyzed by flow cytometry, and dynamic changes of serum levels of cytokines, complement, and immunoglobulin were assayed by enzyme-linked immunosorbent assay. Receiver operating characteristic (ROC) curve and concordance index (C-index) were used to compare the survival predictive ability. *Results.* There was a transitory decrease followed by a steady increase for $CD4^+$ T cell, $CD8^+$ T cell, NK cell, IL-2, C3, C4, and IgG while a reverse trend was detected for Treg cell, IL-6, and IL10 after IRE. The alteration of $CD8^+$ T cell between D3 and D7 was identified as a prognostic factor for both overall survival (OS) and progression-free survival (PFS). The values of ROC curve (AUC) and C-indexes of the alteration of $CD8^+$ T cell for OS and PFS were 0.816 and 0.773 and 0.816 and 0.639, respectively, which were larger than those of other immune or inflammation-based indexes. *Conclusions.* This study presented the first evidence of IRE-based immunomodulatory in patients with LAPC. The alteration of $CD8^+$ T cell between D3 and D7 showed relatively good performance and could be used as an effective tool for prognostic evaluation for LAPC patients after IRE.

1. Introduction

Pancreatic adenocarcinoma is a lethal disease with extremely poor prognosis, which also represented the seventh and sixth leading causes of cancer-related death in the world and in China, respectively. The 5-year survival rate is only 5% [1, 2]. Surgical resection is the only chance to obtain curative treatment while it is only suitable for less than 20% of patients with this disease [3]. Approximately 40% of new cases are diagnosed with locally advanced pancreatic cancer (LAPC), which is characterized by the involvement of major vascular structures, such as celiac trunk, superior mesenteric artery, leading to unresectable but nonmetastatic diseases [4]. Currently, the treatment for LAPC remains a huge challenge due to the poor prognoses of this disease. Limited responses and little impact on survival or life were achieved after the standard treatments, which was mainly systemic chemotherapy [5, 6]. Moreover, the high rates of adverse events due to the toxicity of chemotherapy limited the use and promotion of treatment, such as the combination chemotherapy of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX), even though it was shown to display some progression in improving the survival of patients with LAPC [7–9]. Therefore, it is necessary to evaluate new treatment to optimize common therapeutic approaches. Nowadays, local therapies were shown to improve the prognosis of LAPC patients with varying degrees of success [10].

Irreversible electroporation (IRE), a nonthermal ablation technique, is established as a local ablative therapy for patients with LAPC with promising outcomes of increasing overall survival (OS) from 12 months to 25 months [11]. It is a novel local destructive method based on the transmission of high voltage currents through the tumor via needles, leading to cell membrane defects and apoptotic death [12, 13]. Additionally, during the induction process of apoptotic death by IRE, the structure and composition of the tumor microenvironment are changed, inducing an intense inflammatory cell response, which is characterized by the infiltration of immune cells [14]. It was shown that this IRE-induced immunomodulatory was not only limited to the ablated areas, but also a systemic reaction [15]. Thus, IRE could be regarded as a potential immunomodulatory treatment and might induce extensive changes of immune cells or indexes after ablation.

So far, data is rare on the predictive factors of IRE outcome in patients with LAPC. For this novel and powerful treatment of LAPC, further prognostic markers are urgently needed to choose patients with relatively better prognosis. Moreover, early information of the efficacy of treatment during the first days after IRE would be highly appreciated as therapy may be intensified by other treatments, such as immune therapy, chemotherapy, and radiotherapy, while the regular evaluation of therapy by imaging is only done about 1 month after IRE treatment. For the candidates of predictive factors, circulating biochemical markers may be the promising ones, for their relationship with cancer disease, the immediate therapy effect, and the immunological response of the organism to treatment. More importantly, as failures were achieved for the immune-checkpoint therapies in pancreatic cancer due to the low rates of neoantigen expression and mutation events [16], exploring the alterations and evaluating the prognostic effect of immune cells and indexes might open the prospect of using immune-checkpoint therapies in patients with LAPC.

Here, immunomodulatory effect of IRE was examined by analyzing alterations of several immune cells and indexes in patients with LAPC. We aimed to evaluate the response to IRE therapy during the early treatment phase and identify their role in prognosis.

2. Materials and Methods

2.1. Patients. This study was retrospectively designed. Consecutive patients who were newly diagnosed with LAPC at Sun Yat-sen University Cancer Center between August 2015 and August 2017 were included in this study. The inclusion criteria were as follows: (1) pathologically confirmed pancreatic adenocarcinoma and radiologically confirmed LAPC. LAPC was defined per the seventh edition of the AJCC staging system for pancreatic cancer, which describes LAPC as arterial encasement of either the celiac axis or superior mesenteric artery or unreconstructable superior mesenteric or portal vein involvement, with no evidence of metastatic disease from abdominal and thoracic computed tomography [17, 18]; (2) IRE therapy as the initial treatment. A total of 11 patients were excluded based on the following exclusion criteria: (1) other treatments, including surgical resection and RFA before IRE (seven patients); (2) existing metastatic implants before IRE (one patient); (3) heart arrhythmia and a history of second primary malignant tumors (one patient); (4) missing information of parameters or lost to follow-up (two patients). This study was approved by the Institutional Review Board of Sun Yat-sen University Cancer Center. All procedures performed in present study involving human participants were in accordance with the ethical standards of institutional and/or national research committees and the 1964 Helsinki Declaration and its later amendments or similar ethical standards. Written informed consent was obtained from patients prior to treatment.

2.2. Clinical Data Collection. The following clinical and radiological data were retrieved from medical record archived at Sun Yat-sen University Cancer Center, including age, gender, tumor size, tumor grade, tumor site, white blood cell (WBC) count, platelet (PLT) count, serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), albumin (ALB), total bilirubin (TBIL), indirect bilirubin (IBIL), C-reactive protein (CRP), carcinoembryonic antigen (CEA), and carbohydrate antigen 19-9 (CA19-9). The inflammation-based indexes, including neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), prognostic index (PI), and modified Glasgow Prognostic Score (mGPS), were also entered into this study. The thresholds for the clinical or radiological variables were used as the cutoff values. With the cutoff value of 1.47 and 165.29, NLR and PLR were associated with the optimal Youden indexes for OS and progression-free survival (PFS) prediction, respectively. The defined score of other inflammation-based indexes, such as PI and mGPS, had been described in previous studies [19].

2.3. Treatment Procedure. The NanoKnife IRE equipment from Angiodynamics System (Queensbury, NY, USA) was used. General anesthesia with deep neuromuscular block was adopted. To create an electric field around the tumor, 3 to 6 probes were used according to the size and location of the tumor. Ultrasound was used to guide the placement of all probes, and adequate space between probes was then confirmed. The generator unit software was used to analyze the probe configuration data of the ultrasound and provided optimal voltage and pulse length delivery. If the tumor size was larger than 1.5 cm in the axial plane, a pull-back technique with the same procedure was performed to cover the entire area of ablation.

2.4. Sample Collection. All blood samples were collected before the hypothesis of this study was known. The blood samples were collected using Na-heparin plasma tubes from enrolled patients before IRE (preOP) and then on days 3 (D3) and 7 (D7) after IRE. Isolation of peripheral blood mononuclear cells (PBMCs) was processed immediately using Hypaque-Ficoll (Promega) and frozen in liquid nitrogen in 5% (v/v) plus 95% (v/v) autologous serum [20].

2.5. Flow Cytometry Analysis. Frozen PBMCs were thawed in a 37°C water bath and then cultured overnight at 37°C in RPMI-1640 (Gibco BRL) supplemented with 5% human AB type serum and labeled with FITC-, APC-, and/or PE-conjugated murine anti-human monoclonal antibodies. The CD3, CD3CD4, CD3CD8, CD3CD16CD56, and CD4CD25 phenotype of lymphocytes were sequentially analyzed by flow cytometry (FACS caliber, 4 color system, BD Bioscience, CA, US).

2.6. Assays of Immune Parameters. The quantitative sandwich enzyme immunoassay technique (ELISA kit, R&D system, Minneapolis, MN) was adopted to measure serum concentrations of cytokines, including IL-2, IL6, IL-10, interferon-γ (IFN-γ), and tumor-necrosis factor (TNF). During the procedure of measure, 50 to 100 μl of assay diluent was added to the 96-well polystyrene microplate, which was precoated with murine monoclonal antibody against IL-2, IL-6, IL-10, IFN-γ, and TNF. Serum samples were incubated at 37°C for 2 hours and then the plates were aspired and washed three times. Same incubation was repeated after 200 micoliters of conjugate was added. Then, plates were incubated at 37°C for 20 to 30 minutes after 200 μl of substrate solution was added. Finally, 50 μl of stop solution was added to the plates. A microplate reader (ClinicalBio 128c, Austria) was used to read optimal density (OD) within 30 minutes at 450 nm wavelength, whose references were set to 550 and 620 nm.

A Beckman ARRAY 360 System (Beckman Coulter, Galway, Ireland) was used to evaluate the concentrations of several humoral immune parameters, including C3, C4, IgA, IgM, and IgG. Specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). After the incubation, the microplate reader (ClinicalBio 128c, Austria) was used to read OD within 30 minutes at 450 nm wavelength, whose reference was set to 630 nm [20].

2.7. Follow-Up. The follow-up procedure was performed in accordance with previous publications and recommendations [21, 22]. OS was defined as the duration from treatment until death or the last follow-up. PFS was defined as the duration from treatment until the date when disease progression was diagnosed or until the last follow-up. The last follow-up was completed on September 30, 2018.

2.8. Statistical Analysis. Continuous variables were compared using an independent sample t-test and the Mann-Whitney U test. Binary categorical variables were compared using the chi-square test. OS and PFS curves were analyzed using the Kaplan-Meier method, and differences between the groups were identified using the log-rank test. Univariate analysis was performed to assess the significance of parameters. Multivariate analysis was performed using the Cox regression model for the variables that were found to be significant in the univariate analysis, and the corresponding 95% confidence intervals (CIs) were calculated. ROC curves and C-indexes were used to compare the survival predictive ability. Two-tailed P values < 0.05 were considered statistically significant. All statistical analyses were performed using the R statistical package (R software version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Patient Characteristics. In the present study, a total of 34 patients with LAPC were retrospectively included in this study. All patients have received IRE therapy. There were 18 (52.9%) female patients and 16 (47.1%) male patients. The median age was 59.5 years (range 45-73 years). Patient characteristics were summarized in Table 1. Large size and moderate differentiation were the most commonly seen features of tumors. Most patients had lower values of inflammatory indexes, such as PLR, PI, and mGPS, while patients with higher values of NLR occupied the majority of all patients. For the whole study cohort, there were only 4 patients whose TBIL was higher than 100 umol/L. Complications after IRE treatment in patients with LAPC were also evaluated (Table 2). The most frequently reported complications were pain (3 of 34 patients) and hypotension (3 of 34 patients).

3.2. Modulation of Circulating Immune Cells. To investigate how IRE influences circulating immune cells, these cells were phenotypically characterized by evaluating the absolute number of helper T cell ($CD4^+$ T cell, identified as $CD3^+CD4^+$), cytotoxic T cell ($CD8^+$ T cell, identified as $CD3^+CD8^+$), regulatory T cell (Treg, identified as $CD4^+CD25^+FoxP3^+$), and natural killer cell (NK cell, identified as $CD3^-CD16^+CD56^+$) before (preOP) and after IRE treatment (D3 and D7). It was shown that the absolute numbers of $CD4^+$ T cell ($p<0.05$), $CD8^+$ T cell ($p<0.05$), and NK cell ($p<0.01$) were decreased immediately after IRE (D3), followed by a steady increase in the next few days (D7) ($p<0.001$). However, the trend for Treg cell reversed between preOP and D7 ($p<0.05$). The NK cell showed the most dramatic inverse effect for each time interval. Huge alterations of $CD4^+$ T cell and $CD8^+$ T cell were observed while there was a significant decrease in the ratio of $CD4^+$ T cell to $CD8^+$ T cell from D3 to D7 ($p<0.05$) (Figure 1).

3.3. Modulation of Circulating Cytokines and Humoral Immune Parameters. For a more complete understanding of the IRE-associated alteration of immune, analyses of the plasma concentration of several cytokines were conducted. Marked changes were observed for interleukin-2 (IL-2) ($p<0.05$), IL-6 ($p<0.001$), and IL-10 ($p<0.01$). IRE dramatically increased circulating IL-6 and IL-10 at D3 but these decreased at D7 (all $p<0.05$). Although no changes of IL-2 at D3 were observed, there was a significant increase from D3 to D7 ($p<0.05$). On the contrary, IRE did not significantly alter plasma concentration of IFN-γ and TNF ($p>0.05$). Moreover, we analyzed the plasma concentration of several general humoral immune parameters (complement: C3 and C4; immunoglobulin: IgA, IgG, and IgM). C3, C4, and IgG notably decreased immediately after IRE (D3) (all $p<0.05$) but significantly increased within one week (all $p<0.01$). There were no significant changes for concentration of IgA and IgM (all $p>0.05$) (Figure 2).

Table 1: Characteristics of patients with LAPC undergoing IRE therapy.

Characteristics		Number	Percentage (%)
Patients		34	100
Age (years)	≤ 60	19	55.9
	> 60	15	44.1
Gender	Female	18	52.9
	Male	16	47.1
Tumor size (cm)	≤ 2	1	2.9
	2~4	19	55.9
	>4	14	41.2
Tumor grade	Well	2	5.9
	Moderate	19	55.9
	Poor	13	38.2
Tumor site	Head	17	50.0
	Body / Tail	17	50.0
WBC (∗109)	≤ 10	30	88.2
	> 10	4	11.8
HGB (g/L)	≤ 120	10	29.4
	> 120	24	70.6
PLT (∗109)	≤ 300	29	85.3
	> 300	5	14.7
ALT (U/L)	≤ 40	25	73.5
	> 40	9	26.5
AST (U/L)	≤ 40	28	82.4
	> 40	6	17.6
ALP (U/L)	≤ 100	18	52.9
	> 100	16	47.1
GGT (U/L)	≤ 45	18	52.9
	> 45	16	47.1
ALB (g/L)	≤ 40	3	8.8
	> 40	31	91.2
TBIL (umol/L)	≤ 20.5	26	76.5
	> 20.5	8	23.5
IBIL (umol/L)	≤ 15	30	88.2
	> 15	4	11.8
CRP (ng/L)	≤ 3	25	73.5
	> 3	9	26.5
CEA (ng/mL)	≤ 5	20	58.8
	> 5	14	41.2
CA19-9 (U/ml)	≤ 35	8	23.5
	> 35	26	76.5
NLR	≤ 1.47	9	26.5
	> 1.47	25	73.5
PLR	≤ 165.29	22	64.7
	> 165.29	12	35.3
PI	0	25	73.5
	1	9	26.5
mGPS	0	29	85.3
	1	4	11.8
	2	1	2.9

LAPC, locally advanced pancreatic cancer; IRE, irreversible electroporation; WBC, white blood cell count; PLT, platelet count; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase; ALB, albumin; TBIL, total bilirubin; IBIL, indirect bilirubin; CRP, C-reactive protein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; PI, prognostic index; mGPS, modified Glasgow Prognostic Score.

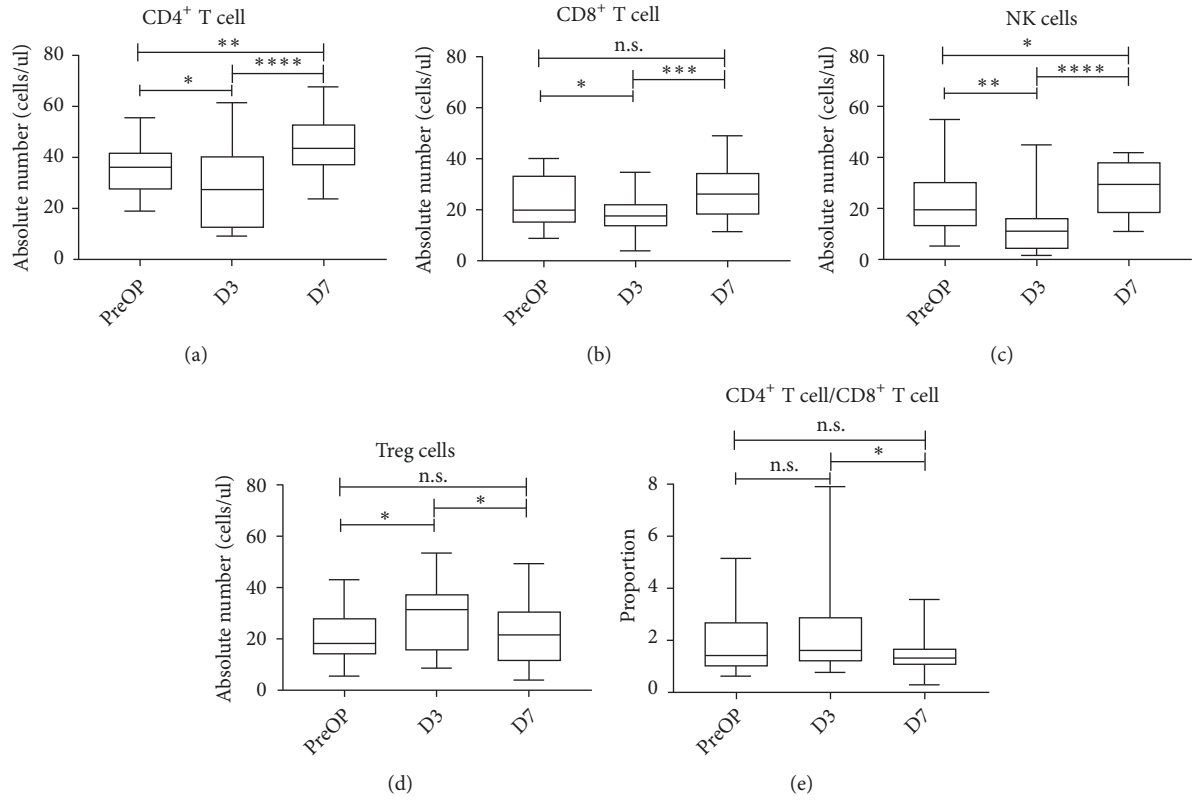

Figure 1: Distribution of serum concentration of CD4+ T cell (a), CD 8+ T cell (b), NK cell (c), Treg cell (d), and the ratio of CD4+ T cell/CD8+ T cell (e) before, 3 days, and 7 days after IRE therapy, indicating medians, interquartile range, 5th and 95th percentiles, and extreme values. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. NK cell: natural kill cell; Treg cell: regulatory T cell; IRE: irreversible electroporation.

Table 2: Complications after IRE treatment in patients with LAPC.

Complications	Number
Hypotension	3
Hypokalemia	2
Fatigue	2
Vomiting	1
Diarrhea	2
Thrombosis	2
Ascites	1
Pain	3
Muscle weakness	1

Abbreviations as in Table 1

3.4. Comparison of Survival Stratified by Changes of Immune Cells and Parameters. In the whole study cohort, there were 27 (79.4%) patients alive at the end of follow-up. The cumulative 1-year and 2-year OS rates were 69.9% and 52.4%, respectively. To evaluate the prognostic value of immune cells and parameters, the elevated or decreased group of these variables was defined by the threshold, which was the median value of the alterations between D3 and D7. In the subgroup analyses for OS, patients with an increase of $CD4^+$ T cell (p=0.047), $CD8^+$ T cell (p<0.001), and NK cell (p=0.013) or a decrease of Treg cell (p=0.015) had significant better OS than others. There were no significant differences with regard to OS when it was stratified by changes of cytokines, including IL-2, IL-6, and IL-10 (all *p>0.05)*. In addition to these variables, alteration of C3, C4, and IgG did not lead to significant differences in OS (all *p>0.05)* (Figure 3). Regarding PFS, significant survival benefit could be obtained from an increase of $CD8^+$ T cell (p=0.048) while the alterations of other immune cells or parameters were not significantly associated with PFS (Figure 4).

3.5. Univariate and Multivariate Analyses of OS and PFS. In Cox regression analysis, the increase of $CD8^+$ T cell was associated with increased OS and PFS [Elevated vs Nonelevated, OS, HR=0.039, 95%CI, 0.002-0.780, p = 0.034; PFS, HR=0.418, 95%CI, 0.138-0.954, p=0.049] in all patients. Moreover, there were no other prognostic factors for OS and the remaining one prognostic factor for PFS was NLR (> 1.47 vs ≤ 1.47, HR=3.425, 95%CI, 1.002-12.616, p=0.046) (Table 3).

3.6. Comparison of Predictive Value of the Immune Cells and Inflammation-Based Indexes. ROC curves were used to compare the sensitivity and specificity of survival prediction among the immune cells, parameters and inflammation-based indexes (Figure 5). The values of AUC of alteration

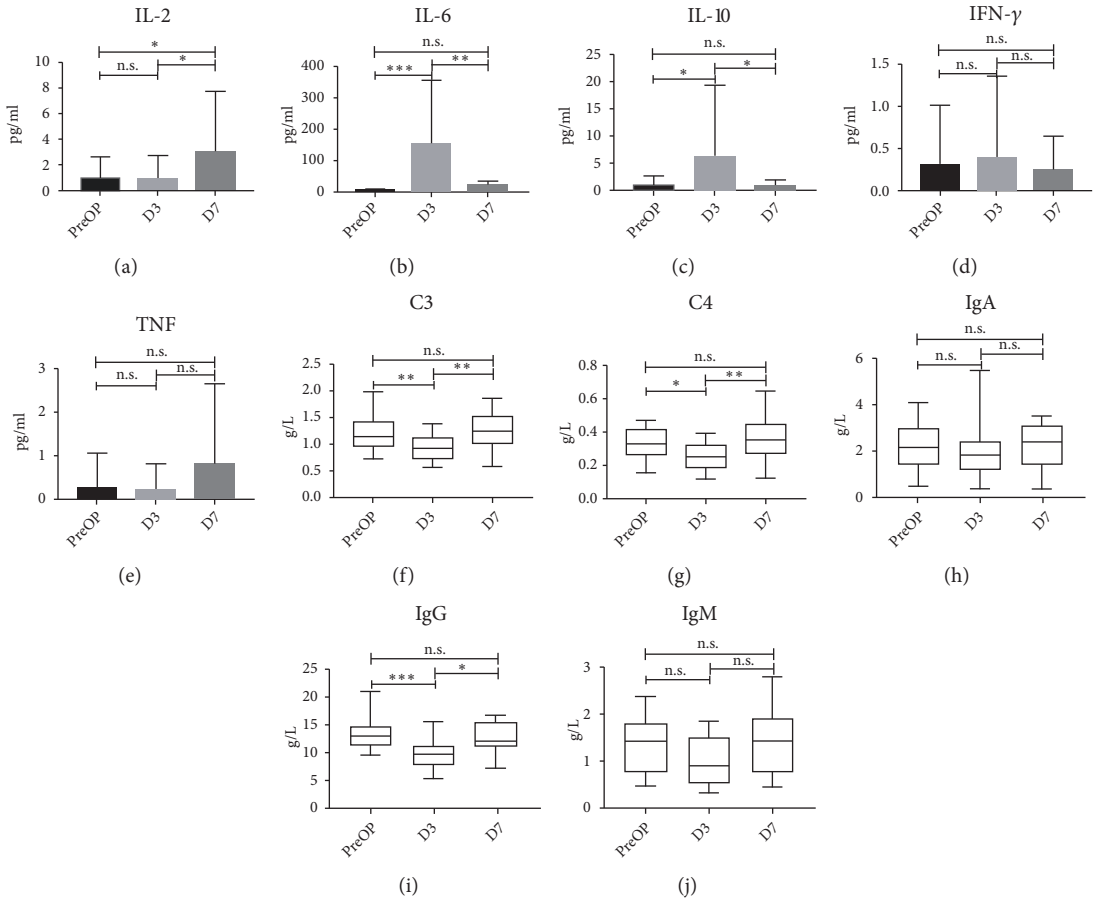

Figure 2: Distribution of serum concentration of IL-2 (a), IL-6 (b), IL-10 (c), IFN-γ(d), TNF (e), C3 (f), C4 (g), IgA (h), IgG (i), IgM (j) before, 3 days, and 7 days after IRE therapy. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. IL: interleukin; IFN-γ: interferon-γ; TNF: tumor-necrosis factor; C3: complement 3; C4: complement 4; IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; IRE: irreversible electroporation.

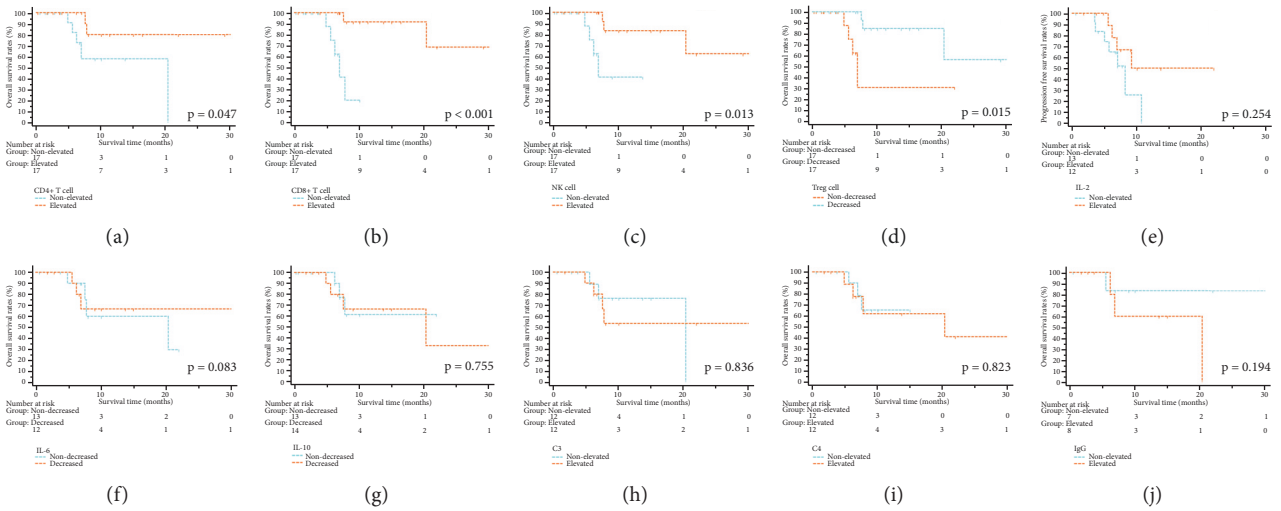

Figure 3: The survival curves of overall survival stratified by immune cells and parameters. Alteration of CD4+ T cell (a), CD 8+ T cell (b), NK cell (c), Treg cell (d), IL-2 (e), IL-6 (f), IL-10 (g), C3 (h), C4 (i), and IgG (j). NK cell: natural kill cell; Treg cell: regulatory T cell; IL: interleukin; C3: complement 3; C4: complement 4; IgG: immunoglobulin G; IRE: irreversible electroporation.

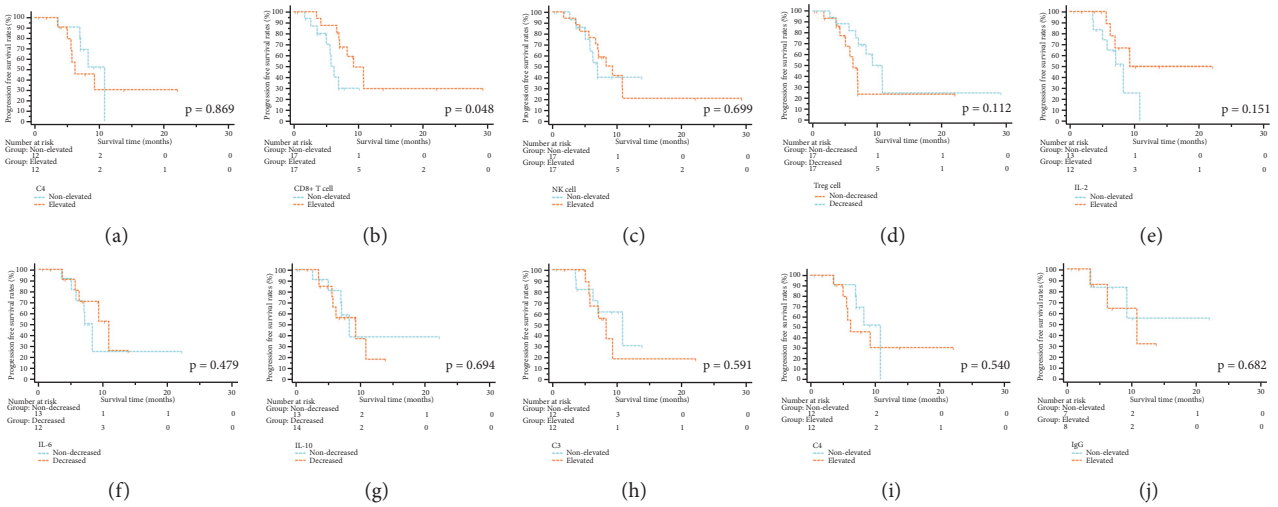

Figure 4: The survival curves of progression-free survival stratified by immune cells and parameters. Alteration of CD4+ T cell (a), CD 8+ T cell (b), NK cell (c), Treg cell (d), IL-2 (e), IL-6 (f), IL-10 (g), C3 (h), C4 (i), and IgG (j). NK cell: natural kill cell; Treg cell: regulatory T cell; IL: interleukin; C3: complement 3; C4: complement 4; IgG: immunoglobulin G; IRE: irreversible electroporation.

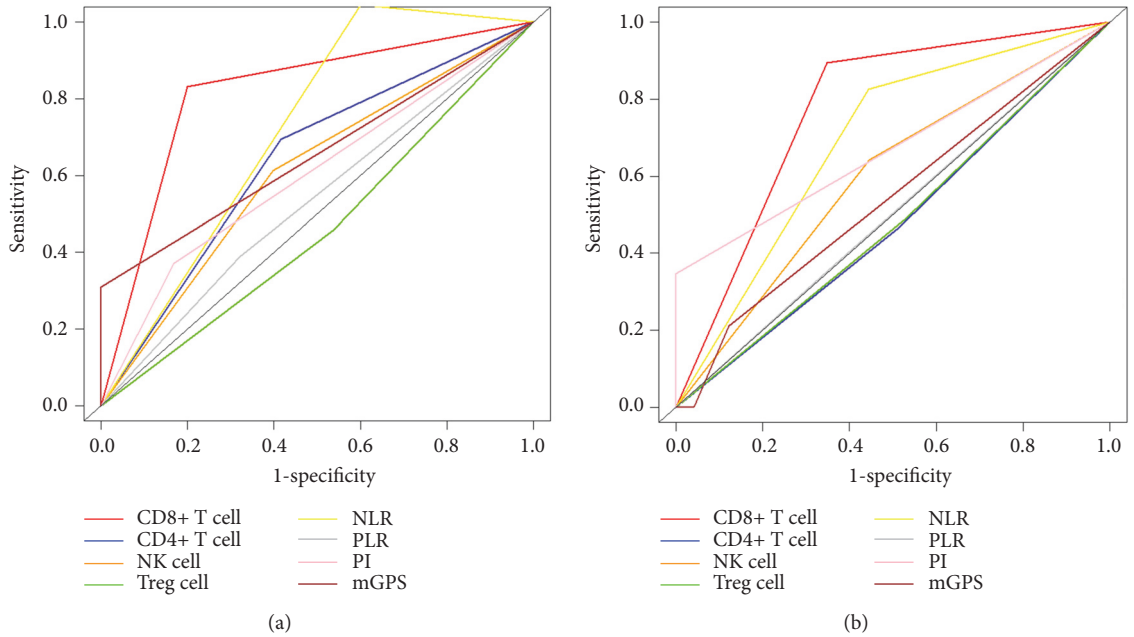

Figure 5: Comparison of ROC curves of alteration of immune cells, cytokines, or inflammation-based indexes, for predicting OS (a) and PFS (b) in patients with LAPC after IRE therapy. ROC: receiver operating characteristic; OS: overall survival; PFS: progression-free survival; LAPC: locally advanced pancreatic cancer; IRE: irreversible electroporation.

of $CD8^+$ T cell for OS and PFS prediction were 0.816 and 0.773, respectively, which were both higher than those of other immune parameters or inflammation-based indexes (Table 4). In terms of comparisons of C-indexes for OS prediction, the value of alteration of $CD8^+$ T cell was 0.816 (95%CI 0.711-0.921), which was higher than that of other factors. In terms of PFS prediction, $CD8^+$ T cell also displayed relatively high value of 0.639 (95%CI 0.523-0.755), showing significant better predictive power (Table 5).

4. Discussion

In this study, an immunomodulatory effect was demonstrated by altering lymphocytes, cytokines, and humoral immune parameters in patients with LAPC after IRE. It was the first evidence for IRE-based immune modulation in LAPC patients. It was shown that there was a transitory decrease followed by a steady increase for $CD4^+$ T cell, $CD8^+$ T cell, NK cell, IL-2, C3, C4, and IgG while a reverse trend was observed

Table 3: Univariate and multivariate analyses of OS and PFS in patients.

Characteristic		OS						PFS					
		Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
		HR	95%CI	P	HR	95%CI	P	HR	95%CI	P	HR	95% CI	P
Age (years)	≤ 60 / > 60	0.753	0.163-3.377	0.711			NI	0.988	0.379-2.577	0.981			NI
Gender	Female / Male	8.535	0.994-72.563	0.055			NI	1.535	0.577-4.084	0.391			NI
Tumor size (cm)	>4 / 2~4 / ≤ 2	1.405	0.405-4.876	0.592			NI	1.019	0.412-2.522	0.967			NI
Tumor grade	Poor / Moderate / Well	2.754	0.668-11.354	0.161			NI	0.910	0.391-2.117	0.827			NI
Tumor site	Head / Body / Tail	3.778	0.743-19.204	0.109			NI	0.848	0.393-1.830	0.674			NI
WBC ($*10^9$)	≤ 10 / > 10	0.733	0.087-6.164	0.775			NI	1.019	0.412-2.522	0.967			NI
HGB (g/L)	≤ 120 / > 120	0.544	0.121-2.445	0.427			NI	1.202	0.377-4.280	0.777			NI
PLT ($*10^9$)	≤ 300 / > 300	1.588	0.289-8.721	0.595			NI	0.477	0.107-2.116	0.330			NI
ALT (U/L)	≤ 40 / > 40	0.995	0.191-5.181	0.995			NI	0.409	0.117-1.431	0.162			NI
AST (U/L)	≤ 40 / > 40	0.033	0.000-67.662	0.382			NI	0.596	0.168-2.112	0.423			NI
ALP (U/L)	≤ 100 / > 100	0.832	0.185-3.742	0.811			NI	0.522	0.193-1.417	0.202			NI
GGT (U/L)	≤ 45 / > 45	1.404	0.310-6.365	0.660			NI	0.418	0.136-1.289	0.129			NI
ALB (g/L)	≤ 40 / > 40	0.572	0.066-4.939	0.611			NI	0.924	0.206-4.149	0.918			NI
TBIL (umol/L)	≤ 20.5 / > 20.5	0.033	0.000-67.662	0.382			NI	0.596	0.168-2.112	0.423			NI
IBIL (umol/L)	≤ 15 / > 15	0.040	0.000-95.774	0.530			NI	1.148	0.328-4.022	0.829			NI
CRP (ng/L)	> 3 / ≤ 3	8.328	1.443-48.063	0.018	2.458	0.048-125.03	0.654	1.024	0.275-3.817	0.972			NI
CEA (ng/mL)	≤ 5 / > 5	0.999	0.193-5.161	0.999			NI	2.106	0.805-5.512	0.129			NI
CA19-9 (U/ml)	≤ 35 / > 35	0.865	0.165-4.554	0.868			NI	2.635	0.573-11.523	0.178			NI
$CD4^+$ T cell variation	Elevated/Non-elevated	0.214	0.041-1.127	0.069			NI	0.920	0.343-2.470	0.869			NI
$CD8^+$ T cell variation	Elevated/Non-elevated	0.056	0.006-0.490	0.009	0.039	0.002-0.780	0.034	0.354	0.122-1.026	0.046	0.418	0.138-0.954	0.049
NK cell variation	Elevated/Non-elevated	0.144	0.025-0.816	0.029	0.184	0.005-6.745	0.357	0.817	0.293-2.277	0.700			NI
Treg cell variation	Decreased/Non-decreased	0.165	0.033-0.820	0.028	1.056	0.026-43.365	0.977	0.452	0.166-1.228	0.119			NI
IL-2 variation	Elevated/Non-elevated	0.393	0.075-2.067	0.270			NI	0.408	0.116-1.438	0.163			NI
IL-6 variation	Decreased/Non-decreased	0.851	0.188-3.841	0.834			NI	0.645	0.190-2.187	0.482			NI
IL-10 variation	Decreased/Non-decreased	1.271	0.281-5.757	0.756			NI	1.262	0.396-4.020	0.694			NI
C3 variation	Elevated/Non-elevated	1.172	0.260-5.292	0.837			NI	1.386	0.419-4.584	0.592			NI
C4 variation	Elevated/Non-elevated	1.201	0.241-5.974	0.823			NI	1.458	0.434-4.893	0.542			NI
IgG variation	Elevated/Non-elevated	4.012	0.411-39.156	0.232			NI	1.459	0.237-8.985	0.684			NI
NLR	> 1.47 / ≤ 1.47	5.967	0.680-52.400	0.107			NI	3.185	1.068-13.621	0.039	3.425	1.002 - 12.616	0.046
PLR	> 165.29 / ≤ 165.29	3.752	0.738-19.073	0.111			NI	1.517	0.552-4.171	0.419			NI
PI	2 / 1 / 0	3.864	0.861-17.333	0.078			NI	1.292	0.439-3.804	0.642			NI
mGPS	2 / 1 / 0	3.264	1.266-8.413	0.014	4.285	0.355-51.673	0.252	0.921	0.284-2.987	0.891			NI

OS, overall survival; PFS, progression-free survival; NI, not included; Other abbreviations as in Table 1

Table 4: Comparison of the values of AUC.

	$CD8^+$ T cell variation	$CD4^+$ T cell variation	NK cell variation	Treg cell variation	NLR	PLR	PI	mGPS
OS	0.816	0.639	0.608	0.460	0.722	0.534	0.601	0.654
PFS	0.773	0.477	0.598	0.480	0.691	0.503	0.674	0.540

Abbreviations as in Table 3

Table 5: Comparison of the values of C-indexes.

		CD8+ T cell variation	CD4+ T cell variation	NK cell variation	Treg cell variation	NLR	PLR	PI	mGPS
OS	value	0.816 (0.711-0.921)	0.698 (0.554-0.842)	0.755 (0.619-0.891)	0.764 (0.636-0.892)	0.632 (0.446-0.818)	0.670 (0.484-0.856)	0.675 (0.478-0.872)	0.693 (0.525-0.861)
CD8+ T cell variation	P value		0.008	0.162	0.210	0.011	0.007	0.068	0.103
CD4+ T cell variation	P value	0.008		0.188	0.164	0.275	0.365	0.409	0.482
NK cell variation	P value	0.162	0.188		0.452	0.112	0.113	0.235	0.281
Treg cell variation	P value	0.210	0.164	0.452		0.081	0.086	0.212	0.252
NLR	P value	0.011	0.275	0.112	0.081		0.343	0.349	0.265
PLR	P value	0.007	0.365	0.113	0.086	0.343		0.485	0.415
PI	P value	0.068	0.409	0.235	0.212	0.349	0.485		0.425
mGPS	P value	0.103	0.482	0.281	0.252	0.265	0.415	0.425	
PFS	value	0.639 (0.523-0.755)	0.520 (0.381-0.659)	0.541 (0.407-0.675)	0.614 (0.485-0.743)	0.661 (0.579-0.743)	0.563 (0.436-0.690)	0.505 (0.382-0.628)	0.475 (0.365-0.585)
CD8+ T cell variation	P value		0.081	0.084	0.368	0.376	0.116	0.020	0.006
CD4+ T cell variation	P value	0.081		0.407	0.148	0.013	0.298	0.423	0.260
NK cell variation	P value	0.084	0.407		0.207	0.023	0.399	0.309	0.200
Treg cell variation	P value	0.368	0.148	0.207		0.264	0.219	0.083	0.014
NLR	P value	0.376	0.013	0.023	0.264		0.055	0.002	0.001
PLR	P value	0.116	0.298	0.399	0.219	0.055		0.257	0.078
PI	P value	0.020	0.423	0.309	0.083	0.002	0.257		0.327
mGPS	P value	0.006	0.260	0.200	0.014	0.001	0.078	0.327	

Abbreviations as in Table 3

for Treg cell, IL-6, and IL10 after IRE. Other circulating cytokines, including TNF and IFN, were also evaluated. In terms of IFN, IFN-γ plays the most important role in its immunostimulatory and immunomodulatory effects, compared with the ability to inhibit viral replication directly, which is the main function of IFN-α [23, 24] or IFN-ß [25]. Therefore, IFN-γ and TNF were analyzed while they both failed to show obvious alteration. In addition, the alteration of $CD8^+$ T cell between D3 and D7 was identified as prognostic factor for OS and PFS and first showed both a convenient and effective prognostic value in patients with LAPC after IRE. When compared with the traditional inflammation-based scores, the alteration of $CD8^+$ T cell exhibited a better predictive value for both OS and PFS.

For LAPC patients after ablation therapy, several studies have revealed the changes of individual counts of T cell and subset ratios [26–28]. Alessandro G et al. compared the concentration of $CD4^+$ and $CD8^+$ T cell before and after radiofrequency ablation (RFA) and revealed an increase of above-mentioned T cells from the third day after treatment [26]. In the study conducted by Ketevan M et al., a more significant decrease in the expression of $CD4^+CD39^+$ T cell was observed after RFA, compared with operation [27]. In animal study, IRE therapy, which induced an increase tumor infiltration of $CD3^+$ cells, was reported to be more effective in immunocompetent tumor than in immunocompromised tumors [28]. Furthermore, Martin et al. reported that IRE induced an obvious decrease in the absolute number of Treg cell in patients with LAPC [29]. Similar to Martin's study, our study showed a transitory increase followed by a remarkable decrease for Treg cell, along with a steady increase of effective T cells and humoral immune parameters after IRE. As an inflammation-inducing treatment, IRE not only directly destroys tumor cells, but also results in a release of tumor-associated neoantigens, which may stimulate the cellular and humoral immune of the body. Then, the numbers of immunocytes and production of humoral immune parameters will increase due to the potentiation of cellular and humoral immune. Moreover, it was shown that heat-shock proteins released from the destroyed tumor cells had an adjuvant effect and acted as an alarm for antitumor T cell-mediated immunity [30]. Therefore, IRE may be a mean of significant effort to overcome the immunosuppressive "cold" tumor microenvironment in LAPC and a potential treatment window of opportunity for immune-check-point therapy was suggested by increasing the effector T cells and decreasing immunosuppressive Treg cells. In addition, for these patients, prior biliary drainage procedure or a hepaticojejunostomy during open procedure was performed. Also, no serious complications, such as abdominal infection and pancreatic fistula, were observed in all patients after IRE therapy. Therefore, the influence of hyperbilirubinemia or infection after IRE on the alteration of immune cells was minimized. Although detained changes of immune cells had been described in patients with LAPC after IRE, the clinical performance of these changes in survival prediction was still unclear, thereby limiting their value.

In the next step of the present study, we evaluated the prognostic factors for OS and PFS and showed that elevation of $CD8^+$ T cell was associated with favourable OS and PFS in LAPC patients after IRE. This can be explained by a stimulated host immune response which might limit the progression and invasion of tumor, and therefore, better survival was achieved. This can be proved by previous studies in which strong relationships were observed between immune toxicity and metastasis [31]. Metastases were more frequently observed in patients with lower density of immune effector cells [32], which was in accordance with our results. Furthermore, the predictive power of the alteration of several immune cells and inflammation-based indexes were compared in this study. It was demonstrated that the alteration of $CD8^+$ T cell was superior to other indexes. In addition, as a robust and economic method, the alteration of $CD8^+$ T cell can be obtained from peripheral blood sample fast and easily and can be used widely in clinical practice. Although there was a correlation between immune cells and inflammation-based indexes [27], the alteration of CD8+ T cell could still provide additional prognostic value in patients with the same levels of inflammation-associated situation. Maybe they can be considered as complements for predicting the prognosis of LAPC patients after IRE. However, a slightly lower value of AUC for CD8+ T cell in PFS prediction suggested that, compared with PFS, maybe OS was affected more greatly by the changes of immune system. Different from tumor-infiltrating $CD8^+$ T cells, which were shown to play more important role in determine local progression, compared with prognosis [33, 34]. LAPC is a systemic disease other a local disease. In the present study, peripheral blood samples were collected before and after IRE treatment and were analyzed by flow cytometry for $CD8^+$ T cells. Therefore, it was thought that the human immune system played a more important role in long-term survival than in local control. Similar to the present study, in the study conducted by Chen et al. [35], it was shown that alteration of $CD8^+$ T cells was the only independent prognostic factor for OS, other than PFS. This may partly explain the different impact on survival from the alteration of $CD8^+$ T cells. However, this difference needed to be further explored by further studies.

The comparison of AUC and C-indexes of the alteration of immune cells or inflammation-based indexes was conducted. Although the alteration of $CD8^+$ T cell exhibited the most significant effects in predicting survival, statistical significance was not observed for the differences between alteration of $CD8^+$ T cell and some other indexes, implying that the need of improvement in predicting short-term survival with the changes of immune cells. Maybe the magnitude of effector T cell was more positively associated with long-term survival than short-term survival [36].

As the first study to compare the changes of immunocytes and to explore the prognostic power of these changes in patients with LAPC after IRE, our study was limited by the small size and retrospective nature. The immunocytes measured in this study did not represent all the components in the microenvironment of LAPC after IRE therapy. Moreover, maybe it is necessary to analyze the immune parameters withdrawn at the moment of progression. A longer follow-up period is also needed for the comparisons of effects of

immunocytes on survival in LAPC patients and an external validation is also needed.

5. Conclusions

The present study showed the first picture of immunomodulatory of IRE in patients with LAPC. Alteration of $CD8^+$ T cell was established as prognostic factor for OS and PFS and showed better prognostic value for survival prediction in LAPC patients after IRE therapy. The changes of $CD8^+$ T between D3 and D7 after IRE could be used as a monitor factor of IRE treatment and a prognostic indicator of survival in LAPC patients after IRE therapy.

Data Availability

The datasets from SYSUCC dataset are available from the corresponding author upon reasonable request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Chaobin He, Jun Wang, Shuxin Sun, and Yu Zhang collected the dataset; Chaobin He, Jun Wang, and Shuxin Sun performed the statistical analyses and wrote the manuscript; Shengping Li designed the study. Chaobin He, Jun Wang, and Shuxin Sun contributed equally to this work.

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Review Article

Advances in Tumor-Stroma Interactions: Emerging Role of Cytokine Network in Colorectal and Pancreatic Cancer

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Cytokines are a family of soluble factors (Growth Factors (GFs), chemokines, angiogenic factors, and interferons), which regulate a wide range of mechanisms in both physiological and pathological conditions, such as tumor cell growth and progression, angiogenesis, and metastasis. In recent years, the growing interest in developing new cancer targeted therapies has been accompanied by the effort to characterize Tumor Microenvironment (TME) and Tumor-Stroma Interactions (TSI). The connection between tumor and stroma is now well established and, in the last decade, evidence from genetic, pharmacological, and epidemiological data supported the importance of microenvironment in tumor progression. However, several of the mechanisms behind TSI and their implication in tumor progression remain still unclear and it is crucial to establish their potential in determining pharmacological response. Many studies have demonstrated that cytokines network can profoundly affect TME, thus displaying potential therapeutic efficacy in both preclinical and clinical models. The goal of this review is to give an overview of the most relevant cytokines involved in colorectal and pancreatic cancer progression and their implication in drug response.

1. Introduction

During the last years, it has been well recognized that cancer is not a single mass of transformed cells, but it is also composed by nonmalignant cells, such as Cancer-Associated Fibroblasts (CAFs), tumor infiltrating cells (T-cells, macrophages, and neutrophils), as well as vasculature with endothelial cells, soluble factors (cytokines and GFs), and the extracellular matrix, which are all together referred to as TME (Figure 1) [1, 2].

The connection between tumor and stroma is now well established and evidence from genetic, pharmacological, and epidemiological data supported the importance of microenvironment in tumor progression. However, several of the mechanisms behind TSIs and their implication in tumor progression remain unclear and need to be evaluated for their potential in pharmacological response.

The crosstalk between cancer cells and the surrounding TME may act through different processes, such as cell-to-cell direct contact, or by soluble factors. Indeed, one of the key players involved in intra- and intercellular communication is cytokines, like GFs and chemokines, which signal through both autocrine and paracrine fashion.

TSI represent one of most relevant contributors to the limited therapeutic success achieved by selectively targeting tumor cells. Indeed, not only does TME promote cancer invasion and metastasis, but it also provides resistance to chemotherapy, and cancer cells upregulate cytokines' expression proportionally to the progression of the disease. Understanding the mechanisms involved in TSI thoroughly in order

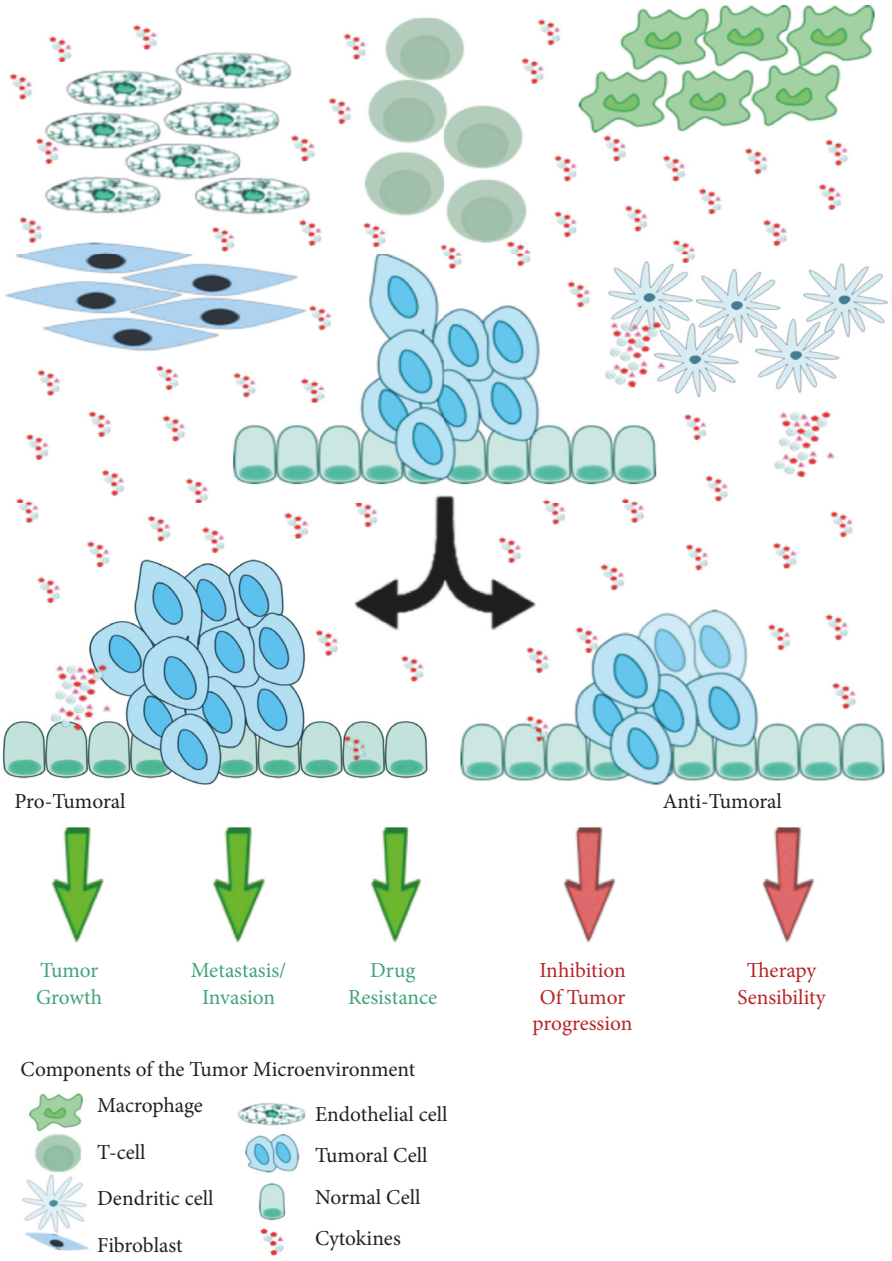

Figure 1: Schematic illustration of the cytokines role in tumorigenesis. Cytokines are released by both tumor and stromal cells, including immune cells like macrophages, B and T lymphocytes, dendritic cells, and endothelial cells and fibroblasts. The binding of cytokines to their receptors on surface of targeted cells causes the activation of intracellular signaling cascades with protumoral and/or antitumoral properties.

to achieve "comprehensive" targeting of both cell autonomous progression mechanisms and TSI in advanced and metastatic colorectal and pancreatic tumor remains crucial [3, 4].

Herein, we will briefly describe current knowledge about the role played by chemokines and GFs in colorectal and pancreatic cancer and their treatment.

2. Cytokines Networks in Cancer

Cytokines are a set of soluble proteins and, through the binding to membrane receptors, they activate signal transduction pathways involved in several physiological and pathological mechanisms, thus providing complex networks of communication. Cytokines are released by both stromal and cancer cells in response to external stimuli; they can be clustered in families comprising GFs, chemokines, angiogenic factors, and interferons [5]. Various stroma cells can express cytokines, including immune cells, such as macrophages, B and T lymphocytes, dendritic cells, and fibroblasts and endothelial cells, thus affecting the behavior of cells around them (Figure 1).

Cytokines are redundant molecules, which regulate similar effects, due to their shared common receptors; moreover, they are pleiotropic, meaning that the cytokines-cytokines receptor interactions can, in turn, regulate a wide range of mechanisms, such as tumor cell growth and progression, angiogenesis, and metastasis [6]. However, several data demonstrated that cytokines can also display antitumoral properties, thereby highlighting a paradigm in cytokine role in affecting both pro- and antitumoral mechanisms (Figure 1) [7, 8]. Representative examples of the pleiotropic and controversial role of cytokines in TSIs are Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF)-α.

In multicellular organisms, IL-6 family plays an important role in communication and regulation of complex processes. Indeed, various cell types are involved in IL-6 secretion in response to different stimuli, such as immune reactions, response to infections, tissue injuries, hematopoiesis, and host defense. Due to the IL-6 involvement in homeostasis, it is not surprising that its uncontrolled signaling is associated with pathological processes like tumor initiation, progression, and metastasis [9, 10]. IL-6 family is composed by different cytokines (e.g., IL-6, IL-11, Oncostatin M (OSM) and Leukemia Inhibitory Factor (LIF)), which display the common ability to bind glycoprotein 130 (gp130) chain. This binding leads to the activation of the canonical IL-6-activated Janus Kinase- (JAK-) Signal Transducers and Activators of Transcription (STAT) pathway and Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) signaling, two of the most deregulated pathways involved in different stages of cancer development and progression [11]. The great complexity involved in IL-6 redundancy and pleiotropy is due to the various homo-/heterodimer receptor associations and subsequent intracellular signaling. gp130 plays a crucial role in signal transduction and its subunit is ubiquitously expressed; however, only IL-6 and IL-11 bind gp130 homodimer, while the other cytokines signal via heterodimers of gp130-LIF receptor or gp130-OSM receptor [12, 13].

TNF-α represents one of the most important activators of the NF-κB "canonical pathway," a master regulator of maximal cytokine expression; this mechanism in turn explains the cytokines autocrine loops with positive feedback [14]. Indeed, the binding of cytokines to their membrane receptors is able to activate transcription factors, which enhances cytokines gene transcription. TNF-α is a cytokine with a molecular weight of 26 kDa and it regulates different mechanisms (e.g., immunity, inflammation, cellular homeostasis, and tumor progression), according to its concentration [15, 16]. The cleavage of a membrane-bound protein (pro-TNF) from TNF-converting enzyme (ADAM17) allows the presence of a mature cytokine, which binds two main membrane receptors [17]. The binding to TNF Receptor-1 (TNFR1) (ubiquitously expressed) leads to the activation of the NF-κB transcription factor, involved in the regulation of antiapoptotic genes (e.g., B-cell lymphoma-XL (Bcl-XL) and inhibitors of apoptosis) [7, 18]. On the other hand, due to its death domain, TNFR1 is able to bind caspase-8 and to activate the subsequent apoptotic pathway (through the activation of executor caspase-3 and -7) [7]. TNFR2 is mainly expressed by immune cells, but its role in cancer cells is less understood. The binding of TNF-α to TNFR2 in Colorectal Cancer (CRC) cell lines causes the activation of the phosphoinositide 3-kinase (PI3K)/AKT signaling through the phosphorylation of AKT, thus leading to cell proliferation. Furthermore, it has been shown that TNF-α-TNFR2 binding does not enhance MAPK/ERK signaling, as demonstrated by ERK inactivation [7, 19].

2.1. Chemokines. In the wide range of cytokines that affect TME, chemokines are one of the most interesting classes, due to their multiple roles played in TSI, which include TME's composition and function, tumor progression, and drug response [20].

Chemokines are chemotactic cytokines (8-10 kDa) secreted in several tissue environments and are involved in the regulation of inflammatory processes, in which they play a key role as chemoattractants [21]. To date, it has been demonstrated that there are about 50 types of chemokines and 20 seven-transmembrane-spanning G Protein-Coupled Receptors (GPCRs) in humans [22]. Ligand-receptor binding determines conformational changes, which allow the exposure of epitopes on the intracellular loops and carboxyterminal tail of the receptor; this in turn promotes the coupling with the functional heterotrimeric G proteins, which consist of α, β, and γ subunits. Ligand binding catalyzes the exchange of guanosine diphosphate for guanosine triphosphate on the Gα subunit, which triggers the release of this subunit from the receptor and the G$\beta\gamma$ subunits; consequently, signals are transmitted across the membrane and activate downstream effectors [23]. Chemokine binding to GPCRs also leads to the regulation of several both physiological and pathological processes. Indeed, during normal immune surveillance, chemokines induce cell polarization and migration of leukocyte, macrophages, and neutrophils in order to induce their homing in tissue injury or infection [24]. In pathological condition, such as cancer and inflammatory diseases, chemokines are able to activate specific signal transduction cascades, which are involved in proliferation, survival, and migration of cancer cells [24]. Besides the canonical chemokines receptors, 4 Atypical Chemokine Receptors (ACKRs) were recently identified: even if they display structural features similar to those of GPCR, they do not signal through the G proteins and they do not activate chemotaxis [25]. Indeed, ACKRs are involved in the regulation of the extracellular bioavailability of chemokines, their intracellular storage, and their cellular distribution in polarized cells [26].

Moreover, tumor cells not only are able to produce soluble chemokines but also overexpress chemokines receptors on their cell surface, thereby upregulating the autocrine mechanisms. In this way, signaling through chemokines is a complex mechanism of communication between tumor cells and TME through both paracrine and autocrine mechanisms [27].

Chemokines- (e.g., CXCL12 and IL-8-) GPCR binding mainly activates the MAPK/ERK signaling cascade and its downstream effectors involved in cell cycle progression and tumor cells proliferation, such as c-Myc and cyclin-D1 [28, 29]. GPCRs also activate the PI3K/AKT signaling pathway:

once activated, AKT upregulates the oncoprotein Mouse double minute 2 homolog (Mdm2), the key antagonist of the p53 tumor suppresser gene, thereby promoting tumor cell survival [30].

One of the most relevant and characterized axes is CXC Ligand-12 (CXCL-12) (also known as Stromal Cell-Derived Factor (SDF)1-α)/CXC Receptor-4 (CXCR-4), crucially involved in homing and retention of Hematopoietic Stem Cells (HSC) in the bone marrow [31]. Consistently, food and drug administration approved plerixafor, a competitive inhibitor of CXCR4, for HSC transplantation: indeed, the blockade of CXCL12-CXC4 interaction results in mobilization of $CD34^+$ hematopoietic stem cells to the peripheral blood by restoring bone marrow function [32, 33]. Moreover, it is been documented that CXCL12-CXCR4 axis is involved in a wide spectrum of cancer types, such as breast, colorectal, and pancreatic cancer [34–36]. CXCL12/CXCR4 is also responsible for apoptosis regulation via the activity of the Bcl-2 family members. For example, in acute myeloid leukemia cells, CXCL12-induced CXCR4 activation downregulates Bcl-XL expression, thereby shifting the balance from proapoptotic to antiapoptotic signaling [37]. Although CXCL12 does not contain the ELR (Glu-Leu-Arg) motif, it is one of the most angiogenesis-promoting chemokines [38]. Indeed, CXC chemokines are generally classified as promoter/suppressor of angiogenesis, according to the presence of ELR motif: ELR^+ chemokines (CXCL1, CXCL6, and CXCL8) are positive regulators of angiogenesis; conversely, ELR^- chemokines (CXCL4, CXCL10, and CXCL14) display inhibitory features [39]. However, this classification does not always reflect the real activities performed by chemokines, as demonstrated by the role of CXCL12 in the regulation of vessels formation: indeed, it has been shown that CXCR4-defective mice display impaired vascular formation [40].

Another relevant chemokine axis involved in angiogenic mechanisms is the CXCL8- (also known as IL-8-) CXCR1/2 axis. IL-8 is a proinflammatory CXC ELR^+ chemokine, identified for its role as "neutrophil chemotactic factor": indeed, IL-8 mainly acts as a promoter of chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection, where it promotes their chemotaxis and degranulation [41]. The biological effects of IL-8 are mediated through the binding of IL-8 to two cell-surface receptors, the GPCRs CXCR1 (IL-8RA) and CXCR2 (IL-8RB), which are present in various types of normal as well as tumor cells [42]. These receptors share 77% amino acid homology and retain common structural motifs, suggesting that these genes arose through gene duplication [43]. CXCR1 is activated by IL-8 and granulocyte chemotactic protein-2/CXCL6, whereas CXCR2 can be activated not only by IL-8 but also by many other CXC chemokines [43]. As a potent proangiogenic chemokine, IL-8 signaling induces Vascular Endothelial Growth Factor- (VEGF-) independent tumor angiogenesis: indeed, IL-8 stimulates endothelial cell migration and upregulates the two metallopeptidases MMP-2 and MMP-9 [44, 45]. Moreover, IL-8 is involved in a complex positive feedback loop with VEGF: Martin and colleagues have, indeed, demonstrated that IL-8 upregulates VEGF levels in endothelial cells, thereby activating VEGF receptors (VEGFR), through the transcription factor NF-κB [46].

2.2. Growth Factors. In TME, cells can interact with each other also by the presence of polypeptide GFs, which act through the binding to specific cell-surface receptors, often with kinase activity. GFs are released in TME by different type of cells, such as tumor, endothelial, and mesenchymal cells; moreover, their cognate receptors can be expressed also by cells that are not those that released GFs, thereby representing a complex crosstalk between cells in TME. For example, mesenchymal cells predominantly release hepatocyte Growth Factor, which binds its receptor c-Met on the epithelial cells surface [47].

The general classification of GFs into 10 classes, according to targeted cells and functions, is summarized in Table 1 [48].

The interaction between the GFs and their receptors causes the activation of several intracellular signaling cascades, such as MAPK/ERK, PI3K/AKT, and JAK/STAT pathways, involved in supporting tumor progression and drug resistance. Indeed, GFs are involved in several hallmarks of cancer, such as uncontrolled proliferation, cellular motility, and angiogenesis, and they signal through both paracrine and autocrine mechanisms [49].

Transforming Growth Factor-β (TGF-β) is a multifunctional cytokine that regulates several physiological processes, such as cell development and differentiation, by acting as a negative regulator of tumor growth. Consistent with this role in cell proliferation, elements of this pathway (in particular the transcriptional factor Small Mother Against Decapentaplegic (SMAD)4) are commonly mutated in human cancers, thus promoting cell cycle progression, epithelial-mesenchymal transition, invasion, metastasis, and angiogenesis [50]. Once TGF-β binds its receptors, TβR-II recruits and phosphorylates TβR-I, which in turn phosphorylates its substrate complex SMAD2/3. This phosphorylation causes the dissociation of SMAD2/3 from the membrane, the association of a heterodimeric complex with SMAD4, and the translocation to the nucleus, where they act as transcriptional regulators of target genes, such as proapoptotic genes like BIM [51]. However, during the time, several studies showed that TGF-β also promotes protumoral effects, thereby displaying a controversial role in cancer. Indeed, it has been demonstrated that TGF-β/SMAD4 pathway interacts with the other canonical pathways involved in neoplastic transformation, such as MAPK/ERK and PI3K/AKT pathways, mainly due to phosphorylation events. For example, it has been demonstrated that TGF-β2 is able to activate ERK2 in breast cancer cell lines [52]. Conversely, ERK can phosphorylate SMAD2/3 in the linker region, thereby inhibiting their translocation to the nucleus [53].

Among GFs involved in cancer malignancy, Epidermal GF (EGF) promotes tumor growth and progression through the binding to erbB family receptors. erbB family comprises four transmembrane glycoproteins, with high molecular weight (170 to 185 kDa): EGF Receptor (EGFR) (HER1 or erbB1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). The EGF binding to the cell membrane receptor causes the dimerization of two EGFR monomers, which display tyrosine

Table 1: GF classification.

GFs family	Abbreviation	Receptors	Target cells	Role in cancer
Platelet Derived Growth Factor	PDGF	PDGFRα/β	Epithelial and endothelial	Blood vessel formation, cell migration and metastasis
Vascular Endothelial Growth Factor	VEGF	VEGFR1/2/3 and coreceptors Neuropilin1/2	Endothelial and tumor	Mitogenic stimuli
Epidermal Growth Factor	EGF	EGFR	Mesenchymal, epithelial and tumor	Mitogenic stimuli and metabolism increasing
Fibroblast Growth Factor	FGF	FGFR1-4	Epithelial and mesenchymal	Mitogenic and angiogenic stimuli
Transforming Growth Factor-β	TGF	TGF-β type I (TβR-I), type II (TβR-II) and type III (TβR-III)	Tumor	Tumor suppressor
Insulin-like Growth Factor	IGF	IGF-IR and IGF-IIR	Tumor	Upregulation of metabolism, growth and survival
Hepatocyte Growth Factor	HGF	c-Met	Epithelial	Mitogenic stimuli and angiogenesis
Neurotrophin	n.a.	Tropomyosin receptor kinase (Trk) A/B/C and neurotrophin receptor P75	Tumor	Cell survival (Trk) and death (P75)
Ephrin	Eph	EphrinA/B	Tumor	Tumor suppressor/promoter
Angiopoietins	ANG	TIE-2 tyrosine kinase receptor	Endothelial and tumor	Angiogenesis

kinase activity on several substrates of both MAPK/ERK and PI3K/AKT pathways, in order to regulate biological processes including apoptosis and cellular proliferation [54]. According to this pivotal role in cell growth, mutations in EGFR gene, such as the copy number alteration, are frequently recurrent in cancer, thereby leading to EGFR overexpression and constitutive activation at the surface of tumor cells: for example, HER2 overexpression occurs in 15-30% of breast cancers and in 43-89% of the non-small cell lung carcinomas [55, 56].

Other prominent GFs involved in TSI are VEGF family, which comprise homodimeric soluble glycoproteins with a molecular weight of 45 kDa. VEGF is mainly involved in the angiogenic mechanism through its direct effect on endothelial cells, but over time its implication in promoting mitogenic stimuli in tumor cells has been also demonstrated, via autocrine self-regulation [57, 58]. VEGF-A is the most represented member of the VEGF family, which includes Placental Growth Factor (PLGF), VEGF-B, VEGF-C, and VEGF-D: VEGF-A binds to VEGFR1 and VEGFR2, VEGF-B and PLGF bind only to VEGFR1, and VEGF-C and VEGF-D bind to VEGFR2 [59]. Similar to EGFRs, the binding of VEGF to VEGFR induces the omo- or heterodimerization of receptors and the consequent activation of their kinase domain; the activity of VEGFR is regulated by the presence of two coreceptors: neuropilin-1 (NRP1) and NRP2. NRP1 and NRP2 exhibit 44% aminoacidic sequence identity and their expression is upregulated in cancer, supporting their role in oncogenic processes [58].

3. The Role of Cytokines in Colorectal and Pancreatic Cancer

3.1. Colorectal Cancer. CRC is the second main cause of worldwide cancer death, its pathogenesis is very complex, and it is influenced by multiple factors, associated with lifestyle (e.g., smoke, environmental factors, sedentary lifestyle, obesity, and/or hormones) or related to genetic predisposition (i.e., Chron's disease and/or colon polyps) [60]. Chronic inflammation represents one of the main causes involved in CRC progression and development [61]. A complex cytokines network characterizes CRC TME and, despite the role of inflammation in increasing CRC risk, different studies highlighted the correlation between immunity and a more desirable prognosis [62, 63].

TNF is one of the most characterized cytokines in CRC, probably due to the high presence of its receptors TNFR1 and TNFR2 in intestinal epithelial cells [19, 64]. TNF pathogenesis is associated not only with its levels but also with the specific receptor that it binds: low TNF levels are related to greatest percentage of cell migration but higher level of TNF with the inhibition of the physiologic wound closure, mediated by TNFR2 and TNFR1 binding, respectively [64, 65]. Stillie and her colleagues indeed demonstrated that, despite similar inflammation levels, mice lacking TNFR1 have reduced tumor and dysplasia incidence as compared to TNFR1 wild-type mice [64].

Moreover, even if during the time evidence has controversially highlighted TNF-α as both tumor promoter and suppressor, Grimm and colleagues demonstrated that TNF-α is involved in tumor growth, metastasis, invasion, and it is also correlated with positive lymph node stage and tumor recurrence in metastatic CRC (mCRC) patients [66, 67].

Despite the fact that TGF-β pathway is frequently altered in a high percentage of CRC patients, elevated levels of TGF-β have been observed in organoids derived from CRC patients. Indeed, TGF-β expression in TME is supported by the stromal cells compartment contribution (i.e., CAFs and endothelial cells), thus leading to enhancing the colonization capability of CRC cells at the initial phase of metastasis and consequent poor prognosis [68, 69]. Moreover, pharmacological inhibition of TGF-β signaling in the TME causes the reduction of metastases formation in *in vitro* patient-derived tumor organoids [69].

Elevated levels of IL-6 expression were observed in both serum and tissue of CRC patients [70, 71]. The production of IL-6 is mainly associated with NF-κB activation and the involvement of IL-6 in CRC progression is actually accepted; indeed, a recent study demonstrated the direct correlation between IL-6 levels and Tumor Node Metastasis (TNM) stage and with less histological differentiation [72, 73]. Moreover, a recent meta-analysis confirmed the role of IL-6 levels with poor prognosis of both Overall Survival (OS) and disease-free survival of CRC patients, thus highlighting the role of IL-6 as an important biomarker in CRC diagnosis [74]. It has been further demonstrated that IL-6 is also involved in Microsatellite Instability (MSI), a mechanism observed in around 15% of CRCs [75]. Indeed, Tseng-Rogenski and her colleagues demonstrated the ability of IL-6 to induce MSI in *in vitro* CRC models, through the translocation of hMSH3 from the nucleus to the cytosol, thus blockading DNA mismatch repair [76].

Our group has recently demonstrated that the genetic background of CRC cell lines predicts specific chemokines patterns of expression. In particular, we showed that BRAF-mutation and PTEN-loss status are associated with higher levels of IL-8 production [77]. Indeed, IL-8 is another important cytokine involved in CRC and its levels are correlated with CRC progression and development of liver metastases [78]. Elevated serum levels of several cytokines, mainly released by tumor cells and CAFs, have a prognostic value and are also implicated in tumor aggressiveness and poor response to therapy: consistently, high levels of IL-8 in serum of patients correlate with a more advanced tumor stage [79]. Moreover, Lurje and collaborators demonstrated that germline polymorphisms of genes involved in tumor angiogenesis, such as IL-8 and VEGF, independently predict tumor recurrence in advanced status of CRC patients [79]. VEGF, indeed, represents the predominant angiogenic factor in CRC and preclinical experiments have correlated its expression with tumor progression, principally due to the angiogenesis and metastasis induction [80–82]. Furthermore, VEGF deletion, using somatic or siRNA knockout, leads to increasing of apoptosis and CRC sensitivity to chemotherapy [83, 84]. *In vitro* results were also confirmed in CRC patients: VEGF expression is higher in tumor as compared to normal tissue and elevated levels in tissues are associated with an advanced stage of the disease [85, 86].

3.2. Pancreatic Cancer. Pancreatic cancer is one of the most aggressive tumors characterized by a very poor prognosis and by the refractoriness to conventional therapies [87]. Despite the absence of a strong prognostic factor, different studies are focused on the analysis and identification of the putative role of proinflammatory and angiogenic factors in pancreatic cancer patients. Indeed, both pro- and anti-inflammatory cytokines/chemokines are overexpressed in pancreatic cancer [88]. Ebrahimi and his colleagues demonstrated that serum of pancreatic carcinoma patients displays higher levels of IL-6, IL-10, IL-8, and IL-1RA as compared to serum of healthy patients [4]. In particular, IL-6 levels correlate with weight loss and with a worse prognosis [4, 89]. The importance of IL-6 in pancreatic cancer is due to its release not only by cancer cells but also by stromal cells, thus leading to the progression of pancreatic intraepithelial neoplasia and the development of Pancreatic Ductal Adenocarcinoma (PDAC) [90]. These results also support the relevance of the constitutive activation of STAT3 pathway in affecting a malignant phenotype of pancreatic cancer [91].

In *in vitro* and *in vivo* pancreatic cancer samples, IL-8 overexpression is associated with the increasing production of VEGF and metastatic progression in hypoxic condition, through the MAPK/ERK pathway activation [92]. The IL-8-mediated invasive and migration capability is allowed by cooperation with both SDF1-α in TME and MMP-2 activity [93, 94]. The correlation between IL-8 and clinicopathological status of pancreatic cancer patients is also confirmed by the CXCR1 upregulation in tissue derived from patients, which are characterized by poor prognosis [95].

NF-κB pathway is one of the most activated signaling pathways in PDAC cells and patient-derived tissues and its activation is principally due to TNF-α. Consistent with this evidence, TNF-α levels are high in patients affected by pancreatic cancer and correlate with advanced status of the neoplasia [96, 97]. Moreover, TNF-α affects tumor cell growth and invasion in pancreatic tumor both *in vitro* and *in vivo* [98]. Ringel and colleagues also identified the aberrant expression of ADAM17, the TNF-α processing enzyme, and its role in invasion of both PDAC cell lines and tissue derived from patients [99].

The controversial role of TNF described in CRC is also observed in PDAC: albeit the TNF exposure of tumor-bearing mice increases tumor growth, TNF plays also anti-tumorigenic function through TNFR1. Indeed, the presence of TNFR1 is necessary to ensure better immunosurveillance, mediated by increased infiltration of $CD8^+$ T cells [100].

Another mutation involved in pancreatic tumor progression is undoubtedly associated with SMAD4 gene. This tumor suppressor is inactivated in around 55% of PDAC with the homozygous deletion of both alleles or with the loss of one and the mutation in the other one [101]. SMAD4 is the mediator of TGF-β signaling and its association with tumor growth and metastasis in PDAC is currently known [102, 103]. A recent study from Zhao and colleagues showed the potential prognostic role of TGF-β: indeed, higher serum levels of TGF-β were detected in PDAC patients as compared to healthy patients or to benign pancreatic conditions; levels of TGF-β also identified pancreatic cancer stage (I-II versus III-IV) and correlated to the reduction of survival and poor prognosis [104].

A clinical significance to growth-regulated oncogene-α has been assigned by Lian and collaborators. In a recent study, they observed higher level of this chemokine in pancreatic cancer tissues as compared to normal ones, and the expression was correlated with TNM stage and metastases localization, thus leading to significant poor survival of patients [105].

Despite several evidences on the association of specific cytokines/chemokines and the modulation of pancreatic cancer patient survival, a recent study highlighted the importance of the general inflammatory status definition to develop a better target combination strategy. Indeed, this large prospective clinic-based study showed how combined marker of inflammation coincides with greater mortality in pancreatic cancer patients [106].

4. Involvement of Cytokines Patterns in Cancer Therapeutic Choice

As mentioned above, TME and TSI also increase drug-resistance development of cancer cells, thus leading to the need of better understanding of the mechanisms behind acquired tumor resistance, which remain crucial to determine overall patient benefit [107].

Mutational status in CRC is a strong predictor for OS, not only in the metastatic setting but also in earlier stages, and it is involved in drug resistance development [108]. Furthermore, mutations are often used as a biomarker to select patients who would benefit from a specific therapeutic approach: indeed, in patients with mCRC, OS has improved mainly due to the use of targeted therapies, but survival improvement is linked to proper selection of patients who could benefit from these treatments. For example, only patients lacking mutations in KRAS or NRAS benefit from EGFR monoclonal antibodies (cetuximab and panitumumab) treatment [108]. Indeed, panitumumab is currently used in combination with chemotherapy in first and second line and as a monotherapy in chemorefractory KRAS-wild-type CRC patients [109].

Another biologic therapy targeting angiogenesis in mCRC is represented by bevacizumab, a humanized recombinant monoclonal antibody directed against VEGF-A. Bevacizumab is recommended as first- and second-line treatment in combination with chemotherapy, for KRAS-mutated stage IV mCRC patients. However, several studies showed that the clinical benefit from anti-VEGF therapy appears to be independent of KRAS status and predictive biomarkers of sensitivity/resistance have not been yet identified [110]. A recent study demonstrated that IL-8 polymorphisms (c.-251T>A) correlate with a worse Progression-Free Survival (PFS) in KRAS-mutated bevacizumab-treated mCRC patients, consistent with the role of IL-8 in angiogenesis and thus representing an escape mechanism from VEGF-targeted treatment [111].

During the last years, an increasing number of evidences have highlighted the role of IL-8 as a putative prognostic/predictive biomarker in CRC. For example, Lurje and

colleagues demonstrated that germline polymorphisms of IL-8 (T2251A) and VEGF (C+936T) are associated with a higher risk of developing tumor recurrence in stage III CRC patients [79]. Furthermore, Rubie and her colleagues showed that IL-8 levels have a prognostic value and are also implicated in tumor aggressiveness and poor response to therapy: indeed, they demonstrated that IL-8 production is associated with CRC progression, including liver metastases development [78]. A significant number of *in vitro* and *in vivo* preclinical studies support the importance of IL-8-CXCR1/2 signaling in promoting tumor progression and multiple small-molecule antagonists and humanized monoclonal antibodies are under investigations [112]. Based on this evidence, IL-8 and its receptors CXCR1/2 could represent a novel therapeutic target in CRC to sensitize cancer cells toward chemotherapy [113]. Indeed, treatment with an inhibitor of CXCR2, SCH-527123, alone and in combination with oxaliplatin, is effective in synergistically inhibiting proliferation and angiogenesis and enhancing chemosensitivity in CRC cells and xenografts [113].

Matsusaka and colleagues investigated also the correlation between IL-6 (rs2069837, rs1800795) and STAT3 (rs744166, rs4796793) polymorphisms and the outcomes in a phase III mCRC trial of first-line bevacizumab-based chemotherapy, thus demonstrating that IL-6 genotype may be a useful predictive and prognostic biomarker in mCRC patients [114]. Even if IL-6/STAT3 signaling is involved in CRC progression, clinical trials that target IL-6 pathway are currently missing. After the failure of anti-IL-6 antibodies and the controversial results of chimeric murine-human monoclonal anti-IL-6 antibody siltuximab, the anti-IL-6R antibody tocilizumab and the small JAK1 and 2 inhibitor ruxolitinib were developed, but no clinical trial has been developed for cancer treatment [115].

TME and stroma are the most therapeutic barriers in drug response of pancreatic cancer by affecting treatment responses and PDAC patients survival [116]. IL-6/JAK/STAT axis represents a key pathway involved in PDAC progression. Indeed, Xing and collaborators recently demonstrated that IL-6 silencing causes increasing of apoptosis, thus reducing tumorigenicity of cancer cells. Moreover, IL-6 downregulation by gene-silencing enhances the sensitivity of pancreatic cells to gemcitabine [117].

Due to the described evidence of TNF-α implication in pancreatic cancer progression, Egberts and his group investigated the effects of the chimeric monoclonal antibodies infliximab and etanercept on PDAC cells in both *in vitro* and *in vivo* models. Although no significant effects on cell proliferation and invasiveness were observed *in vitro*, strong effects on reducing number of liver metastases were detected in orthotopic xenotransplantation mice models [98]. However, even if TNF-α seems to be relevant in PDAC patients, a phase I/II study for the combination of chemotherapy (gemcitabine) and TNF-α-inhibitor (etanercept) failed to demonstrate a synergism in PDAC patients [118].

A recent phase II clinical trial with the combination of gemcitabine and galunisertib, a TGF-β inhibitor, showed synergistic effects of the two drugs, as demonstrated by an improvement of OS and PFS in stage II to stage IV unresectable PDAC patients [119].

5. Conclusions

Even though it is now well established that TME, with inflammation and inflammatory mediators (such as chemokines, GFs, and angiogenic factors), plays an important role in promoting tumor progression, metastasis, and drug resistance, many of the mechanisms underlying TSIs are to be identified. Indeed, interactions among cancer cells and between cancer cells and the surrounding microenvironment can affect the sensitivity of tumor cells to targeted therapy/chemotherapy. Understanding the role of cytokines in TSIs could be crucial to predict pharmacological responses to specific antagonists and to build the rationale for novel therapeutic combinations in cancer treatment.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Michele Milella and Ludovica Ciuffreda equally contributed to this work.

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Research Article

Identification of Transcriptional Signatures of Colon Tumor Stroma by a Meta-Analysis

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Background. The tumor stroma plays pivotal roles in influencing tumor growth, invasion, and metastasis. Transcriptional signatures of colon tumor stroma (CTS) are significantly associated with prognosis of colon cancer. Thus, identification of the CTS transcriptional features could be useful for colon cancer diagnosis and therapy. *Methods*. By a meta-analysis of three CTS gene expression profiles datasets, we identified differentially expressed genes (DEGs) between CTS and colon normal stroma. Furthermore, we identified the pathways, upstream regulators, and protein-protein interaction (PPI) network that were significantly associated with the DEGs. Moreover, we analyzed the enrichment levels of immune signatures in CTS. Finally, we identified CTS-associated gene signatures whose expression was significantly associated with prognosis in colon cancer. *Results*. We identified numerous significantly upregulated genes (such as *CTHRC1*, *NFE2L3*, *SULF1*, *SOX9*, *ENC1*, and *CCND1*) and significantly downregulated genes (such as *MYOT*, *ASPA*, *KIAA2022*, *ARHGEF37*, *BCL-2*, and *PPARGC1A*) in CTS versus colon normal stroma. Furthermore, we identified significantly upregulated pathways in CTS that were mainly involved in cellular development, immune regulation, and metabolism, as well as significantly downregulated pathways in CTS that were mostly metabolism-related. Moreover, we identified upstream TFs (such as SUZ12, NFE2L2, RUNX1, STAT3, and SOX2), kinases (such as MAPK14, CSNK2A1, CDK1, CDK2, and CDK4), and master metabolic transcriptional regulators (MMTRs) (such as HNF1A, NFKB1, ZBTB7A, GATA2, and GATA5) regulating the DEGs. We found that CD8+ T cells were more enriched in CTS than in colon normal stroma. Interestingly, we found that many of the DEGs and their regulators were prognostic markers for colon cancer, including *CEBPB*, *PPARGC1*, *STAT3*, *MTOR*, *BCL2*, *JAK2*, and *CDK1*. *Conclusions*. The identification of CTS-specific transcriptional signatures may provide insights into the tumor microenvironment that mediates the development of colon cancer and has potential clinical implications for colon cancer diagnosis and treatment.

1. Background

The tumor stroma is an important component of the tumor microenvironment (TME) and plays key roles in the tumor development [1]. Stromal cells are composed of many different types of cells, including vascular endothelial cells, pericytes, adipocytes, fibroblasts, osteoblasts, chondrocytes, extracellular matrix (ECM), and bone-marrow mesenchymal stromal cells [2]. The tumor stroma can promote ECM remodeling, cellular migration, neoangiogenesis, invasion, immunosurveillance evasion, and drug resistance of tumors [3]. Colorectal cancer (CRC) is the fourth most common cancer and a leading cause of cancer mortality worldwide [4]. Transcriptional signatures of CRC stromal cells have been associated with poor prognosis in CRC [5]. Isella et al. demonstrated that the gene signatures of CRC stromal cells (cancer-associated fibroblasts, leukocytes, and endothelial cells) were significantly upregulated in the stem/serrated/mesenchymal transcriptional subtype of CRC which had a poor prognosis [6]. Calon et al. showed that

the CRC stromal transcriptional signatures correlated with disease relapse [5]. These prior studies exhibited the significant roles of tumor stroma in CRC growth, invasion, and metastasis.

In this study, we performed a meta-analysis of three colon tumor stromal transcriptome datasets using the bioinformatics approach. We identified differentially expressed genes (DEGs) between colon tumor stroma (CTS) and normal stroma. On the basis of these DEGs, we identified their associated pathways, upstream regulators, and protein-protein interaction (PPI) network and certain prognostic markers that were associated with survival of colon cancer patients. We also analyzed the enrichment levels of immune signatures in CTS. This study provides insights into CTS molecular features that could have clinical implications for colon cancer diagnosis and treatment.

2. Methods

2.1. Datasets. We searched the NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) using the keywords "colon cancer," "stroma," and "tumor stroma" and identified three CTS gene expression profiles datasets (GSE31279, GSE35602, and GSE46824) [7–9]. In survival analyses, we used the TCGA colon cancer dataset (https://portal.gdc.cancer.gov/) and a SurvExpress (http://bioinformatica.mty.itesm.mx/SurvExpress) built-in dataset (colon metabase) [10]. A summary of these datasets is shown in Supplementary Table S1.

2.2. Identification of DEGs between CTS and Normal Stroma. We used the web tool Network Analyst [11] to identify the DEGs between CTS and normal stroma. The ComBat method [12] in the tool was utilized to remove batch effects from the three CTS datasets (Supplementary Figure S1). Each individual dataset was normalized by base-2 log transformation and quantile normalization, and the R package "limma" was utilized to identify the DEGs between CTS and normal stroma. A meta-analysis of the three datasets was performed using Cochran's combination test [13]. The false discovery rate (FDR), calculated by the Benjamini–Hochberg method [14], was used to adjust for multiple tests. We determined the DEGs with a threshold of absolute combined effect size (ES) >0.82 and FDR<0.05.

2.3. Gene-Set Enrichment Analysis. We performed gene-set enrichment analysis of the DEGs by GSEA [15]. The KEGG pathways significantly associated with the upregulated and the downregulated DEGs were identified (FDR < 0.05), respectively.

2.4. Identification of Transcription Factors (TFs), Kinases, and Master Metabolic Transcriptional Regulators (MMTRs) That Are Significantly Associated with the DEGs. To link gene expression signatures to upstream cell signaling networks, we used eXpression2Kinases [16] to identify the upstream TFs and kinases that regulate the DEGs and utilized iRegulon [17] to identify the MMTRs of the DEGs.

2.5. Identification of PPI Network of the DEGs. We employed Network Analyst [11] to construct a PPI network of the DEGs [11]. Two types of modules (function-first modules and connection-first modules) of the PPI network were extracted. The function-first modules (FFMs) were constructed by pathway enrichment analysis and the connection-first modules (CFMs) were identified by the random walk-based algorithm [18].

2.6. Comparison of the Enrichment Levels of CD8+ T Cells between Two Classes of Samples. The enrichment level of CD8+ T cells in a sample was evaluated by the expression level of *CD8A*. We compared the enrichment levels of CD8+ T cells between two groups of samples using Student's *t*-test.

2.7. Identification of DEGs between High-Stroma-Content and Low-Stroma-Content TCGA Colon Cancer Samples. We used ESTIMATE [19] to quantify the intratumoral stromal content (stroma score) of TCGA colon cancer samples. We identified the DEGs between high-stroma-content (stroma score > median) and low-stroma-content (stroma score < median) tumors using Student's *t*-test.

2.8. Survival Analyses. We compared the overall survival (OS) and the disease-free survival (DFS) of colon cancer patients classified based on gene expression levels (expression levels > median versus expression levels < median). Kaplan-Meier survival curves were used to show the survival differences, and the log-rank test was utilized to evaluate the significance of survival differences. The individual prognostic genes were identified and were fitted in a multivariate Cox regression model. SurvExpress [10] was used for the multivariate survival analysis.

3. Results

3.1. Identification of DEGs between CTS and Normal Stroma. We identified 694 DEGs between CTS and normal stroma by the meta-analysis. These DEGs included 295 downregulated and 399 upregulated genes in CTS (Supplementary Tables S2 and S3). Figure 1 shows the top 25 upregulated and top 25 downregulated genes in CTS ranked on the basis of the combined ES (the detailed results of statistical analysis for the top 10 upregulated and top 10 downregulated genes in CTS are shown in Supplementary Tables S4). *CTHRC1,* a gene involved in vascular remodeling, bone formation, and developmental morphogenesis, was upregulated in CTS with the highest ES. It has been shown that *CTHRC1* could promote human CRC cell proliferation and invasion by activating Wnt/PCP signaling [20]. This gene also plays an important role in promoting ovarian cancer cell adhesion, migration, and metastasis through the activation of integrin β3/FAK signaling [21]. *NFE2L3*, a gene regulating the cell cycle progression in colon cancer [22], was upregulated in CTS with the second highest ES. Interestingly, both *CTHRC1* and *NFE2L3* have been indicated as useful biomarker candidates for CRC diagnosis because of their overexpression in adenomas and CRC relative to normal tissue [23]. *SULF1*,

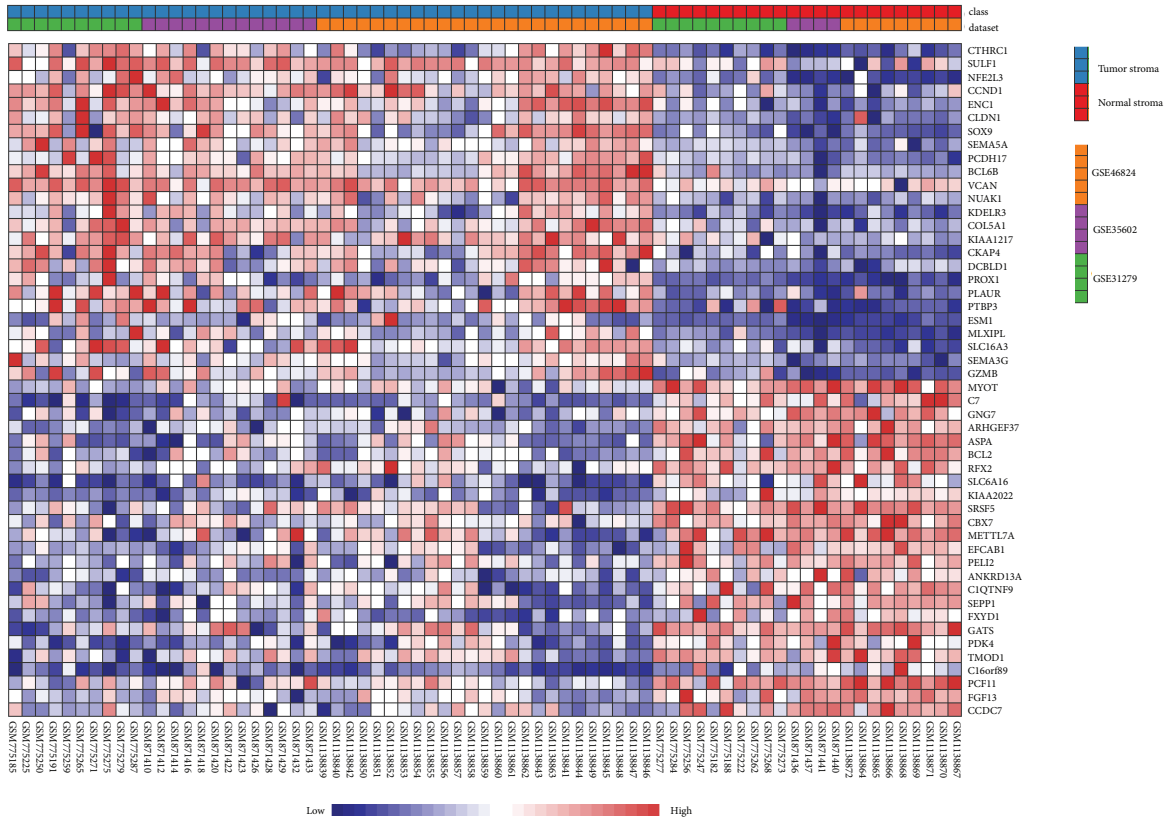


Figure 1: Gene expression pattern of the top 25 upregulated and top 25 downregulated genes in colon tumor stroma (CTS) relative to colon normal stroma ranked on the basis of the combined effect size (ES) identified by Network Analyst [11].

whose expression in tumor stroma is a prognostic marker in advanced pancreatic cancer [24], was upregulated in CTS with the third highest ES. The overexpression of this gene has been associated with a poor prognosis in urothelial carcinoma [25]. *SOX9*, the gene upregulated in CTS with the fourth highest ES, has been shown to be overexpressed in CRC and its overexpression was an independent adverse prognosticator in CRC [26]. Some other genes upregulated in CTS have been demonstrated to be overexpressed in CRC and their expression was negatively associated with CRC prognosis, such as *ENC1*, *CCND1*, V*CAN*, *SEMA5A*, and *NOS3* [27–31]. Interestingly, both *PCDH17* and *BCL6B* were upregulated in CTS, while they had reduced expression in CRC [32, 33]. It indicates that *PCDH17* and *BCL6B* could be specifically expressed in CTS cells but not in colon cancer cells.

Many of the significantly downregulated genes in CTS have been associated with CRC [34–37]. For example, *MYOT*, *ASPA*, and *KIAA2022* were downregulated in CRC [34], the downregulation of *ARHGEF37* was associated with a poor prognosis in CRC [35], higher expression levels of *BCL-2* were correlated with a better survival prognosis in CRC [36], and *PPARGC1A* was a negative predictor for CRC prognosis [37].

Altogether, a number of the abnormally expressed genes in CTS compared to colon normal stroma identified by the meta-analysis have been associated with CRC pathology and prognosis.

3.2. Identification of Pathways Significantly Associated with the DEGs. GSEA [15] identified 44 KEGG pathways that were significantly associated with the upregulated genes in CTS. These pathways were mainly involved in cellular development (p53 signaling, Wnt signaling, apoptosis, Notch signaling, focal adhesion, endocytosis, ECM-receptor interaction, cell adhesion molecules, adherens junction, tight junction, gap junction, and regulation of actin cytoskeleton), immune regulation (leukocyte transendothelial migration, complement and coagulation cascades, natural killer cell mediated cytotoxicity, Toll-like receptor, chemokine signaling, and cytokine-cytokine receptor interaction), and metabolism (purine metabolism and pyrimidine metabolism) (Figure 2, Supplementary Table S5). Previous studies have shown that some of these pathways were significantly associated with colon cancer [38–41]. For example, the Wnt and Notch pathways were associated with colon cancer development [38, 39]. The cytokine-cytokine receptor interaction pathway was significantly enriched in CRC [34]. The ECM and ECM-associated proteins [39], the glycosaminoglycan metabolism, and chondroitin sulfate/dermatan sulfate metabolism pathways played key roles in mediating tumor microenvironment [40, 41].

In addition, GSEA identified six KEGG pathways that were significantly associated with the downregulated genes in CTS (Supplementary Figure S2). Most of these pathways were metabolism-related, including purine metabolism, histidine

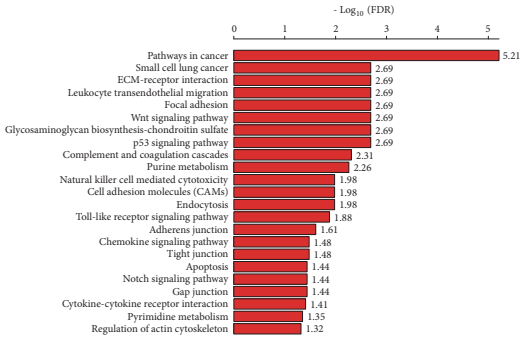


Figure 2: *Significantly upregulated KEGG pathways in CTS relative to colon normal stroma identified by GSEA [15]*. FDR: false discovery rate.

metabolism, glycine, serine, and threonine metabolism, and drug metabolism-cytochrome p450. These pathways have been associated with colon and other cancers [42–44]. For example, impaired purine metabolism was associated with the progression of cancer [42]. Histidine metabolism could boost cancer therapy [43]. Cytochrome P450 enzymes were associated with the metabolism of anticancer drugs and their expression was associated with a poor prognosis in CRC patents [44].

3.3. Identification of Upstream TFs, Kinases, and MMTRs Significantly Associated with the DEGs. We identified 11 significant upstream TFs regulating the DEGs, including SUZ12, NFE2L2, RUNX1, ESR1, STAT3, TCF3, FOSL2, SALL4, AR, SMC3, and SOX2, of which the genes encoding RUNX1 and SALL4 were upregulated in CTS (Figure 3(a)). Most of these TFs have been associated with colon cancer [45–49]. For example, SUZ12 was the most significant upstream TF which could contribute to the CRC development [45]. RUNX1 mutations were associated with the CRC risk [46]. TCF3 and FOSL2 were associated with the tumorigenesis of CRC [47, 48]. The overexpression of SOX2 was associated with the progression and a poor prognosis in colon cancer [49].

Moreover, we identified 124 significant protein kinases that regulate the DEGs (Figure 3(b), Supplementary Table S6). These kinases mainly included cell cycle regulation kinases (CDKs), signaling MAP kinases (MAPKs, MAP2Ks, and MAP3Ks), and ribosomal kinases (RPS6KA1, RPS6KA3, and RPS6KA5). MAPK14 was the most significant upstream kinase negatively regulating the formation of colitis-associated colon tumors [50]. Furthermore, we constructed a TF-kinase interaction network of these TFs and kinases (Figure 3(c)). In the network, the most connected TFs included SUZ12, NFE2L2, RUNX1, STAT3, FOSL2, AR, SMC3, ESR1, and TCF3, and the most connected kinases included MAPK14, CDK1, CSNK2A1, CDK2, MAPK3, HIPK2, ERK1, and CDK4. It indicates that the cell cycle regulation may play a pivotal role in CTS.

MMTRs are interesting biomarkers and targets for metabolism-targeted cancer therapy [51]. We identified 9 (HNF1A, NFKB1, ZBTB7A, ATF6, TEAD4, TFAP2B, JAZF1, FNTB, and EP300) and 12 (PKNOX2, GATA2, MAPK10, TEAD1, TOX, MEF2A, GATA5, ELK1, MAZ, NHLH1, ATF1, and RAD21) MMTRs for the upregulated and the downregulated genes in CTS, respectively (Supplementary Table S7), and built the regulatory networks associated with these MMTRs (Figure 4). In the networks, ATF6 (activating transcription factor 6), a TF regulating unfolded protein response during endoplasmic reticulum (ER) stress, targeted 163 upregulated genes, and PKNOX2 (PBX/knotted 1 homeobox 2), which plays key roles in regulating cell proliferation, differentiation, and death, targeted 131 downregulated genes. Interestingly, two members of the GATA family of TFs (GATA2 and GATA5) were the MMTRs that regulated the downregulated genes in CTS (Figure 4(b)).

Altogether, the identification of upstream TFs, kinases, and MMTRs significantly associated with the DEGs may provide insights into the TME that mediates the development of colon cancer.

3.4. CD8+ T Cells Are More Enriched in CTS than in Normal Stroma. We compared the enrichment levels of CD8+ T cells between CTS and normal stroma and found that CD8+ T cells showed significantly higher enrichment levels in CTS than in normal stroma (Student's t-test, p=0.016) (Figure 5). This suggests an antitumor immune response activity in the TME of colon cancer.

3.5. Identification of Prognostic Factors in Colon Cancer Based on the DEGs and Their Upstream Regulators. We investigated the association between the transcriptional signatures of CTS and survival prognosis (overall survival (OS) and disease-free survival (DFS)) in the TCGA colon cancer dataset. The transcriptional signatures included the top 10 upregulated and top 10 downregulated genes in CTS on the basis of ES, 45 hub genes (≥3 degrees) from the zero-order PPI network of the DEGs (Supplementary Table S8), and the genes encoding 11 TFs, 124 kinases, and 21 MMTRs regulating the DEGs. We found that the expression of many of these transcriptional signatures was significantly associated with the survival of colon cancer patients. For example, the expression of *CEBPB*, a gene significantly upregulated in CTS and a hub node in the PPI network, had a significant negative correlation with OS in colon cancer (Figure 6(a)). The negative correlation between *CEBPB* expression and survival has also been demonstrated in other cancer types, such as high-grade serous ovarian cancer [52]. *PPARGC1* was significantly downregulated in CTS and was a hub node in the PPI network, while its expression had a significant positive correlation with OS in colon cancer (Figure 6(a)). *PPARGC1A* was indicated as a tumor suppressor in colon cancer [53] and ovarian cancer [54], as well as a negative prognostic biomarker for CRC [37]. Our data indicate that the deregulation of these genes in CTS is prognostic for colon cancer patients.

Among the upstream regulators (TFs, kinases, and MMTRs) of the DEGs, the expression of *STAT3*, *RPS6KA5*, *IKBKE*, *ERBB2*, *MTOR*, and *NFKB1* had a positive correlation with OS in colon cancer, while the expression of *CDK1*, *CDK5*, and *BRD2* had a negative correlation with OS in colon cancer (Figure 6(a)). The deregulation of these genes has been

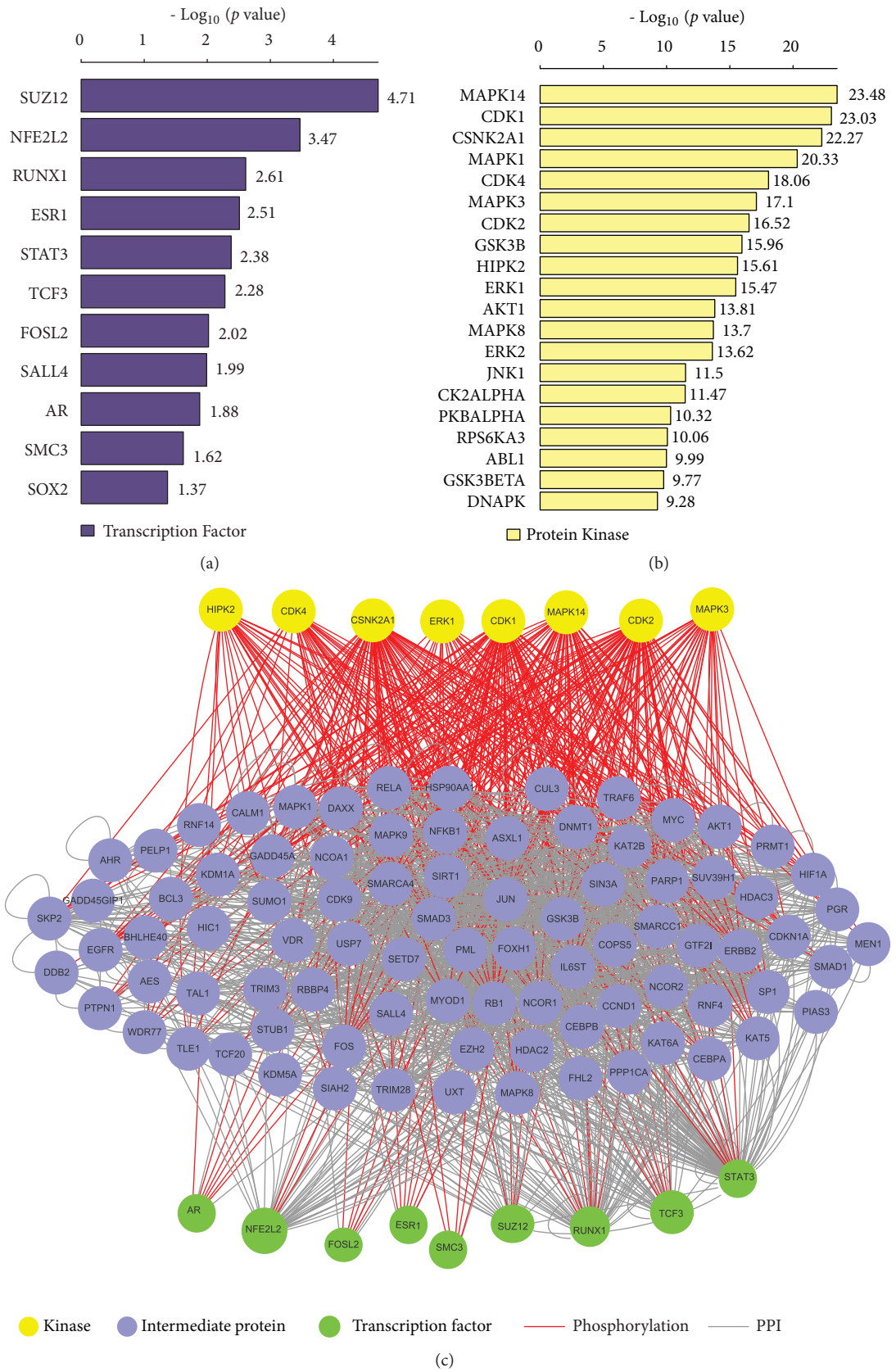
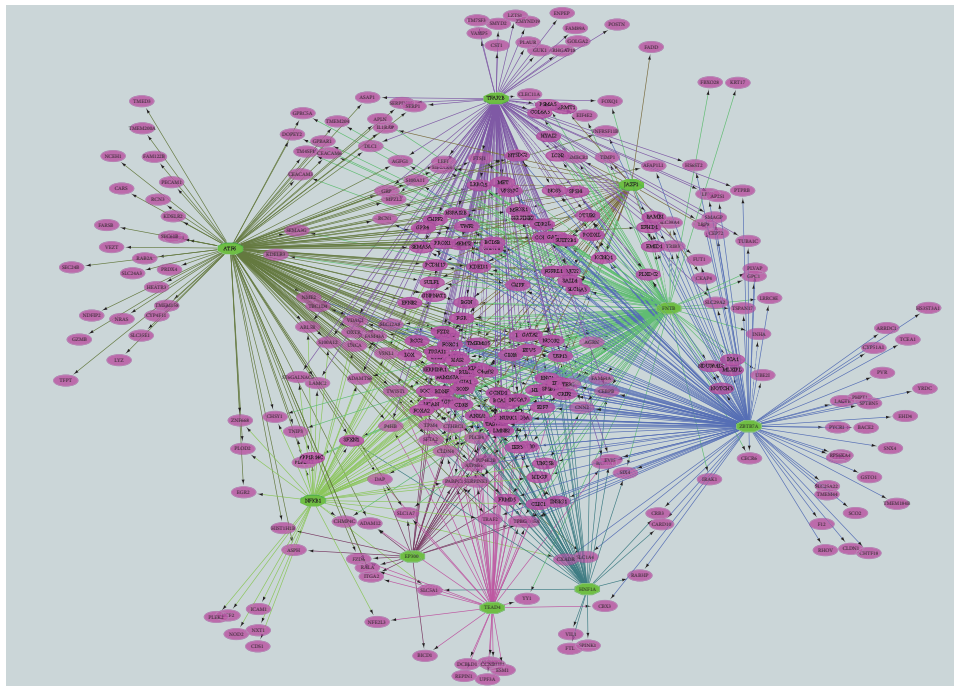
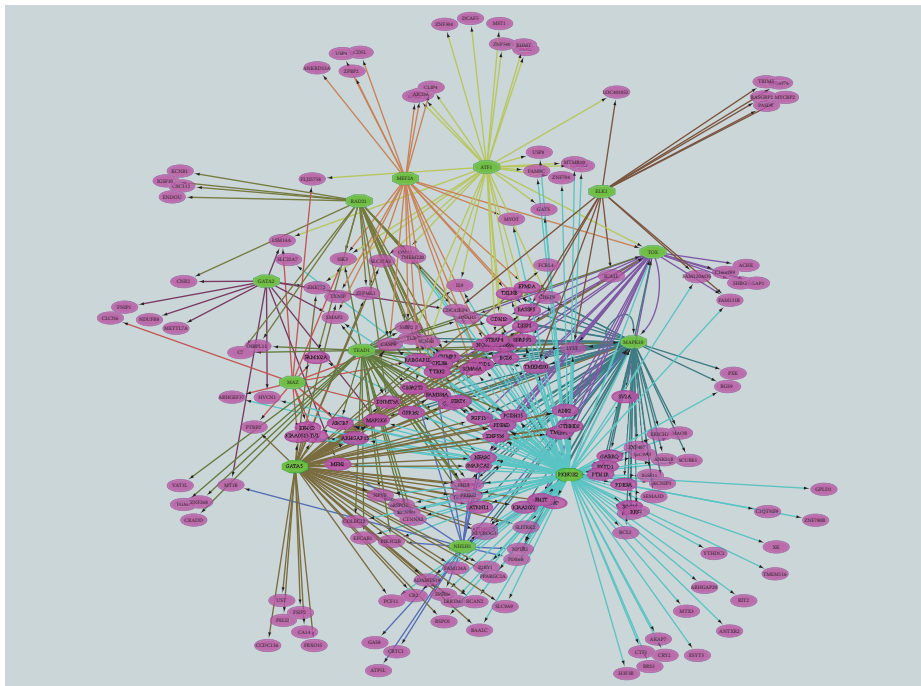


Figure 3: *The significant upstream transcriptional factors (TFs) and kinases that regulate the differentially expressed genes (DEGs) between CTS and colon normal stroma identified by eXpression2Kinases [16].* (a) Significant upstream TFs regulating the DEGs. (b) Significant upstream kinases regulating the DEGs. (c) A TF-kinase interaction network of the significant upstream TFs and kinases regulating the DEGs.



(a)



(b)

Figure 4: *Regulatory networks of the master metabolic transcriptional regulators (MMTRs) and their targeted differentially expressed genes (DEGs) between CTS and normal stroma identified by iRegulon [17].* (a) Regulatory network of the MMTRs and their targeted upregulated genes in CTS. (b) Regulatory network of the MMTRs and their targeted downregulated genes in CTS. The green color octagon indicates MMTRs and purple color oval indicates DEGs.

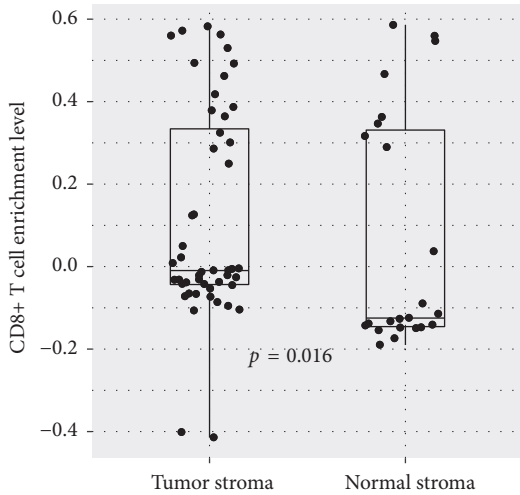


Figure 5: *CD8+ T cells have significantly higher enrichment levels in CTS than in colon normal stroma*. Student's *t*-test *p* value is shown.

associated with tumor progression in a wide variety of cancer types [55–60].

In addition, we identified 18 transcriptional signatures of CTS whose expression was significantly associated with DFS in colon cancer individually (Supplementary Figure S3). These genes included *CEBPB*, *BCL2*, *PAN2*, *NOS3*, *FTL*, *ARHGEF37*, *SMC3*, *EP300*, *JAK2*, *RPS6KA3*, *RPS6KA1*, *PRKACA*, *HIPK1*, *HIPK2*, *MAPK8*, *GSK3A*, *CLK2*, and *CDK3*. It indicates that these CTS transcriptional signatures could be biomarkers for colon cancer relapse.

Furthermore, we used the multivariate analysis to validate the association between the prognostic CTS transcriptional signatures and survival using the colon metabase data [10]. For OS analysis, a total of 482 patients were split into two groups: high-risk group (N=241) versus low-risk group (N=241) based on the prognostic index (Supplementary Figure S4A). As expected, the high-risk group had worse OS than the low-risk group (Figure 6(b)). Similarly, for DFS analysis, we divided patients into the high-risk group (N=272) and the low-risk group (N=273) based on the prognostic index (Supplementary Figure S4B) and found that the high-risk group had worse DFS compared to the low-risk group (Figure 6(c)). These results proved the prognostic value of these CTS transcriptional signatures in colon cancer.

4. Discussion

The tumor stroma constitutes an important component of the TME that mediates tumor growth, immune evasion, and metastasis [1]. Thus, it is important to identify molecular features in the tumor stroma. To this end, we performed a meta-analysis of three CTS transcriptome datasets for identifying CTS-associated transcriptional signatures. We identified a number of upregulated and downregulated genes in CTS compared to colon normal stroma. Furthermore, we identified upregulated and downregulated pathways significantly associated with these deregulated genes in CTS. The upregulated pathways were mainly involved in cellular development, immune regulation, and metabolism, and the downregulated pathways were mostly metabolism-related. These results revealed the abnormal alterations of cellular development, immune regulation, and metabolism pathways in CTS. We found that CD8+ T cells were more enriched in CTS than in colon normal stroma, suggesting an immune infiltration microenvironment in CTS. Furthermore, we identified numerous CTS transcriptional signatures whose expression was significantly associated with prognosis in colon cancer, such as *CEBPB*, *PPARGC1*, *STAT3*, *MTOR*, *BCL2*, *JAK2*, and *CDK1*. These transcriptional signatures are mainly involved in immune regulation (*CEBPB*, *STAT3*, and *JAK2*), metabolism (*PPARGC1* and *MTOR*), cell cycle (*CDK1*), and apoptosis (*BCL2*), suggesting that the deregulation of these pathways in CTS may contribute to the altered prognosis in colon cancer.

To verify the association of the identified transcriptional signatures with CTS, we analyzed the TCGA colon cancer dataset. We divided these cancers into high-stroma-content and low-stroma-content groups on the basis of their intratumoral stromal content evaluated by ESTIMATE [19] and found that 153 upregulated genes in CTS had significantly higher expression levels in the high-stroma-content group than in the low-stroma-content group. These genes included 18 hub genes in the PPI network of DEGs and 6 TFs, 40 kinases, and 12 MMTRs encoding genes that regulated the DEGs (Supplementary Figure S5, Table S9). We also found 27 downregulated genes in CTS which had significantly lower expression levels in the high-stroma-content group, including 14 hub genes, and genes encoding 2 TFs, 18 kinases, and 3 MMTRs (Supplementary Figure S5, Table S9). Interestingly, most of the downregulated hub genes in CTS were also downregulated in the high-stroma-content colon cancers (Supplementary Figure S5). These results indicate that many transcriptional signatures of CTS identified by the meta-analysis are tumor stroma-specific. In addition, we found that CD8+ T cells had significantly higher enrichment levels in CTS versus colon normal stroma (Student's *t*-test, p=0.016), as well as in the high-stroma-content colon cancers versus the low-stroma-content colon cancers (Student's

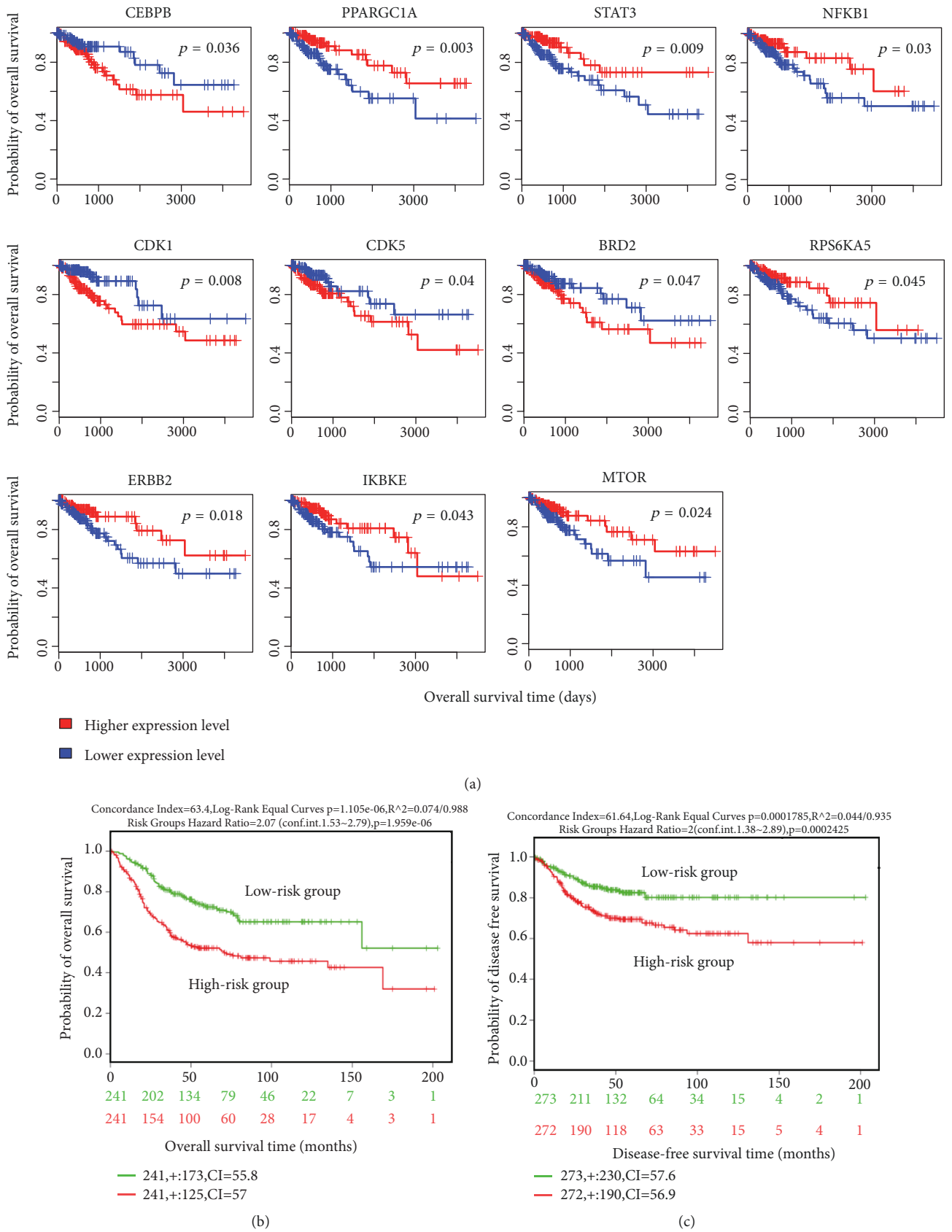


Figure 6: *The CTS gene signatures whose expression is associated with prognosis in colon cancer.* (a) Kaplan-Meier survival curves show the gene signatures whose expression is significantly associated with overall survival (OS) in colon cancer in the TCGA colon cancer dataset (log-rank test, $p<0.05$). (b) Multivariate Cox regression analysis shows that the OS-associated CTS gene signatures are prognostic for OS in colon cancer in a SurvExpress built-in dataset (colon metabase) [10]. (c) Multivariate Cox regression analysis shows that the DFS-associated CTS gene signatures are prognostic for DFS in colon cancer in a SurvExpress built-in dataset (colon metabase) [10]. DFS: disease-free survival.

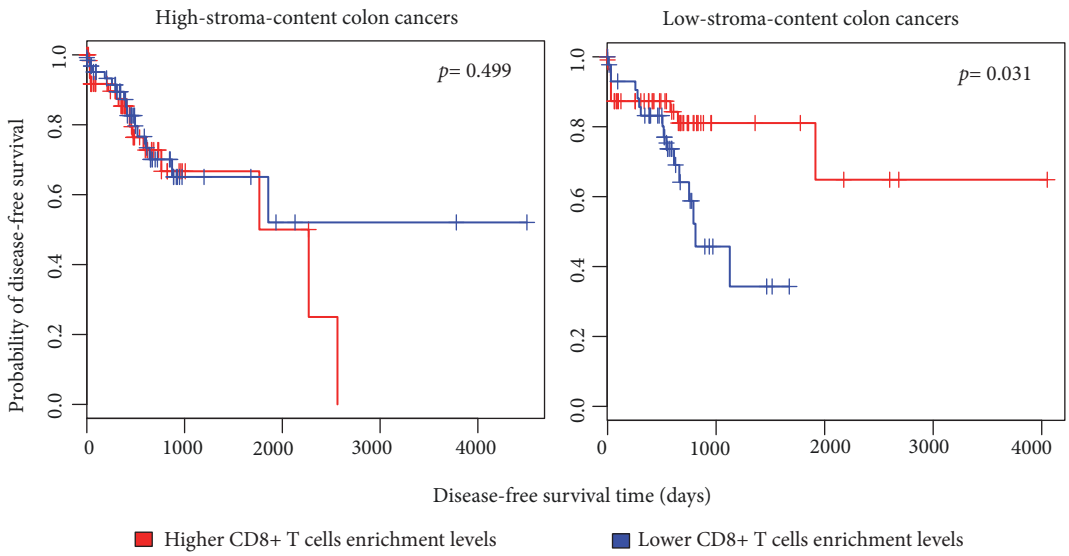


Figure 7: *The higher enrichment levels of CD8+ T cells were associated with better disease-free survival in the low-stroma-content colon cancers, but not in the high-stroma-content colon cancers.* ESTIMATE [19] was used to quantify the intratumoral stromal content (stroma score) of TCGA colon cancer samples. High-stroma-content: stroma score > median; low-stroma-content: stroma score < median.

t-test, $p=3.3*10^{-8}$). It indicates that CD8+ T cells tend to have elevated infiltration in the TME of colon cancer. Interestingly, we found that the higher enrichment levels of CD8+ T cells were associated with better DFS in the low-stroma-content colon cancers, but not in the high-stroma-content colon cancers (Figure 7). It suggests that the immune cells exert an antitumor effect only when they have infiltrated into tumor cells and that the immune cells in the tumor stroma may not have such a direct antitumor effect.

This study has identified a number of CTS-associated transcriptional signatures that could be biomarkers for colon cancer diagnosis and prognosis and may provide therapeutic targets for colon cancer. However, to translate these findings into clinical application, further experimental and clinical validation would be necessary.

5. Conclusions

The identification of CTS-specific transcriptional features may provide insights into the mechanism that mediates the development of colon cancer and thus has potential clinical implications for colon cancer diagnosis and treatment.

Abbreviations

CTS: Colon tumor stroma
DEGs: Differentially expressed genes
PPI: Protein-protein interaction
ES: Effect size
TME: Tumor microenvironment
ECM: Extracellular matrix
TFs: Transcription factors
MMTRs: Master metabolic transcription factors
OS: Overall survival
DFS: Disease-free survival
CRC: Colorectal cancer
GEO: Gene Expression Omnibus
TCGA: The Cancer Genome Atlas
FDR: False discovery rate
GSEA: Gene-set enrichment analysis
FFM: Function-first module
CFM: Connection-first module.

Data Availability

The datasets (GSE31279, GSE35602, and GSE46824) were downloaded from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/), the TCGA colon cancer dataset was downloaded from the website https://portal.gdc.cancer.gov/, and the colon metabase dataset was from SurvExpress (http://bioinformatica.mty.itesm.mx/SurvExpress).

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Md. Nazim Uddin performed data analyses and helped write and prepare the manuscript. Mengyuan Li performed data analyses and helped prepare the manuscript. Xiaosheng Wang conceived the research, designed analysis strategies, and wrote the manuscript. All the authors read and approved the final manuscript.

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Supplementary Materials

Supplementary Figure S1: illustration of PCA and density plots as validation tools for batch effect removal. Plot of principal components: (A) before batch effect removal and (B) after batch effect removal. Plot of density: (C) before batch effect removal and (D) after batch effect removal. The multidimensional scaling of the datasets demonstrates that, before the batch effect adjustment, each dataset obviously separated from all the others, whereas, after batch effect adjustment, samples from all the datasets are incorporated clearly. Supplementary Figure S2: six KEEG pathways that are significantly associated with the downregulated genes in colon tumor stroma relative to normal stroma. Supplementary Figure S3: the genes (DEGs and their upstream regulators) whose expression is significantly associated with disease-free survival in colon cancer (log-rank test, $p<0.05$). Supplementary Figure S4: patients divided into the high-risk group and the low-risk group based on the prognostic gene signatures identified. A. Overall survival. B. Disease-free survival. Supplementary Figure S5: numbers of overlapping genes between the DEGs between colon tumor stroma and normal stroma and their upstream regulators and the DEGs between high-stroma-content and low-stroma-content colon cancers. UP TCGA: upregulated differentially expressed genes between high-stroma-content and low-stroma-content TCGA colon cancer samples and DOWN TCGA: downregulated differentially expressed genes between high-stroma-content and low-stroma-content TCGA colon cancer samples. Supplementary Table S1: a summary of the datasets used in this study. Supplementary Table S2: upregulated genes in colon tumor stroma versus colon normal stroma. Supplementary Table S3: downregulated genes in colon tumor stroma versus colon normal stroma. Supplementary Table S4: the top 10 upregulated and top 10 downregulated genes in colon tumor stroma. Supplementary Table S5: 44 KEGG pathways that were significantly associated with the upregulated genes in colon tumor stroma (CTS). Supplementary Table S6: upstream transcription factors and kinases regulating the differentially expressed genes between colon tumor stroma and normal stroma. Supplementary Table S7: master metabolic transcriptional regulators (MMTRs) (iRegulon normalized enrichment score NES > 3.0) regulating the differentially expressed genes between colon tumor stroma and normal stroma. Supplementary Table S8: hub genes in the protein-protein interaction network of the differentially expressed genes between colon tumor stroma and normal stroma. Supplementary Table S9: overlapping genes between the DEGs between colon tumor stroma and normal stroma and their upstream regulators and the DEGs between high-stroma-content and low-stroma-content colon cancers. *(Supplementary Materials)*

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Review Article

Epigenetic Influences in the Obesity/Colorectal Cancer Axis: A Novel Theragnostic Avenue

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The World Health Organization (WHO) considers that obesity has reached proportions of pandemic. Experts also insist on the importance of considering obesity as a chronic disease and one of the main contributors to the worldwide burden of other nontransmissible chronic diseases, which have a great impact on health, lifestyle, and economic cost. One of the most current challenges of biomedical science faces is to understand the origin of the chronic nontransmissible diseases, such as obesity and cancer. There is a large evidence, both in epidemiological studies in humans and in animal models, of the association between obesity and an increased risk of cancer incidence. In the last years, the initial discovery of epigenetic mechanisms represents the most relevant finding to explain how the genome interacts with environmental factors and the ripple effects on disease pathogeneses. Since then, all epigenetic process has been investigated by the scientific communities for nearly two decades to determine which components are involved in this process. DNA/RNA methylation and miRNA are classified as two of the most important representative classes of such epigenetic mechanisms and dysregulated activity of such mechanism can certainly contribute to disease pathogenesis and/or progression especially in tumors. This review article serves to highlight the impact of DNA/RNA methylation and miRNA-based epigenetic mechanism activities in the interplay between obesity and the development and clinical significance of colorectal cancer.

1. Introduction: Background and Clinical Importance

The World Health Organization (WHO) considers that obesity has reached pandemic proportions: more than 1900 million adults are overweight and, of these over 650 million of them, obesity [1]. Epidemiological experts also insist that obesity must be considered as a chronic disease and one of the main contributors to the worldwide burdens of other nontransmissible chronic diseases, such as autoimmune, inflammatory, neurodegenerative, and cardiovascular diseases, including diabetes or cancer [2, 3]. One of the most relevant challenges that biomedical science is trying to solve is finding the pathogenic mechanism of chronic noncommunicable diseases of metabolic origin, such as obesity and cancer. There is a large evidence of the linking between obesity and cancer. This link has also been supported by animal experiments, where obesity and cancer have been modified by dietary types [4]. Indeed, a strong relationship has been observed between adiposity and the risk of suffering from up to 13 different types of cancer, although there is a substantial heterogeneity between the different studies [5, 6].

During tumorigenesis, adipocytes that are found near to cancer cells suffer several morphological and biochemical alterations and are implicated in developing of the Cancer-Associated Adipocytes (CAAs) which influence cancer cell malignancy. CAAs located close to the invasive front acquire different fibroblast-like features. Lipids secreted by

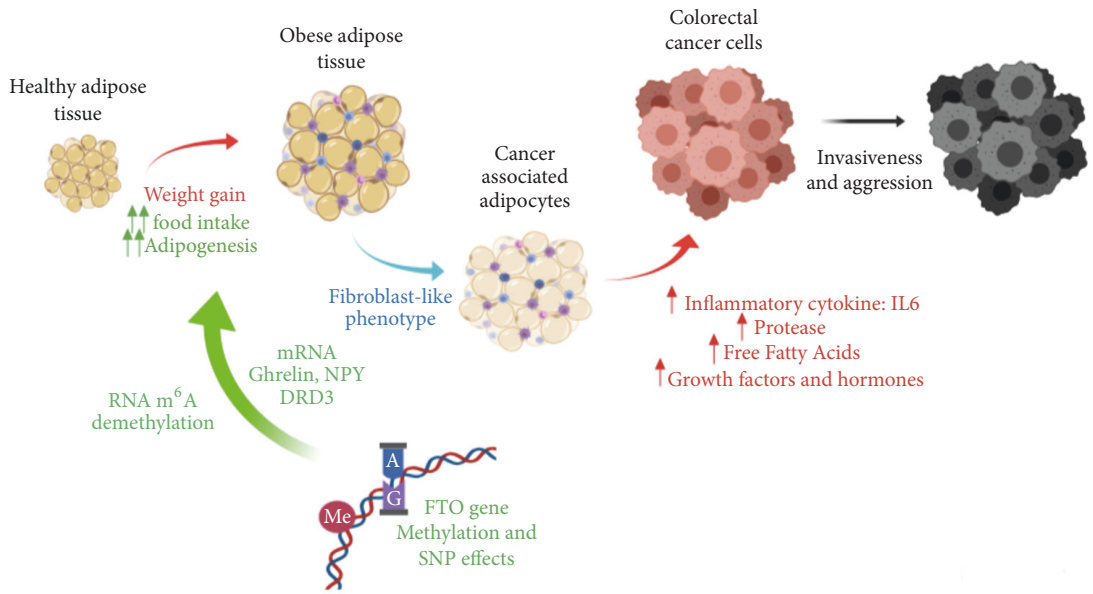

Figure 1: Proposed mechanisms linking FTO gene, obesity, and cancer. The weight gain, malfunctioning of the FTO gene leading to increase food intake and adipogenesis process could develop obesity, especially abdominal obesity. It is also linked to adipocyte hypertrophy and hypoxia. The hypertrophied adipose tissue acquires endocrine characteristics like fibroblasts, which produce an increase of adipokine and hormone secretion profile, proteases, and free fatty acids that may promote the stimulation of a microenvironment favorable for not only tumorigenesis, but acquire new properties as invasiveness and aggression. Abbreviations: m6A: N6-Methyladenosine; NPY: Neuropeptide Y; DRD3: Dopamine Receptor type D3; FTO: Fat-mass and obesity-associated; SNP: Single-nucleotide polymorphism; IL6: Interleukin 6.

adipocytes are transferred to cancer cells and used for energy production through beta-oxidation. The loss of expression of differentiation markers in CAAs such as adiponectin or leptin and the increased secretion of proinflammatory cytokines as Interleukin 6 (IL-6) and tumor necrosis factor (TNF) generate a permissive niche for tumor growth and dissemination by stimulating adhesion, migration, and invasion proprieties of malignant cells (Figure 1). Moreover, the rapid expansion of adipose tissue produces oxygen deficiency and promotes angiogenesis improving the tumor spreading [7, 8].

In particular, the new location of the CAAs transforms them in highly metabolic cell that secrete greater amounts of cytokines associated with insulin resistance [9]. In any case, the ectopic fat depots were mostly associated with paracrine effects in the tumor microenvironment. The ectopic local adiposity corresponds to inflammation and was mainly associated with colon and pancreatic cancer, breast tumorigenesis, and hepatocellular carcinoma [10, 11]. On the other hand, the systemic ectopic fat, also known as "central adiposity", corresponds to visceral adipose tissue (VAT), is responsible for altered levels of sex steroids, insulin resistance, and chronic inflammation, and increased risk of colorectal neoplasia [12, 13].

2. Epigenetics and Epitranscriptomics Mechanism of *FTO* Gene in Obesity and Colorectal Cancer: A Potential Biomarker

Epigenetic processes, including DNA methylation, modification of histones and noncoding RNAs of different size and function, change genes expression without modifying the DNA sequence. They are sensitive to external (e.g., diet and physical activity), internal (hormones and inflammation markers), and genetic factors and reversible and can be passed on to later generations. Two large-scale epigenetic studies have identified a large number of DNA methylation loci associated with Body Mass Index (BMI) [14]. Clearly, more evidence is needed to determine a role (causal) of epigenetic processes in obesity but assessing the epigenome at the right time in life and in the relevant tissues is an important barrier for human studies [14]. Recent studies in genetic association analyses show that alterations in DNA methylation are predominantly the consequence of adiposity, rather than the cause [15].

Different polymorphisms of the *FTO* (fat-mass and obesity-associated) gene have been consistently associated with obesity. The great success on a large scale of the genome-wide association studies (GWAS) was the discovery that the *FTO* locus could regulate the expression of several genes despite their distance in the linear sequence of DNA to influence body weight, through the regulation of epigenetic mechanisms related to obesity [16]. However, the molecular mechanisms responsible for the effect of *FTO* gene on obesity are not known yet. Recent genome studies reveal that genetic variants in this gene are associated not only with human adiposity and metabolic disorders, but also with several cancers including breast cancer, endometrial cancer, pancreatic cancer, and colorectal cancer, since it can activate several signaling and hormonal pathways to increase cancer incidence. These hormones include ghrelin, neuropeptide Y, oxytocin, and leptin. Therefore, the FTO polymorphisms

could exert an influence on hormonal balance and physiologic factors and might increase cancer risk (Figure 1) [17–21]. However, the link between FTO polymorphism, hormonal metabolism, and cancer risk is yet uncertain.

Epigenomics connects genomics with environmental factors in the pathogenesis of several diseases. In fact, another emerging concept in the regulation of gene expression is that some modified nucleotides are found internally in mRNA. These modifications constitute a new concept of epitranscriptomic code. The initial concept of epitranscriptome was introduced with the transcriptomic scale mapping of N6-methyladenosine (m^6A), which revealed that it is found in at least 25% of all mRNAs, typically near stop codons [22]. In fact, the most significant advance on *FTO* gene research is the recent discovery of FTO protein as the first m^6A RNA demethylase [23]. This finding provides solid evidence that the dynamic and reversible enzymatic modification of m^6A in RNA can act as a new epitranscriptomic marker [24]. Recent and consistent studies show that the biological functions of a number of cancers can be regulated at the epitranscriptional level, indicating the pharmaceutical potential of these studies. Indeed, m^6A process has been associated with cancer, contributing to the self-renewal of cancer stem cell, promotion of cancer cell proliferation, and resistance to radiotherapy or chemotherapy, and playing a crucial role in the metabolism of mRNA and regulation of noncoding RNAs in cancer cells [25].

3. Experimental Framework of Current Research: DNA Methylation in Obesity and Colorectal Cancer

Sometimes, it is necessary to go beyond the sequences of the genes to understand better the regulatory mechanisms of their expression, because, often, genomic studies do not identify the causes and etiologies of many diseases. One of the important mechanisms that explain the regulatory elements of the genes is the epigenetic modifications in DNA such as methylation, which consist in the addition of a methyl group to position 5 of the cytosine bases, which generates 5-methylcytosine (m^5C). This process can take place passively, without methylation of the newly synthesized chain after DNA replication, or actively, by mechanisms that do not depend on replication. Active demethylation of DNA has just begun to be studied, along with the discovery of another methylated variant of cytosine, 5-hydroxymethylcytosine (hm^5C), and the enzymes that catalyze its generation from m^5C [26]. There is currently an adequate level of understanding about m^5C and its impact on gene expression, but the products of DNA demethylation are not known in detail, whether they are only intermediates or epigenetic marks by themselves. Therefore, their study will reveal in a novel way the implication of epigenetics in cancer and obesity processes.

There have been published some studies that show abnormal distributions of differentially methylated regions (DMR) overlap, such as CpG hypermethylated islands, which may explain the epigenetic instability that drives the onset of cancer in individuals with obesity [27]. These studies identify a potential epigenome mark of obesity related to breast and colorectal cancer that could be useful for precision medicine in the management of these diseases considering adiposity as a relevant risk factor [28]. These results also show that the molecular heterogeneity of colorectal cancer can be influenced by modifiable risk factors such as obesity. Strikingly, by performing a comprehensive GWAS of the DNA methylation that interrogates 485000 CpG sites, it has been demonstrated for the first time the existence of a specific methylome profile in colorectal cancer associated with excess of adiposity. The epigenome-wide analysis identified 46 unique genes that exhibited differential methylation in the promoter region and island or shore, which could be established as an epigenetic signature of obesity-related colorectal cancer [28].

Epigenetic gene inactivation in transformed cells involves many "belts of silencing". One of the best-known lesions of the malignant cell is the transcriptional repression of tumor-suppressor genes by promoter CpG island hypermethylation. Currently, the scientific community are beginning to understand a great deal of epigenetic silencing of tumor-suppressor genes in human cancer by CpG island promoter hypermethylation. In the process of completing the molecular dissection of the entire epigenetic machinery involved in methylation-associated silencing, such as DNA methyltransferases, methyl-CpG binding domain proteins, histone deacetylases, histone methyltransferases, histone demethylases, and specially the identification of many more hypermethylation-silenced miRNA genes with tumor-suppressor function in human cancer, the epigenetic silencing of these miRNAs will become an excellent target [29]. The importance of hypermethylation events is already in evidence at the bedside of cancer patients in the form of cancer detection markers and chemotherapy predictors, and in the approval of epigenetic drugs for the treatment of hematological malignancies. In the very near future, the synergy of candidate gene approaches and large-scale epigenomic technologies will yield the complete DNA hypermethylome of cancer cells. In fact, it has been recently published that adipose tissue may be a key factor in colorectal cancer development [29].

The low vitamin D levels in colorectal cancer and high expression of vitamin D receptor in adipose tissue may, at least in part, mediate this relationship by modifying adipose tissue DNA methylation and promoting inflammation. Although more studies are needed to discover the precise mediators and mechanisms that determine this relationship, the possible mediation of adipose tissue in colorectal cancer should be born in mind to create new treatments and preventive strategies for colorectal cancer [30, 31].

The recent concept of the implication of epigenetic variations in numerous pathologies has directed attention towards the development of new markers for its detection. Therefore, it has become necessary to develop new analytical tests to identify epigenetic changes through molecular biomarkers related to the active or silenced state of the regions in which they are found, in order to detect changes in methylation, both in DNA and in RNA, whose manifestations can be revealed in advance even of the development of the disease. Every cell, including small and early cancerous lesions, leaves

a record of its physiological state that is reflected in the fragments of DNA and RNA, proteomic and circulating metabolic products. The molecules in circulation make up a file of biomarkers, which constitute a potential reservoir of diagnostic information for the complete organism, whose clinical use depends on the sensitivity of the detection and quantification methods of methylated DNA/RNA from liquid biopsies [32, 33]. Despite the urgent clinical need, the number of epigenetic biomarkers developed so far is really low. Given its stability, the methylation of the CpG islands constitutes a very favorable epigenetic biomarker, which allows designing methods to detect the signals of methylated DNA from specific regions of the genome, usually not methylated. However, the understanding of epigenetic factors may play a role in the search for new biomarkers, necessary to define obesity and cancer in a more precise way [33].

4. MicroRNAs: Understanding the Mechanism of Action

The MicroRNAs (miRNAs) consist of the most clinically important noncoding RNA families and have been investigated by the scientific communities for nearly two decades, since the initial discovery of the RNA interference (RNAi) pathway by Fire and Mello in 1998 [34]. Structurally, once within the cytoplasm each miRNA consists of a single RNA strand, self-folded into a duplex, with one of the duplex ends containing the active (mature) 19-22 nucleotide-long miRNA sequence [35]. Mature miRNAs, once incorporated into the RNAi machinery, act as agents of posttranscriptional gene regulators by binding with near-complementarity to the 3′-UTR of their designated target transcripts with consequent inhibition of ribosomal activity that typically occurs during the process of translation [35].

This regulatory function by miRNAs revealed a further level of complexity in the nature of gene regulation within a vast spectrum of physiological processes and, consequently, highlighted the influence of miRNAs in human disease pathology and progression and/or inhibition, whenever such miRNAs exhibit dysregulated activity [36]. In addition, the sheer fact that there exist over 2600 validated miRNAs in humans is a reminder of the intricate complexities underlying gene regulatory processes, where the dysregulated expression of an individual miRNA or a whole network of miRNAs could contribute to disease pathogenesis and/or progression, especially in tumors [37].

5. miRNA Influences in Obesity

The first indications of miRNA involvement in the regulation of lipid metabolism date back to 2006, where the study conducted by Esau and colleagues elucidated that miR-122 upregulation led to increased plasma cholesterol levels, together with a decrease in lipid metabolic activities, in murine study models [38]. Furthermore, the study carried out by Takanabe and colleagues in 2008 provided the first breakthrough to identify a direct association of miRNA dysregulated activity with obesity [39]. In this study, miR-143 was recognized to be upregulated within mesenteric fatty tissues of mice undergoing a high-fat diet and was also correlated with plasma leptin levels, with leptin being already established as a major adipocytokine [39]. Other initial discoveries to confirm the link between miRNA dysregulation and obesity include the study performed by Xie and colleagues in 2009 [40]. The study involved the employment of miRNA microarrays to analyze differing miRNA expression profiles for over 370 miRNAs during the adipogenesis of adipocytes and preadipocyte 3T3-L1 cells deriving from leptin deficient ob/ob, diet-induced obese mice [40].

The conclusions of this seminal study were that miR-143 and miR-103 upregulation both lead to exacerbated adipogenesis, with additional evidence provided by the correlated increases in adipogenesis markers and triglyceride production in such murine in vivo models [40]. The first human study on the implication of miRNA activity in obesity was conducted in the same year by Kloting and colleagues [41]. In this particular study, multiple fat deposit samples were collected from overweight and obese patients and analyzed for a panel of 155 differing miRNAs using Real Time qPCR [41]. The overall results of this study revealed that the concomitant expression pattern of seven miRNAs (miR-17-5p, miR-132, miR-99a, miR-134, miR-181a, miR-145, and miR-197) is highly correlated with the main diagnostic features for obesity, including factors such as adipose tissue morphology and unique metabolic parameters [41].

The first reporting of the link between circulating miRNA expression and obesity was by the Wang study in 2013 [42]. The results of this investigation revealed that circulating miR-130b levels were correlated with obesity in murine in vivo obesity models and in obese human volunteers [42]. In addition, miR-130b is typically secreted by adipocytes during the process of adipogenesis, with miR-130b also having the capacity to downregulate the expression of key muscular target genes such as peroxisome proliferator-activated receptor γ coactivator 1 alpha (PPARGC1A), leading to a reduction in lipid oxygenation capacity within muscles [42].

In 2014, Shao and colleagues revealed the initial connection between miR-33 and *FTO* expression [43]. This study elucidated that miR-33 in chickens was encoded on intron 16 of the sterol regulatory binding transcription factor 2 (SREBF2) and that the utilization of miR-33 antagonists in primary chicken hepatocytes led to exacerbated FTO transcript expression levels, suggesting that *FTO* is a direct target gene for miR-33 activity [43].

In 2015, Chu and colleagues highlighted the influence of miR-181a on lipid regulatory activities [44]. The study revealed that miR-181a exerts its lipid regulatory function by directly targeting isocitrate dehydrogenase 1 (IDH1), one of the key metabolic enzymes of the tricarboxylic acid cycle and promotor of lipid synthesis genes [44]. Another seminal study conducted in the same year by Wagschal and colleagues focused on cholesterol/triglyceride homeostasis-regulating miRNAs [45]. This large study involved a GWAS of over 188,000 individuals for 69 miRNAs located in close proximity to single-nucleotide polymorphisms (SNPs) associated with hyperlipidemia-inducing genes [45]. The results of this study identified four miRNAs (miR-128-1, miR-148a, miR-130b, and miR-301b) to regulate the transport

of cholesterol-lipoproteins, namely, low-density lipoprotein receptor and the ATP-binding cassette A1 (ABCA1) [45]. These findings were further confirmed for miR-148a and miR-128-1 through in vivo murine models of obesity by overexpression/antisense targeting of such miRNAs [45].

More recently, the study by O'Neill and colleagues revealed the utility of analyzing the circulating expression level of miR-758-3p as a means for discerning between obesity and metabolic syndrome [46]. Further analysis for this novel circulating miRNA obesity biomarker, involving miRNA mimics/antagonists transfected in liver hepatocellular carcinoma cell line (HepG2 cells), demonstrated that miR-758-3p acts directly on the cholesterol efflux regulatory protein/ATP-binding cassette transporter type A1 (CERP/ABCA1) protein expression levels in a concomitant manner [46]. Another recent study, conducted by Castaño and colleagues, successfully identified a unique plasma exosome-based miRNA expression profile for obesity within murine in vivo models for obesity [47]. The expression profile, which consists of miR-122, miR-192, miR-27a-3p, and miR-27b-3p, was also found to be linked to the induction of glucose intolerance and hepatic steatosis within such murine in vivo models [47].

6. miRNA Influences in Colorectal Cancer

The study described above, conducted by Kloting and colleagues, could also be deemed to shed the first rays of light on the influence of miRNAs in obesity and cancer interactions, since miR-17-5p would be later recognized by the scientific community to be a member of the most notorious oncogenic polycistronic miR cluster, namely, the miR-17-92 polycistronic cluster, with such members being highly upregulated in a wide spectrum of human cancer models [41, 48].

Focusing on colorectal cancer, the level of influence by miRNA dysregulated activity has also been widely reported by the scientific community in the last 15 years, ever since the first reporting by Michael and colleagues in 2003 [49]. Among the more recent publications focusing on this research niche, the study by Xu and colleagues analyzed mRNA and miRNA expression profile datasets to identify regulatory networks that specifically control colorectal cancer (CRC) tumorigenesis [50]. This integrated omics analytical approach highlighted a miRNA-mRNA regulatory network that played a pivotal role in orchestrating the mitogen-activated protein kinase (MAPK) signaling pathway and downstream target genes concerned with cell fate [50]. A similar study focusing on the MAPK signaling pathway and its orchestration by miRNAs identified 13 miRNA-mRNA direct associations, including Transforming Growth Factor Beta Receptor 1 (TGFBR1) affected by miR-6071 and miR-2117 [51]. Another study, conducted by Ruhl and colleagues, revealed that miRNA-451a was consistently upregulated following a single dose of 2 Gy or 10 Gy gamma-radiation within murine colorectal cancer (CRC) models [52]. Such an expression profile for miR-451a also correlated with a downregulation of Calcium-binding protein type 39 (CAB39) and BRCA2-interacting transcriptional repressor (EMSY), the latter two genes both acing as CRC biomarkers for poor prognosis [52]. The investigation carried out by Gao and colleagues elucidated the roles played by miR-888 in CRC from a total of 126 patients [53]. The results of this study, following Kaplan-Meier analysis and log-rank testing, revealed that miR-888 upregulation was collated to overall reduction in survival and disease-free survival from CRC, and such upregulation was also discovered within CRC biopsy tissues [53]. Consequently, this indicates the possible exploitation of miR-888 as a potential CRC prognosis biomarker [53].

miRNAs can also act as tumor suppressors within most human cancer models. This is also reflected in CRC, where a whole spectrum of miRNAs acts as regulators of the differing phenotype characteristics of CRC that make the tumor more aggressive and life threatening. The comprehensive review by Mizuno and colleagues gives a detailed representation of the Let-7 family of miRNAs that are typically downregulated in CRC cases [54]. In addition, the recent study conducted by Ke and colleagues elucidated miR-202-5p as a novel tumor-suppressor miRNA in CRC [55]. This investigation also concluded that miR-202-5p exerts such a tumor-suppressing role by direct regulation of a downstream oncogene (SWItch/Sucrose nonfermentable (SWI/SNF) related, matrix associated, actin dependent regulator of chromatin subfamily c member 1) that was previously established to be associated with CRC metastasis and tumor expansion properties [55]. More recent reports highlighting novel miRNA tumor suppressors in CRC include the study by Zhang and colleagues [56]. This particular study identified miR-1258 to be downregulated in CRC biopsies and representative cell lines, though its artificial upregulation led to an induction of cell cycle arrest at the G0/G1 phase in vitro and in vivo, together with inhibition of tumor cell proliferation through direct modulation of E2F Transcription Factor 8 (E2F8) [56].

7. miRNA Influences in Obesity-Linked Colorectal Cancer

The scientific reporting of miRNA activities involved in regulating physiological and molecular pathway links between obesity and colorectal cancer (CRC) is relatively scarce, though such observations are evermore on the increase (Table 1).

The initial findings by Olivio-Marston and colleagues in 2014 examined the possibility of diet-induced obesity to have a tumorigenic effect on CRC within the azoxymethane murine model [57]. In addition, calorie restriction (30% reduction of required daily calorific intake) was also applied on the same murine models to observe any possible converse relationships between diet and CRC [57]. Following a 10-week treatment, the murine group subjected to die-induced obesity had a 2.5-fold increase in CRC prevalence in comparison to the calorie-reduction murine group [57]. Furthermore, a total of 18 miRNAs were highly dysregulated within the diet-induces obesity murine group (eight upregulated and 10 downregulated) [57]. Following a shortlisting and consequent validation using Real Time Quantitative PCR (RT-qPCR) techniques, three miRNAs were validated to be upregulated in diet-induced obesity murines (miR-425, miR-196, and miR-155) and six miRNAs validated as downregulated within

Table 1: List of clinically significant miRNA-driven interplays between obesity and colorectal cancer development/disease progression.

miRNA/s involved	Functional role of miRNA/s (when up-regulated)	Affected pathways and/or gene/s	References
miR-425	Detrimental	Obesity development	[57]
miR-196			
miR-155			
miR-150			
miR-351			
miR-16			
let-7	Detrimental	Obesity development	[57]
miR-34			
miR-138			
miR-4443	Detrimental	NCOA1 and TRAF4	[58]
miR-101c	Detrimental	Tet1	[59]
miR-27b			
miR-130b	Detrimental	PPAR-γ	[60]
miR-138			

the same murine group (including miR-150, miR-351, miR-16, let-7, miR-34, and miR-138) [57].

In 2016, Meerson and Yehuda discovered a precise molecular interplay that placed miRNA activity as pivotal intermediates for the cross-communication between obesity and CRC [58]. This study consisted of treating colorectal cancer human cell lines (HCT-116, HT-29, DLD-1) with leptin and insulin, followed by miRNA expression profiling screens for approximately 800 miRNAs and consequent RT-qPCR validation of any shortlisted miRNA found to be dysregulated [58]. The validated results elucidated that miR-4443 was upregulated in two CRC cell lines after leptin/insulin exposure, with the DLD-1 cell line not demonstrating the same effect due to its lack of leptin receptor expression [58]. Consequent transfection of miR-4443 mimic within the HT-116 cell line resulted in a severe reduction in tumor invasion/proliferation properties [58]. Moreover, concomitant leptin treatment and miR-4443 transfection led to a severe downregulated expression level for Nuclear Receptor Coactivator 1 (NCOA1) and Tumor Necrosis Factor receptor-associated factor 4 (TRAF4), as both are confirmed target genes for miR-4443 and also previously established in orchestrating tumor metastasis properties [58]. Ultimately, this study comprehensively exposes the roles performed by miRNAs such as miR-4443, where in this case miR-4443 acts as a tumor-suppressor miRNA in CRC due to leptin/insulin signaling [52]. However, obesity-induced leptin and/or insulin resistance could thwart such tumor-suppressing activity by miR-4443 on TRAF4/NCOA1 and lead to potentiating the risks for CRC clinical presentation in obese individuals [58].

Continuing in this line of research, the investigations carried out by Tie and colleagues in 2017 revealed further details on the level of miRNA interplay with hypercholesterolemia and CRC [59]. Employment of two murine models (ApoE-/- and C57BL/6) allowed for analysis of any correlations between a high cholesterol diet and CRC incidence in murines [59]. Following the induction of the C57BL/6 murine group to a high cholesterol diet, azoxymethane was administered to both groups for induction of CRC [59]. The results of this phase of the study revealed that incidences of CRC were almost twofold higher in the C57BL/6 murine group that was fed a high cholesterol diet [59]. Further analyses revealed that such hypercholesterolemia led to oxidative stress-related triggering of a miR-101c expression level, consequently inducing a direct downregulation of Tet1 within hematopoietic stem cells and ultimately reducing the expression levels of multiple genes associated with natural killer T cells (NKT) and gamma-delta T-cell development [59]. This study therefore represents an excellent portrayal of how, in this case, an obesity comorbidity status (hypercholesterolemia) led to specific miRNA dysregulations (miR-101c) that ultimately led to a reduction in the efficiency of immune-surveillance against CRC through epitranscriptomic modifications of T-cell differentiation genes [59].

Another scientific report of importance was the recent publication by Motawi and colleagues in 2017 [54]. This study mainly focused on the effects of the dysregulated expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) in obesity [60]. Blood samples were collected from four CRC patient/control groups (34 CRC obese, 36 CRC lean, 22 obese control, and 24 lean control), with consequent serum analysis for circulating miRNA profiling and including peripheral blood mononuclear cell (PBMC) PPAR-γ expression level and degree of its promoter methylation [60]. The results of this study elucidated a major upregulated expression level for miR-27b, miR-130b, and miR-138 within CRC and obese patients [60]. Furthermore, PPAR-γ expression was also found to be downregulated within the same patient groups, consequently inferring the miRNA interplay to lead to increased CRC risks within patients through a downregulatory action by such a miRNA combination on PPAR-γ expression [60].

8. Perspective and Future Directions

There are already a number of reputable industry leaders in miRNA therapies such as miRNA therapeutics, miRagen Therapeutics, Santaris pharma, or Regulus Therapeutics. They are currently entering clinical trials, using miRNAs for the treatment of several diseases, including cancer. The purpose of miRNA therapies is mainly using miRNAs that normally downregulate several target-oncogene, as possible drugs. The loss of function of these miRNAs might alter the expression of target-oncogene. Therefore, it could initiate tumor formation. These miRNAs should function as an inhibitor that antagonize (by sequence complementarity) the upregulated oncomiR in order to artificially lower the oncomiR's expression level to "normal" levels. Alternatively, the patient can be treated with miRNA mimics in order to artificially increase the expression levels of downregulated tumor-suppressor miRNAs. In both cases, a bespoke drug delivery system is employed to assure safe passage of such miRNA mimics/inhibitors through the bloodstream and allow uptake at the site of action [61].

Approximately 20 clinical trials against cancer have currently been conducted using miRNA-based therapeutics [62]. miRNAs are involved in many critical processes such as tumor initiation, progression, and invasion. All of the recent published evidence suggests that inhibition of some overexpressed oncogenic miRNAs could provide a robust strategy for cancer therapy. Beg and colleagues in 2013 designed the first miRNA replacement therapy on human clinical trials for the treatment of advanced or metastatic liver cancer. miR-34 has been presented as a powerful tumor suppressor by the biopharmaceutical company "miRNA Therapeutics", as MRX34, a liposomal miR-34a mimic. This drug can be used in a wide variety of cancers. The MRX34 phase 1 is now being conducted as a clinical trial in liver cancers. However, this clinical trial was stopped in 2016 due to multiple immune adverse events [63].

The gene-miRNA-diet interaction could be a promising target to consider miRNA as drug therapies in the obesity-related cancer axis. A study conducted in prostate cancer cell lines found that genistein (isoflavone) was able to upregulate ADP–Ribosylarginine-Hydrolase (ARH1), a suppressor tumor, by downregulating miR-221 and miR-222 [64]. Curcumin inhibits miR-21, invasion, and metastasis in colorectal cancer [65]. Curcumin also induces the apoptosis of gastric cancer cell lines through upregulation of miR-33b [66]. miR-27a, a miRNA released from adipose tissue cells, promotes the proliferation of liver cancer [67]. There are several evidences on the interaction between genes, diet, obesity, and cancer. However, more studies in obesity-linked colorectal cancer on interaction-communication via miRNA must be considered to increase interest in developing novel miRNA-based theragnostic strategies to counteract and/or mitigate the potentially life-threatening outcomes of untreated colorectal tumors within afflicted obese patients and provide a possible target for diagnosis and therapy.

Enhancing awareness among the scientific community about the prospect of miRNA influences is crucial to consider miRNA therapies in obesity-linked colorectal cancer tumorigenesis and its clinical progression. Research articles pertaining to the identification and validation of such crucial miRNA biomarkers in this bespoke niche of cancer patients are still very low and, consequently, it is necessary to increase the spotlight and eventually increase the level of global research efforts for discovering novel miRNA influences of obesity-linked colorectal cancer. Ultimately, additional research will eventually lead to novel miRNA-based theragnostic tools that can benefit the cancer patient within the clinical setting in the not-too-distant future. Currently, we have enough knowledge about the role of miRNA as a diagnostic tool, but we must gather all the efforts to turn them into a therapeutic tool.

Although miRNA-based therapeutics and diagnostics are still in their infancy, the degree of bespoke therapeutic efficacy and enhanced patient risk-stratification precision offered by such technology will certainly grow to the point where novel diagnostic kits and drug treatments for the treatment of all conditions influenced by miRNA dysregulations will become the mainstay clinical tools of the consultant oncologist by the end of the next decade. Recent technological advances could allow a high performance to obtain the specific profiles of miRNA (fingerprint) at a very precise level, in a cost-effective manner in the diagnosis, treatment, and monitoring of obesity and cancer. The low cost of data generating could lead us to the Big Data Era. The availability of these large datasets provides unprecedented opportunities but also poses new challenges for data mining and more accurate analysis.

9. Conclusions

Even though the current level of evidence is at its infancy, epigenetics mechanism involved to regulatory elements influences within all aspects of human cell physiology pathways can never be underestimated. In this review, we aimed to shed light on merely how epigenetics modifications could induce molecular orchestration that interplays between obesity and colorectal cancer tumorigenesis and development. Within the short-term future, the authors are highly confident that further evidence will be revealed by the global scientific community to further confirm the paramount roles played by DNA methylation and miRNAs within the obese individual, both for inducing comorbidities and severe disease pathologies and for possible prophylaxis and treatment measures.

Conflicts of Interest

The authors declare that they have no conflicts of interest. They agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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