ADVANCEMENTS IN IMMUNOLOGY AND IMMUNOTHERAPY FOR BREAST CANCER

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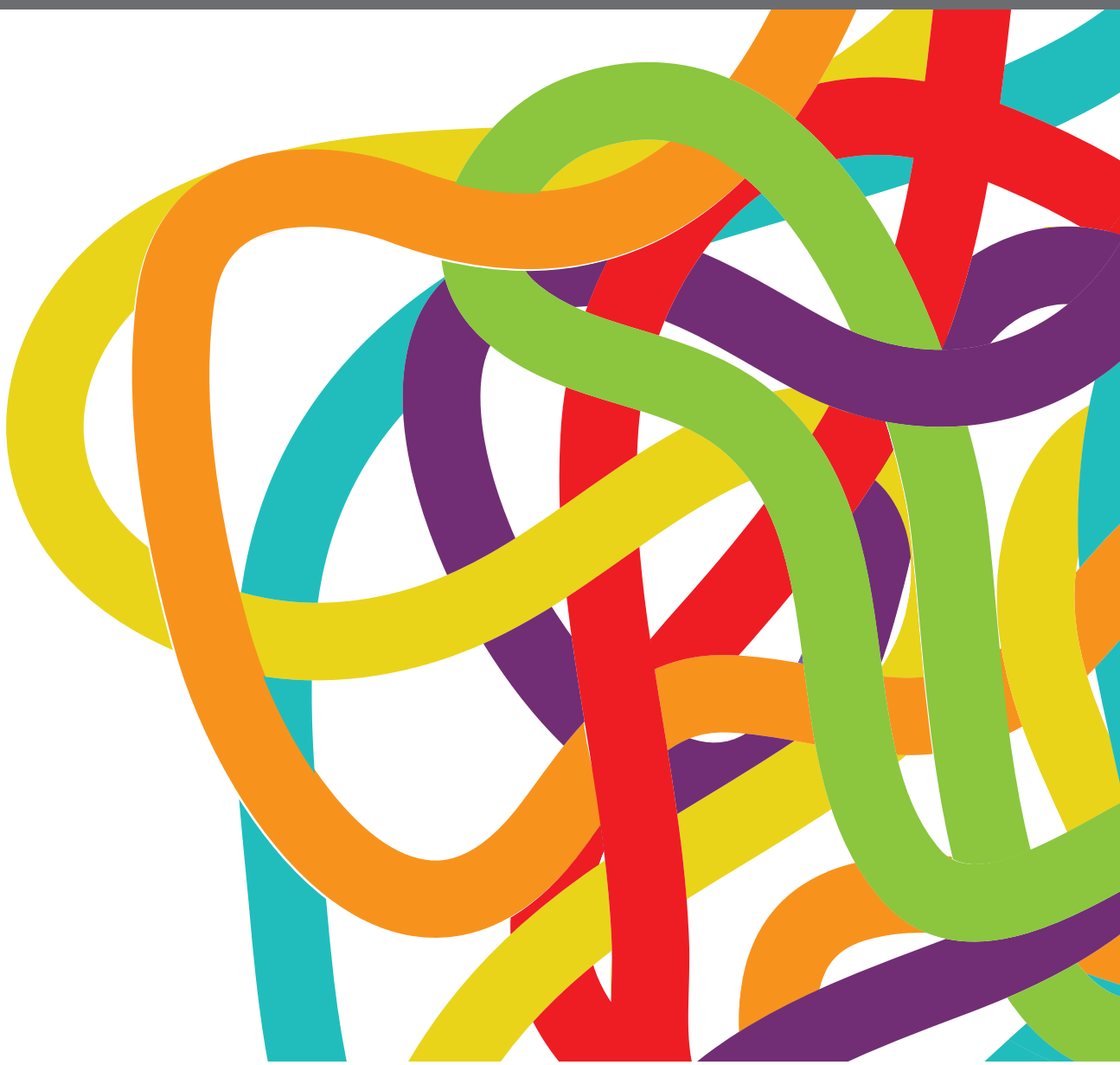

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ADVANCEMENTS IN IMMUNOLOGY AND IMMUNOTHERAPY FOR BREAST CANCER

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Editorial: Advancements in immunology and immunotherapy for breast cancer

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Editorial on the Research Topic
Advancements in immunology and immunotherapy for breast cancer

Breast cancer is a common disease in women in worldwide. Although conventional approaches such as surgery, radiotherapy chemotherapy, and endocrine therapy contribute to the submission of early breast cancer, they are limited by specificity and toxicity in advanced metastatic breast cancer. Accumulated results regarding immunogenicity and immune response in breast cancer have led to the development of immunotherapy for patients with early-stage as well as advanced breast cancer (1). As Chen et al. summarized, the therapeutic effects of immuno-oncology (IO) for breast cancer have been observed but are still limited. Among the subtypes of breast cancer, triple-negative breast cancer (TNBC) has the most aggressive features and the conventional treatment has been limited to chemotherapy. As Ji et al. have described, immunotherapies for breast cancer are focused on TNBC with the combined immunotherapy, including immune checkpoint inhibitors (ICIs), plus chemotherapeutic drugs, molecular target drugs, or radiation. However, there is still room for improvement as new strategies for IO are in progress as mentioned by Qiu et al. The combined agents might have direct cell toxicity, release immunomodulatory factors from dying tumor cells, induce the infiltration of immune cells, and suppress immune regulatory cells. Optimal combinations and sequences of immune-based therapies should be determined.

Programmed death-ligand 1/programmed cell death-1 (PD-L1/PD-1) represent a valuable therapeutic target, but Cong et al. reported that T-cell immunoglobulin mucin-3 (TIM3), another immune checkpoint molecule, is a potential target for IO. The PD-L1 test is the mainstream for companion diagnostic of ICIs, but the development of

biomarkers is required to predict prognosis and/or responses to IO to maximize the clinical benefit of ICIs. Shang et al. examined tumor infiltrating lymphocyte (TIL) and PD-L1 expression in relation to effectives of HER2-targeted therapy. High TIL infiltration before NAC was a strong predictive marker for pathological complete response (pCR) which was consistent with the results of the previous study (2). PD-L1 expression on tumor cells is regulated in the tumor-intrinsic and -extrinsic manner. PD-L1 expression before NAC represents a naïve anti-tumor response while PD-L1 after NAC is the result of the immune contexture in tumors treated with anti-cancer agents. The novel technologies of cancer research are in the advance stages and Magbanua et al. summarized liquid biopsy for circulating tumor DNA (ctDNA) analysis. For urothelial carcinoma, ctDNA proved to be a promising biomarker to predict clinical outcomes in patients with adjuvant atezolizumab (3) and this dynamic molecular biomarker was warranted.

For immuno-related biomarkers, Chang et al. have examined immunoglobulin lambda constant 2 (IGLC2) in TNBC and the low gene expression of IGLC2 was correlated with a poor prognosis and malignant features of TNBC. IGLC2 may contribute to inflamed gene expression profiles but it is still obscure as to why this humoral immune marker is related to cellular anti-tumor immunity. Accumulated results indicated that the presence of B cells and tertiary lymphoid structure (TLS) in tumors were associated with a favorable outcome in patients treated with immunotherapy and this relationship between humoral and cell-mediated immunity gradually became clear (4). Innate immunity – related molecule of CRAR2, the second receptor of complement 5a (C5a), was also a potential biomarker for immune response. Zhu et al. reported that C5AR2 expression was a poorer prognostic factor in breast cancer, especially the ER-positive subtype. Monocyte-macrophages are heterogenous and divided into two subtypes for anti-tumor M1 and pro-tumor M2 macrophages. Tumor-associated macrophages (TAMs), which created an immune-suppressive environment, tended to express the markers for M2 macrophage. Expression levels of C5AR2 were positively correlated with the infiltration of M2 macrophages but negatively correlated with the infiltration of M1macrophages. Liu et al. reported that the high gene expression of GOLT1B, encoding a golgi vesicle transporter protein, was a negative prognostic factor. This gene may contribute to the infiltration of immune cells. The expression level of GOLT1B was negatively correlated with CD8-positive effector T cells, CD4-positive helper T cells, regulatory T cells, and positively correlated with M2 macrophages and neutrophils. These results indicated that both C5rAR2 and GOLT1B were a potential negative predictive biomarker in relation to pro-tumor immunity. Xu et al. identified GW-8510, a CDK2 inhibitor, as an anti-tumor response enhancer using the bioinformatics manner. TNBC cancer cell treatment with GW-8510 increased the level of cleaved caspase-3 and N-terminal fragments of GSDME, which induced pyroptosis, a lytic programmed cell death. Pyroptosis cells released damaged associated molecular patterns (DAMPs), which augmented an anti-tumor response in tumor microenvironment (TME). Neoantigen sources were mainly on single nucleotide variants (SNVs) and small insertion-deletion (indel), which are a potential target for immunotherapy (5). The FDA has already approved pembrolizumab for metastatic solid tumors with tumor mutation burden-high (≧ 10 mut/Mb) or microsatellite instability-high. Zhou et al. reported that PIK3CA is a highly mutated gene and the highest source of neoantigens. Breast cancer in the elderly or breast cancer with ER-positive, HER2-negative yield higher SNV-derived neoantigens. Recent results from Chandran, et al. also demonstrated that mutant PIK3CA-derived public neoantigens had immunogenicity and therapeutic potential (6).

The understanding of molecular and cellular dynamism in TME is required for the development of IO biomarkers. Patysheva et al. highlighted the relation between the response to neoadjuvant chemotherapy (NAC) and circulating monocyte-phenotypes. NAC recruited CD163-positive monocyte-derived macrophages in TME and the circulating CD14pos/lowCD16-positive HLA-DR-positive monocyte in the base-line was associated with NAC efficacy. The accumulation of CD163-positive TAM may result from active recruitment by anti-cancer agents or an adaptive response to inflammation reaction induced by NAC. TIL (CD8-positive T cell) -infiltration which is a favorable prognostic immune marker for breast cancer, especially in TNBC (7, 8). Zhou et al. examined the infiltration of immune cells in three matched samples for normal, primary, and oligometastatic sites. Among matched tissues, immune cell infiltration was less in oligometastatic sites compared to primary sites. Higher CD3 in the intratumor oligometastatic lesion was correlated with better PFS and higher CD4 in the same lesion and was related to better OS in TNBC/HER2-postive breast cancer. CD4-positive T cells modulate cellular (Th1) and humoral (Th2) immunity but intratumor CD4-positive T cells can mediate anti-tumor cytotoxicity in a direct and indirect manner (9). The therapeutic effects of IO depend on how the immune suppressive status of TME is overcome. As regulatory T cells (Tregs) play a major role in the immunosuppression of TME, the targeting of Treg is a promising approach to augment the anti-tumor response (10). Liu et al. summarized Treg-biology and the rationale for Treg-targeting treatment in breast cancer.

Vitorino et al. summarized gut microbiota which influences immunotherapy response. Melanoma patients with "good microbiota" experienced the benefit from IO while the fecal transplantation of good microbiota could overcome resistance to IO (11). Pathogen-associated molecular patterns (PAMPs) from

microbiota may activate local gut-innate immunity but it is still unclear why they can induce systemic CD8 T cell-based anti-tumor immunity. Further study is required to elucidate the contribution of gut microbiota as related to systemic anti-tumor immunity.

In conclusion, anti-PD-(L)1 is now FDA approved for TNBC, both in the neoadjuvant and metastatic setting, in combination with chemotherapy. We do not really know why chemotherapy should synergize with immunotherapy, but this treatment may have broad effects on TME-mediated immunosuppression. Future work should integrate both tumor-cell intrinsic and extrinsic determinants of responsiveness to immunotherapy. This would enable biomarkers for improved patient selection as well as new resistance mechanisms that could be co-targeted in combination strategies. Liquid biopsies could enable accelerated development of treatments as well as tailor treatments for potential real-time response.

Author contributions

Original draft preparation: TS. Review and editing: RS and LF. All authors contributed to the article and approved the submitted version.

Conflict of interest

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REVIEW
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The Emerging Role of T-Cell Immunoglobulin Mucin-3 in Breast Cancer: A Promising Target For Immunotherapy

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Cancer treatment through immune checkpoint receptor blockade has made significant advances in the recent years. However, resistance to the current immune checkpoint inhibitors (ICIs) has been observed in many patients, who consequently do not respond to these treatments. T-cell immunoglobulin mucin-3 (Tim-3) is a novel immune checkpoint molecule emerging as a potential therapeutic target for cancer immunotherapy. Epidemiologic findings reveal that genetic polymorphisms in the Tim-3 gene are associated with increased susceptibility to breast cancer. In patients with breast cancer, Tim-3 is expressed both on immune and tumor cells. Accumulating evidence demonstrates that Tim-3 can notably affect breast cancer treatment outcome and prognosis. Therefore, Tim-3 is being regarded as a high-potential target for improving breast cancer therapy. In this review, we summarize the role of Tim-3 in breast cancer and the regulation mechanisms of Tim-3 to furnish evidences for future research and therapy.

Keywords: breast neoplasm, T-cell immunoglobulin mucin 3, prognosis, regulation, immunotherapy

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INTRODUCTION

Breast cancer is the most common malignant tumor and the leading cause of cancer-associated mortality among women (1). Although comprehensive therapies exist, patient response to the treatments significantly varies, which partly attributed to varying antitumor immune responses (2). Immunotherapy is being recognized as a key therapeutic modality for cancer and represents one of the most promising therapies. An increasing body of evidence suggests immune checkpoint molecules, such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin-3 (Tim-3, also known as Hepatitis A virus cellular receptor 2 [HAVCR2]), and lymphocyte activation gene-3 (LAG-3) are crucial regulators of immune escape and have critical roles in maintaining immune stability. This supports the development of immune checkpoint-targeting based therapeutic strategies (3). Following the success of immune checkpoint inhibitors (ICIs) in melanoma in 2010, multiple monoclonal antibodies against CTLA-4, PD-1, and programmed cell death 1 ligand 1(PD-L1) have been trialed and approved in solid tumors (4). Patients with metastatic breast cancer have shown an objective response rate of 21.4-39.4% receiving treatment with ICIs in clinical trials (5–7), indicating immunotherapy against the ICIs is a promising efficiency in metastatic breast cancer. However, many patients are still resistance to these targeted therapies (8). The second interim

analysis of IMpassion130 indicates no significant difference in overall survival (OS) between atezolizumab plus nab-paclitaxel group and placebo plus nab-paclitaxel group in locally advanced or metastatic triple-negative breast cancer (TNBC), although it suggests a clinically OS benefit in patients with PD-L1 immune cell-positive disease (9). Therefore, intensive research on other inhibitory receptors is being conducted. Recent findings show that Tim-3 is part of a module that contains multiple coinhibitory receptors, which are coexpressed and coregulated on dysfunctional or "exhausted" T cells in cancer (10). A study showed that resistance to anti-CTLA-4 or anti PD-1/PD-L1 inhibitors is compensated by upregulation of other immune checkpoints, such as Tim-3 (11). Consequently, Tim-3 has gained prominence as a potential candidate for cancer immunotherapy. Blocking Tim-3 with other checkpoint inhibitors has been shown to enhance antitumor immunity and suppress tumor growth in several preclinical tumor models (12). These promising results indicate that Tim-3 could be a target for tumor therapy.

As a type I transmembrane protein, Tim-3 was discovered during attempts to identify new cell surface molecules for Th1 and Tc1 cells that produce IFN-γ (13). The Tim-3 locus, along with Tim-1 and Tim-4 loci, is located at 11B1.1 in the mouse genome and at 5q33.2 in the human genome (14). All Tim family molecules, except Tim-4, include a C-terminal cytoplasmic tail with a conserved tyrosine-based signaling motif. Unlike other checkpoint receptors such as PD-1 and T-cell immunoreceptor with Ig and ITIM domains (TIGIT), Tim-3 does not contain the classic inhibitory immunoreceptor tyrosine-based inhibition or immunoreceptor tyrosine-based switch signaling motifs in its cytoplasmic tail (15). Tim-3 inhibits cell proliferation, attenuates effective cytokine synthesis, and promotes apoptosis of activated T cells, by interacting with its ligands that bind to the Tim-3 extracellular immunoglobulin V domain (16). Four distinct ligands for Tim-3 have been identified currently: galectin-9 (Gal-9), phosphatidylserine (PtdSer), high-mobility group protein B1 (HMGB1), and CEACAM-1. A previous review has described the various interaction mechanisms between Tim-3 and its ligands (10).

Tim-3 is significantly upregulated in breast tumor tissues than in the normal tissues (17, 18), and is extremely highly expressed in basal-like and HER2-enriched breast cancer (19). Therefore, targeting Tim-3 has received much attention, particularly in TNBC. Tim-3 is not only expressed on IFN-γ-producing T cells, FoxP3$^+$ Treg cells, macrophages, and dendritic cells (12), but also overexpressed on breast tumor cells (20, 21), which is associated with poor prognosis in breast cancer (20). The current review will focus on the emerging roles of Tim-3 in breast cancer and its regulating mechanisms with the aim to inform future research and therapeutic strategies.

GENETIC POLYMORPHISMS IN TIM-3 INCREASE SUSCEPTIBILITY TO BREAST CANCER

Single nucleotide polymorphisms (SNPs) represent a very common genetic variation in the human genome (22). SNPs in genes regulating DNA mismatch repair, cell cycle regulation, metabolism, and immunity are associated with genetic predisposition to cancer (22). Previous findings have shown that multiple polymorphisms in the promoter region (−574G/T, −882C/T, −1516G/T, and −1541C/T) and in the coding region (+4259T/G, amino acid substitution: Arg to Leu) of the Tim-3 gene were associated with several types of malignant tumors such as non-small-cell lung cancer (23), pancreatic cancer (24), and gastric cancer (25).

Tim-3 gene polymorphism is also involved in breast cancer susceptibility and disease progression. The rs10053538 GT+TT genetic variant of Tim-3 is associated with increased genetic predisposition to breast cancer and faster progression (26). The rs10053538 GT+TT genotype is associated with higher Tim-3 expression and increased lymph nodes metastasis (26). Another study showed that the +4259T/G SNP in the Tim-3 gene is a genetic risk factor for the progression and prognosis of invasive breast cancer (27). This study reported a significantly higher prevalence of the +4259T/G genotype and the +4259G allele among patients with breast cancer than among the controls. Moreover, the +4259T/G polymorphism correlated with a higher expression of the cell proliferation index, Ki-67, in patients with metastasis than those without (27). Therefore, genetic polymorphisms in Tim-3 also play a critical role in breast cancer tumorigenesis and progression (**Figure 1**), which is likely because Tim-3 could suppress the immune response of T cells to tumors.

THE ROLE OF TIM-3 EXPRESSION ON IMMUNE CELLS IN BREAST CANCER

Tim-3 was initially considered to be expressed only by T cells. However, it is now known to be expressed by multiple cell types including T cells (21, 28), dendritic cells (DCs) (29), macrophages (30), myeloid-derived suppressor cells (MDSCs) (31), NK cells (10), stromal cells (19), and vascular endothelial cells (32). Comparing the results of single-cell RNA sequence analysis between breast cancer and normal cells demonstrates that Tim-3 is predominantly expressed on myeloid cells (33). The expression of Tim-3 on multiple immune cell types explains its widespread inhibition in the tumor microenvironment.

A study including 109 patients with TNBC reported expression of Tim-3 in tumor-infiltrating lymphocytes (TILs) from all patients including 17 with <5% stained TILs, 31 with 6%-25% stained TILs, 48 with 26%-50% stained TILs, and 13 with >51% stained TILs (34). In this study, a higher Tim-3 level significantly correlated with younger patients, high proportion of TILs, higher tumor stage, high PD-1 and PD-L1, but with a positive prognosis (34). Two studies further assessed the role of Tim-3 expressed on intra-epithelial TILs (iTILs) and stromal TILs (sTILs) in breast cancer. One study showed that patients with breast cancer with Tim-3$^+$ iTILs (≥ 1%) represent a minority of cases (11%), with a predilection for basal-like breast cancers. Tim-3$^+$ sTILs (≥2%) represented 20% of cases and included more non-basal cases. The presence of Tim-3$^+$

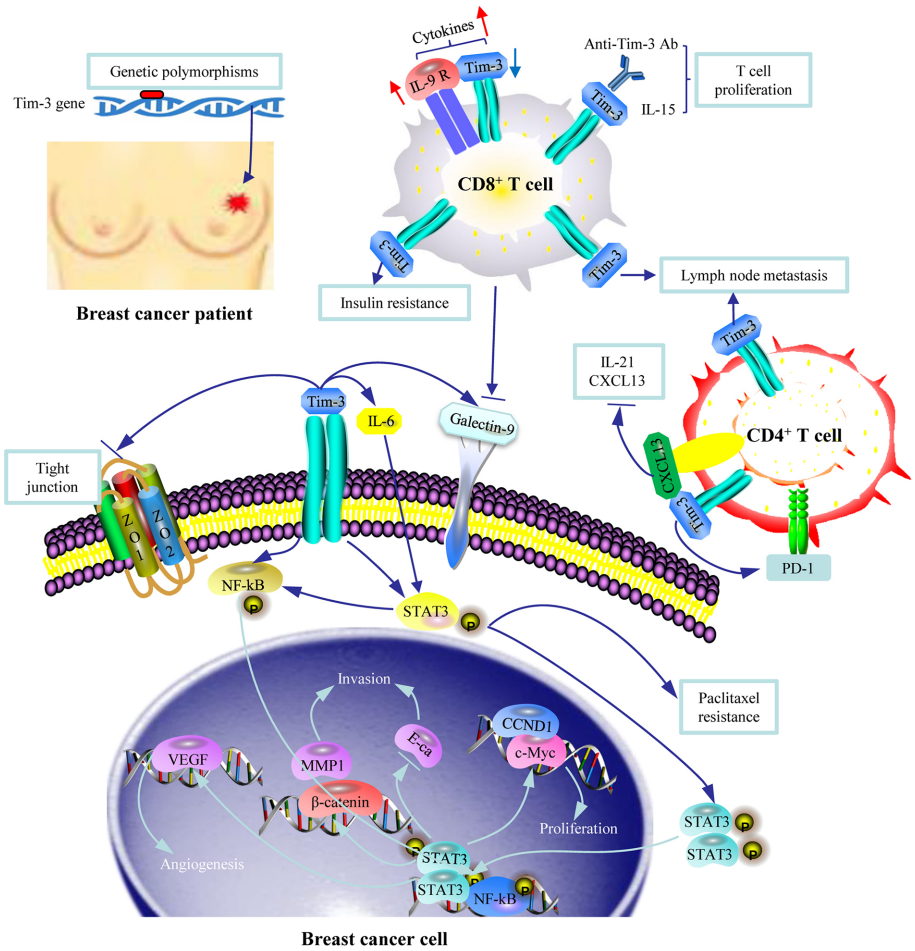

FIGURE 1 | An illustration of the role of Tim-3 in breast cancer. Genetic polymorphisms in Tim-3 are associated with susceptibility to breast cancer. Tim-3$^+$ CD8$^+$ T cells and Tim-3$^+$ CD4$^+$ T cells are associated with risk of lymph node metastasis through Tim-3 mediated immune escape. Tim-3 is involved in insulin resistance. Tim-3 overexpression in breast cancer cells promotes cell proliferation, migration, invasion, tubal formation, and enhanced chemoresistance to paclitaxel by activating the NF-κB/STAT3 signaling pathway. The Tim-3-galectin-9 (Gal-9) pathway is involved in tumor progression given the surface-based Gal-9 protects breast carcinoma cells against cytotoxic T cell-induced death.

iTILs was highly correlated with stromal TILs and other immune checkpoint markers (PD-1$^+$ iTILs, LAG-3$^+$ iTILs and PD-L1$^+$ tumors) (35). Another study showed that luminal A and luminal B breast cancer were associated with higher expression of Tim-3 in sTILs compared to HER2-positive and triple-negative subtypes, but without effect on disease-free survival (DFS) (36) (**Supplementary Table 1**).

Several studies have explored the effect of Tim-3 on CD8$^+$ T cells for it plays a central role in mediating anti-tumor immunity. Tim-3 expression on CD8$^+$ T cells was higher in invasive ductal carcinoma tissue than in normal tissue and correlated with lymph node metastasis, WHO grade, and molecular subtypes in cancer (21). Another study assessed the association of Tim-3 expression on T cells from tumor-draining lymph nodes with breast cancer progression. The authors reported that the frequency of Tim-3$^+$ CD8$^+$ T cells was associated with a higher tumor grade and was significantly higher in patients with more involved lymph nodes than in those with fewer involved nodes (37). The underlying mechanism for this could be the Tim-3-mediated inhibition of the proliferation and activation of CD8$^+$ T cells. Another study supported this view, reporting that IL-15-costimulated tumor infiltrating CD8$^+$ T cells exhibited stronger early proliferation and IFN-g production, which attenuated in the later stages owing to the upregulation of Tim-3 signaling (38). Addition of the Tim-3 ligand Gal-9 significantly suppressed IL-15 costimulation, whereas blocking Tim-3 enhanced it (38). Moreover, compared to IL-9R low CD8$^+$ T cell subset, the IL-9R high subset was characterized by a lower expression of inhibitory molecules Tim-3, PD-1, and killer cell lectin-like receptor G1 (KLRG-1) *ex vivo* and lower IFN-γ after stimulation, which may render the IL-9R high CD8$^+$ T cells less susceptible to signaling mediated by inhibitory ligands, thus leading to higher cytokine expression (39). In addition, Tim-3 on naïve and central memory (CM) CD8$^+$ T subsets is associated with breast cancer insulin

resistance (IR). IR+ patients presented a significantly lower PD-1$^+$ Tim-3$^-$ frequency in CD8$^+$ T subsets compared to those without (40) (**Figure 1**) (**Supplementary Table 1**).

The expression of Tim-3 in CD4$^+$ T cells was also upregulated in breast cancer (28), and correlated with metastatic lymph node load (37), suggesting its importance in suppressing the immune microenvironment. The Tim-3 level in circulating Tfh (CD4$^+$CXCL13$^+$follicular helper T) cells in patients with breast cancer was significantly elevated, which was a Tfh exhaustion marker. Compared to Tim-3$^-$ Tfh cells, Tim-3$^+$ Tfh cells expressed a higher level of PD-1, decreased chemokine CXCL13 and cytokine IL-21 levels, and contained fewer proliferating cells. Naive B cells cocultured with Tim-3$^+$ Tfh cells resulted in significantly lower IgM, IgG, and IgA expression than those cocultured with Tim-3$^-$ Tfh cells, demonstrating that a reduction in Tim-3$^+$ Tfh required B cell involvement. Moreover, the percentage of Tim-3$^+$ Tfh cells in resected breast tumor tissues was much higher than in autologous blood, which also suggests a participation of Tim-3$^+$ Tfh cells in tumor microenvironment (41) (**Figure 1**).

Another study revealed that Tim-3 expression was also localized to macrophages and cDCs in tumors and normal tissues, with the highest levels consistently found on the CD103$^+$ cDC1 subset (29). Tim-3 expression by intratumoral CD103$^+$ DCs regulates chemokine expression during paclitaxel treatment and promotes paclitaxel resistance (29) (**Supplementary Table 1**).

THE ROLE OF TIM-3 EXPRESSION ON TUMOR CELLS IN BREAST CANCER

Tim-3 is not only expressed on immune cells but also overexpressed in multiple types of malignant tumors, such as lung cancer (42), gastric cancer (43), colon cancer (44), hepatocellular carcinoma (45), renal cell carcinoma (46), bladder urothelial carcinoma (47), cervical cancer (48), and breast cancer (20, 21, 49). The ubiquitous expression of Tim-3 on tumor cells strongly indicates its potential role in tumor progression. A meta-analysis showed that a high expression of Tim-3 in solid tumors is associated with a significantly shorter OS (50). However, a high level of Tim-3 expression is associated with better prognosis in several tumor types. Tim-3 expression in renal cell carcinoma is associated with longer progression-free survival and OS (51), whereas low Tim-3 expression levels in tumor tissue is associated with poor prognosis in metastatic prostate cancer (52). Similarly, downregulation of Tim-3 promotes invasion and metastasis of colorectal cancer cells (53). These seemingly contradictory findings imply tumor-type dependent role of Tim-3, which necessitates exploring the role of Tim-3 in breast cancer.

Studies have shown that the expression of Tim-3 on breast cancer cells was significantly higher compared to that on normal tissue [98% *vs* 13% (21), and 42.9% *vs* 18.2% (20)]. Tim-3 expression level on tumor cells was correlated with age ≥45 years (21), greater number of axillary lymph node metastases (21), more advanced clinical stage (20, 21), higher Ki-67 index (20), and a lower 5-year survival (20). Based on the different molecular biology of breast cancer, it would be desirable to explore the expression of Tim-3 in tumors by subtype as well as in primary and metastatic tumors in the future. Several basic research studies explored the mechanism underlying the negative role of Tim-3 in breast cancer. Tim-3 overexpression in breast cancer cells promotes cell proliferation, migration, invasion, and tumor-associated tubal formation and enhances chemoresistance to paclitaxel by activating the NF-κB/STAT3 pathway and its downstream genes (cyclin D1, matrix metalloproteinase-1, vascular endothelial growth factor, and E-cadherin). Tim-3 also deteriorates tight junctions by downregulating zona occludens (ZO)-2, ZO-1, and occludin, which further accelerates tumor progression (54). Another study supported the aforementioned findings by reporting that downregulation of Tim-3 in breast cancer cells inhibited their proliferation, migration, and invasion and promoted their apoptosis (20). Furthermore, breast tumors expressed higher levels of both Tim-3 and Gal-9 compared to healthy tissues, and these proteins were colocalized. The surface-based Gal-9 could protect breast cancer cells against cytotoxic T cell-induced cell death (49) (**Figure 1**).

The expression of Tim-3 in tumors could also interact with immune cells in tumor microenvironment (TME) and promote tumor progression. STAT3 signaling was shown to pay a role in immune cells and promoted immunosuppressive function in the TME (55). Tim-3 overexpression in breast cancer cells activated the STAT3 signal pathway, and then maybe converged in both tumor promotion and immunosuppression, such as the crosstalk between tumor cells and immune cells (56).

THE ROLE OF TIM-3 IN BREAST CANCER PROGNOSIS

The immune microenvironment is strongly correlated with the prognosis of cancer, even in the early-stage ductal carcinoma *in situ* (57). A meta-analysis including 7284 patients with different types of malignant tumors suggested that Tim-3 is an independent prognostic factor for poor OS (58). However, the prognostic role of Tim-3 in breast cancer is different depending on the type of Tim-3 expressing cells.

A study on the effect of the gene expression level of Tim-3 on breast cancer survival by analyzing the KM-plotter database revealed that patients with high Tim-3 expression had a significantly worse relapse-free survival (RFS). OS displayed a similar trend but without statistical significance (20). Another study described a 7 nuclear receptors-based risk score which could effectively predict breast cancer OS. In this study, immune cell infiltration differed significantly between the high-risk and low-risk groups, of which Tim-3 and PD-1 were enhanced in the high-risk group, indicating that the poor prognosis of patients in the high-risk group could be because of the suppression of the immune microenvironment (59). However, another study analyzed the RNA-seq data in the Cancer Genome Atlas (TCGA) database and found that overexpression of Tim-3

correlated with improved OS in breast cancer (17). The differing prognostic outcomes could be attributed to the differing associations between Tim-3 expression with prognostic outcomes by breast cancer subtype. This hypothesis is supported by a subgroup analysis which showed that a high Tim-3 level was associated with worse RFS in luminal A and luminal B subtypes, but improved RFS in basal breast cancer. With regard to OS, high Tim-3 levels were associated with a worse prognosis in luminal A subtype but a better prognosis in the basal subtype of breast cancer (20). Supporting this observation, another study showed that elevated Tim-3 expression significantly correlated with improved RFS in estrogen receptor (ER)-negative or progesterone receptor (PR)-negative breast cancer (17). Meanwhile, other studies showed that both gene (19, 60) and protein (33) expression levels of Tim-3 had no association with survival in patients with breast cancer. These results may partly explain the poor efficacy of anti-immunization checkpoint drugs used as monotherapies in the treatment of breast cancer.

The expression of Tim-3 on immune cells also affects the prognosis in breast cancer. Although a higher expression of Tim-3 on TILs is associated with poor clinical and pathologic features, such as younger patients, high tumor stage, high PD-1, and high PD-L1, patients with high Tim-3 in TILs have better DFS and OS in TNBC (34). Similarly, another study reported the presence of Tim-3^+ iTILs as an independent favorable prognostic factor in the whole cohort and among ER-negative patients (35). In contrast, Tim-3 positivity in stromal regions after neoadjuvant chemotherapy (NAC) was significantly associated with poor prognosis in TNBC (61).

Tim-3 also has predictive value for the therapeutic outcomes in breast cancer. Patients with a high level of Tim-3 expression had more favorable survival outcome after adjuvant chemotherapy or systemic treatments than those with a low level of expression. Of note, increased Tim-3 expression was significantly associated with better RFS in patients treated with chemotherapy than those not (17). This could be partly attributed to the expression of Tim-3 being significantly associated with infiltrating immune cells such as infiltrating $CD8^+$ T cells, T cells (general), B cells, monocytes, and tumor-associated macrophages (TAMs) (17).

REGULATION OF TIM-3 EXPRESSION IN BREAST CANCER

Understanding the regulatory mechanisms of Tim-3 in breast cancer would be of great value for future research and treatment strategy. Previously reported molecules or transcription factors affecting Tim-3 expression include T-bet (62), MEK (63), c-Jun (64), and nuclear factor interleukin 3 regulated (65) in T cells, T-bet in HCV-infected monocytes or macrophages (66), Hif1-α in brain damage (67), and CB2 cannabinoid receptors in ischemic microglial cells (68). Recently, few novel regulating mechanisms of Tim-3 in breast cancer have been identified.

Several novel factors including micro-RNA, cytokines, TNF receptors, and chemotherapy were recognized to regulate Tim-3 expression in immune cells. Treatment of $CD8^+$ T cells with a miR-149-3p mimic attenuated markers of T-cell exhaustion and downregulated mRNAs encoding Tim-3, PD-1, B- and T-lymphocyte attenuator and Forkhead Box P1 (69). In contrast, T-cell proliferation and activation cytokines (IL-2, TNF-α, and IFN-γ) were upregulated after treatment with the miR-149-3p mimic. Treatment with an miR-149-3p mimic reverses $CD8^+$ T cell exhaustion and promotes the $CD8^+$ T lytic activity on 4T1 mouse breast tumor (69). Tumor-secreted cytokines also regulate the expression of Tim-3 in T cells. Tim-3 expression significantly increased on activation of prostaglandin E2 (PGE2) and cyclic AMP signaling pathways. A study revealed elevated Tim-3 expression in Jurkat T cells on exposure to breast tumor cell-conditioned media through the interaction between PGE2 and its receptor EP4 (70). Another study revealed that glucocorticoid-induced TNF receptor expressed in lymphocytes in breast cancer was associated with immune checkpoint markers (Tim-3, PD-1, PD-L1and LAG-3) and T-cell markers (CD8 and FoxP3), indicating that it could also regulate the expression of Tim-3 (71). Ly6GmiLy6Clo $CD11b^+$ $CXCR2^+$ subpopulation ($CXCR2^+$ MDSCs) predominantly proliferates and is recruited in the tumor microenvironment during breast cancer progression. $CXCR2^+$ MDSCs promote breast cancer progression by directly inducing cancer cell epithelial-mesenchymal transition and indirectly promoting T-cell exhaustion by upregulating the expression of immunosuppressive molecules Tim-3, PD-1, PD-L1, LAG-3, and CTLA-4 on $CD4^+$ or $CD8^+$ T cells and inducing exhaustion of the activated T cells *via* IFN-γ (72). A study reported that plasma concentrations of some immune checkpoint markers varied as a function of age: Tim-3, Gal-9, and sCD25 levels were elevated, whereas 4-1BB (CD137) and PD-L1 levels were attenuated in advanced age (73). Furthermore, Victor Sarradin et al. evaluated the immune biomarkers of paired pre- and post-NAC tumor samples in the tumor (no-pathologic complete response, no-pCR) or tumor bed area (pCR), and found that Tim-3 positivity (≥ 1%) was significantly increased after NAC with increases occurring more frequently in no-pCR than in pCR TNBC patients (51.4% *vs* 31%) (61). Another study showed fewer $CD4^+$ T-cells expressing Tim-3 and increased PD-1 and Tim-3 expression on $CD8^+$ T cells following NAC (74). This observation could be attributed to the differences in the activation status of circulating $CD4^+$ and $CD8^+$ T cells after NAC or differences in the effect of chemotherapeutic drugs on cytokine production by the T cells (74).

Several studies have also evaluated the regulatory factors of Tim-3 in breast cancer tissues, including CpG islands, N6-methyladenosine (m6A) RNA methylation, and chemotherapy. Vertebrate CpG islands represent a dispersed but coherent DNA sequence class whose members function as genomic platforms for regulating transcription at their associated promoters (13). CpG islands in the promoter region of Tim-3 were significantly hypomethylated in breast tumor tissue than in normal tissue (18). In addition, decreased binding of H3K9me3 and H3K27me3 was observed in the promoter loci of Tim-3 in tumor tissues. Therefore, both DNA and histone modifications

are involved in the upregulation of Tim-3 in breast tumor tissue (18). However, in peripheral blood mononuclear cells of patients with breast cancer, PD-L1 and TIGIT expressions could be regulated by DNA methylation epigenetic machinery; however, no changes in Tim-3, CTLA-4, and LAG-3 expressions were observed compared to those in healthy donors (75). N6-methyladenosine (m6A) RNA methylation plays critical roles in tumorigenesis and cancer immunoregulation (76). By analyzing the RNA sequencing data of 24 main m6A RNA methylation regulators in patients with breast cancer from TCGA, 2 subgroups of RNA methylation (RM1 and RM2) were identified. Of the 2, RM2 presented greater RNA methylation modification compared to RM1, and RM2 was associated with significantly better OS. One of the reasons why RM2 was associated with better prognosis was because RM2 was associated with higher expressions of HLA-A and higher numbers of tumor-infiltrating $CD8^+$ T cells, helper T cells, and activated NK cells but lower expressions of Tim-3, PD-L1, PD-L2, and CC chemokine receptor 4 (CCR4). The aforementioned results suggest that m6A RNA methylation could regulate the expression of Tim-3 in breast cancer (77). Chemotherapy could also regulate the expression of Tim-3 in breast cancer. Using whole-transcriptome sequencing and whole-exome sequencing with 37 metastatic breast cancer samples, the authors found that HER2 expression and taxane treatment correlated positively with a high expression of HAVCR2 (Tim-3), PDCD1 (PD-1), CD274 (PD-L1), CD276 (B7-H3), CTLA-4, indoleamine 2,3-dioxygenase 1(IDO1), and LAG-3 (78), supporting that HER2 expression and taxane treatment could regulate the expression of Tim-3 in breast cancer (**Figure 2**).

THE THERAPEUTIC SIGNIFICANCE OF TIM-3 IN BREAST CANCER

In recent years, immune checkpoint blockade and vaccines administered in combination with other treatments have emerged as potential breast cancer treatments (79). ICIs, either alone or in combination with other therapies, have created new paradigm in tumor treatment (79). ICIs have significant advantage over conventional therapies, but only a fraction of patients benefit from the current ICIs, and the response rates remain relatively low (80). The coblockade of PD-1 and PD-L1 upregulates the coexpression of Tim-3 and LAG-3 on $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^+$ $FoxP3^+$ $Helios^+$ Tregs in TNBC, indicating that the emergence of compensatory inhibitory mechanisms leads to acquired TNBC resistance against PD-1/PD-L1 blockade (28). Therefore, current research efforts are exploring the possible beneficial effects of blocking Tim-3 as a therapy for cancer.

A few basic research studies suggest that blocking Tim-3 may have remarkable therapeutic value in breast cancer. Tim-3 expression is significantly upregulated on γδ T cells during their *ex vivo* expansion, and these γδ T cells with overexpressed Tim-3 exhibit an increased susceptibility to apoptosis. The combined use of a Tim-3 inhibitor and MT110 (anti-CD3× anti-EpCAM) could enhance the anti-tumor effect of the adoptively transfused γδ T cells, which have clinical implications for the design of new anti-tumor regimens (81). Another study revealed that the outgrowing transgenic T cells exhibit an exhausted phenotype characterized by PD-1 and Tim-3 upregulation and failed to control tumor growth in the absence of costimulatory signals. However, by

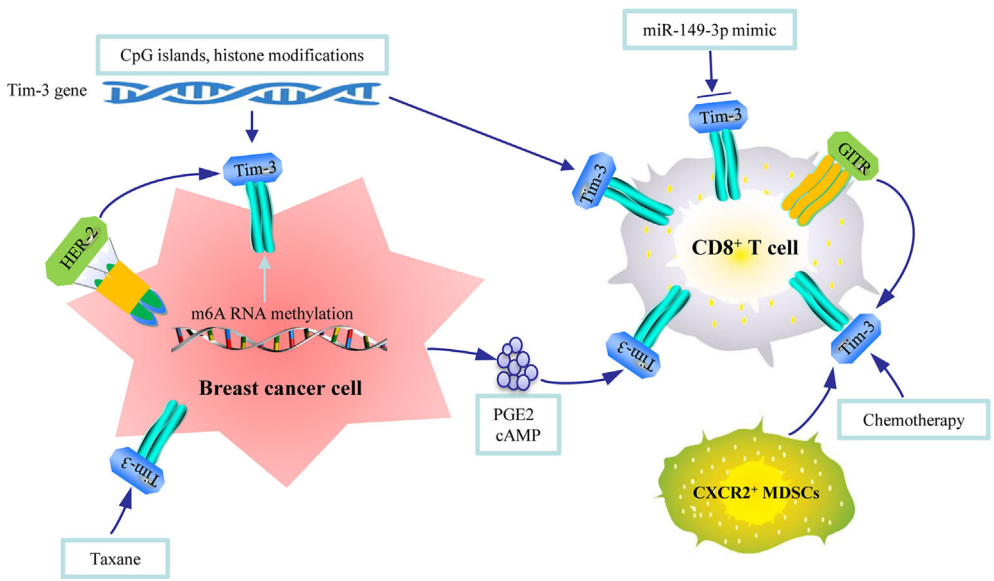

FIGURE 2 | An illustration of the regulation mechanisms of Tim-3 in breast cancer. CpG islands and histone modifications in Tim-3 gene, N6-methyladenosine (m6A) RNA methylation, epidermal growth factor receptor 2 epidermal growth factor receptor 2 (HER2) expression, and taxane can regulate the expression of Tim-3 in breast tumor tissues. Micro RNA, cytokines released by tumor cells, TNF receptor, MDSCs, and chemotherapy can regulate the expression of Tim-3 in immune cells.

coexpressing 2G CAR.MUC1 (signal 1-activation + signal 2-costimulation) and 4/7ICR (signal 3-cytokine), transgenic T cells selectively expanded at the tumor site and produced potent and durable tumor control (82). In addition, inhibiting or blocking Tim-3 enhances the effect of chemotherapy for breast cancer. The study by de Mingo Pulido et al. (29) showed that intratumoral $CD103^+$ DCs highly express Tim-3. Anti-Tim-3 antibody promotes CXCL9 expression by these DCs, which enhances the function of $CD8^+$ T cells and thereby improves paclitaxel's therapeutic effect in breast cancer murine models of triple-negative and luminal B diseases. Another study reported similar findings that the combination of paclitaxel and *Ganoderma lucidum* spores exhibited improved tumor control through recovery of the exhausted TILs by inhibiting the expressions of immune checkpoints (Tim-3 and PD-1), whereas paclitaxel alone evidently increased CTLA-4 expression (83).

Another study reported that, clinically, patients with increased plasma Tim-3 or CTLA-4 expression after treatment initiation experience greater benefit from camrelizumab (anti-PD-1 immune checkpoint inhibitor) with apatinib (vascular endothelial growth factor receptor-2 inhibitor) in advanced TNBC (84). Furthermore, Tim-3-negativity is significantly associated with a pCR after NAC, whereas Tim-3 positivity on TILs is associated with a worse chemotherapy response (85) (**Figure 3**). These findings indicate that combined immune checkpoint inhibitor therapies *via* Tim-3 blockage may increase sensitivity to chemotherapy and enhance its effect (8, 11). However, $Tim\text{-}3^+$ $CD8^+$ was not associated with pCR in paired breast cancer samples before and after NAC in a prospective cohort (n = 50) (86). Therefore, further study is required to evaluate the therapeutic effect of blocking Tim-3 in breast cancer. Several ongoing prospective and planned clinical trials have been initiated in solid tumors with several Tim-3 antibodies, including TSR-022 (NCT02817633), MBG453 (NCT02608268), and LY3321367 (NCT03099109) (10, 87). Most of these anti-Tim-3 antibodies are being tested in combination with anti-PD-1/PD-L1 mAbs. Bispecific antibody targeted at both Tim-3 and PD-1 is also being tested (88). Importantly, early data have shown that the combination is well tolerated without dose-limiting toxicity. The results of these ongoing clinical trials should be significant contributions to breast cancer therapy in the future.

So far, most studies indicated that blocking Tim-3 may have remarkable anti-tumor effect. A recent study performed an RNA sequencing analysis and explored the changes in signaling pathway caused by Tim-3 blockade in tumor-infiltrating

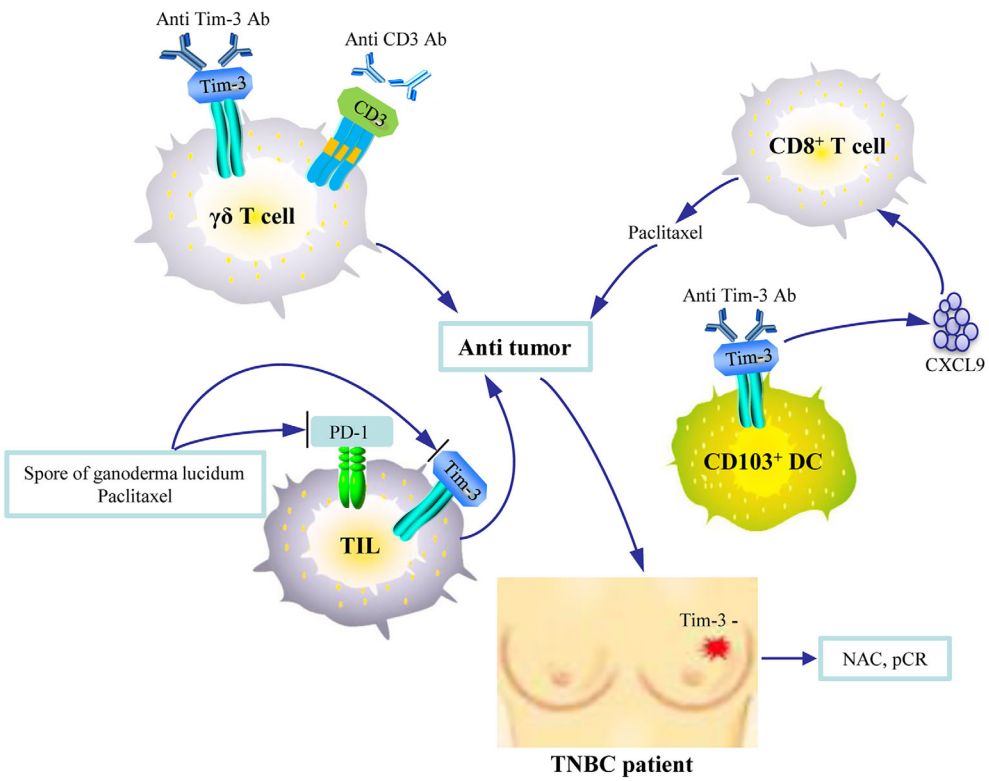

FIGURE 3 | An illustration of the therapeutic significance of Tim-3 blockade in breast cancer. The combined use of Tim-3 inhibitors and anti-CD3 agents enhances the anti-tumor activity of the adoptively transfused γδ T cells. Anti-Tim-3 antibody promotes CXCL9 expression by $CD103^+$ DCs, which enhances the function of $CD8^+$ T cells and improves paclitaxel's therapeutic activity. The combination of paclitaxel and *Ganoderma lucidum* spores enhances tumor control by allowing the recovery of the exhausted tumor infiltrating lymphocytes (TILs) by inhibiting Tim-3. Tim-3-negativity on TILs is associated with pathologic complete response (pCR) after neoadjuvant chemotherapy (NAC) in patients with triple-negative breast cancer (TNBC).

immune cells (80). The results show that Tim-3 blockade enhances anti-tumor immunity by upregulating genes through means such as acetylation, cell differentiation, apoptosis, TGF-β signaling, immune response, negative regulation of angiogenesis, activation of the IFN-γ-mediated pathway, and mitogen-activated protein kinase signaling that favor immune cell proliferation and activation and enhance T-cell cytotoxicity (80). Furthermore, it suppresses tumor angiogenesis, growth, invasion, and metastasis by downregulating genes associated with transcriptional regulation, integrins, cell proliferation, cancer related-pathways, JAK-STAT signaling, angiogenesis, negative regulation of apoptosis, and Wnt signaling (80). These novel findings further our understanding of the pathways regulated by Tim-3 in breast cancer and provide valuable insights for future research.

CONCLUSION AND PERSPECTIVE

Tim-3 is broadly expressed by different types of cells in breast cancer. It has critical roles in tumorigenesis, tumor progression and predicting prognosis. The biology of Tim-3 is complex depending on the cells it is expressed on or the molecular subtypes of breast cancer. Several novel factors including micro-RNA, cytokines, TNF receptors, CpG islands, N6-methyladenosine (m6A) RNA methylation, and chemotherapy are identified to regulate the expression of Tim-3. Tim-3 blockade induces anti-tumor immune response, inhibits tumor growth, and enhances the effect of chemotherapy. Therefore, Tim-3 in breast cancer could be a promising target in tumor treatment.

Currently, the therapeutic potential of targeting Tim-3 is being studied in solid tumors. Tim-3 coblockade with other checkpoint receptors is being investigated in clinical studies, and promising results have been reported in patients with anti-PD-1-refractory disease. Therefore, activating cell costimulatory molecules by combining anti-Tim-3 antibodies with other ICIs or with chemotherapy may be of great potential in improving the treatment outcomes of breast cancer in the future. In addition, recent studies showed that simultaneously block TGF-β and PD-L1 pathways had a superior anti-tumor effect compared to the monotherapies (89, 90). YM101, a bispecific antibody that bound to TGF-β and PD-L1, could effectively counteract the biological effects of TGF-β and PD-1/PD-L1 pathway and enhance the anti-tumor activity *in vivo* (89). Similarly, using M7824 to simultaneously target TGF-β and PD-L1/PD-1 immunosuppressive pathways promoted anti-tumor responses and efficacy in murine breast and colon carcinoma models (90). Therefore, based on the above encouraging findings, it may also have potential for developing the anti-TGF-β/Tim-3 bispecific antibody to conquer the resistance to immune checkpoint inhibitors for cancer patients in the future.

AUTHOR CONTRIBUTIONS

YC and GQ conceived and planned the article. YC and JL took the lead in writing the manuscript in consultation with GQ. GC contributed to the drawing the figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.723238/full#supplementary-material

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An Integrated Analysis of C5AR2 Related to Malignant Properties and Immune Infiltration of Breast Cancer

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Background: C5AR2 (GPR77, C5L2) is the second receptor for C5a that is a potent protein generated by complement activation. C5AR2 can mediate its own signaling events and exert significant immunomodulatory effects through those events. However, research of C5AR2 in cancer is limited, and its function remains unclear in breast cancer.

Methods: The expression of C5AR2 and its correlations with prognosis, immune infiltration, tumor mutation burden (TMB), and microsatellite instability (MSI) in more than thirty types of cancers were described through GTEx, TCGA, PrognoScan, TIMER2.0, CCLE, HPA, and TISIDB database. C5AR2 showed strong relationships to those immune marker sets in breast cancer. Otherwise, CCK8 assay and Transwell assay were conducted to illustrate the role of C5AR2 in migration, invasion, and proliferation of breast cancer cells.

Results: Generally, C5AR2 expression differed across most cancerous and noncancerous tissues, and high C5AR2 expression significantly related to poor prognosis in BRCA, GBM, KICH, LAML, LGG, LIHC, PAAD, and STAD. Moreover, C5AR2 expression levels were dramatically correlated with recognized immune infiltration, especially the polarization of macrophages in breast cancer. Gene set enrichment analysis confirmed that C5AR2 participates in regulating multiple signaling pathways involved in tumorigenesis as well as tumor immunity. C5AR2 overexpression facilitated the functions such as migration, invasion, and proliferation in breast cancer cells, which is consistent with bioinformatics analysis.

Conclusions: C5AR2 is involved in immune infiltration and malignant characteristics of breast cancer, which may be a prospective biomarker for breast cancer.

Keywords: C5AR2, breast cancer, malignant, immune infiltration, prognosis

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women (1). The treatment usually includes surgery, radiation therapy, oral or intravenous anticancer drugs, hormone therapy and targeted biological antibodies (2). In Clinical, it is usually classified into four subtypes: Luminal A, Luminal B, HER-2 overexpression, and triple negative breast cancer based on the expression of ER, PR, and HER-2 (3). Due to high heterogeneity of breast cancer, to identify other biomarkers may benefit the diagnosis and therapeutics.

In humans, there are two identified C5a receptors, C5aR1, known as CD88 likewise, and C5aR2, known as GPR77 or C5L2 likewise. Although the C5a–C5aR1 interaction is well-recognized as having proinflammatory and disease-inducing responses, the role of C5aR2 remains hotly debated (4, 5). Since C5AR2 was originally reported in 2000 (6), the last two decades have seen a quantity of studies reported that C5AR2 is accumulating attention for its unique role in dampening C5a signaling, modulating C5aR1 activity, and more recently, interplaying with other pattern recognition receptors and intracellular inflammasomes (7, 8). Reduced inflammatory cell infiltration was caused by deficiency of C5AR2, suggesting that C5AR2 had a critical effect on optimal C5a-mediated cell infiltration (9). The role of C5AR2 in properly controlling C5a is considerable; otherwise, excessive or unresolved C5a production can aggravate a plethora of acute and chronic diseases, such as ischemia-reperfusion injury, rheumatic arthritis, sepsis atherosclerosis, and cancer, even COVID-19 (10–13).

Research of C5AR2 in cancer is limited and controversial. It was reported a strong association of C5AR2 with chemoresistance and poor prognosis across diverse cohorts of patients with lung and breast cancer, together with IL-10 (14). On the contrary, in a melanoma bearing murine model, C5AR2 has a limited yet favorable effect in restricting tumor growth (15). In another AOM/DSS-induced CRC tumorigenesis, C5AR2 deficiency increased tumor progression, indicating that C5AR2 has an anti-inflammatory effect (16). However, the function and mechanism of C5AR2 independent of complement system in breast cancer remains unknown.

In this study, a comprehensive analysis was utilized to elucidate expression, prognosis, immune infiltration as well as correlation with signaling pathways of C5AR2. Immunohistochemistry of clinical samples and cell lines experiments were also conducted and the results were consistent with bioinformatics analysis. This present study may provide novel insights to show the potential of C5AR2 in breast cancer therapy.

MATERIAL AND METHODS

Data Processing and Analysis of C5AR2 Expression

The data of differential expression levels of C5AR2 between cancerous tissues and matched noncancerous tissue was from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and Genotype-Tissue Expression (GTEx, http://gtexportal.org) projects. The expression levels of C5AR2 in 31 normal tissues and 27 tumor tissues were evaluated, and the expression levels between cancerous samples and matched noncancerous ones were compared. Expression data were transformed by Log2and t-tests of two groups were performed for these types of tumor; $P<0.05$ was identified as a statistically significant difference between cancerous and noncancerous tissues.

Survival Analysis

Univariate survival analysis was performed to illustrate the associations between C5AR2 expression and disease-free survival (DSS) in pan-cancer. Using a bipartite method, the expression levels of C5AR2 were distributed into two groups. The Kaplan-Meier plotter database (17) was utilized as well as the PrognoScan database (18). HR, 95% CI, and log-rank *P* values were calculated then displayed.

Analysis of Immune Infiltration

The Tumor IMmune Estimation Resource (TIMER2.0, http://timer.cistrome.org/) is an integrated database designed to systematically analyze immune infiltrations and gene correlations across different tumor types (19). It provides the purity-adjusted spearman's rho in diverse tumors, characterizing immune infiltrates' abundances from the gene expression profiles estimated by CIBERSORT, QUANTISEQ, XCELL, and several other immune deconvolution multiple methods. The infiltrating levels of immune cells were compared between high and low C5AR2 expression cohorts in breast cancer.

Correlation Analysis

To assess the correlations between C5AR2 and tumor mutational burden (TMB) as well as microsatellite instability (MSI), we conducted Spearman's rank correlation coefficient, and the immune scores and gene correlation of each tumor sample were separately counted as well. Once $P<0.05$ and $R>0.20$, correlations were regarded as significantly positive.

Enrichment Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a powerful resource for understanding functions and utilities from molecular-level information. The molecular signatures database (MsigDB) was also demonstrated here for GSEA analysis, using the Hallmark gene set to illustrate specific biological states or processes (20). Once $|NES|>1$, $P<0.05$, $FDR<0.25$, pathways were regarded with significant enrichment. Meanwhile, GO analysis and GSVA analysis were conducted in breast cancer.

Immunohistochemistry

Clinical samples of breast cancer and normal tissues were incubated with rabbit antibody against C5AR2 at 1:100 dilution at 4°C overnight. Then the sections were incubated with HRP-conjugated goat anti-rabbit IgG H&L at 1:400 dilution at room temperature for 1 hour. We used the GTVision III immunohistochemical detection kit to detect immunoreactivity. All fields were observed under the Olympus BX53 microscope. The difference was measured by the intensity and quantity.

Cell Lines and Culture

MDA-MB-231, T47D, MCF7 cell lines were purchased from ATCC, then cultured in accordance with the manufacturer's instructions. Cell transfection was conducted by lentiviral vector and screened by puromycin.

RNA Extraction and qRT-PCR

NucleoZol reagent was used to isolate RNA from cells, and 2×SYBR PreMix EX TaqTM II was used to conduct qRT-PCR in accordance with the manufacturer's instructions. The primer sequences were listed (5'-3'): C5AR2 forward - CTGCTGAC CATGTATGCCAG, reverse- CGCTGAACCGTAGACCACC. β-actin forward- ACCGAGCGCGGCTACAG, reverse- CTTAATGTCACGCACGATTTCC. Results were calculated based on the $2^{-\triangle\triangle CT}$ method.

CCK8 Assay

Cells were plated in 96-well plates, and during the following seven days, cell proliferation was measured daily by Cell Counting Kit-8 reagent in accordance with the manufacturer's instructions. Using a microplate reader, the absorbance was measured at the indicated time at 450 nm.

Transwell Assay

Fifty thousand breast cancer cells were seeded in the transwell, using the serum-free medium, and in the bottom 24-well plate, the medium with 10% fetal bovine serum was added. For invasion assay, diluted matrigel was pre-prepared. After incubation for 24 hours, cells on the upper membrane of the transwell were wiped off. Cells on the lower membrane of the transwell were fixed, stained, and then imaged and counted.

Western Blot

Protein samples were quantified firstly and separated by page electrophoresis, then transferred to the special PVDF membranes. After membranes were blocked with 5% milk, then incubated with antibody. Signals were finally detected by chemiluminescence kit and imaged.

Statistical Analysis

The Student's t-test (two-tailed) was conducted in contrast between two groups. Spearman's rank correlation test was used to obtain the P values and partial correlation values. Results with $P<0.05$ were considered as statistically significant, and significance is shown as *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$.

RESULTS

Differential Expression of C5AR2 Between Samples of Tumor and Normal Tissues

Physiologic C5AR2 expression was first evaluated across 31 normal tissues from the GTEx database (**Figure 1A**). It was in blood and spleen tissues that C5AR2 expression levels were the highest. However, they were quite lower in most other normal tissues. To figure out correlations of C5AR2 expression with cancer, we then evaluated and compared C5AR2 expression levels between different cancers and matched noncancerous samples. Results from the TCGA and GTEx database indicated that C5AR2 mRNA expression levels were dramatically elevated in BRCA, CHOL, ESCA, GBM, HNSC, KICH, LAML, LGG, LIHC, PAAD, and THCA tissues, while lower in ACC, BLCA, COAD, KIRC, KIRP, LUAD, LUSC, OV PRAD, SKCM, TGCT and UCS tissues confronted with that in normal ones (**Figure 1B**). Further, the protein expression levels were detected by immunohistochemical (IHC) staining in 45 paired breast cancer tissues. The results also showed higher C5AR2 expression level in the breast cancer tissues than the paired adjacent tissues (**Figure 1C**).

Notably, C5AR2 expression levels were diverse in subtypes of breast cancer, and it was much higher in Lumina A and Lumina B (ER-positive) than in HER2 and Basal (ER-negative) in the TIMER2.0 database (**Figure S1**). In addition, the comparisons of C5AR2 mRNA expression between the paired cancerous and noncancerous samples from the TCGA database were exhibited in **Figure S2**. C5AR2 mRNA expression levels were elevated in BRCA, HNSC, LIHC, PCPG, STAD, and THCA. By contrast, C5AR2 mRNA expression levels were declined in BLCA, KIRC, KIRP, LUSC, and THYM. In conclusion, C5AR2 was highly expressed in BRCA, CHOL, ESCA, GBM, HNSC, KICH, LAML, LGG, LIHC, PAAD, PCPG, STAD, and THCA, indicating C5AR2 as a potential tumor target.

Prognostic Value of C5AR2 in Breast Cancer

To figure out how C5AR2 expression correlates with patient prognosis, survival analysis for diverse cancer types from the TCGA database was respectively conducted. Cox proportional hazards model analysis suggested the significant associations between C5AR2 expression and disease-specific survival (DSS) in breast cancer and several other cancer types (**Figure 2A**). Kaplan-Meier survival curves from the Kaplan-Meier plotter database showed that C5AR2 expression levels also had significant associations with overall survival (OS) in several cancer types (**Figure S3**), including BRCA (**Figure 2B**). Considering that C5AR2 expression levels were much higher in Lumina A and Lumina B (ER-positive) than in HER2 and Basal (ER-negative), the PrognoScan database was used as well. The cohort GSE7378 included 100% ER-positive breast cancer samples suggested that elevated C5AR2 expression levels were significantly correlated with poorer DFI in ER-positive breast cancer (**Figure 2C**). In a word, high C5AR2 expression was associated with a poorer prognosis in BRCA, especially the ER-positive breast cancer, in which C5AR2 expression levels were more elevated than in normal tissues, indicating C5AR2 as an oncogene.

Correlations Between C5AR2 Expression and Immune Infiltration, TMB, and MSI

DNA mismatch repair deficiency (MMRd) frequently leads to microsatellite instability-high (MSI-H), then results in the aggravation of tumor mutation burden (TMB). These hypermutation elements contribute to tumorigenesis and are considered as independent predictors of immune checkpoint

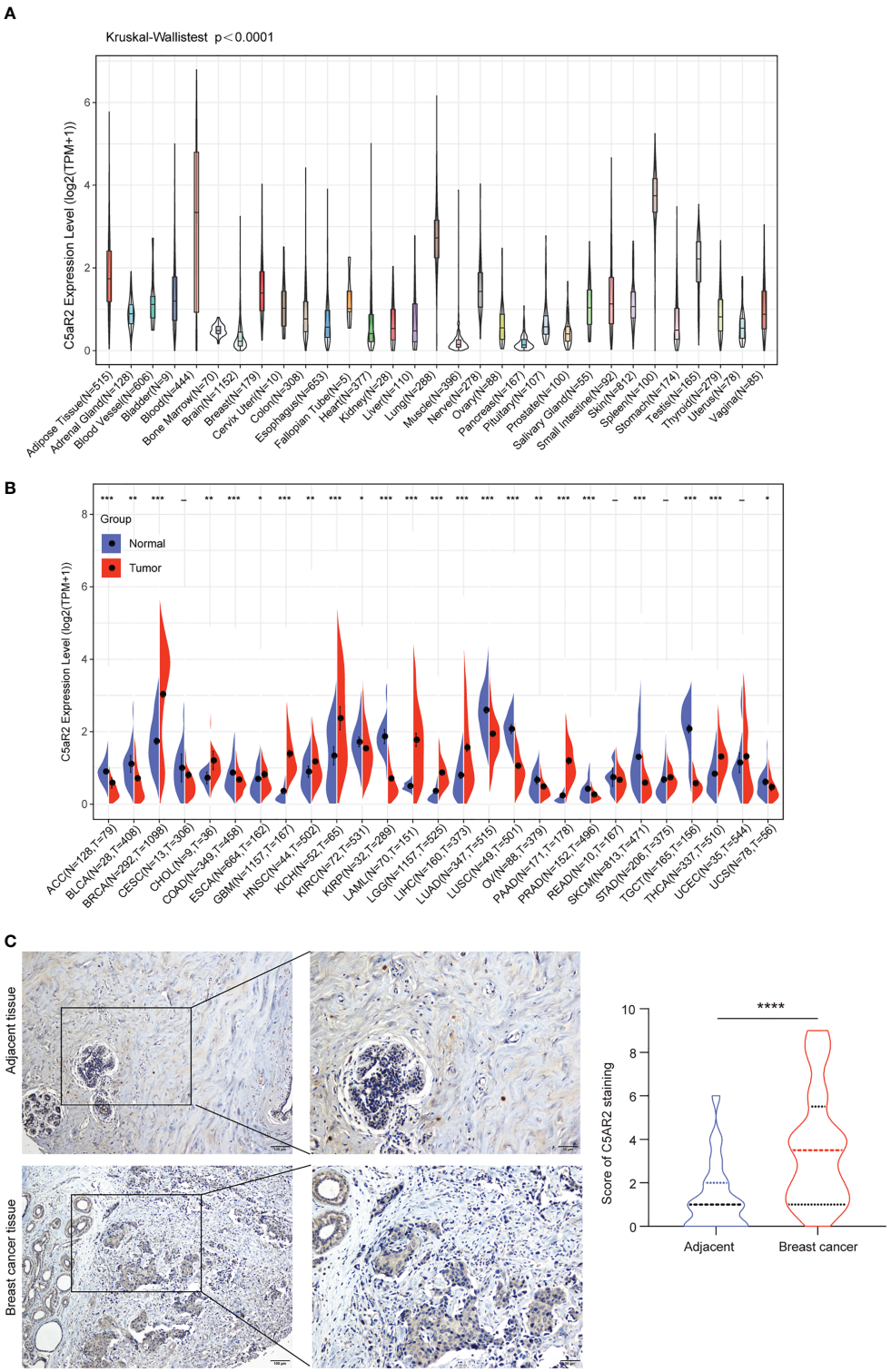

FIGURE 1 | The expression levels of C5AR2 between cancerous and normal tissue samples. **(A)** C5AR2 expression in normal tissues. **(B)** The contrasts of C5AR2 expression between cancerous and noncancerous tissues from TCGA and GTEx data. **(C)** The representative images of C5AR2 staining in breast cancer and adjacent tissues. The protein expression levels of C5AR2 were detected by IHC staining in 45 paired breast cancer and adjacent tissues. Scale bar: 100 μm (left panel) or 50 μm (right panel). The quantitative results were shown in the right. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

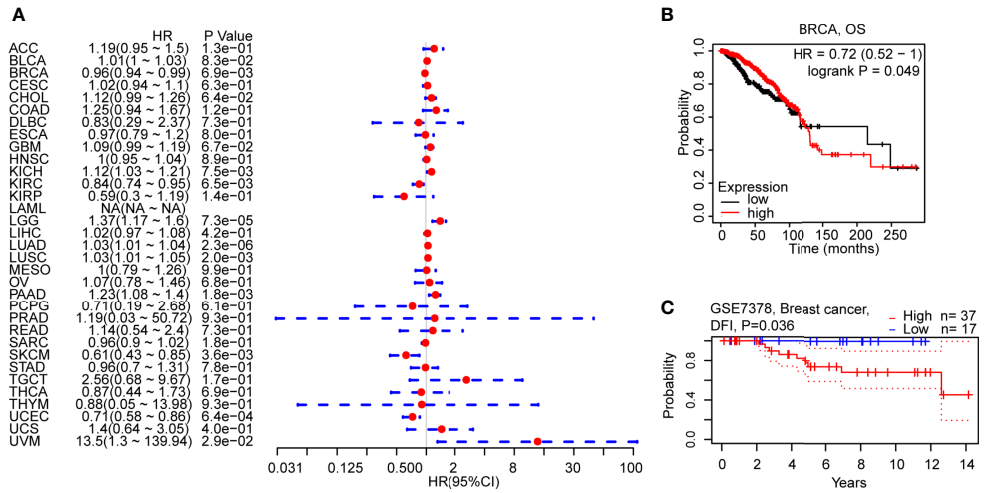

FIGURE 2 | Correlations of C5AR2 expression with patient prognosis. **(A)** Forest plot of correlations between C5aR2 expression level and DSS across diverse tumors in TCGA database. **(B)** Survival curve of OS in BRCA in Kaplan-Meier plotter database. **(C)** Survival curve in the cohorts of GSE7378 in PrognoScan database.

blockade effectiveness (21, 22). Via Spearman's rank correlation coefficient, associations of TMB and MSI with C5AR2 expression were separately analyzed in pan-cancer. The result revealed negative associations in BRCA and GBM (**Figure S4A**). C5aR2 expression levels were negatively related to MSI in BRCA and STAD (**Figure S4B**).

To explore how C5AR2 expression influences immune infiltration, we used the TIMER2.0 database to exhibit the heatmap of correlations of C5AR2 expression levels with various immune infiltrates, including macrophages, monocytes, neutrophils, dendritic cells (DCs), and regulatory T cells (Tregs) in pan-cancer (**Figure S5**). Notably, in breast cancer, C5AR2 expression levels were positively related to immune infiltration of M2 macrophages while negatively related to M0 and M1 macrophages (**Figures 3A–C**). Moreover, the infiltration scores of diverse immune cells in breast cancer patients were evaluated. The infiltration scores of M0 and M1 macrophages were lower, while that of M2 macrophages were elevated in the group of high C5AR2 expression than those in the group of low C5AR2 expression (**Figure 3D**). The results indicated that C5AR2 actively participated in immune infiltration, especially the polarization of macrophages.

Enrichment Analysis

To investigate how C5AR2 expression impact the fate of tumors, GSEA analysis was conducted, dividing the pan-cancer samples into high expression group and low one based on the C5AR2 expression levels, and in separately high and low expression groups, then analyzing the enrichment of signaling pathways or biological states or processes in both KEGG and hallmark datasets. Ranked by NES score, the top fifteen most abundant signaling pathways or biological processes have been listed and demonstrated in **Tables S1** and **S2**, and the top three were shown in **Figure 4**. The results indicated that C5AR2 positively regulates apoptosis, lysosome, peroxisome, fatty acid metabolism, glycosaminoglycan degradation, and other biological processes of KEGG signaling pathways. In hallmark signaling pathways, TNFα signaling *via* NFκB, IL6 JAK STAT3 signaling, IL2 STAT5 signaling, inflammatory response, KRAS signaling up, reactive oxygen species pathway, p53 pathway, and apoptosis were considered as the most enriched. Taken together, C5AR2 widely participated in regulating tumor immunity and metabolic signaling pathways.

To further figure out the biological significance of C5AR2 in breast cancer, GO enrichment analysis of the biological process was conducted, and it suggested that C5AR2 was associated mainly with hormone secretion and transport (**Figure 5A**). We also performed GSVA analysis, and the results revealed that C5AR2 was notably associated with metastasis as well as relapse of breast cancer and the upregulation of ESR1, a proven oncogene in breast cancer (**Figures 5B**).

C5AR2 Overexpression Facilitated the Malignant Behaviors and Oncogenic Signaling in Breast Cancer Cells

C5AR2 expression was most enriched in breast cancer tissues (**Figure 1B**), which was consistent with the results showed in the CCLE database (https://portals.broadinstitute.org/ccle/page?gene=C5AR2), C5AR2 expression levels were highest in a few breast cancer cell lines among solid tumor ones. Then C5AR2 expression levels in multiple breast cancer cell lines were evaluated, and compared with that in T47D and MCF7cells (ER-positive), C5AR2 expression was relatively lower in MDA-MB-231 cells (ER-negative) (**Figure 6A**). To explore how C5AR2 affects the proliferation of breast cancer cells, we overexpressed C5AR2 in MDA-MB-231 cells and validated the success of C5AR2 overexpression in this cell line (**Figure 6B**). The proliferation rates of MDA-MB-231 cells were significantly promoted following C5AR2 overexpression as evidenced by the CCK8 assay (**Figure 6C**). Besides, transwell assay was performed,

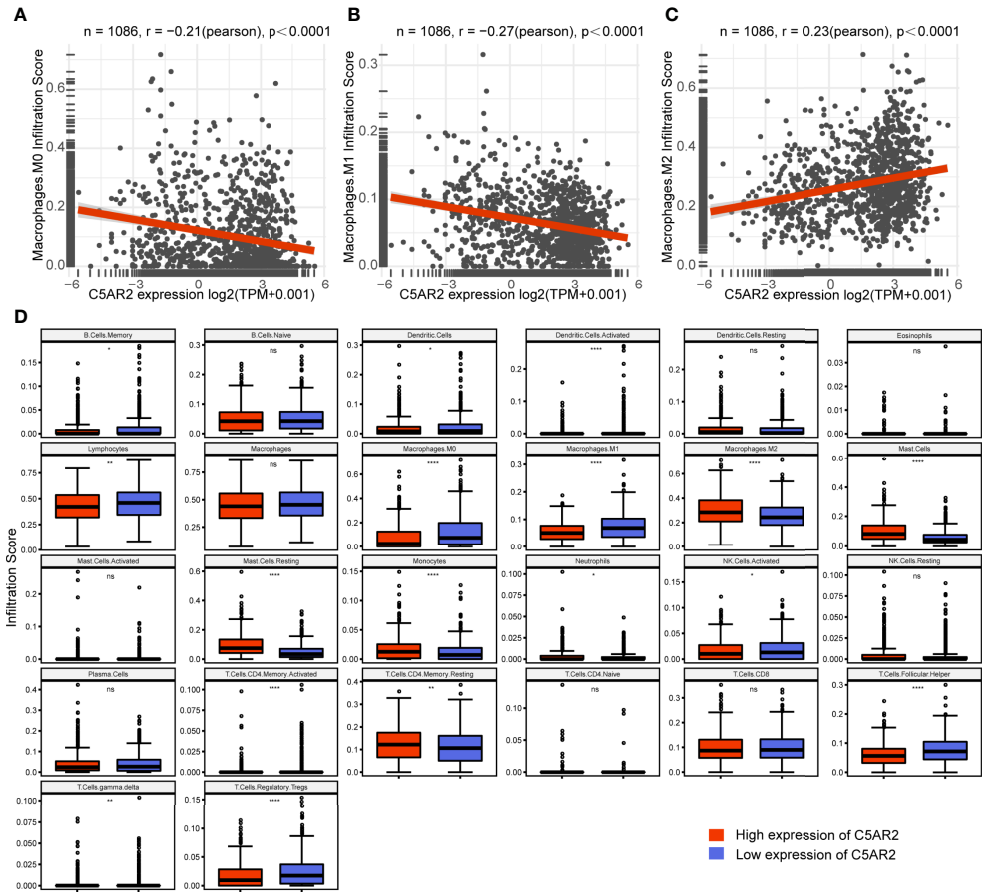

FIGURE 3 | Relationships of C5AR2 expression to immune infiltration in breast cancer. **(A–C)** Relationships of C5AR2 expression to the infiltration scores of macrophages. **(D)** The infiltration levels of 26 tumor-infiltrating immune cells compared in the high and low C5AR2 expression groups in breast cancer. NS, not significant, *P < 0.05, **P < 0.01, and ****P < 0.0001.

and the results revealed that C5AR2 overexpression also significantly enhanced the migratory and invasive capacity of breast cancer cells (**Figure 6D**). Finally, we conducted Western Blot, and the results suggested that in MDA-MB-231 cells, C5AR2 overexpression led to the obviously upregulated levels of MMP2 and MMP9 (**Figure 6E**), indicating that C5AR2 was related to EMT in breast cancer. In addition, the relationships of C5AR2 to other classic genes in key signaling pathways in breast cancer were analyzed through the TIMER database (**Figure 6F**). As shown in **Figure 6G**, C5AR2 expression levels were significantly, strongly, and positively related to MAPK3, STAT3, and NFKB1 and moderately positively related to the three other genes (PIK3CB, CTNNB1, MTOR). Overall, C5AR2 promotes the proliferation, migration, invasion, and activation of oncogenic pathways in breast cancer cells.

DISCUSSION

As a target for therapeutic intervention, the complement cascade is becoming increasingly attractive to numerous academic and pharmacy corporations that have focused on projects that exploit this system to discover new drugs in inflammatory disorders (23). Although C5aR1 is highly regarded as pro-inflammatory and pathogenic in a multitude of inflammatory diseases and C5AR2 is also involved in those inflammatory diseases, including gout, sepsis, hidradenitis suppurativa, and type 2 diabetes, the nature of C5AR2 appears to be much more nuanced and multifaceted (24–27). More and more discoveries have made it clear that complement proteins exist in the tumor environment and impact tumor progress (28). Analyze of C5AR2 in cancer is scarce and mostly focused on knockout mice models. Here we first demonstrated its profiles of expression, prognosis, immune infiltration, malignant properties, and functional signaling in breast cancer.

In this present study, expression levels of C5AR2 in different cancer types and normal samples were evaluated using the TCGA and GTEx databases, indicating that in pan-cancer, there were distinct differences of C5AR2 expression across cancerous and normal tissues. C5AR2 expression was increased in BRCA, CHOL, ESCA, GBM, HNSC, KICH, LAML, LGG, LIHC, PAAD, PCPG, STAD, and THCA, while

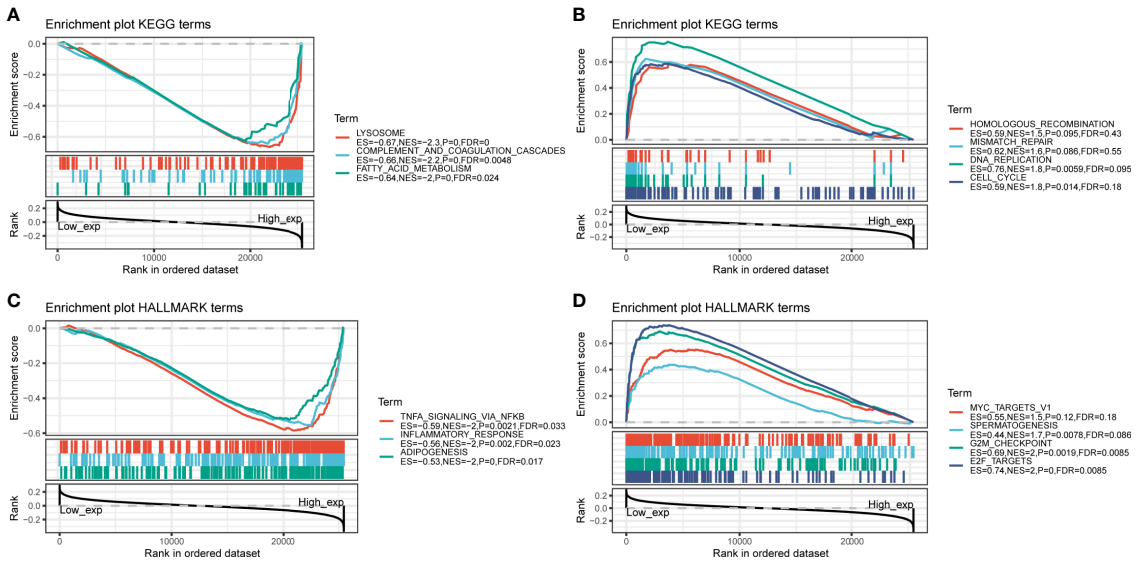

FIGURE 4 | GSEA analysis of C5AR2 related to signaling pathways and biological states or processes in KEGG and hallmark datasets. **(A)** The top three rankings of signaling pathways in the KEGG dataset by samples of high C5AR2 expression. **(B)** The top three rankings of signaling pathways in the KEGG dataset with the low C5AR2 expression. **(C)** The top three rankings of signaling pathways in the hallmark dataset by the high C5AR2 expression sample. **(D)** The top three rankings of signaling pathways in the hallmark dataset with low C5AR2.

decreased in ACC, BLCA, COAD, KIRC, KIRP, LUAD, LUSC, OV PRAD, SKCM, TGCT, THYM, and UCS confronted with adjacent normal controls. Notably, C5AR2 expression levels were much higher in Lumina A and Lumina B (ER-positive) than in HER2 and Basal (ER-negative). Strong correlations were shown between elevated C5AR2 expression and poorer prognosis in BRCA (ER-positive), indicating that C5AR2 has a malignant biological character as well as specific prognostic value, and it may be an oncogene in breast cancer.

One more important discovery is the relationships of C5AR2 expression to immune infiltration. C5AR2 was mainly expressed on macrophages, e.g. Kuffer cells, and mesenchymal cells, e.g. Ito cells in the HPA database (https://www.proteinatlas.org/ENSG00000134830-C5AR2/celltype), then the abundance of these two immune cells infiltrating in the tumor

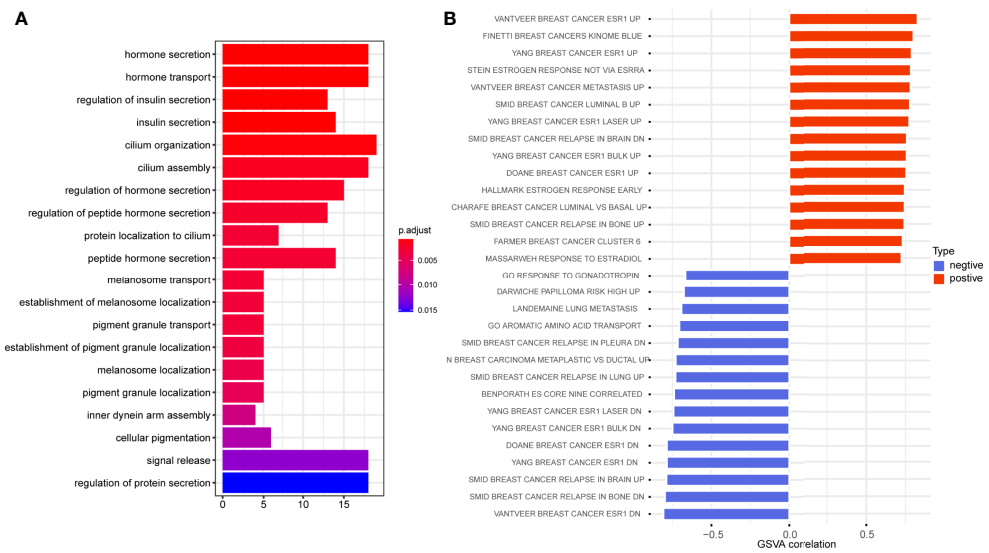

FIGURE 5 | GO enrichment analysis **(A)** and GSVA analysis **(B)** of C5AR2 related to biological processes in breast cancer.

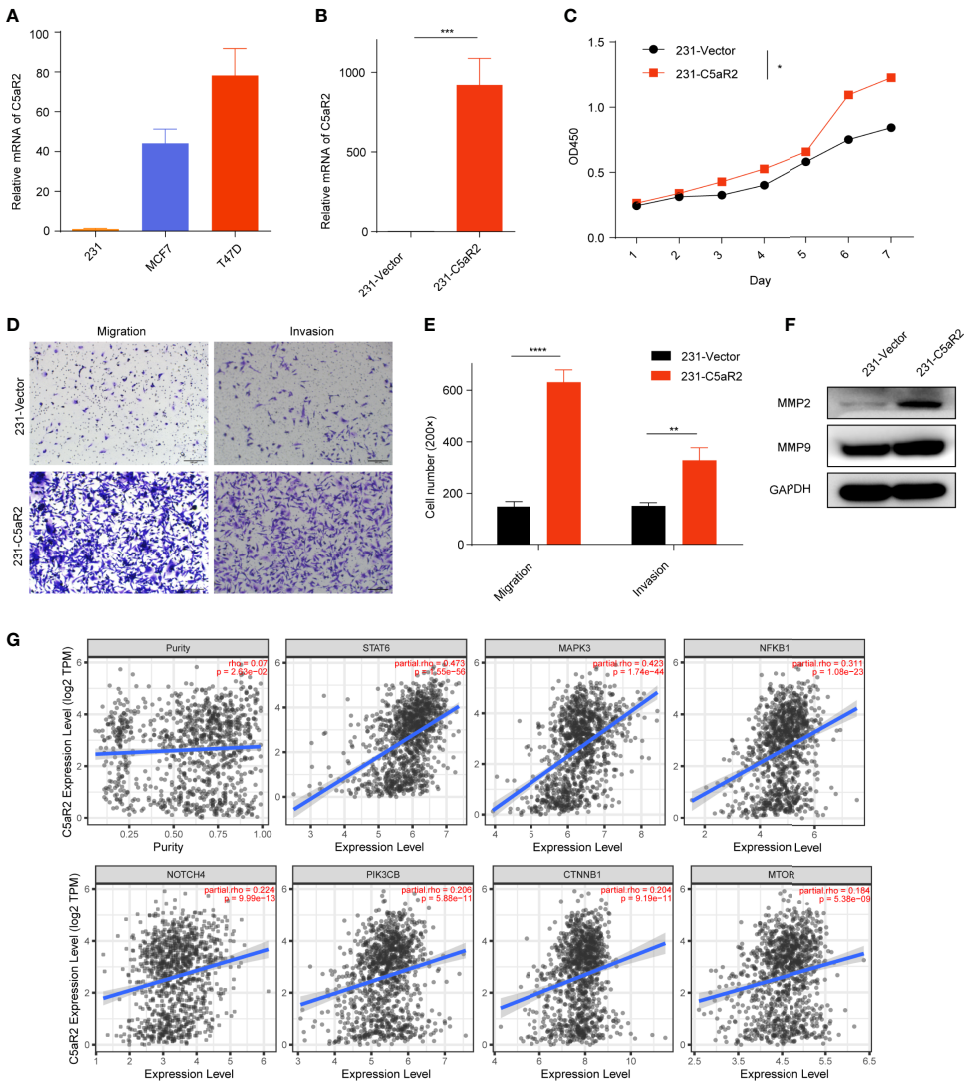

FIGURE 6 | Overexpression of C5AR2 facilitated the migration, invasion, and proliferation in breast cancer cells. **(A)** C5AR2 expression levels were measured in various breast cancer cell lines through qRT-PCR. **(B)** MDA-MB-231 cells were transfected with C5AR2 overexpressed plasmid, and the level of C5AR2 was measured through qRT-PCR. **(C)** The proliferation of MDA-MB-231 cells was measured through CCK8 assay. **(D, E)** The migration and invasion of MDA-MB-231 cells were measured through transwell assay. **(F)** MMP2 and MMP9 expression levels were measured through Western Blot in control and C5AR2 overexpressed MDA-MB-231 cells. **(G)** Correlation between C5AR2 and certain classic genes in key signaling pathways in breast cancer through TIMER database. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

microenvironment may be indirectly reflected by C5AR2 expression levels. In this study, C5AR2 expression showed remarkable relationships to immune infiltrating levels of multiple immune cells, especially the CAFs and macrophages. A previous study revealed that in primary human macrophages, C5AR2 possessed pleiotropic functions (29). In this study, we noticed that C5AR2 was involved in the polarization of macrophages, and C5AR2 expression was positively associated with M2 macrophages and negatively with M1 macrophages in breast cancer. We analyzed the relationships of C5AR2 expression levels to TMB and MSI as well, and the results suggested that C5AR2 might have a synergy effect with known immune checkpoints. Nevertheless, according to TISIDB (30), several published reports were summarized (http://cis.hku.hk/TISIDB/browse.php?gene=C5AR2), and no significant difference in C5AR2 expression levels was found between responders and non-responders to immunotherapy. Taken together, we supposed that the promotion of the polarization of M2 macrophages by C5AR2 leads to an accelerative effect in tumor initiation or development in breast cancer.

Meanwhile, GSEA analysis was performed, revealing that C5AR2 was widely involved in metabolic pathways and biosynthesis in pan-cancer, including TNFα signaling *via* NFκB, IL6 JAK STAT3 signaling, IL2 STAT5 signaling,

inflammatory response, KRAS signaling up, reactive oxygen species pathway, p53 pathway, and apoptosis. In this present report, the gene co-expression analysis revealed the relationship of C5AR2 expression to the activation of oncogenic signaling such as NF-kB. And a previous study revealed that persistent NF-kB activation was maintained by complement signaling *via* C5AR2 (14). GO analysis in breast cancer was conducted, and the results revealed the association between C5AR2 and hormone secretion and transport. Since endocrine therapy is one of the basic methods for treating hormone receptor-positive breast cancer, C5AR2 may play a role in curative effect (1). We used GSVA analysis as well and noticed that C5AR2 was significantly associated with metastasis as well as relapse of breast cancer and the upregulation of ESR1, a proven oncogene in breast cancer involved in endocrine resistance (31).

Then we demonstrated cell experiments to confirm C5AR2 facilitates the migration, invasion, and proliferation in breast cancer cells. C5AR2 expression levels were higher in T47D and MCF7cells (ER-positive) than in MDA-MB-231 cells (ER-negative). Here, C5AR2 overexpression in MDA-MB-231 cells was performed, which promoted migration, invasion, and proliferation. C5AR2 overexpression also upregulated the expression levels of MMP2 and MMP9, which were reported as oncogenes correlated with metastasis and invasion in various cancers (32). MMP2 also activates TGF-β to promote epithelial-mesenchymal transformation (EMT), while by releasing vascular endothelial growth factor (VEGF), MMP9 promotes tumor angiogenesis (33, 34). The results indicated the role of C5AR2 in the metastasis and invasion of breast cancer.

There are some shortcomings and inadequacies in the present study. Firstly, this complex analysis has been done for the first time and no good comparable data is available. Moreover, if experimental validation of C5AR2 knockdown in T47D or MCF7 cells is performed as well, the hypothesis will be more convincing, together with the experiments of C5AR2 overexpression in MDA-MB-231 cells.

In summary, our present study provides insights into the malignant properties of C5AR2 and its potential role in tumor immunology, suggesting that C5AR2 can stand as a prospective biomarker in breast cancer.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YZ, XW, and YX conceived of the presented idea and carried out the experiments. YZ and LC performed the analytic calculations. YZ wrote the initial manuscript. PD and JC reviewed the manuscript. WH supervised all the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.736725/full#supplementary-material

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Distribution Characteristics and Prognostic Value of Immune Infiltration in Oligometastatic Breast Cancer

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Background: To assess the distribution characteristics and the prognostic value of immune infiltration in female oligometastatic breast cancer patients.

Methods: We retrospectively analyzed the clinicopathological data of oligometastatic breast cancer (OMBC) patients diagnosed between June 2000 and January 2020. Immune markers were quantified by immunohistochemistry on FFPE tissues in paired normal breast tissues, primary breast cancers and oligometastatic lesions. Survival analyses were performed using the Kaplan-Meier curves and Cox-proportional hazards model.

Results: A total of 95 female OMBC patients visited Sun Yat-sen University Cancer Center between June 2000 and January 2020, and 33 of them had matched normal breast tissues, primary cancers and oligometastatic lesions and were reviewed in immune infiltration analysis. CD8 of primary tumors had a higher expression than that in matched normal tissues. The expressions of CD8 and FOXP3 were higher in the primary sites than that in the oligometastatic lesions. CD3, CD4 and CD8 were significantly lower in the intratumoral regions than that in the peritumoral regions both in primary and oligometastatic lesions. Notably, the high percentage of CD3 in the intratumoral oligometastatic lesions predicted the longer PFS and OS, and higher CD4 in the same lesions also predicted a better OS. There was obviously positive correlation between CD4/CD3 and Ki-67 in primary cancers and negative correlation between CD4/CD3 and ER in oligometastatic sites.

Conclusion: We explored immune distribution and evolution in time and space in OMBC to provide new understandings for biological behaviors of this disease and further divided patients in different prognosis.

Keywords: immune infiltration, primary tumor, oligometastatic lesion, intratumoral, peritumoral, prognostic value, oligometastatic breast cancer

INTRODUCTION

Breast cancer remains the most commonly diagnosed female malignant tumor with the highest incidence and mortality in 2020 worldwide (1). Distant metastasis/recurrence and its complications are the main cause of breast cancer-specific mortality. Approximately 20-30% of breast cancer patients may occur metastases after diagnosis and primary tumor treatment (2, 3), and the 5-year overall survival (OS) rate of metastatic breast cancer (MBC) patients is only 25% (4). MBC is heterogeneous both biologically and clinically in terms of proclivity for certain sites and disease burden (5). The oligometastatic breast cancer (OMBC) represents a special condition (6) and develops in about 1-10% of new MBC (7, 8). Oligometastatic disease, as a low volume metastatic disease, is defined as a state with limited number and size of metastatic lesions (up to five for breast cancer) in the 4th ESO-ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC4) (9). With the improvement of the insights of oligometastasis, this disease is further classified into induced/genuine oligometastatic disease, repeat/de-novo oligometastatic disease and synchronous/metachronous oligometastatic disease (10). Due to the potential curability, oligometastatic disease aims to achieve a complete remission status and a long-term patients' survival (11, 12). However, since no biomarker for the identification of patients with different prognoses is clinically available, the evaluation of oligometastatic disease is based solely on imaging findings and this manifestation on imaging could represent different clinical scenarios and might require different treatment strategies.

Although breast cancer is long considered as a poorly immunogenic cancer (13), the immune system plays a pivotal role in growth and development of breast cancer (14). Immunosurveillance provides an important first defense against tumor cells, on the other hand, immune responses can also lead to tumor progression by impairing tissue microenvironments and accumulating virulent cells through immunoediting (15, 16). The quantitative and qualitative differences of tumor-infiltrating lymphocytes (TILs) are associated with breast cancer progression and survival (17, 18). The high percentage of CD3+ T cells is related to better outcomes by inducing a more robust antigen-experienced, antitumor immune response (19). CD4+ T cells are divided into CD4+ T-helper 1 (Th1) cells and CD4+ T-helper 2 (Th2) cells, the former facilitates antigen presentation and predicts favorable prognoses (20), while the later inhibits cytotoxic T lymphocytes (CTLs) function, promotes an anti-inflammatory immune response, and enhances tumor growth (21). CD8+ CTLs are essential for tumor destruction. Furthermore, the immune contextures of the different compartments also have a correlation to their potential function and clinical effect. Differential densities of CD8+ and CD163+ cells in the intratumoral and peritumoral compartments are found to have significant prognostic value for clinical outcomes (22). In addition, programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), as immunotherapeutic targets, have also attracted much attention (23–25).

The relatively limited extension of disease suggests that appropriate treatment strategies can potentially cure these OMBC patients. However, the identification of reliable predictive markers able to stratify patients with different prognosis is still a challenge. The characterization of host immunity is closely related to the clinical effectiveness and prognosis of breast cancer. Monitoring immune responses in matched normal breast tissues and tumor lesions to follow their evolution along the disease progression may allow the identification of biomarkers potentially indicative of the different clinical outcomes. Therefore, to give new insights and improve the prognostic stratification, we analyzed the distribution characteristics and prognostic value of immune markers in matched normal breast tissue, primary tumor and metastatic lesions for OMBC.

Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CTLs, cytotoxic T lymphocytes; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; MBC, metastatic breast cancer; OMBC, oligometastatic breast cancer; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PFS, progression free survival; PR, progesterone receptor; SPSS, Statistical Package for the Social Sciences; TAMs, tumor-associated macrophages; Th1, T-helper 1; Th2, T-helper 2; TILs, tumor-infiltrating lymphocytes.

MATERIALS AND METHODS

Patient Population

Patients with breast cancer at Sun Yat-sen University Cancer Center between June 2000 and January 2020 were retrospectively reviewed. Inclusion criteria were as follows: female breast cancer patients with histologically confirmed diagnosis; metastatic disease diagnosed by pathology; no more than 5 metastatic lesions identified by imaging, including contrast-enhanced computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or positron emission tomography/computed tomography (PET/CT); patients with sufficient pathological tissue to perform immunohistochemistry (IHC). Exclusion criteria were as follows: any malignancies besides breast cancer; evidences of hematological or autoimmune diseases; receipt of immune-related drugs within 3 months before tumor biopsy; induced oligometastatic disease (patients with a history of polymetastatic disease); or repeat oligometastatic disease (patients with a previous diagnosis of oligometastatic disease). Clinicopathologic information was retrieved from medical records, including age, TNM stage of primary disease, the time from primary disease to oligometastasis, oligometastatic sites and treatment strategy (including local and systemic therapy) for OMBC, and pathologic analysis of primary and oligometastatic lesions. Oligometastatic disease was defined as a situation in which disease occurred in no more than 5 metastatic sites and this state lasted for more than 6 months (the patients included was a relatively strict oligometastatic status rather than a pre stage of poly-metastasis). Progression free survival (PFS) and OS were

defined as time from diagnosis of oligometastasis to the disease progression and to death (all causes), respectively. All patients were followed-up until death or study data cutoff (May 2021). The study was approved by the Ethical Committees of Sun Yat-sen University Cancer Center (NO.: B2020-319-01) and individual consent for this retrospective analysis was waived.

Immune Assessment by Immunohistochemistry

The expression of immune markers (PD-1, PD-L1, CTLA4, CD3, CD4, CD8, FOXP3, CD68 and CD163) was quantified by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) tissues in paired patient-matched normal breast tissue, primary tumor and oligometastatic lesions. Consecutive 4-μm tissue sections were cut from blocks selected for the presence of representative tumor tissue and immunohistochemistry staining was performed in one batch per marker to prevent intensity differences. The expression of PD-1 was assessed for tumor cell and the CD68 and CD163 double-stained cells were considered as M2 tumor-associated macrophages (M2-like TAMs). CD3, CD4, CD8, FOXP3, PD-L1 and CTLA4 expression was quantified for lymphocytes in normal breast tissue and the primary/metastatic lesions, the latter were divided into the peritumoral and intratumoral by hematoxylin-eosin stain. All markers staining was reported as the percentages of positive cells per slide. The percentages were averaged from two observers and used as the final score for every sample. The two observers discussed the results to reach a consensus if there was a discrepancy (>20% difference in score).

Immunohistochemical investigations were conducted according to the standard streptavidin-biotin-peroxidase complex method. Paraffin-embedded, formalin fixed sections were dewaxed with xylene, rehydrated by graded ethanol, rinsed using deionized water, and then blocked with 3% hydrogen peroxide for 10 min at room temperature. Antigen retrieval was performed by high-pressure-cooking the samples in a 10 mM citrate buffer (pH 6.0) for 4 min. Slides were blocked with 5% normal goat serum for 30 min at room temperature and subsequently incubated with primary antibody at 4°C overnight. The primary antibodies (anti-PD-1, clone UMAB199, OriGene Technologies; anti-PD-L1, clone E1L3N, Cell Signaling Technology; anti-CTLA4, clone UMAB249, OriGene Technologies; anti-CD3, clone LN10, OriGene Technologies; anti-CD4, clone EP204, OriGene Technologies; anti-CD8, clone SP16, OriGene Technologies; anti-FOXP3, clone UMAB248, OriGene Technologies; anti-CD68, clone KP1, OriGene Technologies; anti-CD163, clone 10D6, OriGene Technologies) were diluted following manufacturer's protocols. Secondary goat anti-mouse/rabbit antibodies (PV-6000, OriGene Technologies) were used to detect primary antibodies. The sections were counterstained with hematoxylin.

Statistical Analysis

The continuous variables were described by median and range and the categorical variables were showed with percentages. The cutoff values for immune markers were recommended by Xtile. The median value was as the cut-off value if no appropriate cutoff value was proposed by Xtile. Spearman's correlation coefficient or Chi-square test's Phi coefficient served to assess the correlation among the investigated markers. The Wilcoxon signed-rank test and Kruskal-Wallis One-Way ANOVA were used for the statistical analysis of variation in immune infiltration data between different tissues. The impact of the extent of immune infiltration on PFS and OS was calculated by Kaplan-Meier curves. The Cox-proportional hazards model was carried out to evaluate the simultaneous influence on PFS and OS of all covariates. For all tests, P values less than 0.05 were considered statistical difference, and all P values were tested two sided. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS), version 25.0; Xtile, version 3.6.1 and GraphPad Prism, version 6.0.2.

RESULTS

Clinicopathological Characteristics

A total of 95 female OMBC patients visited Sun Yat-sen University Cancer Center between June 2000 and January 2020, and 33 of them had matched normal breast tissues, primary cancers and oligometastatic lesions and were collected in subsequent immune infiltration analysis (**Figure 1**). The clinicopathological data of 95 patients were summarized in **Table 1**. All patients were genuine oligometastatic disease and de-novo oligometastatic disease. The pathological subtype of primary sites was all invasive ductal carcinoma (IDC). 12.6% (12/95) of the patients were synchronous oligometastatic disease, while the remaining 87.4% (83/95) were metachronous disease. Liver, lung and brain were the main oligometastatic sites, accounting for 40.0%, 29.5% and 27.4%, respectively. There were 40 hormone receptor (HR)+ (human epidermal growth factor receptor 2) HER2- breast cancer, 40 HER2+ cancers and 10 triple negative breast cancer (TNBC) patients based on the primary tumor. The median time to oligometastasis from initial diagnosis of breast cancer was 21.19 months. The median PFS and OS after oligometastatic disease were 16.73 and 162.74 months, respectively. The median follow-up time after the diagnosis of primary breast cancer was 61.0 months, and the median follow-up time after diagnosis of oligometastasis was 33.5 months. Among 95 patients included, 91 patients were performed the systemic therapy. A total of 73 (76.8%) patients received the local treatments, including surgery, radiotherapy and interventional therapy, and surgery was the main treatment strategy, accounting for 75.3%.

We further explored the impact of conventional clinicopathological factors on the survival of OMBC. Kaplan-Meier survival curves analysis suggested that progesterone receptor (PR) of oligometastatic lesions had close links with OS (P=0.006) (**Figure 2**), not PFS (P=0.734). Unfortunately, no independent impact factor was found for PFS and OS after oligometastasis on multivariate analysis (factors with P<0.05 and other important clinicopathological factors were included).

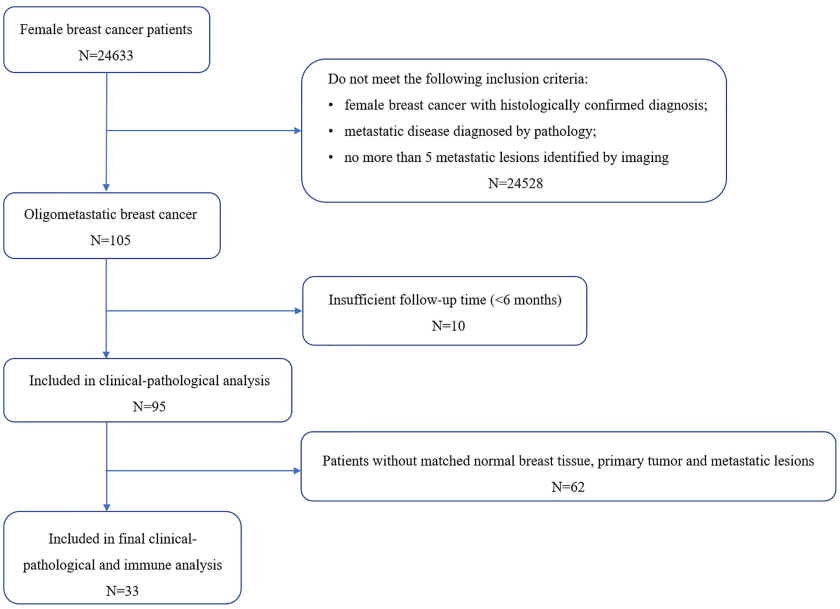

FIGURE 1 | A flow chart outlining included patients' selection.

Correlation Between the Clinicopathological and Immune Markers

We interrogated the correlation between the clinicopathologic factors and immune markers. Stratified of oligometastatic sites, we found that the expressions of CD3 (P=0.001), CD4 (P=0.001) and CD8 (P=0.011) were different in the brain, lung and liver oligometastatic samples. In the peritumoral, the expressions of CD3, CD4 and CD8 were the most abundant in liver, followed by lung and brain shown in **Supplementary Table 1**.

In primary cancer, CD4/CD3 was positively correlated with Ki-67 (the intratumoral: r=0.410, P=0.042; the peritumoral: r=0.414, P=0.029) and negatively correlated with PD-L1, but the number of positive cases of PD-L1 was small (only one sample expressed PD-L1 in the primary sites and two expressed PD-L1 in the metastatic lesions). In oligometastatic lesions, the strongest negative correlation was observed between CD4/CD3 and estrogen receptor (ER) (the intratumoral: r=-0.533, P=0.004; the peritumoral: r=-0.420, P=0.023). In addition, CD4/CD3 in

TABLE 1 | Clinicopathological characteristics of 95 female oligometastatic breast cancer patients.

Factor	Median (range)/number (frequency)
Age at diagnosis of oligometastasis (year)	48 (25-72)
T stage (1-2/3-4/unknown)	74 (77.9%)/18 (18.9%)/3 (3.2%)
N stage (positive/negative/unknown)	73 (76.8%)/21 (22.1%)/1 (1.1%)
Molecular subtype of primary site (HR+HER2-/HER2+/TNBC/unknown)	40 (42.1%)/40 (42.1%)/10 (10.5%)/5 (5.3%)
Oligometastatic type (synchronous/metachronous) [a]	12 (12.6%)/83 (87.4%)
Oligometastatic site (liver/lung/brain/others)	38 (40.0%)/28 (29.5%)/26 (27.4%)/3 (3.2%)
Molecular subtype of metastatic site (HR+HER2-/HER2+/TNBC/unknown)	36 (37.9%)/40 (42.1%)/12 (12.6%)/7 (7.4%)
Systemic therapy after oligometastasis [b] (yes/no)	91 (95.8%)/4 (4.2%)
Local treatments of oligometastatic lesions [c] (yes/no)	73 (76.8%)/22 (23.2%)
PFS (months)	16.73 (6.0-120.4)
OS (months)	162.74 (7.5-233.8)

[a]*Synchronous oligometastatic disease was referred to maximum 6 months interval between diagnosis of oligometastatic disease and primary cancer diagnosis, metachronous oligometastatic disease was referred to more than 6 months interval between diagnosis of oligometastatic disease and primary cancer diagnosis.*

[b]*Among 95 patients included, 91 patients were performed the systemic therapy. 39 patients were HR+/HER2- breast cancer of primary tumor, and 20 patients were conducted the chemotherapy and 19 were carried out the endocrine therapy. 33 patients (84.6%) received anti-HER2 targeted therapy in HER2+ primary breast cancer and all 8 TNBC patients received chemotherapy.*

[c]*Local treatments of oligometastatic lesions included surgery, radiotherapy and interventional therapy.*

HR, hormone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; PFS, progression free survival after oligometastasis; OS, overall survival after oligometastasis.

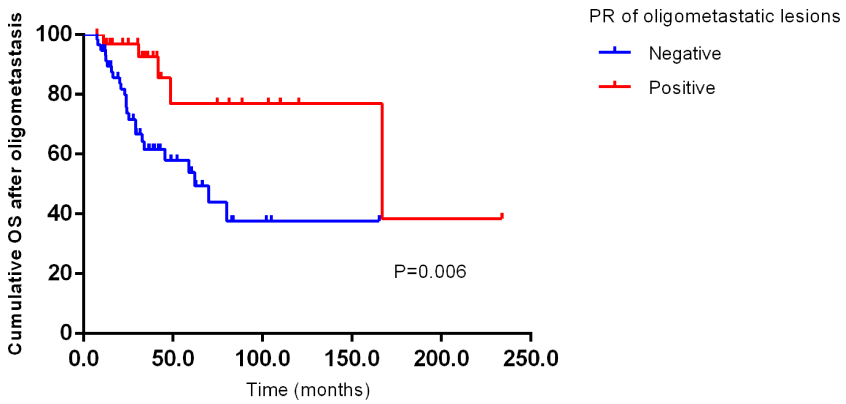

FIGURE 2 | Kaplan–Meier curve for OS of oligometastatic breast cancer patients stratified by PR of oligometastatic lesions. PFS, progression free survival after oligometastasis; OS, overall survival after oligometastasis.

peritumoral oligometastatic lesions was also inversely related to PR (r=-0.049, *P*=0.007) (**Supplementary Table 2**).

Distribution Difference of Immune Infiltration

The different distribution of infiltrating immune cells among matched tissues and matched regions were shown in **Figure 3**. No tumor cells expressed PD-1 either in primary or metastatic lesions. The expression of CD8 (the intratumoral *P*=0.017, the peritumoral *P*<0.001) in primary sites and CD3 (*P*=0.003) and CD4 (*P*=0.004) in the peritumoral primary sites were higher than that in normal breast cancer (**Figure 3A**). For paired tumor tissues (**Figure 3B**), the percentage of intratumoral CTLA4 was higher in oligometastatic lesions than primary tumor (*P*=0.043). The higher expression of CD8 and FOXP3 were in primary breast tumors than that in oligometastatic sites both in the intratumoral and peritumoral (primary vs oligometastatic tissue: CD8: the intratumoral *P*=0.031, the peritumoral *P*<0.001; FOXP3: the intratumoral *P*=0.039, the peritumoral *P*=0.012). Further, we also compared the distribution of intratumoral and peritumoral immune infiltrating cells. For primary and oligometastatic tissue, CD3, CD4 and CD8 were less in the intratumoral than that in the peritumoral (intratumoral vs peritumoral tissue: CD3: the primary *P*=0.002, the metastatic *P*=0.001; CD4: the primary *P*=0.001, the metastatic *P*=0.025; CD8: the primary *P*=0.002, the metastatic *P*=0.025). The expression of PD-L1 and M2-like TAMs in these two regions were not significant difference both in primary and metastatic tissue. CD68 single positive cells was different (*P*=0.034) in primary and oligometastatic lesions.

Considering the close relationship between TNBC/HER2+ breast cancer and immune microenvironment, we performed the subgroup analysis of TNBC and HER2+ breast cancer. There were 19 TNBC (n=5) and HER2+ (n=14) breast cancer patients in 33 patients with immune analysis. In TNBC and HER2+ subgroup, the distribution differences of immune indexes were mainly concentrated in CD3, CD4, CD8 and FOXP3, and the characteristics was similar to the total population (**Figure 4**). The higher percentages of CD3 and CD8 in primary sites and CD4 in the peritumoral primary sites were found than that in normal breast cancer. CD8 and FOXP3 were higher in primary breast tumors than that in oligometastatic sites. In primary and oligometastatic tissue, CD3 were less from the intratumoral than that from the peritumoral.

Prognostic Value of Immune Markers

The prognostic values of immune markers for OS and PFS in all 33 oligometastatic breast cancer patients were shown in **Figure 4**. The PFS rates were 47% at 1 year, 28% at 2 years, and 23% at 3 years; corresponding OS rates were 88%, 84%, and 78%. The median PFS for all 33 patients was 17.24 months, and the median OS was 162.00 months, which was similar to that of the overall 99 OMBC patients.

Patients with low percentage of CD3+ immune cells in the intratumoral oligometastatic lesions had worse PFS (*P*=0.016) and OS (*P*=0.004) than did those with high percentages. Similarly, there was a statistical difference that low CD3+ T cells in the peritumoral metastatic lesions also predicted worse PFS (*P*=0.028) and OS (*P*=0.017). For OS, in addition to CD3, high CD4+ immune cells in the intratumoral metastatic lesions predicted better clinical outcomes (*P*=0.018). The expression of CD3, CD4 and CD8 in normal breast tissue and primary lesions had no prognostic value for PFS and OS after oligometastasis (**Figure 5**). CTLA4, PD-L1, FOXP3+ immune cell and M2-like TAMs in 3 types of matched tissues did not predict the clinical outcomes in OMBC patients. CD68 or CD163 single positive cells had no prognostic value in these patients.

In the subgroup analysis of 19 TNBC and HER2+ breast cancer, CD3 still maintained its predictive value and the low expression of CD3 in the intratumoral primary lesions (*P*=0.015) and peritumoral oligometastatic lesions (*P*=0.040) had worse OS than did those with high expression (**Figure 6**).

DISCUSSION

It is now well appreciated that immune microenvironment plays a critical role in the evolution of breast cancer. Oligometastatic

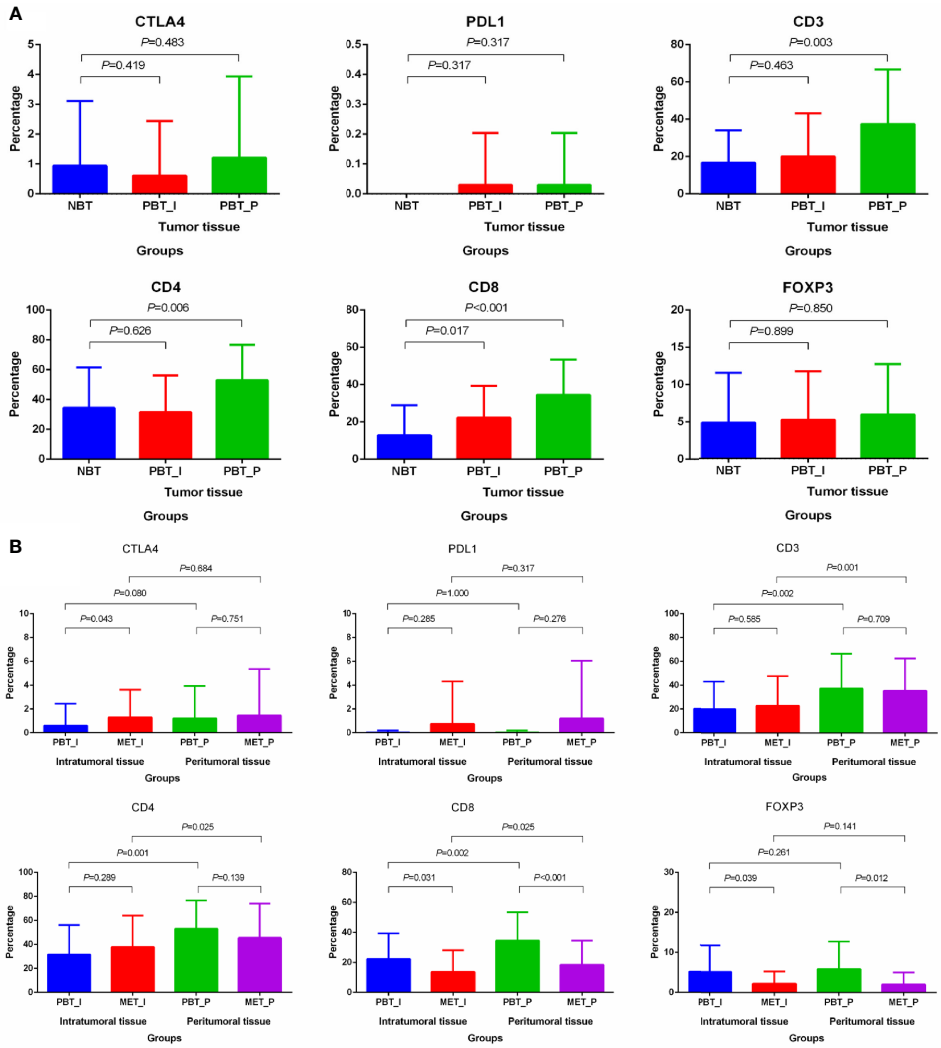

FIGURE 3 | Differential distribution of immune markers in matched normal breast tissues, primary cancers and oligometastatic lesions. **(A)** Differential distribution of immune markers in normal tissue and primary lesions. **(B)** Differential distribution of immune markers in primary and metastatic lesions. NBT, normal breast tissue; PBT_I, intratumoral regions of primary breast tissue; MET_I, intratumoral regions of oligometastatic lesions; PBT_P, peritumoral regions of primary breast tissue; MET_P, peritumoral regions of oligometastatic lesions.

tumors, as a potentially curable state, are given more attention recently. Studies of the breast tumor microenvironment have largely focused on tumor mutational and transcriptional landscapes in primary and conventional polymetastatic breast cancers (26). Our study is novel in two main regards: (1) we examined three cohorts of matched normal tissues, primary breast cancers and oligometastatic lesions, allowing us to discern immune changes in the whole evolution of OMBC and (2) we further divided the same samples into the intratumoral and peritumoral regions to refine the distribution of immune infiltration in the different areas. We explored the changes of immune infiltration in time and space to extend the current cognition of OMBC and to increase the prognosis stratification, and hoped to provide reference for individual therapy.

We detected large-scale differences in the immune microenvironment in the paired primary and oligometastatic lesions, as well as normal breast tissue and primary breast cancer. Most of the markers expressed in immune cells were lower in oligometastases compared with primary tumors in varying degrees, which was consistent with previous studies on polymetastases (27, 28). Of them, the expressions of CD8 and FOXP3 were substantially lower in the oligometastatic sites than that in the primary sites both in the intratumoral and peritumoral regions. Cytotoxic T cells, identifiable by CD8 expression, recognize cells that present foreign antigens in association with the major histocompatibility complex class I molecule through a specific interaction between the presented antigen and the T-cell receptor (29). Cytotoxic T cells, as a major effector component of the adaptive immune system, can act on

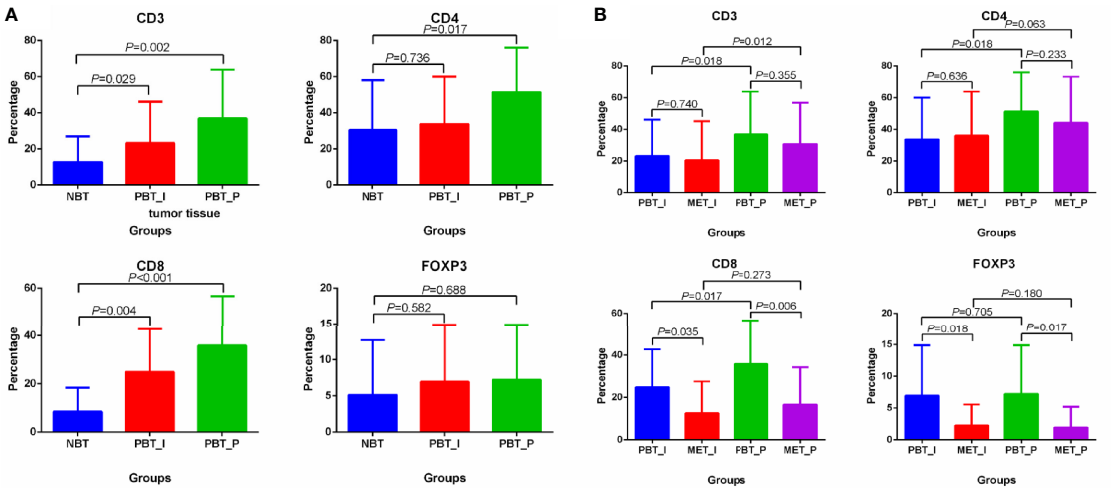

FIGURE 4 | Differential distribution of CD3, CD4, CD8 and FOXP3 in matched normal breast tissues, primary cancers and oligometastatic lesions in TNBC and HER2+ breast cancer. **(A)** Differential distribution of CD3, CD4, CD8 and FOXP3 in normal tissue and primary lesions. **(B)** Differential distribution of CD3, CD4, CD8 and FOXP3 in primary and metastatic lesions. NBT, normal breast tissue; PBT_I, intratumoral regions of primary breast tissue; MET_I, intratumoral regions of oligometastatic lesions; PBT_P, peritumoral regions of primary breast tissue; MET_P, peritumoral regions of oligometastatic lesions.

tumor cells which can present atypical antigens (30, 31), which also partly explained why the CD8 of the primary lesions was higher than that of the normal tissues. Regulatory T cells, which express FOXP3, promote tumor growth by inducing host tolerance against tumor antigens by attenuating the T cell-mediated immune response against the tumor cells and enabling them to evade the antitumor immune response (32). Although these two functionally distinct subsets of T cells exerted paradoxical effects in immune response, the expression CD8 and FOXP3 depleted probably due to a decrease in the overall immune level in oligometastatic lesions. In terms of the regional distribution of immune infiltration, CD3, CD4 and CD8 were significantly lower in the intratumoral than that in the peritumoral both in primary and oligometastatic lesions. This implied a difference in the distribution significance of the immune cells between the intratumoral and peritumoral regions of oligometastatic breast cancer, which might be due to the difference in intensity of the immune response at the two regions (33). Colorectal liver oligometastasis was the best understood tumors on oligometastasis and the findings also showed a lower expression of immune markers (CD3, CD8 and FOXP3) and a lower TILs density in the intratumoral regions of liver metastases than in the peritumoral regions, which was in line with our results (33–35). And immunosuppression might be promoted by a high tumor burden in the intratumoral microenvironment (36, 37).

The analysis of prognostic implications of lymphocytic subsets and density demonstrated that the high percentage of CD3 in the intratumoral oligometastatic lesions predicted the longer PFS and OS, and higher expression of CD4 in the same lesions was related to a better OS. CD3 is expressed on the surface of mature T cells and is associated with better outcomes based on previous studies (38, 39). The role of CD4+ TILs in breast cancer is complex and the numbers and cell subsets of CD4+ T cells dynamically changed with breast cancer progression (40, 41). Preclinical researches showed that CD4 + T cells changed their dominant subsets from Th1 in the early stages to Treg and Th17 cells in the late stages of the cancer progression (42), interestingly, oligometastatic disease was proposed as an intermediate state between localized and systemically metastasized disease. The less specific and, perhaps, biologically irrelevant total CD3 and, occasionally, complex and dynamic CD4 density may offer prognostic information in oligometastatic setting, while the individual stromal and intratumoral lymphocytic subset markers may not so. While immunohistochemistry improves accuracy of these markers expressed on lymphoid cells to assess the clinical importance of subtyping lymphocytes, at the present time any added value from these markers is unclear (43). From the biological aspect, none of the CD3, CD4, and other immunophenotypes can be considered as a surrogate of the extreme heterogeneity and functional diversity of these lymphocytic populations in the tumor microenvironment (44). Since the immune contexture in breast carcinomas and methodological limitations, it appears that less specific markers offer more information than the more specific but still partly understood ones.

The balance among various immune cells was also worthy of attention, which reflects the immune response in the tumor microenvironment, TIL ratios may be also a predictor of clinical outcome. In our study, there was obviously positive correlation between CD4/CD3 and Ki-67 in primary cancer and negative correlation between CD4/CD3 and ER in oligometastatic cancer. The quantitative balance between different subsets of TILs is also revealed by the immune cell ratio, which may be more reliable to indicate the immunologic response status on the tumor microenvironment. Highly proliferating metastatic tumors, possibly because proliferation is related to higher levels of genomic aberrations and, therefore to produce the neoantigens, may attract T cells (45). Perhaps, adding proliferation index, as Ki-

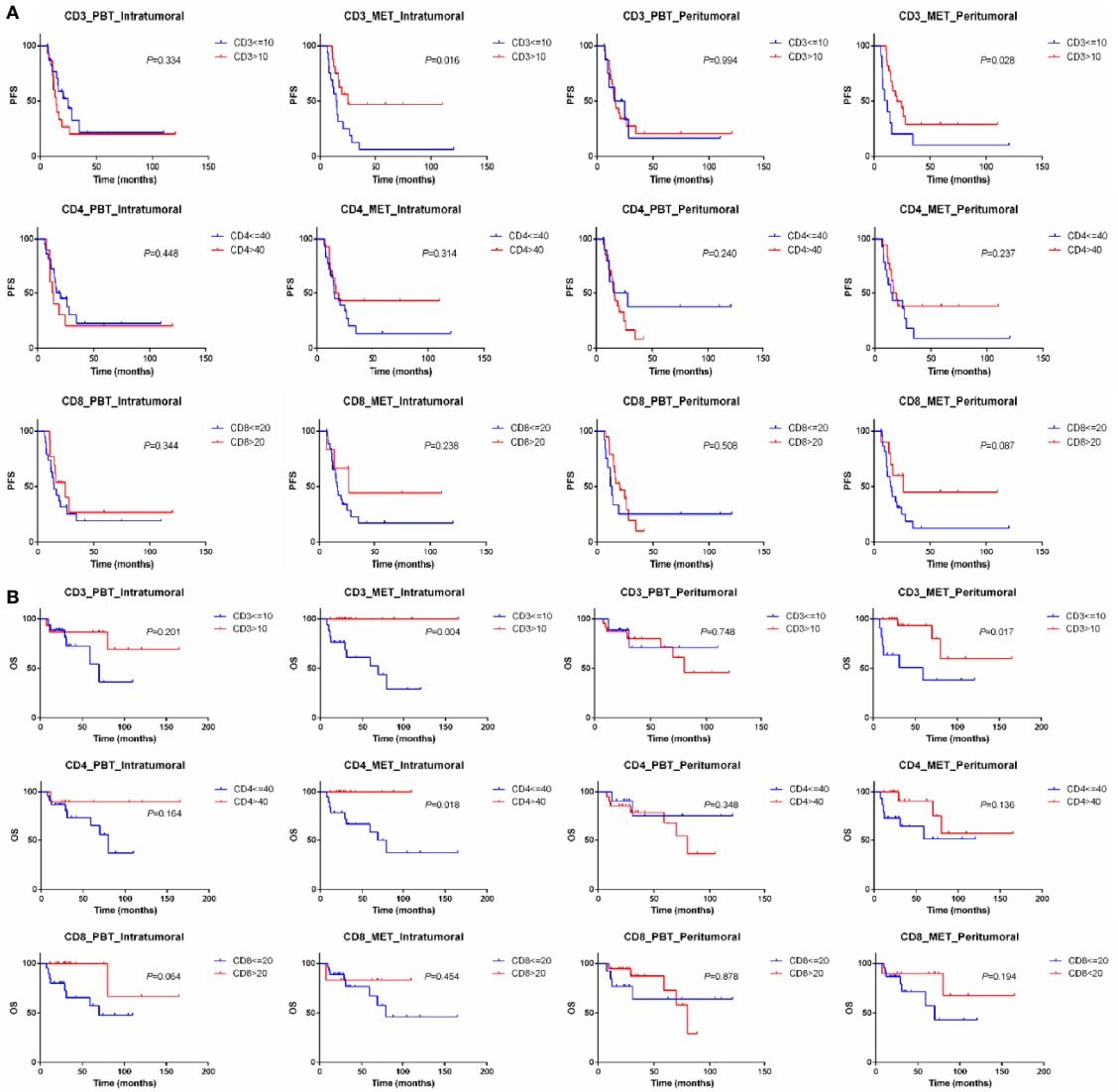

FIGURE 5 | Kaplan–Meier curve for PFS **(A)** and OS **(B)** of oligometastatic breast cancer patients stratified by the expression of CD3, CD4 and CD8. PBT, primary breast tissue; MET, oligometastatic lesions.

67, to the known associations between immune infiltration and subtypes may expand the knowledge of characterizing the status of host anti-tumor immune response, which needs to be taken into account in breast cancer therapeutics. Hormone receptor positive tumors have less TIL. The decreased lymphocytic infiltrate may be due to the expression of the estrogen receptor which has been shown to both promote a Th2 immune environment and decrease MHC class II expression in breast cancer cells (46, 47).

Considering different types of oligometastatic disease, all 95 OMBC patients included were genuine oligometastatic disease and de-novo oligometastatic disease, which reduced the heterogeneity of the population in terms of biological behavior and drug response of this disease (10). We did not further classify OMBC patients with simultaneous and metachronous metastases for the following reasons: 1) No consensus approached about the interval between diagnosis of primary cancer and oligometastasis to differentiate between synchronous and metachronous disease, especially synchronous disease (48). 2) The view that synchronous oligometastatic disease was associated with a worse prognosis than metachronous oligometastatic disease (49) were not confirmed by all studies (50). In addition, we explored to add time index, namely the time from diagnosis of oligometastatic disease to disease progression more than 6 months, to the current definition of oligometastasis to ensure a relatively strict oligo-metastatic status rather than a pre stage of poly-metastasis.

Our study also has several limitations. This is a small retrospective study of patient-matched pairs of primary and oligometastatic tumor samples from breast cancer. Our results

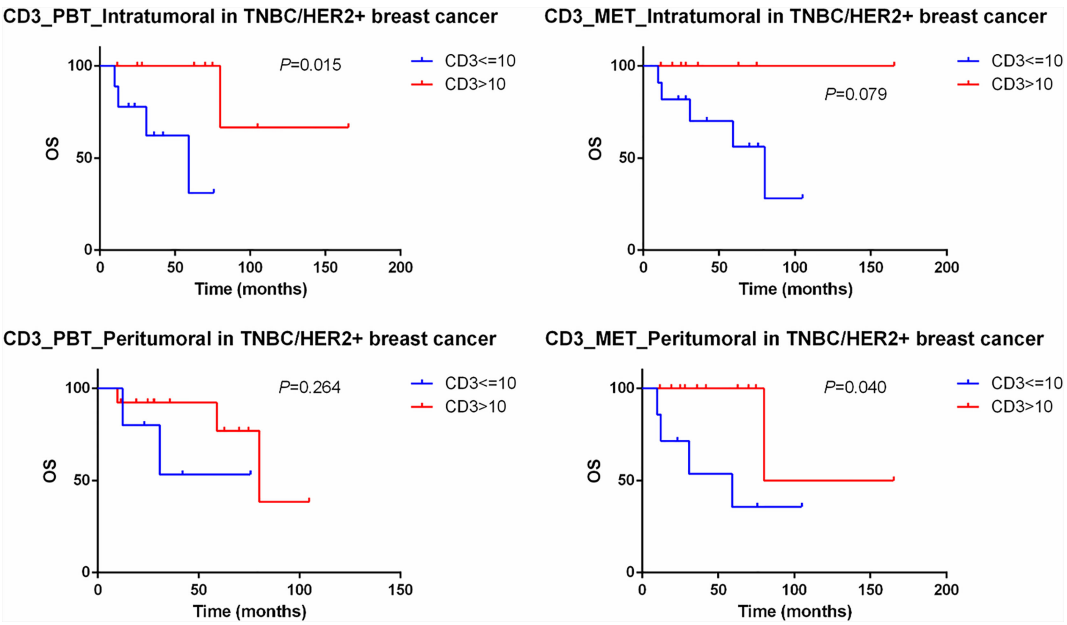

FIGURE 6 | Kaplan–Meier curve for OS of TNBC and HER2+ oligometastatic breast cancer patients stratified by the expression of CD3. PBT, primary breast tissue; MET, oligometastatic lesions.

should be interpreted with caution and a larger number of OMBC are needed to test the strength of our findings. Second, the receipt of other treatments before biopsy of oligometastasis, such as surgery, radiotherapy and chemotherapy, may have influenced the expression of immune cells in our patients. Third, the gene and RNA test were not carried out due to the limitation of specimen. The implementation of multiomics analysis can well explain the difference of immune infiltration in the multiple level. Despite these limitations, our study clearly highlights on the evolution and involvement of immune infiltration in the progression from a primary tumor to its oligometastatic cascade in breast cancer patient. In addition, we shed light on the prognostic values of immune markers and provided new insights for biological behaviors of the disease and further individualized treatment in OMBC.

Increasing attention has been paid to oligometastatic tumors due to the potentially curable possibility. We discerned immune changes in the whole evolution of OMBC and further refined the distribution of immune infiltration in the different regions. In addition, we found that high expression of CD3 in the intratumoral oligometastatic lesions predicted the longer PFS and OS. We improved the stratification of prognosis and provided new insights for biological behaviors of the disease and further individualized treatment in OMBC patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committees of Sun Yat-Sen University Cancer Center (NO.: B2020-319-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception and design: DZ, KJ, FX, and SW. Administrative support: FX and SW. Provision of study materials or patients: DZ, KJ, RH, QL, and WX. Collection and assembly of data: DZ, KJ, RH, QL, and WX. Data analysis and interpretation: DZ, KJ, ML, CZ, and QZ. Manuscript writing: all authors. Final approval of manuscript: all authors.

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REVIEW
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Research Progress on the Role of Regulatory T Cell in Tumor Microenvironment in the Treatment of Breast Cancer

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The tumor microenvironment (TME) is a complex ecosystem comprised of cancer cells, stromal cells, and immune cells. Analysis of the composition of TME is essential to assess the prognosis of patients with breast cancer (BC) and the efficacy of different regimes. Treg plays a crucial role in the microenvironment of breast cancer subtypes, and its function contributes to the development and progression of BC by suppressing anti-tumor immunity directly or indirectly through multiple mechanisms. In addition, conventional treatments, such as anthracycline-based neoadjuvant chemotherapy, and neo-therapies, such as immune-checkpoint blockades, have a significant impact on the absence of Tregs in BC TME, thus gaining additional anti-tumor effect to some extent. Strikingly, Treg in BC TME revealed the predicted efficacy of some therapeutic strategies. All these results suggest that we can manipulate the abundance of Treg to achieve the ultimate effect of both conventional and novel treatments. In this review, we discuss new insights into the characteristics of Treg in BC TME, the impact of different regiments on Treg, and the possibilities of Treg as a predictive marker of efficacy for certain treatments.

Keywords: regulatory T cell, tumor microenvironment, breast cancer, immunotherapy, neoadjuvant treatment

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BACKGROUND

In 1995, Sakaguchi et al. (1) described T cells (Tregs) as CD4+ CD25+ T cells with immunosuppressive effects on the human immune system. Tregs can suppress effector T cell responses as well as the activity of other immune cells, such as mast cells, dendritic cells, and B cells; thus, they are involved in cellular activation, maintenance of immune homeostasis (2), and allergy,

Abbreviations: MHC, major histocompatibility complex; CTLs, cytotoxic T lymphocyte; TME, tumor microenvironment; Treg, regulatory T cell; BC, breast cancer; TILs, tumor-infiltrating lymphocytes; OS, overall survival; APCs, antigen-presenting cells; TCR, T cell receptor; JAK, Janus kinase; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; STAT5, signal transducer and activator of transcription 5; mTORC2, mTOR complex 2; FOXO, Forkhead box O; tTreg, thymus-derived Treg; pTreg, peripheral Treg; Tconv, conventional T cells; TCGA, The Cancer Genome Atlas; AJCC, American Joint Committee on Cancer; Th1, T helper type 1; Th2, T helper type 2; CNA, copy number alteration; BCSS, breast cancer specific survival; pCR, pathological completed response; ORR, objective response rate; NAC, neoadjuvant chemotherapy; CTK, cyclophosphamide; TKIs, tyrosine kinase inhibitors; ADCC, antibody-dependent cellular cytotoxicity; CDK4/6, cyclin-dependent kinases 4 and 6; DNMT1, DNA methyltransferase 1; TGF- β, transforming growth factor-β; IL-2R, interlukin-2 receptor.

while in malignant tumors they promote tumor progression by suppressing anti-tumor immunity (3, 4). The tumor microenvironment (TME) is a collective term for a complex ecosystem composed of heterogeneous cancer cells, stromal cells, and immune cells rather than a simple homogeneous population of cancer cells. Specifically, the immune cells in the TME consist of different cells, such as CD8+ CTLs CD4+ Th cells and Treg. However, the TME is relatively unique in different cancers. Among the TME of breast cancer (BC), tumor-infiltrating lymphocytes (TILs) are probably the most representative and studied component of BC and provide insights into the immunogenicity of breast cancers (5). However, when tumors are clinically detected, this immune response is, in most cases, unable to stop the cancer progression because tumors have developed the immune constructive process. Several studies have shown that, in primary breast cancer, Treg (6–8) infiltration of BC is associated with immune tolerance and leads to overall survival (OS) prognosis. Considering the important role of Treg in BC TME, it is necessary to evaluate the unique properties of Treg in BC TME by studying its onset, progression, and anti-immune mechanism. Many breast cancer drugs used today have also been shown to have direct or indirect effects on immunity, thus altering cancer progression. Therefore, we want to investigate the impact of these mechanisms on Treg. If these mechanisms can alter the abundance of Treg in BC TME, can Treg predict the effect of mechanisms, and can Treg abundance be used as a prognostic marker in BC patients? Here we will also discuss the latest advances in knowledge related to these questions.

THE DEVELOPMENT OF Treg IN BC TME

Treg development begins in self-reactive thymocytes selected through high-affinity interactions with major histocompatibility complex (MHC) class II molecules expressed by thymic antigen-presenting cells (APCs) (4). A fraction of CD4+ CD8- thymocytes that receive strong T cell receptor (TCR) stimulation *via* self-antigen peptide–MHC complexes acquires the expression of CD25 (also known as IL-2Rα), which functions to increase the affinity for the interlukin-2 receptor (IL-2R) subunit CD122 (also known as IL-2Rβ). The IL-2–CD25 dimer then recruits CD122, followed by the common cytokine receptor γ-chain (γc). Subsequently, these three subunits make up a trimeric receptor expressed on Treg (9, 10). Upon IL2 and IL-2R binding, signaling occurs *via* multiple intracellular pathways, including the Janus kinase (JAK)–STAT pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway, and the mitogen-activated protein kinase pathway (11–13), wherein subsequent signaling *via* signal transducers and transcription activator 5 (STAT5) emits IL-2R signaling, leading to the expression of FOXP3, which confers various Treg cell-specific features to the cells, including the production of high levels of immune-suppressive molecules (14–16). In addition, signaling *via* the co-stimulatory receptor CD28 contributes to the commitment of a fraction of T cells in the thymus to the Treg cell lineage by inducing epigenetic and additional differentiation events in these cells (17–20). This commitment process involves many molecules; however, PI3K, AKT, and mTOR form a common intracellular signaling hub for TCR, CD28, and IL-2R that activates AKT through PI3K and mTOR complex 2, leading to the modulation of many cellular targets, including the forkhead box O family transcripts that are critical for Treg cell lineage commitment (21–23). As shown in **Figure 1**, we visualize the development process.

Thymus-derived Treg (tTreg) (formerly known as natural TREG—nTreg cells) and peripheral Treg (pTreg) cells (known as induced Treg—iTreg—cells when induced *in vitro*) are two types of Treg generated at different sites (4, 24). tTreg cells are generated through high-affinity contact with their own peptide MHC class II complexes in the thymus that are generated as a functionally mature T cell subpopulation. Under certain specific conditions, peripheral conventional T cells (Tconv) can differentiate into Treg cells in the presence of transforming growth factor-β (TGF-β) and are termed pTreg cells (25–31). However, whether this process requires the involvement of IL2 is unclear. Several studies supported the theories that IL-2 plays a key role in promoting TGF-β-mediated $Foxp3^+$ expression in $CD4^+$-naïve T cells, although it cannot induce Foxp3 alone (32–34).

There is compelling evidence that PD-ligand 1 (PD-L1) plays a key role in the induction and maintenance of pTregs, leading to pTregs amplification in TME, which then inhibits T cell responses to tumors (35–38). *In vitro*, PD-L1 can induce Tregs in the absence of TGF-β, suggesting that PD-L1 signaling can promote pTreg development (36). *In vivo*, blocking PD-L1 signaling abrogates induction in a tumor-induced Treg transformation model even in the presence of TGF-β (39). The internal mechanism can be attributed to the reduction of the Akt signaling pathway, which is essential for pTreg cell development (40). The specific development and infiltration process of tTregs and pTregs are presented in **Figure 2**.

These two subgroups share similar phenotypic characteristics and suppressive function in response to T cell-mediated immune response and cancer. Although some minor differences are found between these two groups, such as mRNA transcript and protein expression, epigenetic modification, and stability, it is still difficult to distinguish them, so the term Tregs can, by default, be used directly to refer to $FOXP3^+$ Tregs (41). Treg cells are chemo-attracted to the BC TME, where they can recognize their cognate antigens, be activated, and proliferate. The chemotaxis of Treg cells to the TME is mediated by combinations of chemokines and their receptors (for example, CCL22–CCR4, CCL28–CCR10, CXCL12–CXCR4, CCL5–CCR5, and/or CCL1–CCR8). They differ in different cancers (42–45). Especially in BC, CCR5, CCR8, CCR10, CX3CR1, CXCR3, and CXCR6 are stably and differentially expressed by tumor-resident Treg cells at the mRNA and protein levels (46, 47). While CCR4 was highly expressed by both tumor and peripheral blood Treg cells, CCR7 and CCR9 were downregulated in the Treg of TME. CCR5, CCR2, CXCR3, and CXCR6 were highly expressed by both tumor Tconv and Treg. However, CCR8 was found to be only highly enriched in tumor

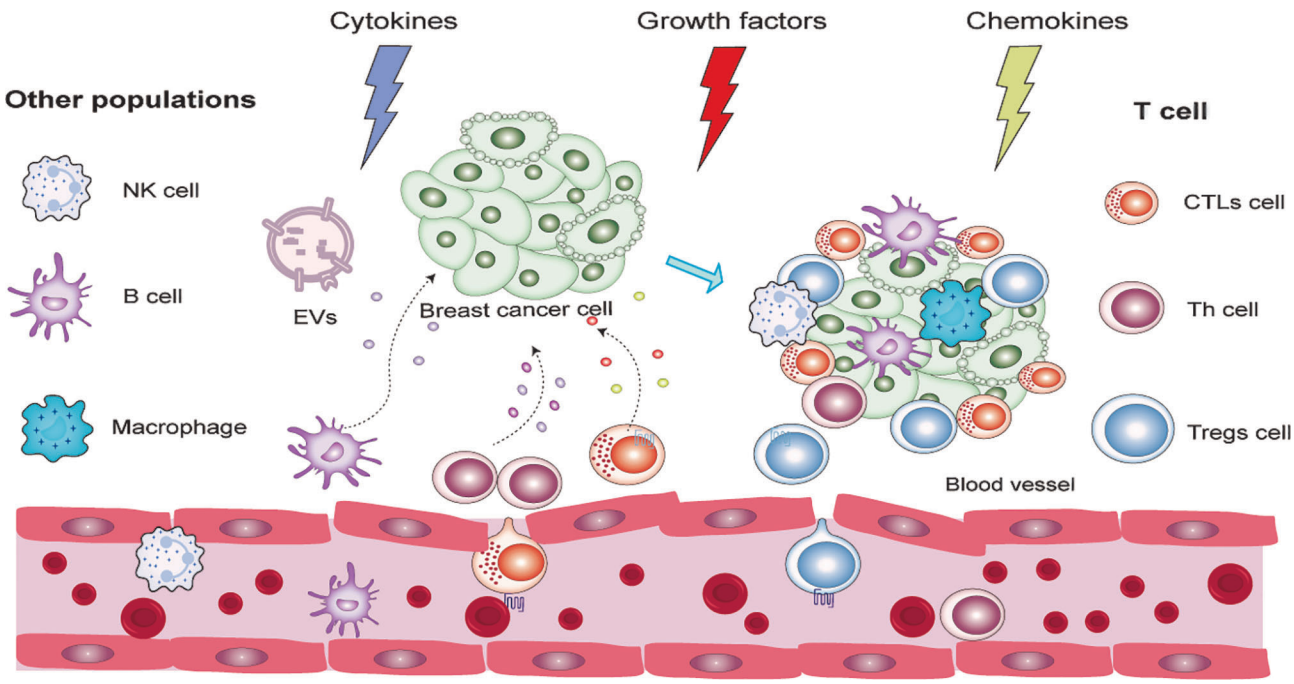

FIGURE 1 | Graphic representation of the development of Tregs. The location of origin of Tregs consisted of the thymus and secondary lymphoid tissue. The process involved in the thymus includes the selection of high-affinity CD25+ Treg cells and the expression of FOXP3 and other essential receptors expressed on the membrane through a complex signal transduction. The other process taking place in the secondary lymphoid tissue is attributed to the binding of PD-1 and PD-L1 and the cytokine TGF-β, but the inner mechanism remains unclear.

Treg cells and were much less abundant in Tconv cells, suggesting that Treg and Tconv cells may embrace both distinct and shared pathways to maintain their chemotaxis to the breast tumor microenvironment (47). In BC mouse models, blocking chemotactic signaling using antibodies or small molecules targeting CCL1–CCR8 reduces Treg cell accumulation in BC TME (46). Interestingly, these Treg cells recruit chemokines that can be produced not only by macrophages and tumor cells in TME (42–45, 47) but also by dysfunctional CD8+ T cells in TME that exhibit defective IL-2 production, such as CCL1 and CCL22.

Immune cell infiltrations are greatly heterogeneous between tumor types, and they can be located in different parts of the tumor, such as the center, margins, or adjacent lymphoid structures (48). High levels of Tregs in the periphery and TME were reported in peripheral and TME of breast (49), gastrointestinal tract (50), living carcinoma (51), pancreatic (52), and ovarian carcinoma (43). However, more Tregs infiltrate in TME than in adjacent normal tissue and peripheral blood in patients with primary breast cancer (47, 49, 53, 54). Notably, the density of Treg cells in the TME does not always correlate with matched peripheral blood (55). Within TME, Tregs were mainly distributed in the interstitial (also called mesenchymal) compartments and around the edges of BC infiltration (56). Interestingly, the specific TME in BC confers different characteristics to Treg cells. In a study by G Plitas et al. (47), the gene expression pattern of tumor-resident Treg resembled that of normal breast tissue but differed from that of corresponding activated or memory T cells isolated from peripheral blood, suggesting that the TME and its surrounding healthy regions are the main determinants of the gene expression characteristics of tumor and tissular Treg. TME usually contains large numbers of overexpressed immunosuppressive Treg cells of molecules, such as CTLA4 (57), PD-1 (58), LAG-3 (59), TIM-3 (60), and TIGIT (61), which are essential for their suppressive function (62). There have been many hypotheses on the composition of Treg in TME since Green et al. (63) who found amphiregulin to be expressed by Treg cells in a model of murine lung cancer. A more reliable conclusion is that Treg cells within TME in human cancer patients can be (i) tTreg recruited to the tumor site from outside the tissue and actively expanding (64) and/or (ii) a pool of pTreg derived from Tconv cells in periphery (64, 65) and/or, possibly, (iii) local expansion of tissue-resident Treg and/or (iv) Tregs converted from original TME- resident Tconv in TME. However, in BC TME, the difference of TCR sequence among blood and tumoral Tregs and Tconv cell was analyzed by Palita et al. (47). These analyses revealed a low TCR repertoire overlap between normal tissue and tumoral Treg cell and between intratumoral Treg and Tconv, which argue against hypotheses (iii) and (iv). However, both normal tissue and tumor Treg subsets contained large, expanded clones (47), similarly to the activated CD45RO+ (pTreg) but not to the resting CD45RA+ Tregs (tTreg) in peripheral blood, supporting hypothesis (ii) and denying hypothesis (i). These results together validated the possibility that, in breast cancer TME, the majority of Tregs in

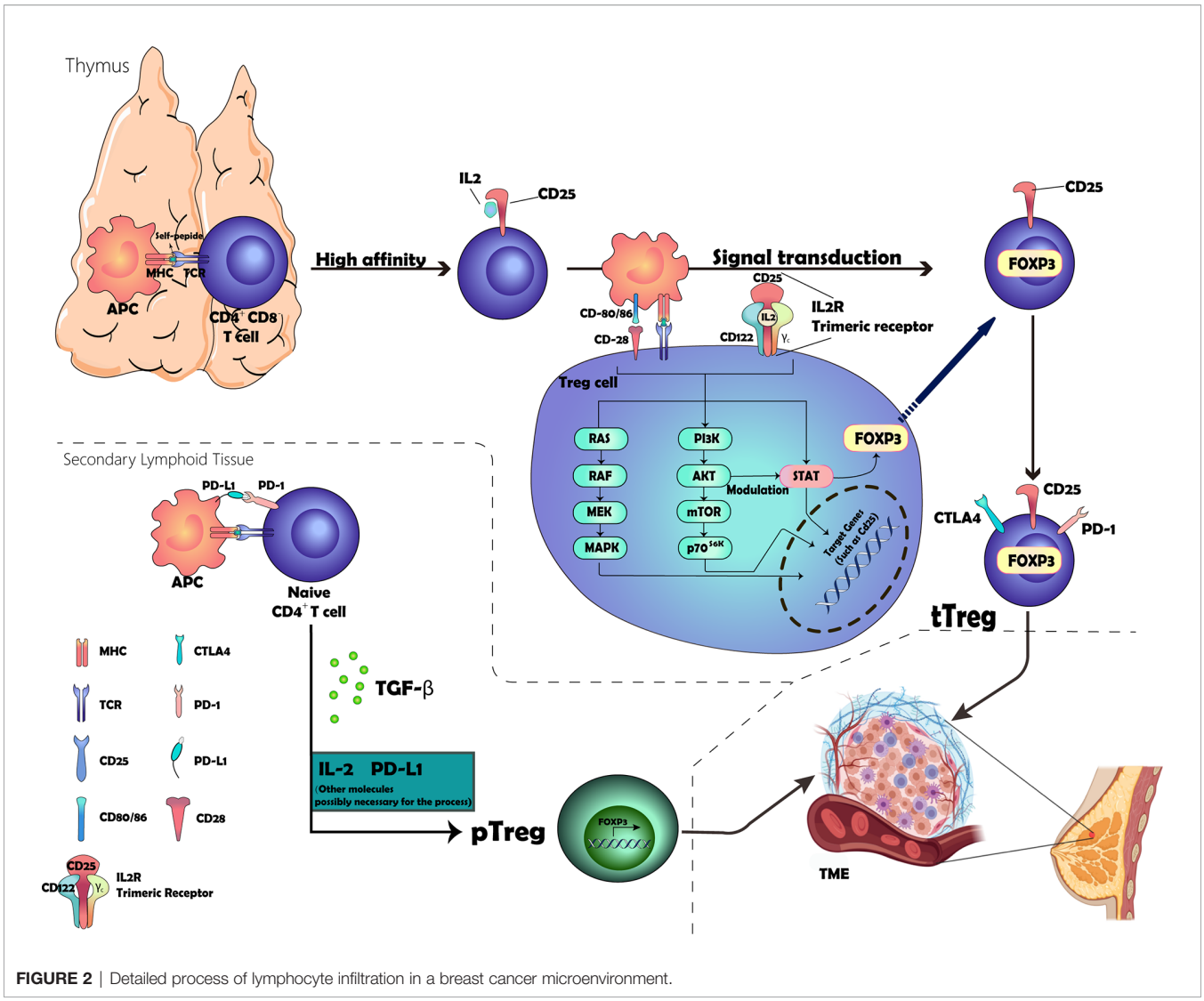

FIGURE 2 | Detailed process of lymphocyte infiltration in a breast cancer microenvironment.

breast tumors were initially recruited from the periphery (lymphoid tissue and blood), after which their phenotypic and functional features were shaped by the local environment. On the other hand, in certain circumstances like the late stages of cancer progression, the Th1 cells may convert to Tregs, thus promoting cancer development and progression, consequently conferring negative prognostic effects on breast cancer patient outcomes (66, 67).

DIFFERENCES IN TME BETWEEN DIFFERENT SUBTYPES OF BC

The TME of BC is relatively unique among the different subtypes. The immune infiltrate is heterogeneous and can be located in different parts of the tumor, such as the core (the center), the infiltrative margin, or the adjacent tertiary lymphoid structures. As for TILs, the most-studied component, it was higher in HER2+ and TNBC than in tubulointerstitial BC subtypes, as demonstrated by a secondary analysis of several clinical trials, such as FinHer (5), NeoALTTO (68), GeparQuattro (69), etc. Specifically, in a recent TNBC study (70), TME within TNBC is classified as immunoreactive subtype or "immune-cold" subtype by microdissection of tumor tissue. The CD8+ T levels are high, and PD-L1 was amplified, indicating a good effect of TME. However, in the "immune-cold" subtype, TME showed a negative expression of CD8+ T cells instead of the B7 family co-suppressor molecule B7-H4, which could suppress the effects of T cell effector function and infiltration. This result suggests that Her2-positive and Luminal BC can also be classified into subtype, and we can select the immune response subtypes for immunotherapy.

TME is diverse, but its signature is associated with primary cancer tissue, suggesting a link between BC and tissue-resident Tregs (71). Treg enrichment is thought to be reflected in more BC

with a higher histological grade (47, 72), invasive characteristics of the tumor (73, 74), and BC subtypes (6, 56, 72). The Treg infiltration rate increases in the order of Luminal A < Luminal B < Luminal HER2 < HER2-enriched < basal-like breast cancer. TNBC had the highest proportion of CD4+ T cells among the subtypes of breast cancer, and thus Treg cells transformed by Tconv were particularly prominent. In addition, the higher number of Tregs in the HER2-enriched BCs is partly explained by the higher level of chemokines, cytokines (75, 76), and TGF-β (77, 78) present in TME. However, a recent study of Masanori Oshi overturned these theories (79). According to The Cancer Genome Atlas database, the abundance of Tregs in primary tumors was not related with BC subtype, American Joint Committee on Cancer staging, or Nottingham pathological grade. Strikingly, the Treg infiltrating order of subtypes was entirely consistent with the order in which the PD-L1 expression rate increased (72), indicating that chemokines, cytokines, and/or immune checkpoint may be the inner factor that determines Treg infiltration instead of these clinical characteristics. To date, besides *in vitro* or animal models, the correlation between PD-L1 expression in tumor cell and the amount of Tregs in TME has been evaluated in patients with gastric and colorectal carcinoma (80, 81). However, in BC, this correlation remains controversial. However, as mentioned above, basal-like breast cancer with a higher level of CD8+ T cells expresses amplified PD-L1 (70), so this correlation is likely to be present in all BC subtypes.

THE ROLE OF TREG IN THE TME OF DIFFERENT BC SUBTYPES AND THE ESTABLISHMENT OF TARGETING Treg TREATMENT

There are several mechanisms Treg can perform to suppress immune cells (82), such as (i) releasing granzyme B and perforins to induce the apoptosis of effector cells (83), (ii) negative signaling to T cells through conversion of ATP to AMP, thereby inhibiting T cell proliferation and IL-2 formation (1, 84), (iii) interacting with B7 expressed by responder T cells through the CTLA-4 (85, 86), and (iv) secreting cytokines, IL-10 and IL-35, which are the key suppressive cytokines for Treg production to inhibit antitumor immunity and favor tumor growth by reducing effector expansion and cytokine production (IFNc and TNFa) (87). The effect of TGF-β1 on the generation of pTreg was well defined, but the suppressive function of TGF-β1 is still unknown. Three recent letters on TGF-β1 were published, two of which (88, 89) claimed that TGF-β1 did not work, but Stephen-Victor et al. (90) insisted that their debate can be attributed to the difference of gene editing. The conclusion from the study of Stephen can be attributed to the fact that they did not ablate the tgfb1 gene successfully but, rather, reverse it, which made the chromosomes fragile and triggered the mutant mice to death. Overall, Treg cells suppress strong antitumor immunity, thereby impeding an effective immune response to tumors. In addition to direct immunosuppressive activity, Treg cells can also inhibit the development of high endothelial venules by suppressing the self-amplification loop activated by mouse T cell (91, 92). Thus, the absence of Treg cell promoted the development of high endothelial venules, which have an important role in lymphocyte recruitment (91), representing a novel role of Treg cell in TME.

Considering these mechanisms of Treg action in TME, appropriate methods can be used to inhibit their anti-immunity effect. First of all, we can reduce the number of infiltrating Tregs while preserving the peripheral Treg—for example, anti-chemokines like anti-CCR4 mAb (93) and anti-CCR8 (94) treatments specifically depleted Treg in TME, with the result that Treg depletion will contribute to the activation of APC and upregulate CD80/86 expression to enhance the presentation of autoantigens and tumor antigens to Tconv cells, and these activated Tconv cells can then further activate the APCs. This positive loop inhibits anti-tumor immunity and inhibit tumor growth. It is worth noting that the CCR8 expression within Treg is exclusively on Treg cells in breast cancer (26), and the enrichment of CCR8 expression has been correlated with poor prognosis in patients with various types of cancer, including breast cancer and melanoma (47). Targeting CCR8 mAb may be a more effective therapeutic strategy than anti-CCR4 mAb.

In addition to anticancer factors, antagonizing cytokines that regulate Treg factors in TME may be another promising approach to inhibit Treg action—for example, TGF-β1 has a strong impact on pTreg production with insignificant immune-suppressive effect, so anti-TGF-β1 is highly likely to reduce Tregs. A study performed in melanoma has shown that the combination with anti-CTLA-4 mAbs and the TGF-β1 receptor serine/threonine kinase inhibitor galunisertib directly inhibited the generation of pTreg, increased the CTL/Treg ratio, and decreased the indoleamine 2,3-dioxygenase expression of APCs in tumor-draining lymph nodes (95). The fusion protein (M7824) combined by anti-PD-L1 and anti-TGF-β1 was also investigated in some studies (96), and M7824 exhibited a good effect in reducing Treg on patients with clinical benefits. Considering the suboptimal effect of anti-PD-1/PD-L1 or anti-CTLA-4 in the treatment of breast cancer, the addition of anti-TGF-β1 is still under investigation if it enhances the overall effect of improving anti-tumor immunity, and further studies are needed to evaluate Treg after using this drug.

In addition, it is even more important to inhibit Treg infiltration by targeting the molecules that perform the primary function—for example, anti-CTLA-4 is applied to stop the process of downregulating B7 expression on APCs. An increasing number of studies have shown the antibody-dependent cellular cytotoxicity (ADCC) effect of anti-CTLA-4 on Tregs based on this theory (97–99), while clinical responders of anti-CTLA-4-(ipilimumab)-treated melanoma patients can also achieve a depletion effect of Treg (100). Unlike CTLA-4, anti-PD-1 could not be included in our targeted Treg group despite the fact that it has been shown to be an effective option for treating cancer patients. This is because PD-1 is an

auto-inhibitor of PD-1-expressing cells, and therefore inhibition of PD-1 in $CD4^+$ T cell enhances the function of PD-1-expressing T cells and Treg cells (101), with the overall effect of increasing anti-tumor immunity. This phenomenon can be explained by the hypothesis that anti-programmed death-1 (PD1)/PD-L1 mainly targets PD-1^{hi} Tconv cells and has a greater effect on these cells than on Treg cells. Considering the characteristics of Tregs and the great differences between anti-Treg treatments, the anti-BC immunity strategy can be tailored to be an effective combination of immunotherapies and other targeted therapies.

Treg INTERACTIONS WITH A VARIETY OF CELLS

TME provides an environment for residing Treg to interact with their other immune cells, fibroblasts as well as vascular endothelium in TME. The interaction between these cells in TME nurture direct contact or indirect signals that promote or inhibit breast cancer growth, invasion, angiogenesis, and metastasis.

The mutual communication between Treg and Tconv is mainly indirect. First of all, the CTLA-4 on Treg can capture its ligands CD80 and CD86 on APCs, thus impairing their ability for co-stimulation of Tconv cells (85, 102, 103). The loss of co-stimulation makes Tconv more vulnerable to Treg suppression, and these Tconv with high-affinity TCRs will die by apoptosis (104). In addition, the competition of Tconv against IL-2 and other cytokines (57, 84) and the conversion of ATP into AMP (1, 84) are other indirect reactions that prevent optimal T cell activation.

The high abundance of Treg was also associated with increased infiltration of M2 macrophages and T helper type 2 (Th2) cells and decreased infiltration of T helper 1 (Th1) cells (79, 105). Similarly, in one of our unpublished original papers, CIBERSORT algorithm was used to test the correlation between Treg and macrophages in BC. We found that Treg was positively related to macrophage 0 (M0) but negatively correlated with macrophage 1 (M1). The negative correlations between M1 and Treg can be attributed to the suppressing M1-to-Treg contact (106) and/or inhibiting effect of soluble factors like TNF secreted by M1 on the accumulation of Tregs in TME (107). Some studies have demonstrated that TNF produced by M1 can diminish the suppressive activity of Treg cells through the NF-κB pathway (108, 109).

Carcinoma-associated fibroblasts are abundant in TME and involve many cancerous features such as tumor cell proliferation, angiogenesis, drug resistance, and metastases (110, 111). In BC, their enhanced role in tumor invasion and metastases is more pronounced. In addition, cancer-associated fibroblasts (CAFs) are able to secrete chemokines and cytokines, such as TGFβ, CXCL12, VEGF, and IL6, which stimulate cancer cell proliferation, epithelial–mesenchymal transition, and migration (112–115). The interaction between Treg and fibroblast in TME is also well identified. In a study of Costa et al. (116), multicolor flow cytometry and principal component analysis were performed to classify CAFs into four subtypes. Notably, the most representative subtype, CAF-S1, characterized by a high expression of the six fibroblast markers (FAP, integrin b1/CD29, aSMA, S100-A4/FSP1, PDGFRb, and CAV1) except CAV1, was positively found to be correlated with the number and function of Tregs but negatively correlated with CD8+ T lymphocytes. The internal mechanism was also well studied, namely, that CAF-S1 secrets CXCL12, which attracts Tregs and retains these cells through OX40L, PL-L2, and JAM2. In addition, CAF-S1 increases T lymphocyte survival and promotes their differentiation into Tregs *via* B7H3, CD73, and DPP4.

The interaction between Treg and vascular endothelial cells is a two-way process. Vascular endothelial cells can lessen the infiltration of Treg through chemical signals and physical barriers; they can also downregulate Treg activity through the production of leptin (117). Correspondingly, Tregs have also been reported to reduce endothelial cell activity and their chemotaxis of T cells (118). First of all, adhesion molecules, such as intercellular adhesion molecule and vascular adhesion molecules, are two main factors that promote T cell infiltration (119, 120).

However, the vascular endothelium cannot upregulate the expression of these two molecules in TME, which leads to the difficulty of T cell penetration. Meanwhile, this low expression can be reversed prophylactically by Treg depletion (121), which can be another mechanism of anti-Treg treatment. Additionally, the vascular endothelium establishes a physical barrier that restricts T cell infiltration. Accordingly, the blockade of the VEGF–VEGFR2 axis reportedly inhibits tumor growth through the decreased recruitment of Treg cells in the BC TME of a preclinical mouse model (122). In gastric cancer, anti-VEGFR2 mAb ramucirumab has already shown to lessen the density of effector Tregs (eTregs) but preserve CD8+T cells in the TME (74). The clinical efficacy of the combination of anti-VEGF–VEGFR2 axis and immune checkpoint blockade has been found in NSCLC (123), gastric cancer (124), RCC (125), etc.

THE HETEROGENEITY OF Tregs IN PERIPHERAL AND TME

The heterogeneity of Tregs was generated during, before, and after the entry of Tregs into BC TME. When Tregs are in the periphery, it can be subdivided according to the difference of transcription factors. Under the appliance of the transcriptional factor FOXP3 and other two surface markers, CD25 and CD45RA, circulating Tregs can be divided into three main groups: fraction I—$CD45RA^+$ $CD25/FOXP3^{lo}$ naive Tregs, fraction II—$CD45RA^+CD25/FOXP3^{hi}$ eTregs, and fraction III —$CD45RA^-CD25/FOXP3^{lo}$ cells, non-Treg. Helios, another transcription factor from Ikaros family, expressed by Treg but not Tconv cells in mice (126), can further classify Treg cells. FrII Treg cells in human blood exclusively express Helios, while both Helios-positive and Helios-negative cells are included in Fr I and Fr III $Helios^+$. It was proposed that the expression of Helios by human Treg cells may promote leukemic cell survival and

angiogenesis in *in vitro* assays (127). Moreover, Helios-negative Tregs were found to have low levels of Treg-specific demethylation region demethylation, so it shows a higher inflammatory cytokine production (128) and lesser suppressive activity (129). Based on these characteristics of Helios in Treg, Helios represents an attractive target for cancer immunotherapy at present. Consistently, it was argued that agonistic anti-glucocorticoid-induced TNFR-related protein (GITR) antibodies could inhibit Helios expression in Treg cells, whereby executing its anti-tumor function (130). Besides Helios, other markers, like TIGIT, CD226 (128), CD15s, HLA-DR, TIM-3, CD177 (47), and ICOS (131, 132), are promising markers expressed by Tregs that have the potential to further classify Tregs based on their function.

Chemokines, such as CCR4, CCR6, CCR8, and CXCR3, have also been used to characterize peripheral Treg. In this review, attention was paid to CCR8 and CD177, which play the critical role exclusively in BC. The study of Plitas (47) has shown that CCR8 was significantly upregulated in intratumoral Treg cells compared with normal adjacent tissue residents and their peripheral counterparts. Obviously, the enrichment of CCR8 is also correlated with a worse prognosis in BC patients (47). Moreover, the ratio of CCR8 and Foxp3 mRNA amounts can be an independent prognostic factor for the survival of BC patients. CCL1 is a known cognate of CCR8 which is highly expressed by intratumoral myeloid cells (47). Stimulating CCL1 can also enhance the suppressive capacity of human Treg cells *in vitro* through the STAT3-dependent pathway (133). As a result, targeting CCR8+ Treg cells through anti-CCR8 mAb or anti-CCL1 neutralizing mAb provides an opportunity for the selective depletion of Treg cells as an immunotherapeutic approach for the treatment of breast cancer. CD177 is another protein associated with cell adhesion and migration, which is highly expressed by Treg cell subsets (10–50% of the total number of Treg cells in breast cancer) (47). The role of CD177 on Treg cells remains to be unclear, and it is very likely that CD177 performs some functions and further subdivides Treg. Compared with CCR8 expressed on all Treg cells, CD177 was found to be expressed highly on a subset of tumor-associated Treg cells through flow cytometry. Moreover, single-cell analyses confirmed that CD177 is expressed highly in some Treg clusters in BC TME (134)..

Upon entry, TME will also remodel Tregs, resulting in a high degree of heterogeneity in genomic, transcriptional programs and chemokine receptor expression within the tumor Tregs despite their strong similarity to effector molecules. Recent work using multiregional genome sequencing of tumors has revealed a high degree of tumoral subclonality difference between spatial regions (135), including breast cancer (136). As for differences in transcriptional programs, single-cell RNA-seq detected differences in the co-expression patterns between Treg subpopulations of checkpoint receptor genes (CTLA-4, TIGIT, and GITR and other co-receptors) in certain Treg subset that can be mutually exclusively expressed in other subsets, indicating a different spatial and functional distribution of these subpopulations. Considering the results mentioned above, it is critical to decipher the inner mechanisms that shape and stabilize the Treg cell phenotype through the whole process of Treg recruitment. This is essential for us to evaluate, *i.e.*, the feasibility and safety of novel therapeutic approaches aiming at targeting a specific Treg target.

THE RELATIONSHIP BETWEEN THE DENSITY OF Treg AND PROGNOSTICS OF PATIENTS WITH BC

As mentioned above, the abundance of Tregs in the TME is not always linked to those in matching peripheral blood, suggesting that the analysis of the TME where T cells directly interact with tumor cells is more essential in studies of cancer immunology. Interestingly, within the TME, the density of intratumoral and stromal Treg infiltration should be assessed separately because they are independent prognostic factors (137). In a study with 1,270 samples of whole-tissue sections, intratumoral infiltration by Tregs is highly correlated with the prognosis of breast cancer (6, 72, 138). Although stromal Treg is sensitive to chemotherapy, intratumoral Treg is a better prognostic predictor of patients with breast cancer (6, 139).

Survival analysis was conducted by some research teams with respect to Treg high- and low-density BC subgroups without considering the subtypes of BC. The mean DMFS, DFS, breast cancer-specific survival (BCSS), OS, and DSS were comparable between the two groups, so the Treg levels did not significantly affect DMFS, DFS, or BCSS (56, 79).. Then how about the correlation within each subtype?

In breast cancer, a high frequency of TILs is associated with poorer survival in patients with ER+ and Her2+ breast tumor (56), while in TNBCs, the most aggressive and immunogenic subtype (140–142), the high incidence of TILs is significantly associated with longer survival (79, 143–145), indicating that the mere presence of TILs is insufficient to precisely predict their influence, and disease progression and clinical outcomes are influenced by TIL subtypes and their biological and functional characteristics rather than their density (146). Bohling and Allison (147) found a possible association between Treg infiltrates with TNBC subtype. According to Joe Yeong (148, 149), patients with TNBC exhibiting high intratumoral Treg density also have significantly longer DFS and OS than those with fewer intratumoral Tregs. In addition, some studies have demonstrated the association between Tregs in TME with HR- and HER2+ (6, 137, 150, 151). Jiang et al. (152) found that an abundant Treg infiltrate had an opposing prognostic significance in HR- and HR+ BC. The prognostic significance of Tregs was associated with HR- tumor status. On the HR- BC subgroup, high Treg showed a favorable effect on BCSS, in contrast to the lack of impact on BCSS among HR+ BCs (56). However, M Gobert et al. and (53) and GJ Bates et al. (8) found that the abundance of Treg has an influence on prognosis in HR+ BCs, while the prognostic value is unfavorable. The relation between Treg and prognostic value in HER2 overexpression is also

controversial. In BC patients where an association between Treg infiltration and HER2 overexpression was discovered, Tregs were mainly linked to poor prognostics, such as higher tumor grade and decreased OS and PFS (6, 150). In addition, Tsang et al. (153) found that TILs were associated with a smaller tumor size in HER2-enriched tumors. However, he considered both cytotoxic CD8+ T lymphocytes and Tregs together as a factor and observed only a correlation between this subtype and the CTL, which could explain why the TIL was associated with a better prognosis. As we have mentioned above, no statistically significant difference was found with respect to Treg in relation to tumor stage, lymph node status, and tumor size. Nonetheless, a lower CTL/Treg ratio was observed among locally advanced BCs as compared to early BCs (56). Moreover, the recruitment of Tregs to TME has been associated with the development of metastases in patients with BC (73, 74, 154–156).

Some immune checkpoints expressed on Tregs also have a certain prognostic value. CTLA-4, expressed on the surface of naive effector T cells and Tregs with a low level, was the first clinically targeted immune checkpoint molecule (157). CTLA-4 has a high affinity toward CD80 and CD86, thereby dampening the stimulatory signals and attenuating T cell activation by interrupting the conventional TCR signaling (158, 159). In the TME, CTLA-4 inhibits immune response and promotes tumor cell survival (159). CTLA-4+ tumor-infiltrating Tregs could also contribute to tumor immune evasion by suppressing antitumor immunity and downregulating CD80/86 expression on APCs (86). A higher expression of CTLA-4 on Tregs in BC TME compared to peripheral blood Treg cells revealed more active and proliferative Treg cells in TME (47). PD-1 and PD-L1 are expressed on the surface of both activated T cells and Tregs. PD-1 and its interactions with PD-L1 play important roles in the tumor evasion of immune responses through different mechanisms, including inhibition of effector T cell proliferation, reducing cytotoxic activity, induction of apoptosis in T cells, and Treg expansion in TME. As we have mentioned above, Treg infiltration is likely to be an unfavorable factor in the HR-positive and triple-negative BC patients. Interestingly, Li et al. (72) noticed that, in the TNBC, PD-L1 was also proved to be an independent unfavorable prognostic factor for OS by multivariate analysis adjusted by age, tumor size, grade, and lymph node status. However, there was nearly no data and study to specifically investigate the abundance of PD-1 and PD-L1 expressed on Treg in BC TME. Considering the unique function of Treg, further studies are warranted to analyze these two molecules on Treg using flow cytometry and other experimental methods. The treatment of breast cancer includes the treatment of local disease with surgery, radiation therapy, and systemic treatment with chemotherapy, endocrine therapy, biologic therapy, or combinations of these. In this section, we will introduce the latest information on the role of Treg in the systemic treatment of BC. We put a great emphasis on both the influence of different regimes on the density and function of Tregs and the impact of Treg on the efficacy of different treatments in preoperative stage. The efficacy marker of drugs or regimens includes pathological completed response (pCR), objective response rate, etc.

THE CORRELATION BETWEEN Tregs AND DIFFERENT THERAPEUTIC STRATEGIES OF BC

CDK4/6

Cyclin-dependent kinases 4 and 6 (CDK4/6) are fundamental drivers of the cell cycle and are required for the initiation and progression of various malignancies. The pharmacologic inhibitors of CDK4/6 have been found to have a significant activity against several solid tumors (160, 161). Their primary mechanism of action is thought to be the inhibition of phosphorylation of the retinoblastoma (RB) tumor suppressor, inducing G1 cell cycle arrest in tumor cells (162). Currently, three CDK4/6 inhibitors have now been approved by the FDA for the treatment of ER-positive metastatic breast cancer: palbociclib (PD0332991), ribociclib (LEE011), and abemaciclib (LY835219). S Goel et al. (163) used murine models of BC and other solid tumors to show that CDK4/6 inhibitors not only induce tumor cell cycle arrest but also promote anti-tumor immunity. Deng et al. (164) indicated that palbociclib or trilaciclib (another CDK4/6 inhibitor) significantly enhances Tconv cell activation, thus contributing to antitumor effects *in vivo*. However, in addition to the effect on Tconv cell, CDK4/6 can also markedly suppress Treg proliferation associated with the reduced activity of the E2F target, DNA methyltransferase 1 (DNMT1) (163). Similarly, in the studies of S Goel et al. (163) and JR Whittlee et al. (165), the flow cytometric analysis of breast cancer in murine revealed that abemaciclib or the combination of fulvestrant–palbociclib did not alter the fractions of most types of TIL but significantly increased the CD3+ T cells and reduced the Tregs in both the TME and periphery. Moreover, the CTL/Treg cell ratio increased significantly in abemaciclib-treated tumors, further suggesting a tipping of the immune balance in favor of anti-tumor immunity (163). In particular, the Treg was more sensitive to CDK4/6 inhibitors compared with other lymphocytes, and this behavior has been related to the high expression in these cells of the proteins of the CDK4/6–cyclin D-RB axis (166, 167) or the reduced activity of DNMT1 (163). Reduced expression of the immune checkpoint receptor PD1 on Tregs was also observed in the study of S Goel, which was consistent with the diminishment of the immune-suppressing function of Treg in BC TME (163), suggesting that CDK4/6 inhibitors may enhance the susceptibility of such tumors to immune checkpoint blockade (53).

Immune Checkpoint Inhibitors

Immune checkpoint blockade is a promising drug working by blocking checkpoint proteins from binding with their partner proteins. In this review, we will focus on three representative ICBs, PD-1/PD-L1 inhibitor, and CTLA-4 blockage. The effect of ICB on BC patients is still under investigation. However, there are several ongoing trials using PD-1/PD-L1 inhibition and/or CTLA-4 blockage in combination with standard anti-HER2 therapy for HER2+ BC—for example, the phase II DIAmOND study is investigating the combination of PD-L1 and CTLA-4

inhibition added to trastuzumab in patients with HER2+ mBC who progressed on prior trastuzumab-based therapy (168). In another trial, Santa-Maria *et al.* found that the response rates to PD-1/PD-L1 and CTLA-4 inhibition were low in all MBC. However, high rates of clinical benefit were observed in TNBC (169) because of their high expression of these IC molecules. To date, most studies revealed the effect of ICBs on T effector cells, and little is known about their effect on Tregs. As mentioned above, Tconv cells and Treg cells in TME similarly express immune checkpoint molecules, including CTLA-4 and PD-1, at levels that are dependent on the TME, indicating that antibodies targeting these proteins could affect both cell types.

The anti-tumor activity of the anti-CTLA-4 blockade was originally hypothesized to depend on the reinvigoration of dysfunctional CTLA-4-expressing Tconv cells (170). However, evidence from several preclinical studies indicate that the anti-tumor effects of these drugs depend on macrophages depleting Treg cells expressing CTLA-4 in the TME through ADCC, thereby increasing the CTL/Treg cell ratio (62, 98, 99), which implies that CTLA-4 blockade can activate anti-tumor immunity in the presence of enough TILs (171). Nonetheless, there is an absence of studies of Treg depletion in BC TME. Thus, further analyses to address the roles of CTLA-4 in Treg cells in BC settings are warranted.

PD-1 inhibits the excessive activation of Tconv cells by suppression of TCR and costimulatory and renders them dysfunctional or exhausted (172–174). As indicated above, Treg and Tconv cells in the TME express comparable PD-1 and are dependent on TCR and CD28 signaling for their survival and function. PD-1 inhibition potentiates the activation and immunosuppressive function of Treg cells. In line with this hypothesis, a study using a mouse model of autoimmune pancreatitis revealed that PD-1-deficient Treg cells had an increased immunosuppressive activity that was sufficient to rescue the auto-immune phenotype, indicating that PD-1 reduces the immunosuppressive function of Tregs (58). Y Togashi *et al.* found that, *in vitro*, anti-PD-1 mAbs enhance Treg cell-mediated immunosuppression using human samples (175). One of the representative anti-PD1, pembrolizumab, effectively blocked PD-1 expression but did not affect the expression of other Treg-related markers. These results suggest that anti-PD-1 mAbs may reverse immune escape by directly blocking the PD-1/PD-L1 interaction instead of altering the Treg phenotype or function (176).

Anthracycline-Based Neoadjuvant Chemotherapy

Anthracycline-based neoadjuvant chemotherapy (NAC) with or without taxanes for the initial treatment of patients with invasive BC is the top preoperative systemic therapy regimen recommended by the National Comprehensive Cancer Network panel. In general, the abundance of TILs in BC TME predicts the response to NAC (177, 178). Moreover, Denkert et al. found that the decreased Treg in TME is also linked to the pCR to NAC (179). However, the correlation between pCR and Tregs before NAC is still controversial. Fangxuan Li et al. (180) found that it has no significant relation with pCR. Nevertheless, in some studies, pCR to NAC is associated with less Treg abundance in TNBC but not in ER-positive/Her2-negative breast cancer (79). To be more specific, Ladoire et al. (181) and Senovilla et al. (182) found that, in patients treated with NAC, it is the increased CTL/Treg cell ratio in TME that can precisely predict pCR. Interestingly, the levels of CD8+ T cells and Tregs decreased during NAC in patients of TNBC (183), which raise a question of whether the dynamics of Treg can predict pCR. Hamy et al. (179) found that the decrease of lymphocyte infiltration during chemotherapy is related to the increase of PCR rate, which may be related to the disappearance of Treg after neoadjuvant therapy, but there are few related studies. Adriamycin is one of the typical anthracycline drugs. In BC, docetaxel can indirectly favor immunosurveillance upon polyploidization (182). Moreover, docetaxel is correlated with a reduced activity of Treg in BC and increases the CTL/Treg cell ratio (184). Nevertheless, little studies are conducted to investigate the influence of docetaxel on BC TME.

Anthracycline-based NAC not only contains anthracycline but also can be added with a series of other cytotoxic agents, including taxanes, platinum, and cyclophosphamide (CTK). These cytotoxic treatments can temporarily overcome the immunosuppressive TME, contributing to greater antitumor immune responses (185). CTK embraces direct alkylating and antiangiogenic properties. It is also reported to modulate the immune system in the host through many mechanisms (186). Sistigu et al. (187) reviewed some of these mechanisms, including Th2/Th1 to Th17 shifts in cytokine production, induction of Th17 cells, enhancement of T cell proliferation, resetting of dendritic cell homeostasis, and, more importantly, inhibition of Tregs. However, depending on the dose administered, the antitumor effects of cyclophosphamide can be either through immunopotentiation or direct cytolytic activity (188). Low-dose CTK contributes to antitumor immunity, whereas high-dose CTK works solely through its cytotoxic effects. Patients with breast cancer and treated with metronomic low-dose CTK were found to have a transient reduction in circulating Tregs, lasting 4 to 6 weeks, and diminished functionality (189). Ghiringhelli et al. also found that low-dose CTK depletes Treg cells in peripheral blood, causing the activation of antitumor immunity (190), and thus patients gained survival benefits more or less. However, low-dose CTK also gives rise to higher lymphocyte-infiltrating BC TME, including Treg, but the repletion of Treg cells abolished the antitumor effect of low-dose CTK to some extent (191), which was consistent with a murine experiment (192). These opposite effects of low-dose CTK on circulating and BC TME Treg beg a question on whether low-dose CTK induces the recruitment of Treg from peripheral blood to the TME. In addition to cyclophosphamide, several studieshave revealedthat other cytotoxic agents can also deplete Treg cells. Nevertheless, these data remain controversial, and further preclinical and clinical studies are needed.

Anti-HER2

HER2-blocking therapies, such as trastuzumab, an IgG1 monoclonal antibody, and/or pertuzumab in combination with chemotherapy, represent the standard first-line treatment for HER2 + BC. In addition to the direct targeting effects on HER2-positive

cells, it has been reported that trastuzumab is able to induce a long-lasting immune response in patients with BC (193), but it is still unclear whether trastuzumab has direct effects on Treg immune subsets. A significant decrease in the number of circulating Treg was revealed in patients treated with transtuzumab (194–196). In addition, the decrease of circulating Treg was associated with an objective clinical response or disease stabilization in patients treated with trastuzumab, and the frequency of Treg increased as the disease progressed during trastuzumab treatment (196). Moreover, the recurrence of BC during trastuzumab therapy highly correlates with an increase in Treg frequency. Taken together, circulating Treg can be a predictive marker for response to trastuzumab of the patients.

Small-molecule tyrosine kinase inhibitor (TKIs) is another highly rational anti-HER2 therapeutic regime targeting the adenosine triphosphate (ATP) binding domains of EGFR family due to the homological structure of the ATP, resulting in inhibiting tyrosine kinase phosphorylation (197). It has achieved extreme success in the treatment of other oncogene-driven malignancies. However, treating HER2-positive BC have fallen short of expectations. Some combination therapies of TKIs showed a higher disease-free survival in HER2+ metastatic breast cancer patients (198, 199). Unfortunately, the outcomes of these studies have been disappointing so far. Classic TKIs, such as a dual HER1/HER2 kinase inhibitor, the HER2/HER3 dimerization inhibitor pertuzumab, and the pan-HER (HER1, 2, and 4) kinase inhibitor neratinib can postpone or overcome anti-HER2 resistance and have yielded clinical advantages combined with chemotherapy, hormone therapy, and/or another HER2-inhibiting agent (200, 201). Unlike pertuzumab only improving the anti-trophic effect of the HER2-block, it was shown by the EGF104900 study, lapatinib also amplifies the trastuzumab-induced ADCC effect (202), indicating that lapatinib is more likely to have an antitumor effect through the depletion of Tregs in TME. Additionally, studies from L Hannesdottir et al. (203) in MMTV-neu animals shed light on the effects of lapatinib on enhancing the antitumor immunity. In the neoadjuvant phase II SOLTI-1114 PAMELA trial (NCT01973660), 151 HER2+ BC patients received lapatinib and trastuzumab, plus hormonal therapy if HR+; no significant difference in immune subpopulation densities in TME was observed. BC treated with trastuzumab or/and lapatinib achieving a pCR showed numerically higher densities of Treg cells (204), which is in accordance with the work of Hannesdottir.

CONCLUSIONS

With the deepening of research on TME in breast carcinoma, analysis on the composition of TME becomes increasingly important for evaluating the prognosis of patients with BC disease and the efficacy of different regimes. As a crucial role in TME, the function of Tregs directly and indirectly suppress the anti-tumor immunity through a variety of cellular interactions. In TME, tTreg and pTreg are recruited through the binding of some certain chemokines and their receptors. However, they cannot be easily distinguished. In BC, Tregs have a significantly distinct prognostic value of BC with different subtypes, and the conclusions of these articles are fairly conflicting with each other. By comparing different theories, Tregs are more likely to be an unfavorable factor of the prognosis of BC as a whole. However, further research or meta-analysis needs to be done to verify this effect. In view of the discovery of the great potential value of Treg, Treg cells are under intense scientific and commercial scrutiny as a novel therapeutic strategy or biomarker for anticancer treatment. Some classic regimes, such as anthracycline-based NAC, anti-Her2 treatment, immune checkpoint inhibitor, and cyclin-dependent kinases 4 and 6 (CDK4/6), proved to have a strong impact on depleting Treg in BC TME through different immunological effects. The link between Treg and the efficacy evaluation of tumor response to different treatments is found in anthracycline-based NAC, anti-HER2 NAC, but the relationship is still unknown in other treatments, which is a potential research field for us to manipulate Treg to reach the highest efficacy of these treatment strategies.

AUTHOR CONTRIBUTIONS

JL wrote the manuscript. XW contributed significantly to manuscript preparation. YD and XY conceived the structure and revised the manuscript. HW complemented relevant content and revised illustration and ZL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Immune Checkpoint Inhibitors Combined With Chemotherapy Compared With Chemotherapy Alone for Triple-Negative Breast Cancer: A Systematic Review and Meta-Analysis

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Background: It is still controversial whether immune checkpoint inhibitors (ICIs) can improve the curative effect when added to original standard chemotherapy treatment for triple-negative breast cancer (TNBC). We compared their antitumor efficacy and adverse effects (AEs) to make a better clinical decision.

Methods: Seven databases were searched for eligible articles. Progression-free survival (PFS), overall survival (OS), and AEs were measured as the primary outcomes.

Results: Nine randomized controlled trials (RCTs) involving 4,501 patients were included. ICI+chemotherapy treatment achieved better PFS (hazard ratio [HR]: 0.78, [0.70–0.86], $p < 0.00001$), OS (HR: 0.86, [0.74–0.99], $p = 0.04$), and complete response (584/1,106 *vs.* 341/825, risk ratio [RR]: 1.38, [1.01–1.89], $p = 0.04$). With the prolongation of survival, the survival advantage of ICI+chemotherapy increased compared with chemotherapy. Subgroup analysis suggested that the addition of ICIs might not have a better effect in Asian patients, patients with locally advanced disease, or patients with brain metastases. In the toxicity analysis, more Grade 3–5 AEs and serious AEs were found in the ICI+chemotherapy group. For Grade 3–5 AEs, more cases of diarrhea, severe skin reactions, pneumonitis, hepatitis, and adrenal insufficiency were related to the ICI+chemotherapy group.

Conclusions: ICI+chemotherapy appears to be better than chemotherapy alone for TNBC treatment, with better OS and PFS. However, its high rates of serious AEs need to be taken seriously.

Systematic Review Registration: PROSPERO Registration: CRD42021276394.

Keywords: chemotherapy, triple-negative breast cancer, meta-analysis, immune checkpoint inhibitors, systematic review

INTRODUCTION

In recent years, breast cancer has been the most common malignancy in women (1). As one of the major subtypes (15–20%), triple-negative breast cancer (TNBC) has the worst prognosis (2). In clinical practice, chemotherapy remains the standard of care (not only in neoadjuvant therapy but also in radical drug therapy) for patients with TNBC (3). However, its poor survival efficacy is not satisfactory for patients and doctors. In recent years, immune checkpoint inhibitors (ICIs) have been incorporated into cancer treatment, and their efficacy has been proven in lung cancer, hepatocellular carcinoma, and gastric cancer (4–6). However, whether ICIs can improve the curative effect when added to original standard chemotherapy treatment for TNBC is still controversial.

In the updated guidelines, ICIs+chemotherapy has been recognized as one of the treatment options for TNBC (7, 8). The KEYNOTE-522 and IMpassion130 trials compared ICIs +chemotherapy with chemotherapy in 2,076 patients with TNBC and suggested that combination therapy prolonged progression-free survival (PFS) and increased the rates of pathological complete response (PCR) (9, 10). Similar results were confirmed by 4 other randomized controlled trials (RCTs) (11–15). However, Bachelot et al.'s, Brufsky et al.'s, and Tolaney et al.'s studies reported that ICIs+chemotherapy could not improve the survival of patients but will cause more adverse effects (AEs) and reduce the quality of life of patients (16–18).

Hence, this meta-analysis of RCTs aimed to compare the efficacy and safety between ICIs+chemotherapy and chemotherapy for TNBC.

MATERIALS AND METHODS

We conducted this study according to the Preferred Reporting Items for Systematic Review and Meta-Analysis guidelines (PRISMA) (**Table S1**) (19). (PROSPERO Registration: CRD42021276394)?.

Search Strategy

Studies were retrieved from the following databases: The Cochrane Library, PubMed, Web of Science, Scopus, EMBASE, ScienceDirect, and Ovid MEDLINE. Studies were retrieval time from inception to May 5, 2021. The MeSH terms and keywords were "Breast cancer", "Immune checkpoint inhibitors", and "Chemotherapy". The search details are included in **Table S2**.

Selection Criteria

The inclusion criteria were as follows: (1) RCTs published in English comparing ICIs+chemotherapy with chemotherapy alone; (2) studies that enrolled patients with TNBC; and (3) the outcomes included survival indicators (OS and PFS), drug responses, and AEs.

The exclusion criteria were as follows: (1) animal studies; (2) meta-analyses and reviews; (3) conference articles; (4) case reports; and (5) studies that did not only enroll patients with TNBC.

Data Extraction

Two investigators extracted the following information independently: the publication year, first author, participant characteristics (quantity, age, etc.), tumor characteristics (histopathology, stage, etc.), antitumor efficacy (OS, PFS, etc.), and number of AEs. All disagreements were resolved by a third investigator.

Outcome Assessments

PFS and OS were the primary outcomes. The subgroup analysis of OS was performed according to age, race, Eastern Cooperative Oncology Group (ECOG) performance status, baseline disease status, metastatic sites, PD-L1 status, neoadjuvant therapy, homologous recombination deficiency (HRD), metastases (brain, bone, liver, or lung), lymph node-only disease, and previous treatment (chemotherapy, taxane, or anthracycline).

Quality Assessment

We assessed the quality of the included RCTs by using the Cochrane Risk of Bias Tool (CRBT) (20) and 5-point Jadad scale (21). We assessed the quality of the results by using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system (22).

Statistical Analysis

When analyzing survival outcomes (PFS, OS, etc.), we used hazard ratios (HRs). When analyzing dichotomous variables (PFSR, OSR, complete response [CR], AEs, etc.), we used risk ratios (RRs). Heterogeneity was evaluated by the I^2 statistic and χ^2 test. A random-effects model was used when heterogeneity was significant ($I^2 < 50\%$ or $p > 0.1$); otherwise, a fixed-effects model was used. Publication bias was evaluated through visual inspection of the funnel plots. A $P < 0.05$ was identified as statistically significant. All analyses were performed using Review Manager 5.3 and SPSS 18.0.

RESULTS

Search Results

Nine RCTs involving 4,501 patients (2,645 patients in the ICI +chemotherapy group and 1,856 patients in the chemotherapy group) were included (9–16, 18) (**Figure 1**). Two studies (14, 16) were conducted in Europe, one (18) was conducted in the USA, and the other five studies were global studies (9–13). The essential information of the included studies is summarized in

Abbreviations: AEs, adverse effects; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CR, complete response; CRBT, Cochrane Risk of Bias Tool; ECOG, Eastern Cooperative Oncology Group; GRADE, Grading of Recommendations Assessment, Development and Evaluation; HR, Hazard ratio; HRD, homologous recombination deficiency; ICIs, immune checkpoint inhibitors; OS, overall survival; OSR, overall survival rate; PCR, pathological complete response; PFS, progression-free survival; PFSR, progression-free survival rate; PRISMA, Preferred Reporting Items for Systematic Review and Meta-Analysis guidelines; RCTs, randomized controlled trials; RR, risk ratio; TNBC, triple-negative breast cancer.

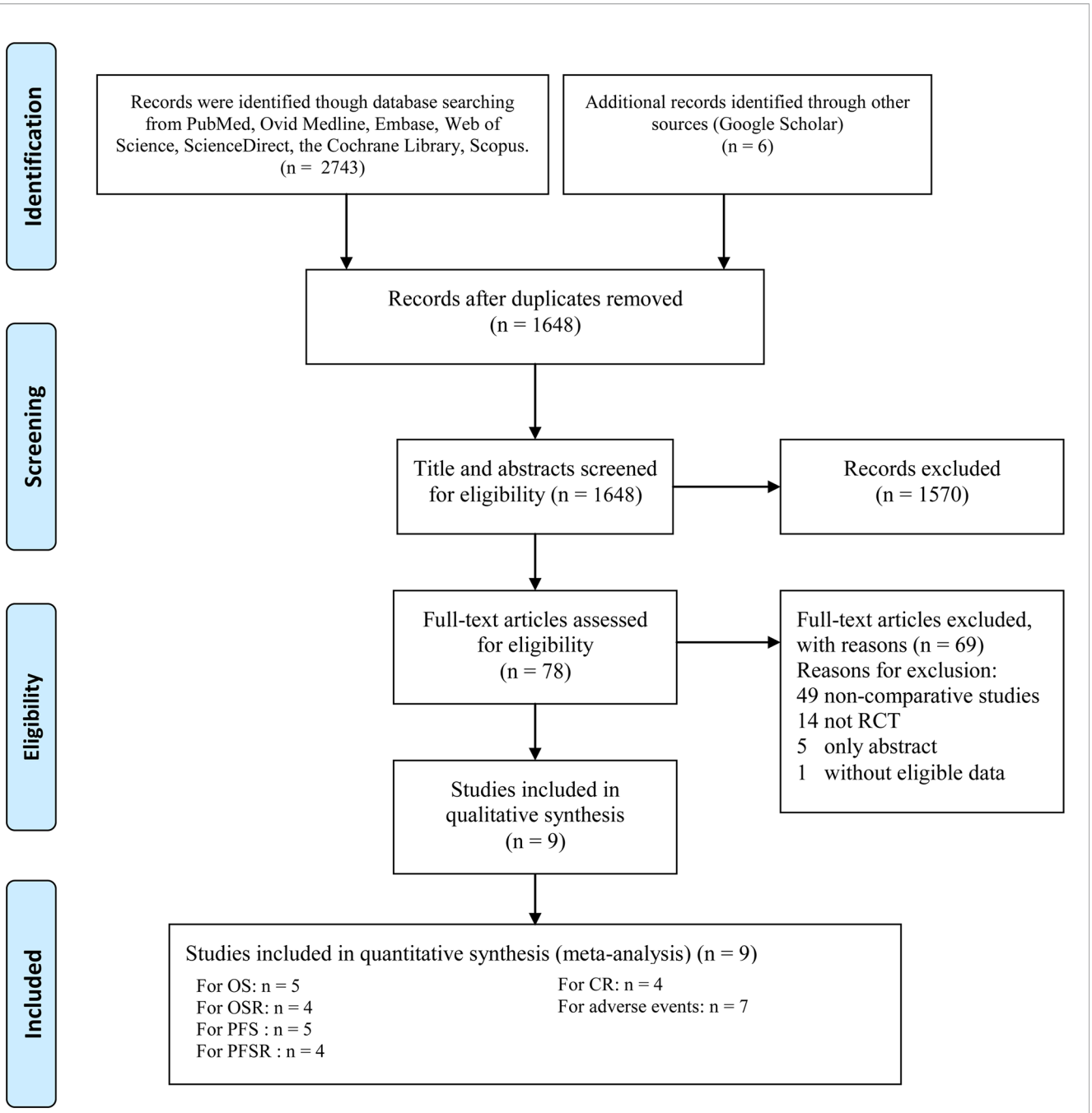

FIGURE 1 | Flow chart of study selection.

Table 1. According to the Jadad scale (**Table S3**) and CRBT (**Figure S1**), all eight RCTs were of high quality. According to the GRADE system, the evidence grades of all the results were medium-high.

Antitumor Efficacy

The ICI+chemotherapy group achieved better OS than the chemotherapy group (HR: 0.86, [0.74–0.99], p = 0.04; **Figure 2**). At all points in time, the overall survival rate (OSR) tended to favor the ICI+chemotherapy group (OSR-6 m, RR: 1.17, [1.13–1.21], $p < 0.00001$; OSR-12 m, RR: 1.08, [1.00–1.17], $p = 0.05$; OSR-18 m, RR: 1.11, [0.99–1.24], $p = 0.07$; OSR-24 m, RR: 1.12, [0.97–1.30], $p = 0.13$; OSR-30 m, RR: 1.20, [1.00–1.44], $p = 0.04$; OSR-36 m, RR: 1.33, [1.06–1.67], $p = 0.01$, **Figure S2**). With prolonged survival time, ICI+chemotherapy had an increasing advantage for OS (**Figures 3A, B**).

The ICI+chemotherapy group achieved better PFS than the chemotherapy group (HR: 0.78, [0.70–0.86], $p < 0.00001$;

TABLE 1 | Characteristics of the included randomized controlled trials.

Study				Period	Groups	Patients (n)	Median age (year)	Stage	PD-L1+	Treatment	Follow-upduration, mo	Design
2021	Miles et al. (15)	IMpassion131	Phase III	2017.08–2019.09	ICIs +Chemotherapy	431	54	Stage IV	191	Atezolizumab, 840mg (d1, 15) + Paclitaxel, 90 mg/m^2 (d1, 8, 15), q4w until PD	8.8	RCT
					Chemotherapy	220	53		101	Paclitaxel, 90 mg/m^2 (d1, 8, 15), q4w until PD	8.5	
2021	Bachelot et al. (16)	SAFIR02-BREAST IMMUNO	Phase II	2016.01–2019.09	ICIs +Chemotherapy	47	56	Stage IV	10	Durvalumab, 10 mg/kg, q2w +Chemotherapy (doctor's choice), until PD	19.7	RCT
					Chemotherapy	35	56.5		8	Chemotherapy (doctor's choice), until PD		
2020	Schmid et al. (9)	KEYNOTE-522	Phase III	2017.03–2018.09	ICIs +Chemotherapy	784	49	Stage II–III	656	Pembrolizumab, 200 mg, q3w +Paclitaxel, 80 mg/m^2, q1w +carboplatin[a], for 12w (first neoadjuvant treatment); followed by Pembrolizumab, 200 mg, q3w+Doxorubicin, 60 mg/m^2, q3w (or Epirubicin, 90 mg/m^2, q3w) +cyclophosphamide, 600 mg/m^2, q3w, for 12w (second neoadjuvant treatment). After definitive surgery, pembrolizumab, 200 mg, q3w for up to 9 cycles.	15.5	RCT
					Chemotherapy	390	48		317	Placebo, q3w+Paclitaxel, 80 mg/m^2, q1w+carboplatin[a], for 12w (first neoadjuvant treatment); followed by Placebo, q3w+Doxorubicin, 60 mg/m^2, q3w (or Epirubicin, 90 mg/m^2, q3w) +cyclophosphamide, 600 mg/m^2, q3w, for 12w (second neoadjuvant treatment); after definitive surgery, placebo, q3w for up to 9 cycles.		
2020	Schmid et al. (10)	IMpassion130	Phase III	2015.06–2017.05	ICIs +Chemotherapy	451	55	Stage IV	185	Atezolizumab, 840 mg (d1, 15) +Nab-paclitaxel, 100 mg/m^2 (d1, 8, 15), q4w until PD	18.5	RCT
					Chemotherapy	451	56		184	Nab-paclitaxel, 100 mg/m^2 (d1, 8, 15), q4w until PD	17.5	
2020	Mittendorf et al. (11)	IMpassion031	Phase III	2017.07–2019.09	ICIs +Chemotherapy	165	51	Stage II–III	78	Atezolizumab,840 mg, q2w +Nab-paclitaxel, 125 mg/m^2, qw, for 12 weeks, followed by Atezolizumab,840 mg, q2w +Doxorubicin, 60 mg/m^2+Cyclophosphamide, 600 mg/m^2, q2w for 8w; after surgery, atezolizumab,1,200 mg, q3w for 11 cycles	20.6	RCT
					Chemotherapy	168	51		76	Nab-paclitaxel, 125 mg/m^2, qw, for 12 weeks, followed by Doxorubicin, 60 mg/m^2+ Cyclophosphamide, 600 mg/m^2, q2w for 8w; after surgery, subsequently monitored for 1 year	19.8	
2020	Cortes et al. (12)	KEYNOTE-355	Phase III	2017.01–2018.06	ICIs +Chemotherapy	566	53	Stage IV	425	Pembrolizumab, 200 mg q3w +(Nab-paclitaxel, 100 mg/m^2, d1, 8, 15, q4w or Paclitaxel, 90 mg/m^2, d1, 8, 15, q4w or	25.9	RCT

(Continued)

TABLE 1 | Continued

Study				Period	Groups	Patients (n)	Median age (year)	Stage	PD-L1+	Treatment	Follow-upduration, mo	Design
										Gemcitabine, 1,000 mg/m²+Carboplatin, d1, 8, q3w) until PD		
					Chemotherapy	281	53		211	Nab-paclitaxel, 100 mg/m², d1, 8, 15, q4w or Paclitaxel, 90 mg/m², d1, 8, 15, q4w or Gemcitabine, 1,000 mg/m²+Carboplatin, d1, 8, q3w until PD	26.3	
2020	Nanda et al. (13)	I-SPY2	Phase II	2015.11–2016.11	ICIs +Chemotherapy	69	50	Stage I–III	–	Pembrolizumab, 200 mg, q3w +Paclitaxel, 80 mg/m2, d1, 7, 14+Doxorubicin, 60 mg/m², d1, 14+Cyclophosphamide, 600 mg/m2, d1,14 for 4 cycles	33.6	RCT
					Chemotherapy	181	47		–	Paclitaxel, 80 mg/m2, d1, 7, 14+Doxorubicin, 60 mg/m2, d1, 14+Cyclophosphamide, 600 mg/m2, d1, 14 for 4 cycles		
2020	Tolaney et al. (18)	–	Phase II	2017.04–2018.08	ICIs +Chemotherapy	44	58	Stage IV	–	Pembrolizumab, 200 mg, q3w +Eribulin,1.4 mg/m², d1, 8, q3w until PD	10.5	RCT
					Chemotherapy	44	57		–	Eribulin, 1.4 mg/m2, d1, 8, q3w until PD		
2019	Loibl et al. (14)	GeparNuevo	Phase II	2016.06–2017.10	ICIs +Chemotherapy	88	49.5	Stage I–III	69	One injection durvalumab, 0.75 g 2w followed by durvalumab 1.5 g, q4w +Nabpaclitaxel, 125 mg/m², q1w for 12w, followed by Durvalumab, 1.5 g, q4w +dose-dense Epirubicin/Cyclophosphamide, q2w for 4 cycles.	–	RCT
					Chemotherapy	86	49.5		69	One injection placebo, 2w followed by placebo, q4w +Nabpaclitaxel, 125 mg/m², q1w for 12w, followed by placebo, q4w+dose-dense Epirubicin/Cyclophosphamide, q2w for 4 cycles.		

RCT, randomized controlled trial; NSCLC, non-small-cell lung cancer; PD-L1+, programmed death ligand 1 positive; PD, progressive disease; ICIs, immune checkpoint inhibitors.
[a]*At a dose based on an area under the concentration-time curve of 5 mg per milliliter per minute once every 3 weeks or 1.5 mg per milliliter per minute once weekly in the first 12 weeks.*

Figure 2). At all points in time, the progression-free survival rate (PFSR) significantly favored the ICIs+Chemotherapy group (PFSR-6 m, RR: 1.09, [0.78–0.1.52], p = 62; PFSR-12 m, RR: 1.26, [0.84–1.88], p = 0.27; PFSR-18 m, RR: 1.26, [0.90–1.75], p = 0.18; PFSR-24 m, RR: 1.35, [0.95–1.91], p = 0.10; PFSR-30 m, RR: 0.2.05, [1.46–2.86], p < 0.0001, **Figure S3**). With prolonged survival time, ICI+chemotherapy had an increasing advantage for PFS (**Figures 3C, D**).

In the subgroup analysis, the favorable tendency of OS did not show significant changes according to age, ECOG performance status, number of metastatic sites, PD-L1 status, neoadjuvant therapy, lymph node-only disease, bone metastases, liver metastases, lung metastases, or previous chemotherapy (chemotherapy, taxane, or anthracycline). The addition of ICIs might have the opposite effect in the subgroups by race (Asian), baseline disease status (locally advanced), and brain metastases (yes) (**Table 2**).

The CR of the ICI+chemotherapy group was higher than that of the chemotherapy group (584/1,106 *vs.* 341/825, RR: 1.38, [1.01–1.89], p = 0.04; **Figure 4**).

Toxicity

In summary, ICI+chemotherapy treatment was related to more Grade 3–5 AEs, treatment-related Grade 3–5 AEs, serious AEs, treatment-related serious AEs, and AEs leading to treatment discontinuation. Total AEs, treatment-related AEs, death, treatment-related death, and AEs leading to dose reduction/dose interruption were comparable between the two groups (**Table 3**).

For total AEs, increases in aspartate aminotransferase (AST) levels, vomiting, cough, rash, pyrexia, pruritus, infusion reaction,

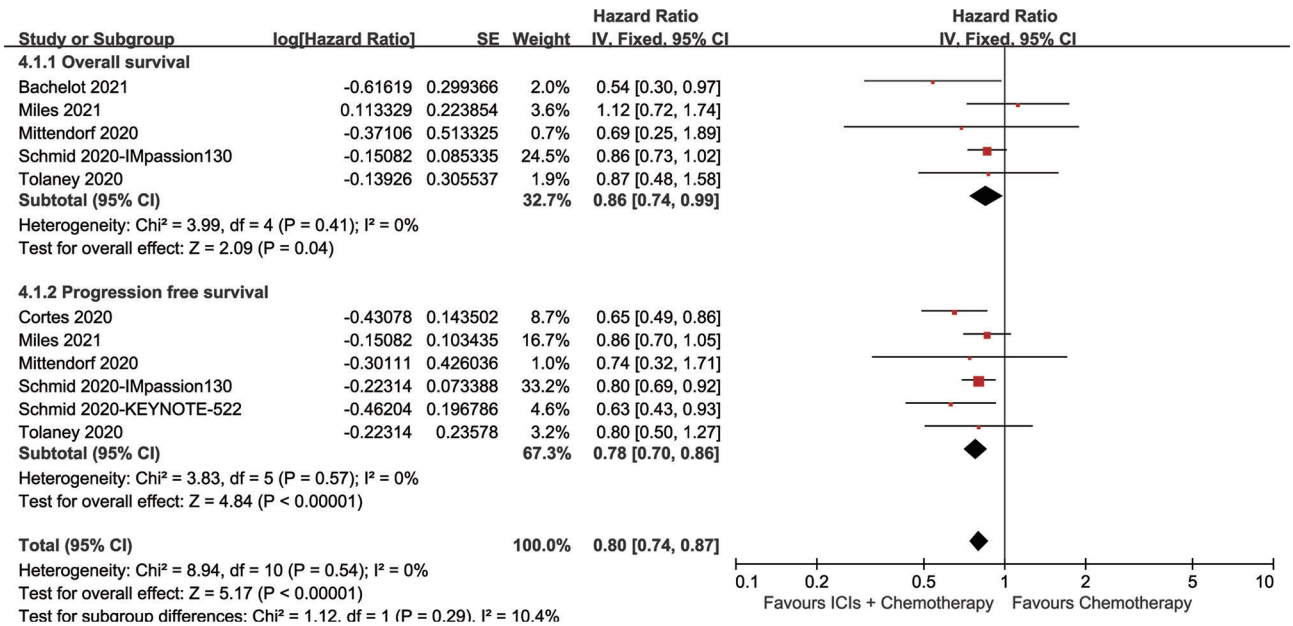

FIGURE 2 | Forest plots of OS and PFS associated with ICIs+Chemotherapy *versus* Chemotherapy.

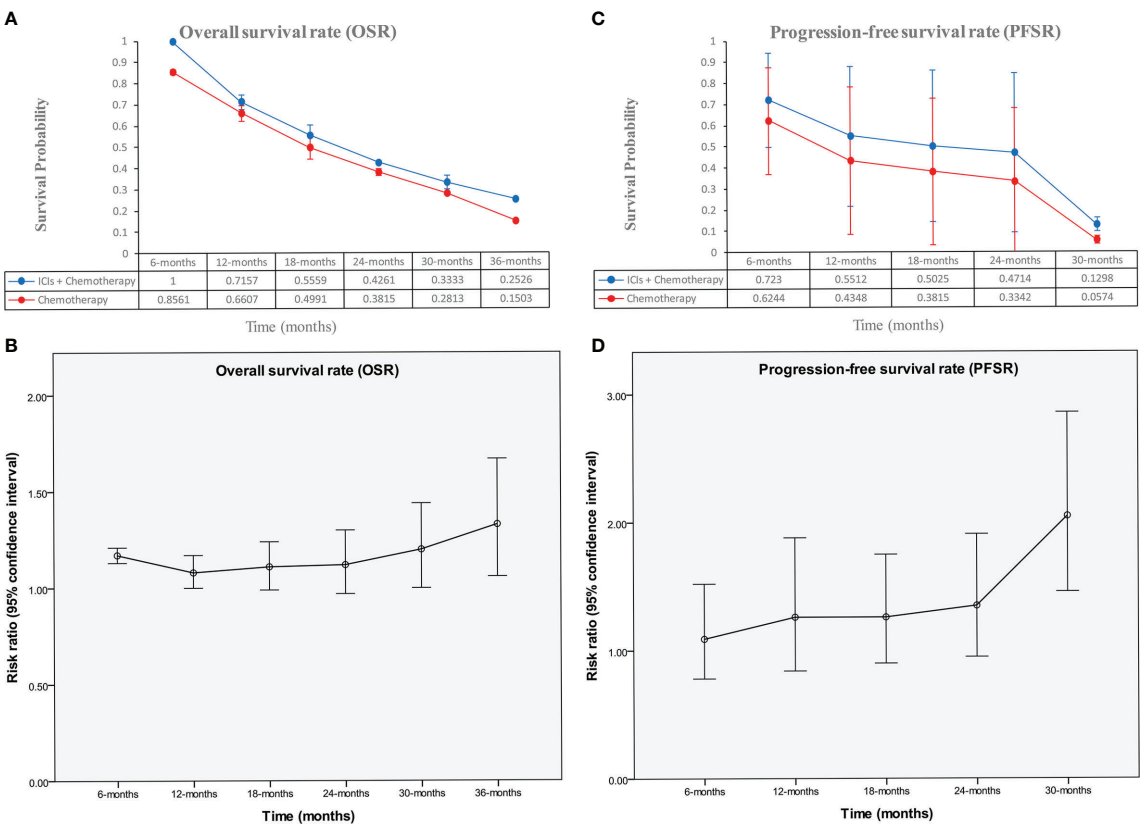

FIGURE 3 | Comparisons of OSR (6–36 months, **A, B**), and PFSR (6–30 months, **C, D**) associated with ICIs+Chemotherapy *versus* Chemotherapy according to survival time.

TABLE 2 | Subgroup analysis for OS.

Subgroups	Included studies	Total	ICIs+Chemotherapy		Chemotherapy		HR (95% CI)
			Events	n	Events	n	
All patients	5	2,056	651	1138	566	918	0.79 (0.63,0.99)
Age							
18–40 years	1	114	44	63	37	51	0.81 (0.52,1.25)
41–64 years	1	569	158	284	170	285	0.88 (0.71,1.10)
>65 years	1	219	53	104	72	115	0.78 (0.55,1.12)
Race							
White	1	609	180	308	198	301	0.80 (0.65,0.98)
Asian	1	161	39	85	34	76	1.17 (0.74,1.87)
Black or African-American	1	58	14	26	21	32	0.75 (0.38,1.49)
ECOG performance status							
0	1	526	127	256	145	270	0.85 (0.67,1.08)
1	1	372	127	193	132	179	0.85 (0.66,1.08)
Baseline disease status							
Locally advanced	1	88	21	46	13	42	1.53 (0.76,3.06)
Metastatic	1	812	234	404	266	408	0.82 (0.90,0.98)
Number of metastatic sites							
0-3	1	673	172	332	194	341	0.83 (0.68,1.02)
4+	1	226	83	118	83	108	0.90 (0.66,1.22)
PD-L1 status							
PD-L1 positive	4	717	206	407	181	310	0.79 (0.63,0.99)
PD-L1 negative	2	562	175	283	179	279	0.56 (0.23,1.38)
Neoadjuvant therapy							
Yes	1	88	25	44	27	44	0.87 (0.48,1.58)
No	4	1,968	626	1,092	539	874	0.86 (0.74,0.99)
Homologous recombination deficiency (HRD)							
Low HRD	1	21	3	10	8	11	0.27 (0.07,1.10)
High HRD	1	31	9	19	9	12	0.71 (0.26,1.89)
Brain metastases							
Yes	1	61	22	30	19	31	1.34 (0.72,2.48)
No	1	841	233	421	260	420	0.83 (0.70,1.00)
Bone metastases							
Yes	1	286	92	145	103	141	0.80 (0.61,1.07)
No	1	616	163	306	176	310	0.88 (0.71,1.09)
Liver metastases							
Yes	1	244	88	126	95	118	0.77 (0.58,1.03)
No	1	658	167	325	184	333	0.88 (0.72,1.09)
Lung metastases							
Yes	1	469	138	227	153	242	0.94 (0.74,1.18)
No	1	433	117	224	126	209	0.80 (0.62,1.02)
Lymph node-only disease							
Yes	1	56	12	33	11	23	0.74 (0.32,1.67)
No	1	843	243	417	266	426	0.88 (0.74,1.05)
Previous neoadjuvant or adjuvant chemotherapy							
Yes	1	570	160	284	166	286	0.92 (0.74,1.15)
No	1	332	95	167	113	165	0.75 (0.57,0.99)
Previous taxane treatment							
Yes	1	461	138	231	136	230	0.95 (0.75,1.20)
No	1	441	117	220	143	221	0.76 (0.59,0.97)
Previous anthracycline treatment							
Yes	1	485	143	243	144	242	1.00 (0.79,1.26)
No	1	417	112	208	135	209	0.71 (0.55,0.92)

PD-L1+, programmed death ligand 1 positive; ICIs, immune checkpoint inhibitors; HR, hazard ratio; CI, confidence interval; OS, overall survival; HRD, homologous recombination deficiency; ECOG, Eastern Cooperative Oncology Group.

hypothyroidism, nail disorders, hypokalemia, hyperthyroidism, pneumonitis, hepatitis, and adrenal insufficiencies were related to the ICI+chemotherapy group. Total AEs greater than 10% are summarized in **Table 4**.

For Grade 3–5 AEs, more cases of diarrhea, severe skin reactions, pneumonitis, hepatitis, and adrenal insufficiencies were related to the ICI+chemotherapy group. Grade 3–5 AEs greater than 1% are summarized in **Table 5**.

Sensitivity Analysis

In the analysis of complete response, PFSR, and AEs, the I^2 statistic was >50%, which suggests significant heterogeneity. By

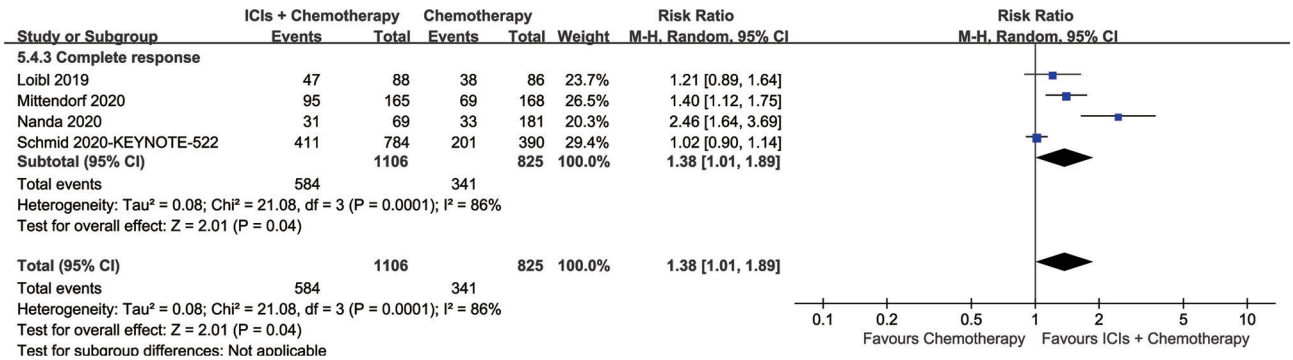

FIGURE 4 | Forest plots of CR associated with ICIs+Chemotherapy *versus* Chemotherapy.

removing each study, the sensitivity analysis suggested that the results were stable and reliable (**Figure S4**).

Publication Bias

No significant publication bias was found based on the funnel plots of survival (**Figure S5A**) and safety (**Figure S5B**).

DISCUSSION

Due to the lack of targets for therapeutic intervention, the treatment of TNBC is challenging (23). Whether immunotherapy can improve the curative effect when added to original standard chemotherapy treatment is still controversial (7, 8). This meta-analysis first compared ICI+chemotherapy with chemotherapy for TNBC treatment. The results suggest that ICI+chemotherapy treatment showed better efficacy in OS, PFS, and complete response. With the prolongation of survival, the survival advantage of ICI +chemotherapy increased compared with that of chemotherapy. In the toxicity analysis, more Grade 3–5 AEs and serious AEs were found in the ICI+chemotherapy group.

Better survival rates were the main benefit for the ICIs+ Chemotherapy group. With the prolongation of survival, the advantage of OS and PFS in the ICIs+Chemotherapy group increased compared with the chemotherapy group. Similar results were confirmed by three large sample RCTs (KEYNOTE-522, IMpassion130 and KEYNOTE-355) (9, 10, 12). The I-SPY2 study and KEYNOTE-522 study suggested that significantly higher rates of CR were achieved in the ICIs +Chemotherapy groups (9, 13). Two reasons may explain the benefit of ICIs+Chemotherapy: (1) ICIs kill tumor cells by activating tumor immunity, which is different from chemotherapy and plays a synergistic role, especially in PD-L1-positive TNBC (9, 24). The antitumor effect may be more significant in early breast cancer than metastatic disease, because the tumor immune microenvironment is more robust (25); and (2) higher CR rates (584/1,106 *vs.* 341/825, RR: 1.38, [1.01–1.89]) were found in the ICIs+Chemotherapy groups, which is very important for the long-term survival of breast cancer patients after surgery (11, 13). Cortazar et al.'s pooled analysis also confirmed the strong association of PCR (no tumor in either breast or the lymph nodes) after neoadjuvant

TABLE 3 | Summary of adverse events.

Adverse events	Studies involved	ICIs+Chemotherapy		Chemotherapy		Risk ratio	95% CI	I^2 (%)	P
		Event/total	%	Event/total	%				
Total adverse events	7	2462/2488	95.95%	1550/1589	97.55%	1.01	0.99-1.03	85	0.41
Treatment-related adverse events	5	1951/2013	96.92%	1255/1325	94.72%	1.02	0.98-1.06	88	0.43
Grade 3-5 adverse events	6	1697/2444	69.43%	901/1545	58.32%	1.14	1.03-1.25	69	0.0006
Treatment-related grade 3-5 adverse events	6	1295/2057	62.96%	724/1369	52.89%	1.09	1.03-1.16	45	0.002
Serious adverse events	2	155/616	25.16%	111/619	17.93%	1.40	1.13-1.74	19	0.002
Treatment-related serious adverse events	4	128/751	17.04%	88/740	11.89%	1.44	1.13-1.85	30	0.003
Adverse event leading to treatment discontinuation	4	123/751	16.38%	76/740	10.27%	1.61	1.24-2.10	46	0.0004
Adverse event leading to dose reduction/ dose interruption	1	194/451	43.02%	173/451	38.36%	1.12	0.96-1.31	–	0.16
Death	3	7/663	1.06%	4/654	0.61%	1.76	0.52-5.97	0	0.37
Treatment-related death	1	2/451	0.44%	1/451	0.22%	2.00	0.18-21.98	–	0.57

ICIs, immune checkpoint inhibitors; CI, confidence interval.

TABLE 4 | Total adverse events an incidence of more than 10% according to combination of two groups.

Adverse events	Studies involved	ICIs+Chemotherapy		Chemotherapy		Total incidence	Risk ratio	95% CI	*I2* (%)	P
		Event/total	%	Event/total	%					
Alopecia	5	1123/2054	54.67%	771/1376	56.03%	55.22%	1.03	0.97-1.09	0	0.33
Nausea	6	1135/2123	53.46%	825/1557	52.99%	53.26%	1.04	0.98-1.10	42	0.23
Infection	1	50/88	56.82%	39/86	45.35%	51.15%	1.25	0.93-1.68	–	0.13
Anemia	6	1004/2123	47.29%	640/1557	41.10%	44.67%	1.05	0.98-1.13	21	0.18
Fatigue	6	882/2123	41.54%	709/1557	45.54%	43.23%	1.04	0.97-1.12	0	0.30
Hyperglycaemia	1	32/88	36.36%	37/86	43.02%	39.66%	0.85	0.58-1.22	–	0.37
Leucopenia	2	101/253	39.92%	96/254	37.80%	38.86%	1.04	0.91-1.19	0	0.61
Neutropenia	5	823/2054	40.09%	484/1376	35.17%	38.12%	1.07	0.98-1.17	29	0.12
Mucositis	1	32/88	36.36%	33/86	38.37%	37.36%	0.95	0.64-1.39	–	0.78
Diarrhea	5	510/1557	32.76%	422/1276	33.07%	32.90%	1.07	0.88-1.29	67	0.52
Peripheral sensory neuropathy	4	241/773	31.18%	281/886	31.72%	31.46%	1.12	0.99-1.28	50	0.30
Nail discolouration	2	71/253	28.06%	72/254	28.35%	28.21%	0.96	0.74-1.25	0	0.79
Taste and smell disorders	1	25/88	28.41%	24/86	27.91%	28.16%	1.02	0.63-1.64	–	0.94
Vertigo	1	24/88	27.27%	22/86	25.58%	26.44%	1.07	0.65-1.75	–	0.80
Aspartate aminotransferase increased	2	78/253	30.83%	54/254	21.26%	26.04%	1.44	1.07-1.92	0	0.01
Constipation	4	378/1488	25.40%	278/1095	25.39%	25.40%	1.03	0.90-1.19	0	0.62
Headache	3	187/704	26.56%	158/705	22.41%	24.49%	1.18	0.99-1.42	0	0.07
Vomiting	5	384/1557	24.66%	258/1276	20.22%	22.66%	1.22	1.06-1.40	14	0.006
Sleep disturbance	1	22/88	25.00%	17/86	19.77%	22.41%	1.26	0.72-2.21	–	0.41
Anorexia	1	20/88	22.73%	19/86	22.09%	22.41%	1.03	0.59-1.79	–	0.92
Rash	5	456/1919	23.76%	262/1315	19.92%	22.20%	1.17	1.02-1.34	10	0.03
Cough	3	174/704	24.72%	132/705	18.72%	21.72%	1.32	1.08-1.61	0	0.007
Elevated alanine aminotransferase level	5	451/2054	21.96%	265/1376	19.26%	20.87%	1.10	0.97-1.26	0	0.15
Arthralgia	3	146/704	20.74%	148/705	20.99%	20.87%	0.95	0.70-1.28	54	0.74
Myalgia	3	150/704	21.31%	132/705	18.72%	20.01%	1.14	0.93-1.40	0	0.22
Asthenia	3	286/1400	20.43%	185/1009	18.33%	19.55%	1.02	0.86-1.20	0	0.82
Decreased neutrophil count	5	404/2035	19.85%	270/1471	18.35%	19.22%	0.98	0.76-1.27	61	0.90
Stomatitis	2	54/253	21.34%	43/254	16.93%	19.13%	1.26	0.88-1.81	0	0.20
Peripheral neuropathy	5	304/1557	19.52%	230/1276	18.03%	18.85%	1.01	0.87-1.18	28	0.87
Decreased appetite	2	118/616	19.16%	113/619	18.26%	18.70%	1.05	0.83-1.32	0	0.69
Epistaxis	2	46/253	18.18%	45/254	17.72%	17.95%	1.03	0.55-1.90	63	0.94
Hot flush	2	49/253	19.37%	41/254	16.14%	17.75%	1.19	0.82-1.72	0	0.36
Bone pain	1	17/88	19.32%	13/86	15.12%	17.24%	1.28	0.66-2.47	–	0.47
Fever without neutropenia	1	16/88	18.18%	12/86	13.95%	16.09%	1.30	0.66-2.59	–	0.45
Pyrosis	1	18/88	20.45%	10/86	11.63%	16.09%	1.76	0.86-3.59	–	0.12
Dyspnoea	3	121/704	17.19%	104/705	14.75%	15.97%	1.16	0.91-1.47	0	0.22
Hand–foot-syndrome	1	11/88	12.50%	16/86	18.60%	15.52%	0.67	0.33-1.36	–	0.27
Pyrexia	2	122/616	19.81%	69/619	11.15%	15.47%	1.78	1.35-2.34	0	<0.0001
Peripheral edema	3	111/704	15.77%	105/705	14.89%	15.33%	1.06	0.83-1.35	0	0.66
Dermatitis	1	13/88	14.77%	12/86	13.95%	14.37%	1.06	0.51-2.19	–	0.88
Insomnia	2	90/616	14.61%	81/619	13.09%	13.85%	1.12	0.85-1.48	29	0.45
Pruritus	3	111/685	16.20%	91/800	11.38%	13.60%	1.57	1.00-2.49	66	0.05
Dysgeusia	2	81/616	13.15%	84/619	13.57%	13.36%	0.90	0.54-1.48	57	0.68
Back pain	3	92/704	13.07%	90/705	12.77%	12.92%	1.02	0.78-1.34	50	0.87
Infusion reaction	3	152/1037	14.66%	55/644	8.54%	12.31%	1.55	1.16-2.08	0	0.003
Dizziness	1	64/451	14.19%	46/451	10.20%	12.20%	1.39	0.97-1.99	–	0.07
Urinary tract infection	1	56/451	12.42%	46/451	10.20%	11.31%	1.22	0.84-1.76	–	0.29
Lacrimation increased	2	30/253	11.86%	27/254	10.63%	11.24%	1.11	0.68-1.82	–	0.66
Dyspepsia	1	16/165	9.70%	21/168	12.50%	11.11%	0.78	0.42-1.43	–	0.42
Paronychia	1	15/165	9.09%	21/168	12.50%	10.81%	0.73	0.39-1.36	–	0.32
Pain in extremity	2	71/616	11.53%	62/619	10.02%	10.77%	1.15	0.83-1.59	0	0.39
Abdominal pain	3	67/704	9.52%	77/705	10.92%	10.22%	0.87	0.64-1.19	0	0.39
Upper respiratory tract infection	1	18/165	10.91%	16/168	9.52%	10.21%	1.15	0.61-2.17	–	0.68

ICIs, immune checkpoint inhibitors; CI, confidence interval.

TABLE 5 | Grade 3–5 adverse events an incidence of more than 1% according to combination of two groups.

Adverse events	Studies involved	ICIs+ Chemotherapy		Chemotherapy		Total incidence	Risk ratio	95% CI	*I2* (%)	*P*
		Event/ total	%	Event/ total	%					
Neutropenia	5	547/2,054	26.64%	319/1,376	23.18%	25.26%	1.02	0.91–1.15	0	0.69
Leukopenia	2	44/253	17.39%	38/254	14.96%	16.17%	1.14	0.79–1.66	39	0.48
Decreased neutrophil count	5	287/2,035	14.10%	181/1,471	12.51%	13.43%	0.90	0.76–1.06	16	0.20
Anemia	6	269/2,123	12.62%	136/1,557	8.73%	10.98%	1.17	0.96–1.42	0	0.11
Febrile neutropenia	3	28/322	8.70%	30/435	6.90%	7.66%	1.28	0.77–2.12	0	0.34
Infection	1	5/88	5.68%	4/86	4.65%	5.17%	1.22	0.34–4.40	–	0.76
Elevated alanine aminotransferase level	5	96/2,054	4.67%	44/1,376	3.20%	4.08%	1.39	0.97–1.99	0	0.08
Bone pain	1	4/88	4.55%	2/86	2.33%	3.45%	1.95	0.37–10.39	–	0.43
Fatigue	6	76/2,123	3.58%	43/1,557	2.76%	3.23%	1.36	0.94–1.97	44	0.11
Hypertension	2	14/616	2.27%	23/619	3.72%	3.00%	0.62	0.32–1.18	30	0.14
Peripheral sensory neuropathy	4	24/773	3.10%	24/886	2.71%	2.89%	1.07	0.62–1.87	0	0.80
Aspartate aminotransferase increased	2	10/253	3.95%	3/254	1.18%	2.56%	3.03	0.91–10.04	0	0.07
Peripheral neuropathy	5	46/1,557	2.95%	25/1,276	1.96%	2.51%	1.58	0.98–2.56	26	0.06
Nail discoloration	2	8/253	3.16%	4/254	1.57%	2.37%	1.86	0.61–5.71	26	0.28
Hand–foot-syndrome	1	1/88	1.14%	3/86	3.49%	2.30%	0.33	0.03–3.07	–	0.33
Nausea	6	46/2,123	2.17%	31/1,557	1.99%	2.09%	0.96	0.33–2.73	67	0.93
Diarrhea	5	37/1,557	2.38%	19/1,276	1.49%	1.98%	1.76	1.01–3.04	7	0.04
Asthenia	3	31/1,400	2.21%	15/1,009	1.49%	1.91%	1.26	0.67–2.35	0	0.47
Hypokalemia	1	11/451	2.44%	4/451	0.89%	1.66%	2.75	0.88–8.57	–	0.08
Infusion reaction	3	21/1,037	2.03%	5/644	0.78%	1.55%	2.26	0.84–6.06	0	0.11
Vomiting	5	26/1,557	1.67%	13/1,276	1.02%	1.38%	1.38	0.72–2.67	0	0.34
Severe skin reaction	5	45/2,320	1.93%	2/1,428	0.14%	1.25%	8.50	2.54–28.46	0	0.0005
Fever without neutropenia	1	1/88	1.14%	1/86	1.16%	1.15%	0.98	0.06–15.38	–	0.99
Injury-poisoning and procedure	1	1/88	1.14%	1/86	1.16%	1.15%	0.98	0.06–15.38	–	0.99
Anorexia	1	1/88	1.14%	1/86	1.16%	1.15%	0.98	0.06–15.38	–	0.99
Mucositis	1	2/88	2.27%	0/86	0.00%	1.15%	4.89	0.24–100.35	–	0.30

ICIs, immune checkpoint inhibitors; CI, confidence interval.

chemotherapy with an improved long-term benefit with respect to OS and DFS, especially in patients with TNBC (26). However, subgroup analysis suggested that addition of ICIs might not have a better effect in Asian patients, patients with locally advanced disease, or patients with brain metastases. Therefore, we suggested that ICIs+Chemotherapy is better than chemotherapy alone with longer survival, especially for patients with PD-L1-positive TNBC.

Higher rate of AEs, especially Grade 3–5/serious AEs, is the main restrictive factor to add immunotherapy to chemotherapy (9, 10). Twenty-one Grade 3–5 AEs greater >2% were reported in the ICIs+Chemotherapy group (neutropenia, leukopenia, decreased neutrophil count, anemia, febrile neutropenia, infection, elevated alanine aminotransferase [ALT] levels, bone pain, increased AST levels, fatigue, nail discoloration, peripheral sensory neuropathy, peripheral neuropathy, hypokalemia, diarrhea, mucositis, hypertension, severe skin reactions, asthenia, nausea, and infusion reactions) compared with twelve in the chemotherapy group (neutropenia, leukopenia, decreased neutrophil count, anemia, febrile neutropenia, infection, hypertension, hand–foot-syndrome, elevated ALT levels, fatigue, peripheral sensory neuropathy, and bone pain). The frequency of AEs was similar as previously reported by Schmid et al. in the updated report of the IMpassion130 trial (23). Hypothyroidism, hyperthyroidism, pneumonitis, hepatitis, and adrenal insufficiency were five AEs of special interest, which were all significantly increased after the addition of ICIs (27). High levels of AEs leading to treatment discontinuation was found in the ICIs +Chemotherapy group (16.38 *vs.* 10.27%), which might decrease antitumor efficacy (10). In the subgroup analysis according to the organs, the addition of ICIs might have a greater impact on the gastrointestinal system, hepatobiliary system, respiratory system, and the thyroid. Therefore, we suggested that although ICIs +Chemotherapy has better survival efficacy, the increase in serious complications deserves attention to improve the lifelong treatment of patients during survival.

However, this meta-analysis had some limitations described as follows: (1) The treatments used in the ICIs+Chemotherapy group and chemotherapy group were different between the groups, which might also increase heterogeneity. (2) Four out of the eight included studies (9, 11, 13, 14) focused on neoadjuvant therapy for early breast cancers, and the other 4/8 studies (10, 12, 16, 18) focused on medical therapy for metastatic breast cancers, and the combined analysis might increase heterogeneity. (3) Only RCTs published in English were included, which might introduce language bias; and (4) significant heterogeneity was found in some analyses (CR, PFSR, etc.), which might decrease the credibility of these results.

ICIs+Chemotherapy appears to be better than chemotherapy alone for TNBC with better OS and PFS. With the prolonged survival time, ICIs+Chemotherapy had an increased advantage for survival. However, the high rates of Grade 3–5/serious AEs, especially immunotherapy-related AEs, need to be taken seriously. However, due to the limitations described above, the results must be confirmed by more large-sample and high-quality RCTs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JH had full access to all of the data in the manuscript and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version. Concept and design: All authors. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: QJ, JD, MH, and NL. Critical revision of the manuscript for important intellectual content: QJ, JH, and WZ. Statistical analysis: QJ, JH, and WZ. Supervision: QJ and JH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.795650/full#supplementary-material

Supplementary Table 1 | PRISMA 2009 checklist.

Supplementary Table 2 | Search strategies.

Supplementary Table 3 | Quality assessment of the included studies according to Jadad scale.

Supplementary Table 4 | GRADE quality assessment for the outcomes of survival, responses, summary of adverse events, total adverse events, and grade 3–5 adverse events.

Supplementary Figure 1 | Cochrane risk assessment associated with ICIs +Chemotherapy *versus* Chemotherapy.

Supplementary Figure 2 | Forest plots of OSR (6–36 months) associated with ICIs+Chemotherapy *versus* Chemotherapy according to survival time.

Supplementary Figure 3 | Forest plots of PFSR (6–30 months) associated with ICIs+Chemotherapy *versus* Chemotherapy according to survival time.

Supplementary Figure 4 | Sensitivity analysis of complete response **(A)**, PFSR **(B)**, and total adverse events **(C)** associated with ICIs+Chemotherapy *versus* Chemotherapy.

Supplementary Figure 5 | Funnel plots of survival summary **(A)** and safety summary **(B)** associated with crizotinib *versus* alectinib.

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The Therapeutic Effectiveness of Neoadjuvant Trastuzumab Plus Chemotherapy for HER2-Positive Breast Cancer Can Be Predicted by Tumor-Infiltrating Lymphocytes and PD-L1 Expression

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Introduction: Neoadjuvant trastuzumab plus chemotherapy may affect programmed death-ligand 1 (PD-L1) expression and tumor-infiltrating lymphocytes (TILs) in HER2-positive breast cancer. Discordant results were shown on the correlation between PD-L1 expression or TILs and the effectiveness of neoadjuvant therapy in HER2-positive breast cancer patients. This study aimed to clarify the predictive value of PD-L1 expression and TILs in neoadjuvant therapy in patients with HER2-positive breast cancer.

Methods: HER2-positive breast cancer cases receiving neoadjuvant treatment (NAT; n = 155) were retrospectively collected from July 2013 to November 2018. Histopathologic analysis of TILs was performed on hematoxylin and eosin (H&E)-stained sections from pre- and post-NAT specimens. The TIL score as a categorical variable can be divided into high (≥30%) and low (<30%) categories. The expression of PD-L1 was detected by immunohistochemistry, and the percentage of positive membranous staining (at least 1%) in tumor cells (PD-L1+TC) and TILs (PD-L1+TILs) was scored.

Results: In our study, 87 patients received neoadjuvant chemotherapy alone and 68 received neoadjuvant trastuzumab plus chemotherapy. Multivariate logistic regression analysis confirmed that lymph node metastasis, high TILs, and PD-L1+TILs in pre-neoadjuvant therapy specimens were independent predictors of pathological complete response (pCR) in neoadjuvant therapy ($p < 0.05$, for all). Among all patients, TILs were increased in breast cancer tissues post-neoadjuvant therapy ($p < 0.001$). Consistent results were found in the subgroup analysis of the trastuzumab plus chemotherapy group and the chemotherapy alone group ($p < 0.05$, for both). In 116 non-pCR patients, PD-L1+TC was

decreased in breast cancer tissues post-neoadjuvant therapy (p = 0.0219). Consistent results were found in 43 non-pCR patients who received neoadjuvant trastuzumab plus chemotherapy (p = 0.0437). However, in 73 non-pCR patients who received neoadjuvant chemotherapy, there was no significant difference in PD-L1+TC expression in pre- and post-neoadjuvant therapy specimens (p = 0.1465). On the other hand, in the general population, the neoadjuvant trastuzumab plus chemotherapy group, and the neoadjuvant chemotherapy group, PD-L1+TILs decreased after treatment ($p < 0.05$, for both).

Conclusion: Higher TIL counts and PD-L1+TILs in pre-neoadjuvant therapy specimens and lymph node metastasis are independent predictors of pCR in patients with HER2-positive breast cancer treated with neoadjuvant therapy. TIL counts, PD-L1+TC, and PD-L1+TILs changed before and after neoadjuvant trastuzumab plus chemotherapy for HER2-positive breast cancer, which may suggest that, in HER2-positive breast cancer, neoadjuvant trastuzumab plus chemotherapy may stimulate the antitumor immune effect of the host, thereby preventing tumor immune escape.

Keywords: HER2-positive breast cancer, neoadjuvant trastuzumab plus chemotherapy, therapeutic effect, PD-L1, TILs

INTRODUCTION

Trastuzumab plus pertuzumab and chemotherapy have been confirmed as the neoadjuvant therapy for stage II–III HER2-positive breast cancer (1). Before 2020, since pertuzumab was not included in medical insurance, many patients with HER2-positive breast cancer still choose trastuzumab plus chemotherapy as neoadjuvant treatment. In HER2-positive breast cancer, neoadjuvant trastuzumab plus chemotherapy can dramatically increase effectiveness compared to chemotherapy alone. However, there were still 25% of patients who showed tumor progression after treatment, thus affecting the prognosis (1–4). Therefore, there is an urgent need to find an accurate and reliable biomarker to predict who will benefit from this treatment. Up to now, several clinical factors, such as lymph node metastasis, tumor size, and hormone receptor (HR) expression, have been correlated with the efficacy of neoadjuvant treatment (NAT) (5, 6). However, choosing NAT based on the above factors does not benefit all patients. Therefore, a molecular marker that can reliably and efficiently assess the effectiveness of NAT is critical in HER2-positive breast cancer.

Programmed death-ligand 1 (PD-L1) is a B7 immune molecule transmembrane protein found in several tumor cells and immune cells, which mediates tumor immunosuppression and is linked to tumor cell immune escape (7, 8). Research shows that trastuzumab can affect PD-L1 expression on $CD8^+$ T cells and cancer cells in HER2-positive breast cancer (9–11). However, another study observed that trastuzumab could downregulate the effects of PD-L1 on cancer cells through HER2 inhibition (10). Furthermore, the PANACEA trial also proposed the hypothesis that trastuzumab can reverse tumor-mediated immunosuppression and activate the local antitumor immune effect (12). Chemotherapy can also cause immunogenic cell death and cellular damage (13). However, the impact of PD-L1 expression on cancer cells and lymphocytes in HER2-positive breast cancer remains unknown.

Tumor-infiltrating lymphocytes (TILs), as immune cells that penetrate tumor tissues, may be associated with immune-mediated tumor–host interaction and antibody-dependent cell-mediated cytotoxicity (ADCC) (14–16). Previous research suggested that increased baseline TILs in patients may be related to the benefit of the anti-HER2 monoclonal antibody trastuzumab (17–20). However, another study showed that high levels of TILs were linked to a lack of benefits from trastuzumab therapy (21). Consequently, the effects of TILs on neoadjuvant trastuzumab plus chemotherapy on HER2-positive breast cancer patients remain a mystery.

Recent research has shown that the active HER2 oncogene regulates the mobilization and activation of tumor-infiltrating immune cells and the therapeutic activity of trastuzumab (22, 23). In several trials, elevated levels of TILs were linked to the benefits of trastuzumab plus chemotherapy. However, experiments on the predictive value of PD-L1 and TILs in the effectiveness of NAT for HER2-positive breast cancer patients showed discordant results. They mainly emphasized the correlation between the expression of PD-L1 or TILs and the efficiency of NAT in the tissue; however, there were no differences in the harmonizing tissues prior to and following NAT.

This research aimed to investigate how TILs and PD-L1 expression in paired tissues changed prior to and following NAT, as well as the relationship between these improvements and the effectiveness of neoadjuvant trastuzumab plus chemotherapy in HER2-positive breast cancer patients.

MATERIALS AND METHODS

Patients

Data were obtained from 155 cases of HER2-positive invasive breast cancer patients at the Shandong Cancer Hospital from

July 2013 to November 2018. Diagnosis of patients was confirmed histologically by core needle biopsy, and the stage of the disease was clarified using ultrasonography (US), bone scintigraphy, and computed tomography (CT). The medical and pathology records of these patients were examined through the hospital medical record system. A flowchart summarizing the patient selection process followed is shown in **Figure 1**. We accessed formalin-fixed, paraffin-embedded tissue samples from NAT patients. A proportion of patients receiving NAT were treated with a taxane-containing regimen along with platinum or an anthracycline. Another proportion of patients received anti-HER2 trastuzumab combined with chemotherapy. The following clinicopathologic variables were acquired: age, tumor dimension, status of the lymph node, initial HR, Ki-67 proliferation index, and neoadjuvant therapy (with and without trastuzumab). Pathological complete response (pCR) was identified as noninvasive breast cancer and axillary lymph nodes remaining after NAT (ypT0 ypN0).

Immunohistochemistry

Immunohistochemical staining was performed on 155 paired breast cancer surgery and biopsy samples with 3.7% neutral formaldehyde, the samples were embedded in paraffin, and 4-μm-thick serial parts were fixed to the samples. This was followed by xylene dewaxing, ethanol graded hydration, ethylenediaminetetraacetic acid (EDTA) antigen repair solution, phosphate-buffered saline (PBS) rinsing (1:50 dilution; clone SP142, Ventana, Shanghai Roche Diagnostic Products Limited Company, Shanghai, China) overnight in a 37°C incubator, treatment with goat anti-mouse/rabbit IgG polymer secondary antibody dropwise, and 3,3′-diaminobenzidine (DAB) development. Contrast dyeing of hematoxylin was performed, followed by ethanol dehydration and sealing. Immunophenotyping was carried out using the following antibodies: anti-ER (clone 6F11; Leica Microsystems, Bannockburn, IL, USA), anti-PR (clone 16; Leica Microsystems), anti-HER2/neu (Ventana 4B5; Ventana Medical Systems, Tucson, AZ, USA), and Ki-67 (MIB-1; Ventana Medical Systems). Estrogen receptor (ER) and progesterone receptor (PR) positivity was defined as staining of ≥1% tumor cell nuclei, while HER2 positivity was evaluated following the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) criteria (24). Briefly, sections with a HER2/CEP17 ratio of ≥2.0 and copy number ≥4.0 or a dual-probe HER2/CEP17 ratio of <2.0 with ≥6.0 HER2 signal per nucleus were determined as positive. A HER2-negative status was defined as a HER2/CEP17 ratio ≥2.0 with <4.0 HER2 signal per nucleus, or a HER2/CEP17 ratio <2.0 and ≥4.0 + <6.0 HER2 signal per nucleus, or a HER2/CEP17 ratio <2 and <4.0 HER2 signal per nucleus. The Ki-67 status was determined by analyzing at least 500 cancer cells per patient. Five high-power-field images were used for each section. Patients were categorized into three groups based on the percentages of Ki-67-positive tumor cells: low, <15%; intermediate, 15%–30%; and high, >30% (25).

PD-L1 Immunohistochemistry

In this study, a two-step immunohistochemistry method was used. The PD-L1 antibody reagent is a rabbit monoclonal antibody (ZZR3). PD-L1 on the tumor cell (TC) membrane or cytoplasm was recorded as PD-L1+TC, and the expression on TILs was recorded as PD-L1+TILs (**Figure 2**). The monoclonal antibody was used to stain breast cancer pathological sections using established methods (26, 27). A 5% increase from 0% to 100% was observed in carcinoma cells with direct membrane PD-L1 expression; less than 1% had a negative markup. For each tumor, the mean PD-L1 labeling was calculated across all cells (28). PD-L1 expression (in percentage) by TILs was also documented in 5% increments and scored as none (0), focal (1+; <5%), moderate (2+; 5%–50%), or severe (3+; 51%–100%). If the PD-L1-positive membrane staining percentage scores of TC and TILs in the tissue after treatment were lower than those before NAT, it was defined as a decrease in PD-L1+TC or a

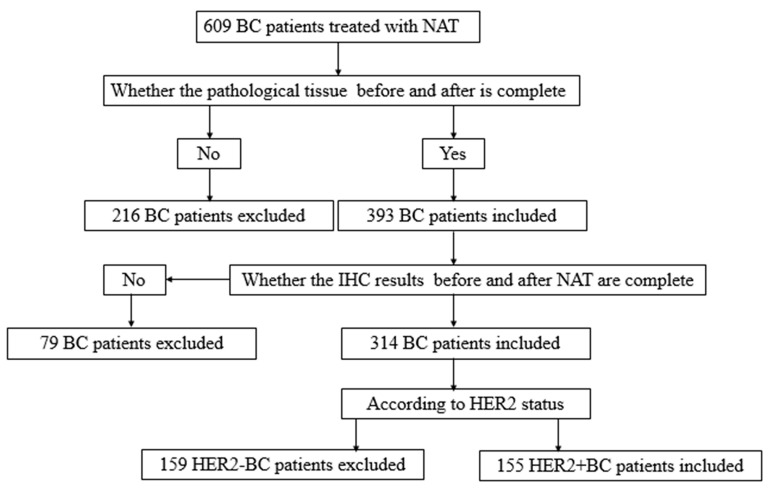

FIGURE 1 | Flowchart explaining the process of patient selection.

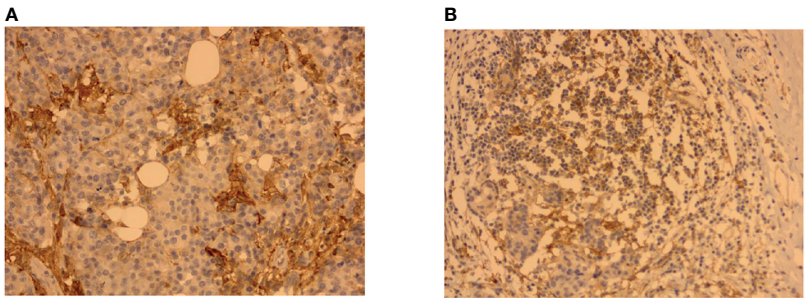

FIGURE 2 | Representative immunohistochemistry (IHC) images showing PD-L1+TC and PD-L1+TILs in breast cancer tissues. **(A)** PD-L1 immunostaining on tumor cells (TC). **(B)** PD-L1 immunostaining on tumor-infiltrating lymphocytes (TILs).

decrease in PD-L1+TILs; otherwise, it was defined as an increase in PD-L1+TC or PD-L1+TIL.

Assessment of TILs

TILs were histopathologically examined using H&E-stained portions from 155 breast cancer samples. The tumor bed was tested and graded in those cases that achieved pCR. Two pathologists (YSG and HY) blinded to the clinical criteria and reactions evaluated the TILs separately. All mononuclear cells including lymphocytes and plasma cells were graded, except granulocytes and other polymorphonuclear leukocytes; invasive lesions and inflammatory infiltration in the matrix of normal breast structures were excluded (29). The TIL count was defined as a percentage estimate of the stromal area adjacent to the tumor that contained mononuclear cells (30). When the TIL score was used as a categorical variable, it was divided into two categories: high TILs (≥30%) and low TILs (<30%) (29). The TIL count of post-NAT surgical resection tissue minus the TIL count of pre-neoadjuvant therapy core needle biopsies represents the change in TILs. If the TIL count of the tissue after treatment was increased compared with that before treatment, it was defined as an increase in TILs; otherwise, it was defined as a decrease in TILs.

Statistical Analysis

The chi-square test was employed to assess the relationships between PD-L1+TC, PD-L1+TILs, TILs, and patients' clinicopathological characteristics. To determine the variables that were substantially correlated with pCR, we applied univariate and multivariate logistic regression analyses. Wilcoxon's non-parametric test was used to compare the changes between the values of PD-L1+TC, PD-L1+TILs, and TILs before and after neoadjuvant treatment. The relationship between PD-L1+TC, PD-L1+TILs, TILs, and disease-free survival (DFS) was determined using the Kaplan–Meier procedure, and the results were compared using the log-rank test. The Cox regression model was adopted to conduct a multivariate study of the prognostic variables. SPSS version 22 was used for all analyses (IBM Corp., Armonk, NY, USA). A $p < 0.05$ was considered statistically significant.

RESULTS

Patients' Characteristics

One hundred and fifty-five HER2-positive breast cancer patients were included in this study. The characteristic features of the study population are listed in **Table 1**. The median age of the patients was 50 years (range = 28–74 years). Most of the patients were older than 50 years (55.5%) at the time of diagnosis. Of the patients, 71 (45.8%) were menopausal. There were 126 (81.3%) patients with a clinical tumor diameter larger than 2.0 cm and 148 (93.5%) patients with clinical lymph node metastases. Sixty-eight patients (43.9%) received neoadjuvant trastuzumab plus chemotherapy, while 87 (56.1%) received neoadjuvant chemotherapy. According to the Miller–Payne scoring system, 39 (25.2%) patients realized pCR and 116 (74.8%) patients were non-pCR.

Expressions of TILs, PD-L1+TC, and PD-L1+TILs in Samples Before and After Neoadjuvant Treatment and their Correlations With Clinicopathological Characteristics

As shown in **Tables 2** and **3**, in samples before neoadjuvant therapy, TILs were negatively associated with the expression of PR, while PD-L1+TILs were negatively associated with the expressions of ER and PR ($p < 0.05$, for all). However, in samples before neoadjuvant therapy, no correlation between PD-L1+TC and age, primary tumor (cT), lymph node involvement (cN), ER status, PR status, or Ki-67 index was found ($p > 0.05$). In samples after neoadjuvant therapy, the expression of PD-L1+TC was negatively correlated with ER ($p < 0.05$). As for the TILs and PD-L1+TILs in samples after neoadjuvant therapy, there was no correlation with age, cT, cN, ER status, PR status, or Ki-67 index.

Correlation of the Expressions of TILs, PD-L1+TC, and PD-L1+TILs With Clinicopathological Factors Including pCR to Neoadjuvant Therapy

As shown in **Table 4**, univariate analysis confirmed that pCR had a positive correlation with cN, high TIL counts, and PD-L1+TILs in specimens prior to neoadjuvant therapy ($p < 0.05$, for all), but

TABLE 1 | Patient characteristics.

Characteristics	*N* (%)
Age (years)	
≤50	69 (44.5)
>50	86 (55.5)
Menstrual status	
Menopause	71 (45.8)
Non-menopausal	84 (54.2)
cT (cm)	
≤2	29 (18.7)
>2	126 (81.3)
cN	
Negative	7 (4.5)
Positive	148 (93.5)
Neoadjuvant treatment	
Trastuzumab+chemotherapy	68 (43.9)
Chemotherapy	87 (56.1)
Neoadjuvant efficacy	
pCR	39 (25.2)
No pCR	116 (74.8)

cT, primary tumor; cN, lymph node involvement; pCR, pathological complete response.

not with age, menstruation, cN, cT, ER, PR, and Ki-67 index in PD-L1+TC before neoadjuvant therapy, PD-L1+TILs after neoadjuvant therapy, TIL changes, or PD-L1+TIL changes ($p > 0.05$, for all). Multivariate logistic regression analysis verified that cN, high TIL counts, and PD-L1+TILs in pre-NAT samples were significantly correlated with pCR ($p < 0.05$, for all).

Changes in TILs, PD-L1+TC, and PD-L1+TILs Before and After Neoadjuvant Treatment

The TIL counts in breast cancer tissues improved after neoadjuvant therapy in all patients ($p < 0.001$). Subgroup analysis of the trastuzumab plus chemotherapy group and the chemotherapy alone group revealed consistent findings ($p < 0.05$). The expressions of PD-L1+TC was reduced in breast cancer tissues after NAT in 116 non-pCR patients ($p = 0.0219$). In 43 non-pCR patients who received neoadjuvant trastuzumab plus chemotherapy, consistent findings were observed ($p = 0.0437$). Although neoadjuvant chemotherapy was given to 73 non-pCR patients, there was no substantial difference in PD-L1+TC expression before and after neoadjuvant therapy ($p = 0.1465$). PD-L1+TILs were downregulated following treatment in the general population, the neoadjuvant trastuzumab plus chemotherapy group, and the neoadjuvant chemotherapy group ($p < 0.05$; **Figure 3**).

Relationship Between Changes in Various Factors and Prognosis

According to the Kaplan–Meier study, only the changes in PD-L1+TC before and after neoadjuvant chemotherapy were related to DFS ($p = 0.0080$). Nevertheless, the transition in TILs and PD-L1+TILs between pre and post-NAT showed no association with DFS ($p > 0.05$; **Figure 4**). A multivariate Cox regression study was performed using the significant clinicopathological variables identified by univariate analysis (cN, cT, and PD-L1+TC before treatment). We did not find the above factors to be independent predictors of DFS ($p > 0.05$, for all; **Table 5**).

DISCUSSION

Numerous experiments have been conducted to investigate the predictors of NAT effectiveness in HER2-positive breast cancer. Until now, no accurate and commonly used biomarker has been discovered, except for a few clinicopathological factors such as

TABLE 2 | Expressions of TILs, PD-L1+TC, and PD-L1+TILs in pre-neoadjuvant therapy specimens and correlations with clinicopathological characteristics.

	TILs			PD-L1+TC Status			PD-L1+TILs		
	High	Low	*p*-value	Positive	Negative	*p*-value	Positive	Negative	*p*-value
Age (years)			0.711			0.598			0.348
≤50	6	63		43	26		22	45	
>50	9	77		50	36		22	63	
cN			0.999			0.155			0.660
Negative	0	7		6	1		1	1	
Positive	15	133		87	61		61	43	
cT (cm)			0.208			0.501			0.961
≤2	1	47		19	10		8	20	
>2	14	62		74	52		36	88	
ER			0.446			0.389			0.001
Negative	5	61		37	29		9	55	
Positive	10	79		56	33		35	53	
PR			0.004			0.749			0.009
Negative	5	85		55	35		18	69	
Positive	10	55		38	27		26	39	
Ki-67 index			0.110			0.249			0.469
Low	0	9		6	3		4	5	
Intermediate	3	61		43	21		19	44	
High	2	68		43	37		20	58	

TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; cN, lymph node involvement; cT, primary tumor; ER, estrogen receptor; PR, progesterone receptor.

TABLE 3 | Expressions of TILs, PD-L1+TC, and PD-L1+TILs in post-neoadjuvant therapy specimens and correlations with clinicopathological characteristics.

	TILs			PD-L1+TC Status			PD-L1+TILs		
	High	**Low**	***p*-value**	**Positive**	**Negative**	***p*-value**	**Positive**	**Negative**	***p*-value**
Age (years)			0.526			0.892			0.193
≤50	13	47		39	12		37	22	
>50	13	62		49	16		37	35	
cN			0.620			0.426			0.971
Negative	2	5		1	1		4	3	
Positive	24	104		87	27		70	54	
cT (cm)			0.997			0.556			0.102
≤2	5	21		15	3		19	8	
>2	21	88		73	25		55	49	
ER			0.583			0.012			0.059
Negative	13	48		30	17		28	31	
Positive	13	61		58	11		46	26	
PR			0.128			0.351			0.784
Negative	13	72		51	19		45	36	
Positive	13	37		37	9		29	21	
Ki-67 index			0.889			0.211			0.413
Low	2	7		9	0		7	2	
Intermediate	10	46		36	14		28	23	
High	14	54		41	14		38	31	

TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; cN, lymph node involvement; cT, primary tumor; ER, estrogen receptor; PR, progesterone receptor.

HER2. The HER2 oncogene can affect the therapeutic effect of trastuzumab by inducing the expression of PD-L1 and the recruitment and activation of TILs, suggesting that TILs and PD-L1 have been linked to trastuzumab efficacy (9, 21, 23, 31). Several studies have confirmed that TILs and PD-L1 have such predictive values in HER2-positive breast cancer patients, but debate is still ongoing (32–34). Most of the previous studies have concentrated on the expression of PD-L1 or TILs in tissues before NAT in HER2-positive breast cancer to predict the effectiveness of neoadjuvant therapy. There is still lack of information on whether the changes in PD-L1 and TILs in the tissues before and after NAT could predict the efficacy of neoadjuvant treatment.

We first tested whether there was any association between the TIL counts and clinicopathological characteristics before and after neoadjuvant therapy. Previous studies have shown that higher TIL counts pre-NAT were significantly associated with more aggressive clinicopathological features, such as higher cT staging, histological grade, and Ki-67 index (35). In our study, we concluded that the TIL counts in tissues before NAT were significantly higher in PR-negative cases. Consistent with previous research (36), no evidence of an association was found between the TIL counts after NAT and age, postoperative staging, cT, cN, distant metastasis, ER and PR status, or Ki-67 index. We have reached conclusions inconsistent with previous studies regarding the relationship between TILs post-NAT and the clinicopathological characteristics. The different TIL evaluation criteria, including only HER2-positive breast cancer types, and the heterogeneity of the histopathological tissues of HER2-positive breast cancer have likely caused the conflicting results.

According to some studies, cytotoxic agents may release tumor antigens and aid in the recruitment of immune cells to the tumor through mediators such as the pro-inflammatory cytokine interferon-c (37). Moreover, by inducing ADCC through immune cells and immunogenic cell death, trastuzumab can increase the density of $CD3^+$ and $CD8^+$ TILs and enhance the antitumor immune response (38). This laid the theoretical foundation for our research. Our study showed a significant increase in TILs following NAT in all patients, prompting us to speculate that NAT may activate the local antitumor immune status.

Inconsistent with our research, a previous research has shown that, in HER2-positive breast cancer treated with neoadjuvant chemotherapy plus trastuzumab, high grades of TILs in tissues before NAT were associated with a significant improvement in the pCR rate (39). We observed that cN, higher TILs, and PD-L1+TILs in specimens before neoadjuvant therapy, but no other clinicopathological factors, were independent predictors of pCR in NAT. Previous studies have confirmed that PD-L1+TILs are regulated through adaptive mechanisms and reflect preexisting immunity, and their expressions may be caused by an organism's strong primary cytotoxic immune attack on tumor neoantigens (34, 40, 41). Therein, chemotherapy and targeted therapy-induced cellular damage and immunogenic cell death will cause a cascade of cellular immune responses, the development of new immunogenic epitopes, antigen cross-presentation, cytokine and chemokine secretion, induction of tumor-specific cytotoxic T cells, and activation of dendritic cells. Similarly, chemotherapy and targeted therapy can also cause a cascade of humoral immune responses (13, 36, 42). This supported a previous theory that chemotherapy and targeted therapy could improve treatment efficacy by increasing the immune activity of patients (43). Furthermore, the FinHER Study showed that the high level of stromal TILs at diagnosis predicted the benefits of trastuzumab adjuvant therapy and proposed that the establishment of a HER2 signal might be the reason for the maintenance of the immunosuppressive microenvironment, However, trastuzumab may break the hypothesis of such an immunosuppressive microenvironment (19). Further research is

TABLE 4 | Correlation of the expressions of TILs, PD-L1+TC, and PD-L1+TILs with clinicopathological factors including pCR to neoadjuvant therapy.

Parameters	Univariate			Multivariate		
	Hazard Ratio	95%CI	*p*-value	Hazard Ratio	95%CI	*p*-value
Age (years)	1.048	0.489–2.244	0.905			
≤50						
>50						
Menstruation	1.349	0.626–2.911	0.445			
Menopausal						
Non-menopausal						
cN	7.344	1.358–39.727	0.021	0.115	0.020–0.659	0.015
Negative						
Positive						
cT (cm)	1.966	0.817–4.732	0.131			
≤2						
>2						
ER	0.326	0.619–2.841	0.467			
Negative						
Positive						
PR	0.553	0.256–1.194	0.132			
Negative						
Positive						
Ki-67 index	0.609	0.277–1.338	0.466			
Low						
Intermediate						
High						
Pretreatment PD-L1+TC	0.635	0.289–1.396	0.258			
Negative						
Positive						
Pretreatment TILs	0.091	0.018–0.463	0.004	0.102	0.020–0.533	0.007
Low						
High						
Pretreatment PD-L1+TILs	0.202	0.066–0.620	0.005	0.272	0.085–0.872	0.028
Negative						
Positive						
TIL change	1.875	0.668–5.266	0.233			
Decreased						
Increased						
Unchanged						
PD-L1+TIL change	3.967	1.051–14.970	0.089			
Decreased						
Increased						
Unchanged						

TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; cN, lymph node involvement; cT, primary tumor; ER, estrogen receptor; PR, progesterone receptor.

urgently needed to investigate the relationship between neoadjuvant trastuzumab plus chemotherapy and the immune microenvironment of HER2-positive breast cancer and whether this treatment can affect the immune microenvironment of local antitumor. Besides, our study proved that PD-L1+TILs in pre-NAT specimens were also an independent predictor of pCR in neoadjuvant treatment. One possible explanation for such findings is that the expression of PD-L1 by immune cells, especially TILs, reflects a robust primary immune response and shows an adaptive response to an intensive primary cytotoxic immune attack on cancer neoantigens (44). In conclusion, higher TILs and PD-L1+TILs in pre-NAT specimens may also forecast the effectiveness of neoadjuvant trastuzumab with chemotherapy for HER2-positive breast cancer, in accordance with our findings. However, more research is needed to explicate the antitumor immune response mechanism of TILs and PD-L1+TILs and to clarify the role of PD-L1+TIL in neoadjuvant trastuzumab combined with chemotherapy.

A basic experiment confirmed two main ways to regulate PD-L1 expression after trastuzumab treatment (10). Firstly, the cytokines released by trastuzumab through external pathways may activate trastuzumab-mediated ADCC, thereby upregulating PD-L1 expression on breast tumor cells. Secondly, a trastuzumab-mediated intrinsic pathway to inhibit HER2 downstream cell signal transduction downregulates PD-L1 expression on tumor cells. These pointed out that this extrinsic pathway is related to trastuzumab resistance and that the internal pathway is related to the antitumor immune effect of trastuzumab. The results of the basic experiment may explain the following conclusions we have reached. In our subgroup analysis, PD-L1 +TC in the neoadjuvant trastuzumab plus chemotherapy subgroup was significantly reduced, and the results were statistically significant. However, no statistically significant reduction in PD-L1+TC was found in the general population and the subgroup of neoadjuvant chemotherapy alone. This result

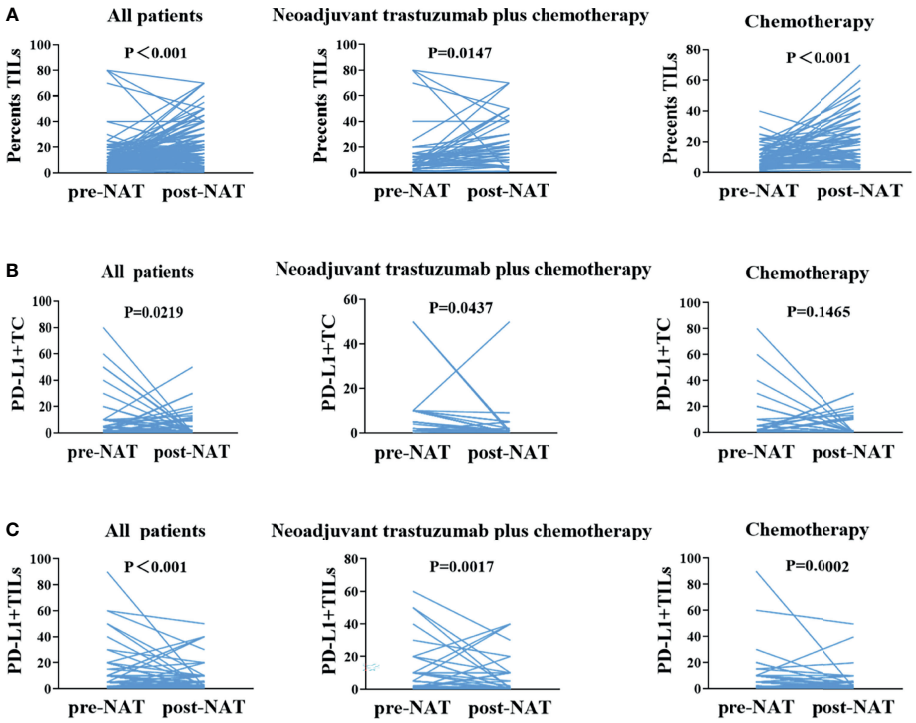

FIGURE 3 | One-to-one correspondence of tumor-infiltrating lymphocytes (TILs), PD-L1+TC, and PD-L1+TILs between pre- and post-neoadjuvant therapy samples for all cases. **(A)** Changes in TILs between before and after neoadjuvant therapy, before and after neoadjuvant trastuzumab plus chemotherapy, and before and after neoadjuvant chemotherapy. **(B)** In non-pCR patients, changes in PD-L1+TC between pre- and post-neoadjuvant therapy, before and after neoadjuvant trastuzumab plus chemotherapy, and before and after neoadjuvant chemotherapy. **(C)** Changes in PD-L1+TILs between pre- and post-neoadjuvant therapy and before and after neoadjuvant trastuzumab plus chemotherapy. *pCR*, pathological complete response.

indicates that neoadjuvant trastuzumab plus chemotherapy may affect the expression of PD-L1+TC through the intrinsic pathway mediated by trastuzumab. Moreover, a study has shown that PD-L1+TC can mediate antitumor immune escape (45). In our study, only the subgroup of trastuzumab combined with chemotherapy showed a decrease in PD-L1+TC after treatment, suggesting that PD-L1-TC is related to the efficacy of neoadjuvant trastuzumab plus chemotherapy. However, the relationship between PD-L1+TC and trastuzumab in truncating tumor immune escape needs further confirmation by basic experiments.

Professor Arlene H. Sharpe has shown that PD-L1 on highly immunogenic tumor cells is enough to promote tumor immune escape and constrain the tumor lysing ability of $CD8^+$ T cells (46). Furthermore, chemotherapy can activate the antitumor

TABLE 5 | Factors correlated with disease-free survival (DFS) in univariate and multivariate analyses.

Clinicopathological Parameters	DFS					
	Univariate Analysis			Multivariate Analysis		
	HR	95%CI	*p*-value	HR	95%CI	*p*-value
cN	0.363	0.089–1.477	0.157	0.491	0.117–2.060	0.331
cT	0.543	0.295–1.002	0.051	0.594	0.318–1.108	0.102
ER	1.156	0.757–1.763	0.502			
PR	1.077	0.699–1.659	0.736			
Ki-67 index	1.037	0.467–2.302	0.378			
Pretreatment PD-L1+TILs	1.009	0.635–1.604	0.969			
Pretreatment TILs	1.320	0.531–3.283	0.550			
Pretreatment PD-L1+TC	0.720	0.463–1.120	0.145	0.744	0.478–1.159	0.191
TIL change	0.623	0.209–1.860	0.808			
PD-L1-TIL change	0.769	0.468–1.266	0.380			

TILs, tumor-infiltrating lymphocytes; PD-L1, programmed death-ligand 1; cN, lymph node involvement; cT, primary tumor; ER, estrogen receptor; PR, progesterone receptor.

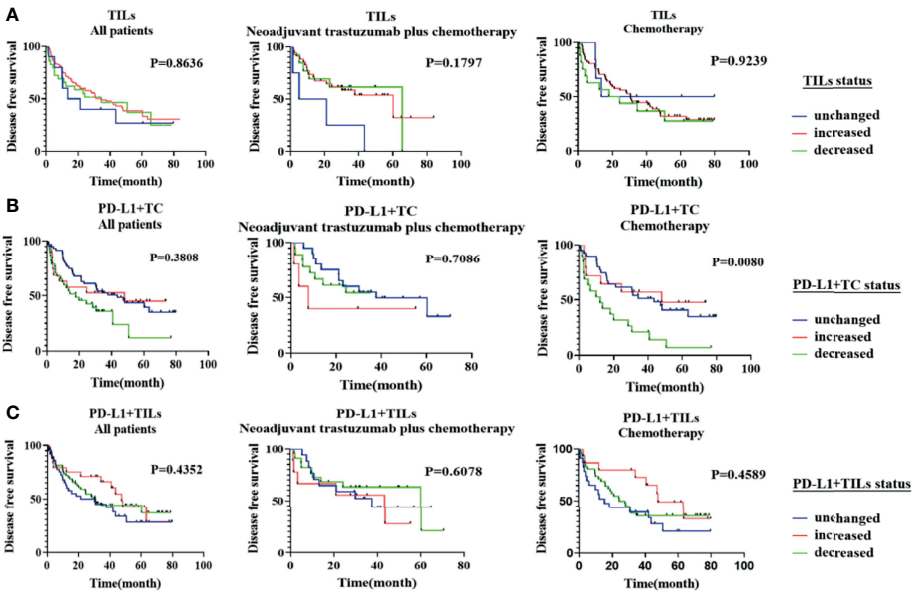

FIGURE 4 | Kaplan–Meier analysis of disease-free survival (DFS) according to the changes in tumor-infiltrating lymphocytes (TILs), PD-L1+TC, and PD-L1+TILs between pre- and post-neoadjuvant treatment. **(A)** The changes in TILs before and after neoadjuvant treatment were not significantly correlated with patients' DFS in all populations, the neoadjuvant trastuzumab plus chemotherapy group, and the neoadjuvant chemotherapy group ($p > 0.05$, for all). **(B)** The changes in PD-L1+TC before and after neoadjuvant chemotherapy were related to DFS ($p = 0.0080$), but the changes in PD-L1+TC in all populations and the neoadjuvant trastuzumab plus chemotherapy population were not related to DFS ($p > 0.05$, for all). **(C)** The changes in PD-L1+TILs before and after neoadjuvant treatment were not significantly correlated with patients' DFS in all populations, the neoadjuvant trastuzumab plus chemotherapy group, and the neoadjuvant chemotherapy group ($p > 0.05$, for all).

immune response. This laid the theoretical foundation for our research hypothesis. We found that, in HER2-positive breast cancer, the TIL counts in post-NAT tissues were increased, but PD-L1+TC was decreased, suggesting that neoadjuvant trastuzumab plus chemotherapy may activate the antitumor immune response, thereby inhibiting tumor immune escape.

Our study has several limitations. Firstly, the low sample size hampered conducting statistical analysis on subtype comparisons and adequately powered multivariate analysis. We also did not investigate other immune-oncologic biomarkers such as CTLA-4 and the expressions of other immune checkpoints in tumor and immune cells. Secondly, the PD-L1 status was based on a single antibody. Due to the significant differences in previous studies using different PD-1/PD-L1 antibodies, our results may be limited by the use of a single antibody. Finally, limited by economic factors, this study only included patients receiving single-target chemotherapy, but failed to show a relationship between TILs, PD-L1+TC, and PD-L1+TILs and the efficacy of neoadjuvant dual-target plus chemotherapy in HER2-positive breast cancer patients. To confirm and endorse our results, larger prospective trials with multi-institution cohorts, homogeneous breast cancer tumor subtypes, and several distinct anti-HER2 treatment regimens are required.

High TILs and PD-L1+TILs in samples prior to NAT and lymph node metastasis can predict the pCR for neoadjuvant treatment in HER2-positive breast cancer patients. Both PD-L1+TILs and TILs were changed in pre- and post-NAT samples of HER2-positive breast cancer, suggesting that the immune microenvironment has a crucial role in neoadjuvant treatment. More studies on the mechanism and prospective clinical verification are required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shandong Cancer Hospital and Institute. The approval number is SDTHEC 201907003 (Shandong, China). The patients/participants provided written informed consent to participate in this study. Written informed consent was obtained from individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HL and MS were responsible for the study design and concept. MS, YC, JC, SY, QT, and XM performed data acquisition. MS

analyzed and interpreted the data. JZ and HY contributed to pathological section reading. MS and HL prepared and edited the manuscript. All authors have read and approved the final version of the manuscript.

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REVIEW
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Clinical Progress of PD-1/L1 Inhibitors in Breast Cancer Immunotherapy

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Breast cancer is a major killer of women's health worldwide. While breast cancer is thought to have lower immunogenicity compared with other solid tumors, combination therapy is able to improve the immunogenicity of the tumor and sensitize breast cancer cells to immunotherapy. Immunotherapy represented by immune checkpoint inhibitors (ICIs) has been largely explored in the field of breast cancer, including both early and advanced disease. Immunotherapy for triple-negative breast cancer (TNBC) has been the most studied, and the PD-L1 inhibitor atezolizumab combined with nab-paclitaxel has been used in the first-line treatment of TNBC. Immunotherapeutic data for human epidermal growth factor receptor-positive and hormone receptor-positive breast cancer are also accumulating. This review summarizes the clinical trial data of ICIs or ICI-containing therapies in different types and stages of breast cancer.

Keywords: breast cancer, immunotherapy, immune checkpoint inhibitor, PD-1, PD-L1

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1 INTRODUCTION

Immunotherapy represented by immune checkpoint inhibitors (ICIs) has become an important strategy for the treatment of malignant tumors. With the increase in the indications of programmed cell death receptor 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) inhibitors, the treatment pattern of many solid tumors has gradually changed (1). However, the development of immunotherapy in breast cancer is relatively slow. Breast cancer (BC) is traditionally considered to be poorly immunogenic. Due to the heterogeneity of molecular subtypes of breast cancer, the immune microenvironment of each subtype is discrepant (2, 3), which is one of the challenges of breast cancer immunotherapy. The further research of tumor immune microenvironment brings new opportunities for immunotherapy of this disease. Based on the Impassion130 study, PD-L1 inhibitor atezolizumab combined with nab-paclitaxel has been approved in the first-line treatment of triple-negative breast cancer (TNBC) (4, 5), which opens up a new window for the treatment of advanced TNBC. The exploration of ICI monotherapy and combination therapy involves multiple disease stages of TNBC. Immunotherapy has also been increasingly explored in human epidermal growth factor receptor 2 (HER2)-positive and hormone receptor-positive breast cancer. Immunotherapy may become a new treatment paradigm for breast cancer. As the existence of heterogeneity in the tumor microenvironment of different molecular types of breast cancer, and the inconsistent efficacy of immunotherapy, we reviewed the current clinical trial evidence for breast cancer immunotherapy according to molecular subtypes.

2 PD-1/L1 INHIBITORS FOR TRIPLE-NEGATIVE BREAST CANCER

TNBC accounts for about 15%–20% of all breast cancers (6). Due to the lack of hormone receptor and HER2 expression, chemotherapy has been the mainstay treatments for TNBC for many years (7). However, suboptimal survival and tolerance of chemotherapy impels the development of novel strategies for treating this difficult-to-treat disease (8). There are several lines of supporting evidence for the potential of immunotherapy in TNBC. High expression of the immunomodulatory genes is associated with better outcomes for patients with TNBC (9). Higher enrichment of tumor-infiltrating lymphocytes (TILs) has been shown to be a prognostic predictor in TNBC (10–12). TNBC cells harbored higher level of PD-L1 expression than non-TNBC cells (13). Based on these rationale, immune checkpoint inhibitors (ICIs) represented by PD-1/L1 inhibitors are increasingly being explored for the treatment of early-stage TNBC and advanced-stage TNBC.

2.1 Neoadjuvant Therapy

2.1.1 Combination of Immunotherapy and Chemotherapy

The phase III KEYNOTE-522 study (14) randomized 1,174 early TNBC patients to the neoadjuvant chemotherapy (carboplatin plus paclitaxel and sequential doxorubicin/epirubicin plus cyclophosphamide) combined with pembrolizumab, a PD-1 inhibitor, followed by adjuvant pembrolizumab, or chemotherapy alone, followed by adjuvant placebo. The results showed that the pathological complete response (pCR) rate was significantly higher in the pembrolizumab plus chemotherapy group (64.8% vs. 51.2%) for the overall population. Moreover, patients with PD-L1 expression, which was assessed by PD-L1 22C3 pharmDx assay, and positive lymph nodes benefited more from pembrolizumab. In terms of event-free survival (EFS), the 18-month EFS rates were 91.3% and 85.3%, respectively, and the hazard ratio (HR) for EFS was supportive of pembrolizumab addition (HR 0.63, 95% confidence interval (CI) 0.43–0.93). The phase III NeoTRIPaPDL1 study (15) randomized 280 patients with early TNBC to atezolizumab (anti-PD-L1) plus carboplatin/nab-paclitaxel arm or placebo plus carboplatin/nab-paclitaxel arm. The results showed that the pCR was not significantly different in neither the overall population (43.5% vs. 40.8%) nor the PD-L1-positive (determined by VENTANA PD-L1 SP142 assay) population (51.9% vs. 48.0%). IMpassion031 (16), a phase III trial, showed that atezolizumab plus nab-paclitaxel and sequential doxorubicin/cyclophosphamide increased pCR rate to 58%, compared with 41% in the chemotherapy group. In the PD-L1-positive subgroup (identified by VENTANA SP142 assay), pCR rate was 20% higher in the atezolizumab group (69% vs. 49%). Treatment-related grades 3–4 AEs were balanced (57% vs. 53%) and treatment-related serious AEs were 23% and 16%, respectively. PD-L1 inhibitor durvalumab addition to sequential taxane–anthracycline chemotherapy was investigated in GeparNuevo (17). In this phase II trial, 117 patients were randomized to the window-phase durvalumab group (durvalumab was administered 2 weeks before the beginning of nab-paclitaxel). The pCR of these patients was 61.0%, compared with 41.4% for the placebo cohorts (OR 2.22, 95% CI 1.06–4.64). While for the nonwindow dosing cohort (n = 57), no advantage of durvalumab was observed over chemotherapy.

2.1.2 Combination of Immunotherapy and Targeted Therapy

The phase II I-SPY 2 trial tested the efficacy and safety of durvalumab plus PARP inhibitor olaparib and paclitaxel compared with paclitaxel alone in the neoadjuvant setting of TNBC treatment (18). In 21 TNBC patients, the estimated pCR rate was 47% with the combination subgroup and 27% with chemotherapy alone subgroup. Further biomarker analysis showed that low CD3/CD8 gene signature ratio, high macrophage/Tc-class 2 ratio, and high proliferation signatures were associated with a higher pCR in the combination arm.

2.2 Maintenance Therapy

2.2.1 Combination of Immunotherapy and Chemotherapy

A phase II RCT named SAFIR02-BREAST IMMUNO compared durvalumab with chemotherapy (paclitaxel, capecitabine, and FEC) in the maintenance therapy for HER2-negative metastatic breast cancer (19). Patients with disease that did not progress after 6 to 8 cycles of first-line or second-line chemotherapy were included. In the exploratory TNBC subgroup analysis, the OS was significantly improved in the durvalumab arm (21 months vs. 14 months, HR 0.54, 95% CI 0.30–0.97); PD-L1-positive (detected by VENTANA PD-L1 SP142 assay) patients benefited more from durvalumab administration than from chemotherapy (HR 0.37, 95% CI 0.12–1.13), while PD-L1-negative patients did not benefit much (HR 0.49, 95% CI 0.18–1.34). In addition, TNBC patients with CD274 gain/amplification could benefit from durvalumab over chemotherapy in OS (HR 0.18, 95% CI 0.05–0.71), compared with patients with CD274 normal/loss (HR 1.12, 95% CI 0.42–2.99).

2.3 First-Line Therapy

2.3.1 Immunotherapy Alone

ICI monotherapy was firstly explored in advanced-stage TNBC treatment. In cohort B of the international phase II KEYNOTE-086 study, pembrolizumab as first-line treatment for metastatic TNBC patients with tumor PD-L1 combined positive score (CPS) ≥1 was evaluated with PD-L1 22C3 pharmDx assay. In this cohort, 84 untreated patients with PD-L1-expressing metastatic TNBC received pembrolizumab 200 mg/3 weeks for up to 2 years. The ORR, PFS, and OS were 21.4%, 2.1 months, and 18 months, respectively. As for grade 3 or higher AEs, the incidence rate was 9.5% (20). The PCD4989g study, an open-label, multicenter phase 1a study, evaluated atezolizumab monotherapy in advanced solid and hematologic malignancies, which enrolled 116 metastatic TNBC patients. In 21 first-line patients, the ORR was 24% and median OS was 17.6 months. Of 116 patients in all lines, grade 3 or above AEs accounted for 21%. Patients with PD-L1 ≥1% had higher ORR and longer OS, and

PD-L1 ≥10% was an independent predictor of better response and survival, with VENTANA PD-L1 SP142 assay being used for quantifying PD-L1 expression (21).

2.3.2 Combination of Immunotherapy and Chemotherapy

Chemotherapy is demonstrated to be capable of enhancing tumor immunogenicity and T-cell-dependent antitumor response (22). Several studies of PD-1/L1 antibodies combined with chemotherapeutic agents have been performed in the first-line treatment of TNBC. The IMpassion130 study assessed the efficacy of atezolizumab in combination with nab-paclitaxel in patients with unresectable, locally advanced, or metastatic TNBC (23, 24). In the overall intention-to-treat (ITT) population, the combination of atezolizumab and nab-paclitaxel resulted in a significant improvement in median PFS (7.2 months vs. 5.5 months; HR 0.80, 95% CI 0.69–0.92); however, no statistically significant increment in median OS was observed in the atezolizumab arm (21 months vs. 18.7 months; HR 0.86, 95% CI 0.72–1.02). The ORR in atezolizumab group was 56.0%, versus that of 45.9% in the nab-paclitaxel group. Furthermore, for the PD-L1 (evaluated by VENTANA PD-L1 SP142 assay) positive subgroup (41% of all patients), the median PFS of combination group and chemotherapy group were 7.5 months and 5.0 months, respectively (HR 0.62, 95% CI 0.49–0.78); the median OS was significantly prolonged in the combination group (25 months vs. 18 months, HR 0.71, 95% CI 0.54–0.94), though that could not be formally concluded owing to the prespecified statistical testing hierarchy. While, for the PD-L1-negative patients, there was no difference in OS between the two groups (19.7 months vs. 19.6 months, HR 0.97; 95% CI 0.78–1.20). Patients receiving atezolizumab experienced more grade 3 or higher AEs (40.4% vs. 30.7%). In March 2019, the FDA approved atezolizumab plus nab-paclitaxel for first-line treatment of locally advanced or metastatic TNBC with PD-L1 ≥1%.However, atezolizumab plus paclitaxel, assessed in IMpassion131 study, failed to improve PFS or OS compared with paclitaxel alone (25). The phase III IMpassion132 study of gemcitabine plus carboplatin/capecitabine with or without atezolizumab as the first-line therapy of TNBC is ongoing (NCT03371017). In the phase III KEYNOTE-355 study (26, 27), pembrolizumab combined with chemotherapy (nab-paclitaxel; paclitaxel; or gemcitabine/carboplatin) as the first-line treatment of advanced TNBC mainly benefited patients with ≥10% PD-L1 expression (detected by PD-L1 22C3 pharmDx assay) disease, with a median PFS of 9.7 months in combination group versus 5.6 months in chemotherapy group (HR 0.66, 95% CI 0.50–0.88).With respect to grades 3–5 AEs, 68.1% patients experienced that in the combination group (2 deaths), in contrast with 66.9% in the chemotherapy group (0 death). However, more patients suffered grades 3–4 immune-related AEs and infusion reactions in the immunochemotherapy group (5.5% vs. 0%).

2.3.3 Combination of Immunotherapy and Targeted Therapy

Hyperactivation of the PI3K/AKT pathway, resulted from the downregulation of *PTEN* gene, is one of the dominant mechanisms of tumor progression (28). Agents targeting the PI3K/AKT pathway may augment the antitumor adaptive immune responses (29). Bases on this rationale, a phase 1b study of combining AKT inhibitor ipatasertib, atezolizumab, and chemotherapy (paclitaxel or nab-paclitaxel) as first-line treatment for locally advanced or metastatic TNBC was performed. The results showed that the ORR reached 54%, with manageable toxicity (30). Antiangiogenic therapy is shown to have a synergistic antitumor effect with anti-PD-1 therapy (31). The phase II WJOG9917B NEWBEAT study evaluated the triple combination of PD-1 inhibitor nivolumab, bevacizumab, and paclitaxel in the first-line treatment for patients with TNBC (n = 18, 32%) or hormone receptor-positive breast cancer (n = 39, 68%) (32). This combination therapy led to an ORR of 83.3% in patients with TNBC, which demonstrated promising synergistic efficacy of VEGF inhibitor addition to immunochemotherapy. Another phase II trial explored PD-1 inhibitor camrelizumab (SHR-1210) combined with apatinib for advanced TNBC patients (n = 34). The results showed that apatinib continuous dosing group (d1–d14 administration) had an ORR and DCR of 47.4% and 68.4%, respectively. In apatinib intermittent dosing group (d1–d7 administration), there was no confirmed ORR, with a DCR of 44.4% and a PFS of 2 months (33). TNBC usually has upregulated MAPK pathway and increased sensitivity to MEK inhibition. MEK inhibitor increases the levels of effector $CD8^+$ T cells in tumors and synergizes with anti-PD-L1 blockade (34). The cohort 1 of COLET study showed that MEK1/2 inhibitor cobimetinib plus paclitaxel could enhance antitumor effects for the first-line treatment of TNBC (35). IMpassion130 illustrated that the combination of atezolizumab and nab-paclitaxel as first-line treatment is effective for TNBC patients (24). Therefore, the cohort 2 of COLET evaluated the efficacy and safety of atezolizumab plus cobimetinib plus nab-paclitaxel or paclitaxel as first-line treatment of locally advanced or metastatic TNBC (36). The results showed that the ORRs were similar between the nab-paclitaxel and paclitaxel arms (29% vs. 34%). Patients with PD-L1-positive disease had numerically higher ORR (44%) and 6-month PFS rate. The safety profile of combined treatments was consistent with the known individual safety profiles.

2.4 Second-Line or Later Therapy

2.4.1 Immunotherapy Alone

In the TNBC cohort of the phase 1b KEYNOTE-012 study, pembrolizumab as the first-line or later treatment yielded an ORR of 18.5%, PFS of 1.9 months, OS of 10.2 months, and ≥grade 3 AEs of 18.8% (37, 38). Cohort A of KEYNOTE-086 tested the efficacy of pembrolizumab as second-line or later therapy of metastatic TNBC. In the total patients (n = 170), the ORR was 5.3%, and PFS and OS were 2.0 months and 9.0 months, respectively; grade 3 or above AEs were 12.9%. The PD-L1 ≥1% population derived similar benefits as the overall patients, with an ORR of 5.7%, PFS of 2.0 months and OS of 8.8 months (39). KEYNOTE-119 compared pembrolizumab with chemotherapy (capecitabine, eribulin, gemcitabine, and vinorelbine) in second-line or third-line setting for metastatic

TNBC patients (40). The results showed that pembrolizumab did not present superior efficacy over chemotherapy, except the exploratory subgroup who had PD-L1 CPS of 20 or higher, with OS being 14.9 months versus 12.5 months (HR 0.58, 95% CI 0.38–0.88). Moreover, the grades 3–5 AEs were lower in the pembrolizumab group (14% vs. 36%). The phase 1b JAVELIN study evaluated avelumab, an PD-L1 inhibitor, in patients with metastatic breast cancer who had received a median of three prior cytotoxic therapies. In the TNBC cohort (n = 58), the ORR was 5.2% in PD-L1 nonselected patients, and patients with PD-L1-positive (assessed by PD-L1 73-10 pharmDx assay) disease had an ORR of 22.2% (41), which further clarified the PD-L1 prevalence is an important predictor of immunotherapy. Additionally, the PCD4989g study showed that atezolizumab monotherapy yielded an ORR of 11% and an OS of 7.3 months for TNBC patients in second-line and beyond setting (21).

2.4.2 Induction Therapy and Sequential Immunotherapy

To date, the timing of immunotherapy dosing remains to be further explored and studied. The TONIC study is a phase II RCT of nivolumab after induction therapy for metastatic TNBC (42). Patients were randomized into induction therapy groups (radiotherapy, doxorubicin, cyclophosphamide, cisplatin) or no induction therapy groups, followed by sequential nivolumab. The results showed that the ORR was 20% in all-line patients, including 23%, 45%, and 32% ORR for 1, 2, and later lines of patients, respectively. The ORR was 8%, 35%, 8%, and 23% in the radiotherapy, doxorubicin, cyclophosphamide, and cisplatin induction groups, respectively, compared with 17% in the noninduction group. Therefore, induction therapy with doxorubicin and cisplatin could improve the sensitivity of TNBC to immunotherapy. Future randomized controlled studies with larger samples comparing the efficacy differences between simultaneous versus sequential administration are expected.

2.4.3 Combination of Immunotherapy and PARP Inhibitor

DNA repair deficiency in cancer cells contributes to immunogenic neoantigens accumulation, and PARP blockade can upregulate PD-L1 expression in breast cancer cells (43). Thereby, the combined treatment of PARP inhibitor and PD-1/L1 inhibitor is a potential strategy to treat breast cancer. The phase II TOPACIO/KEYNOTE-162 trial showed promising antitumor activity of PARP inhibitor niraparib plus pembrolizumab in patients who had received a median of 1 prior line of therapy (0–3) in the metastatic setting (44). The ORR was 21% and DCR was 49%. In 15 *BRCA*-mutated patients, the ORR, DCR, and PFS was 47%, 80%, and 8.3 months, respectively, which were both greater than that of patients with wild-type *BRCA* (11%, 33%, 2.1 months). Furthermore, patients with PD-L1-positive (examined by PD-L1 22C3 pharmDx assay) cancers responded better than those with PD-L1-negative ones (32% vs. 8%). The most common grade 3 or higher AEs were anemia (18%), thrombocytopenia (15%), and fatigue (7%). The breast cancer cohort of the MEDIOLA study (open-label, multicenter, phase I/II) explored the efficacy of PARP inhibitor olaparib combined with durvalumab in advanced *BRCA*-mutated HER2-negative metastatic breast cancer, and the results showed that the ORR reached 63.3%, and the PFS and OS were 8.2 months and 21.5 months, respectively (45).

2.4.4 Combination of Immunotherapy and Immunomodulator

Imprime PGG (Imprime) is a novel immune agonist that activates the innate immune system to reregulate the immunosuppressive tumor microenvironment, activate antigen-presenting cells, and stimulate antigen-specific T-cell activation (46, 47). Preclinical studies showed that Imprime significantly enhanced the antitumor efficacy of ICIs (48). The phase II IMPRIME1 study investigated Imprime addition to pembrolizumab for second-line and later TNBC patients (49). The ORR was 15.9% and PFS was 16.4 months. The 12-month and 18-month OS rates were 57.6% and 36.7%, respectively. Notably, the study observed an ORR of 50% and an OS of 17.1 months in 12 patients who initially had hormone receptor-positive disease but converted to TNBC after endocrine therapy. Grades 3–4 AEs occurred in 6.8% of patients. These data validate the preclinical findings and provide clinical evidence for the immunomodulator-ICI combination in the treatment of TNBC. Large randomized controlled studies are needed to further clarify the advantages of this novel therapy.

Taken together, ICIs have been assessed in multiple settings for TNBC treatment. Two studies of pembrolizumab and atezolizumab in the adjuvant treatment of TNBC (SWOG S1418/NRG BR006, IMpassion030) are recruited (50, 51). The role of immunotherapy in the neoadjuvant treatment of TNBC still needs to be verified by updated EFS and OS data. We could see that the subset of TNBC patients benefited from immunotherapy mainly were these with PD-L1 expression ≥1%. Although the predictive threshold of PD-L1 varies across studies, in general, the benefit may be more pronounced with higher levels of PD-L1 expression. TMB is another predictor of ICI efficacy in TNBC patients. Furthermore, applying immunotherapy at earlier lines was associated with higher response rate. ICI is superior to chemotherapy in the maintenance treatment of metastatic TNBC. ICI monotherapy leads to suboptimal tumor response and patients' survival, and its combination with chemotherapy and (or) targeted therapy is more effective but accompanied by increased incidence of AEs. These findings suggest a meaningful clinical benefits of ICI addition to standard chemotherapy and (or) targeted agents for the treatment of locally advanced or metastatic TNBC. However, several questions such as optimal chemotherapeutic partner and sequence of administration and difference between anti-PD-1 and anti-PD-L1 inhibitors remain unknown. A phase II trial of pembrolizumab versus nivolumab versus atezolizumab, all combined with chemotherapy, for metastatic TNBC treatment is ongoing (NCT03952325). Additionally, according to transcriptomic profile, TNBC can be classified into luminal androgen receptor, immunomodulatory, basal-like immune-suppressed, and mesenchymal-like subtypes (52). Immunomodulatory TNBC is deemed to be sensitive to immune checkpoint blockade therapy (52). However, there are no data on the difference in the

responsiveness of TNBC subtypes to immunotherapy. Further studies of subtypes are needed to select benefited population and achieve precise immunotherapy for TNBC.

3 PD-1/L1 INHIBITORS FOR HER2-POSITIVE BREAST CANCER

Previous studies indicated that substantial quantities of lymphocytic infiltrate in the tumor stroma is associated with achieving a pathological complete response and having improved survival in patients with HER2-positive breast cancer (53–56). High expression of PD-1/L1 and other checkpoint molecules was observed in TILs (56, 57). Trastuzumab, an antibody of HER2, can exert antitumor immune effects through antibody-dependent cellular cytotoxicity and phagocytosis and complement-dependent cytotoxicity (58). Preclinical studies discovered that the combination of ICIs and trastuzumab could reverse trastuzumab resistance (59). Based on these evidences, several clinical trials evaluated the value of ICIs combined with anti-HER2 treatment in HER2-positive advanced breast cancer.

3.1 Second-Line or Later Therapy

3.1.1 Combination of Immunotherapy and Anti-HER2 Treatment

The phase Ib-II PANACEA study investigated the efficacy and safety of pembrolizumab plus trastuzumab in advanced HER2-positive breast cancer resistant to previous multiple lines of trastuzumab-containing therapies (60). Its phase II results showed that in HER2-positive, advanced, heavily pretreated breast cancer patients, the ORR of PD-L1-positive patients (n = 40, selected by PD-L1 22C3 pharmDx assay) was 15%, the median PFS was 2.7 months, the estimated 6-month PFS rate was 25%, and the 12-month PFS rate was 12%; the median OS has not been reached, and the 6-month and 12-month OS rates were estimated to be 87% and 65%, respectively. However, for PD-L1-negative patients (n = 12), no one achieved objective response or disease control, the median PFS was 2.5 months, and the estimated 6-month and 12-month PFS rates were 13% and 0%, respectively; the median OS was 7.0 months, 6-month OS rate was estimated to be 64%, and 1-year OS rate was estimated to be merely 12%. Moreover, patients achieving response and disease control had more TILs in metastatic lesions. In terms of AEs, 29% patients experienced treatment-related grades 3–5 AEs. The phase Ib CCTG IND.229 study tested the combination of durvalumab and trastuzumab in HER2-positive metastatic breast cancer patients who pretreated with trastuzumab, pertuzumab, T-DM1, and lapatinib (61). All enrolled 15 patients had PD-L1-negative (assessed by VENTANA PD-L1 SP263 assay) disease, and evaluable pretreatment and on-treatment tumor biopsies (n = 5) had sparse CD8 cell infiltration. The results showed that none of these patients achieved response and their long-term survival was also disappointing. Another phase Ib study explored the safety and efficacy of pembrolizumab in combination with T-DM1 in patients with HER2-positive metastatic breast cancer previously treated with trastuzumab, pertuzumab, and paclitaxel (62). The results showed that the overall ORR was 20%, PFS was 9.6 months, and DOR was 10.1 months. Additionally, no correlation between the expression level of PD-L1 (examined by PD-L1 22C3 pharmDx assay) or the proportion of TILs and efficacy was observed. The randomized phase II KATE2 study compared the efficacy of T-DM1 combined with atezolizumab with T-DM1 alone in the second-line treatment of HER2-positive breast cancer (63). The results showed that there was no significant difference in PFS between the T-DM1 plus atezolizumab group and the T-DM1 plus placebo group (8.2 months vs. 6.8 months, HR 0.82, 95% CI 0.55–1.23), neither in the PD-L1-positive (diagnosed by VENTANA PD-L1 SP142 assay) subgroup (8.5 months vs. 4.1 months, HR 0.60, 95% CI 0.32–1.11) nor in the PD-L1-negative subgroup (6.8 months vs. 8.2 months, HR 1.02, 95% CI 0.60–1.74). In the PD-L1-positive subgroup, the ORR was higher in the atezolizumab group (54% vs. 33%). However, in PD-L1-negative patients, the ORR was inferior in the combination group (39% vs. 50%). The OS curves of the two groups separated after 1 year of follow-up, and the median OS has not been reached.

From the above studies, the efficacy of PD-1/L1 inhibitors combined with anti-HER2 therapy for heavily pretreated HER2-positive advanced breast cancer seems to be unsatisfactory. There are no reliable markers that can accurately predict the benefited population. Notably, these studies had small sample sizes, included patients who had heavy tumor burden and progressed on multiple prior anti-HER2 therapies, which possibly explained the suboptimal results. In the future, it may be worth to assess the tumor microenvironment, explore practical immune-related predictive biomarkers of efficacy, and apply ICIs combined with anti-HER2 therapy in early-stage or first-line setting of HER2-positive breast cancer.

4 PD-1/L1 INHIBITORS FOR HORMONE RECEPTOR-POSITIVE/ HER2-NEGATIVE BREAST CANCER

Compared with other subtypes, hormone receptor-positive breast cancer is characterized as immunologically cold nature with lower PD-L1 expression, lower levels of TILs, and lower TMB (13, 64, 65). The efficacy of PD-1/PD-L1 inhibitor monotherapy in hormone receptor-positive metastatic breast cancer is limited (41, 66). Therefore, immune combination therapy is an approach to improve the efficacy of immunotherapy in hormone receptor-positive breast cancer. The I-SPY2 study included 52 hormone receptor-positive/HER2-negative patients (18). The combination of durvalumab, olaparib, and chemotherapy was promising for hormone receptor-positive breast cancer patients at high risk of MammaPrint, with a pCR of 28% compared with 14% in the chemotherapy-alone group (18). The phase II CheckMate7A8 (NCT04075604) (67) and phase III CheckMate7FL (NCT04109066) (68) about nivolumab combined with endocrine therapy in the neoadjuvant setting of hormone receptor-positive/HER2-negative breast cancer are enrolling. However, the addition of pembrolizumab to eribulin

for metastatic hormone receptor-positive breast cancer patients who pretreated with 0 to 2 lines of salvage chemotherapy did not improve ORR, PFS, or OS (immature) compared with eribulin alone (69). Cyclin-dependent kinase (CDK) 4 and 6 inhibitors were demonstrated to be capable of increase levels of tumor-infiltrating T cells and yield synergic antitumor efficacy with anti-PD-1/L1 inhibitors in preclinical studies (70, 71). A phase Ib trial assessed the safety and antitumor activity of pembrolizumab plus abemaciclib in endocrine-resistant hormone receptor-positive/HER2-negative patients who were pretreated with 1 or 2 chemotherapy regimens for metastatic disease (72). The results showed that the ORR and DCR was 29% and 82%, respectively. Median PFS reached 8.9 months, and OS reached 26.3 months. Safety was generally tolerable and consistent with known side effects of individual drug.

5 PREDICTIVE BIOMARKERS OF EFFICACY

Current studies suggested that not all patients were sensitive to immunotherapy or immune combination therapy. Therefore, it is essential to explore biomarkers predictive of efficacy to screen beneficiary populations and avoid blind application of expensive but minimally effective agents. Some studies have shed some light on us about selecting sensitive subpopulation.

In SAFIR02-BREAST IMMUNO study, exploratory analyses identified *CD274* gene (encodes the CD274 molecule namely PD-L1) amplification as a potential biomarker of sensitivity to durvalumab (19); however, tumor infiltration lymphocytes (CD8, FoxP3, and CD103) and homologous recombination deficiency did not predict that (19). Exploratory efficacy analyses in IMpassion130 suggested that PD-L1 expressed on tumor-infiltrating immune cells is the most powerful biomarker for predicting survival benefits of immunotherapeutic regimen for patients with untreated advanced or metastatic TNBC (73). In the study by Schmid et al., AKT inhibitor plus atezolizumab and chemotherapy benefited TNBC patients irrespective of PD-L1 status and PIK3CA/AKT1/PTEN alteration status (74). Tolaney et al. found that PD-L1 detected by PD-L1 22C3 assay did not predict the efficacy of pembrolizumab in combination with eribulin in hormone receptor-positive patients (69). Notably, PD-L1 detection approaches differ in studies because of the differences in assays and interpretation standards, which leads to inconsistent PD-L1 prevalence. For example, the *post-hoc* analysis of IMpassion130 found that the PD-L1-positive percentage was 46% for SP142 assay, 81% for 22C3 assay, and 75% for SP263 assay (75). Standardization of detection assays is another challenge of precisely guiding the prescription of immunotherapy. Studies have shown that high tumor mutation burden (TMB) can predict the efficacy of breast cancer immunotherapy (76, 77), but there is no uniform standard for TMB threshold. Although there are some discoveries in biomarker exploration currently, and PD-L1 is recognized, it is still insufficient for tumor microenvironment and biomarker research. Future studies that identify biomarker-defined subgroups are needed to select breast cancer patients that could significantly benefit from immunotherapy.

6 CONCLUSIONS AND EXPECTATIONS

Immunotherapy has developed rapidly in the field of breast cancer, especially in the exploration of TNBC treatment. Immune combination therapy including immunotherapy combined with chemotherapy, targeted therapy, or immune agonists has shown good efficacy and tolerable safety, which is superior to ICI monotherapy. However, there is no consensus on the difference between PD-1/L1 antibodies, optimal partners for combined treatments, the effect of dosing sequence on efficacy, and how long immunotherapy should be administered. The identification of predictive biomarkers of efficacy requires further exploration. Although a correlation between PD-L1 expression level and efficacy has been illustrated in multiple studies, some studies observed that PD-L1-negative patients could also benefit from immunotherapy. There are considerable variations between subtypes (triple-negative vs. other subtypes) and disease settings (early-stage vs. advanced-stage). Overall, the earlier stage immunotherapy is dosed, the higher the response rate. In addition, studies on immunotherapy combined with radiotherapy or local ablation therapy are ongoing. Immunotherapy is promising in the treatment of various types of breast cancer.

AUTHOR CONTRIBUTIONS

FC carried out the primary literature search, drafted and revised the manuscript, and participated in discussions. NC, YG, LJ, and ZL helped modify the manuscript. JC carried out the literature analysis, drafted and revised the manuscript, and participated in discussions. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.724424/full#supplementary-material

Supplementary Table 1 | Main characteristics of cited studies. mTNBC, metastatic triple-negative breast cancer; pCR, pathological complete response; EFS, event-free survival; IDFS, invasive disease-free survival; PFS, progression-free survival; OS, overall survival; ORR, objective response rate; AEs, adverse events; DCR, disease control rate; m, months. *Durvalumab 2 weeks before the beginning of nab-paclitaxel.

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Prospects of Immunotherapy for Triple-Negative Breast Cancer

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In the classification and typing of breast cancer, triple-negative breast cancer (TNBC) is one type of refractory breast cancer, while chemotherapy stays in the traditional treatment methods. However, the impact of chemotherapy is short-lived and may lead to recurrence due to incomplete killing of tumor cells. The occurrence, development, and relapse of breast cancer are relevant to T cell dysfunction, multiplied expression of related immune checkpoint molecules (ICIs) such as programmed death receptor 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) produce immunosuppressive effect. Immunotherapy (namely, immune checkpoint inhibitors, adoptive cellular immunotherapy, CAR-T immunotherapy and some potential treatments) provides new hope in TNBC. This review focuses on the new immune strategies of TNBC patients.

Keywords: triple-negative breast cancers, immunotherapy, immune checkpoint molecules, PD1/PD-L1 pathway, CTLA-4, combination therapy

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INTRODUCTION: TREATMENT AND PROGNOSIS OF TNBCs

According to the statistics of the World Health Organization (WHO), approximately 8.2 million people beings die of most cancers every year, accounting for 13% of international deaths. As one of the oldest tumors in the records of human civilization, breast cancer is the most clinically diagnosed cancer (1). In the classification and classification of breast cancer, breast cancer that does not express estrogen receptor (ER) or the progesterone receptor (PR) and does not amplify ERBB2 [commonly called human epidermal growth factor receptor 2 (HER2)] amplification are categorized as triple-negative breast cancer (TNBC), accounting for 10–20% of all breast cancers. TNBC subtypes were categorized by multi-omics data (2): (1) Intracavity androgen receptor subtype characterized by means of androgen receptor signal (23%); (2) Immunomodulatory (IM) subtype (accounting for 24% of tumors) with excessive immune cell signal and cytokine signal gene expression; (3) A basal-like and immune-suppressed (BLIS) (39%) subtype, characterized with the aid of upregulation of cell cycle, activation of DNA restore and downregulation of immune response genes; and (4) a mesenchymal-like (MES) subtype rich in breast stem cell pathway (15%). In addition, in the clinical patient population, we can see that TNBC is more common in young female patients. The tumor is usually large in size and of high grade, with greater lymph node metastasis at diagnosis, and has a

high biological aggressiveness. Compared with women with other breast cancer subtypes, female with TNBC have higher early distant recurrence rate and worse 5-year prognosis. Therefore, it is very indispensable to obtain the cure purpose at an early stage or manipulate disorder inside the controllable range. Currently, the essential scientific remedies for TNBC consist of surgical resection, chemotherapy, radiotherapy, targeted therapy (3). Conventional chemotherapy drugs, including paclitaxel, anthracycline and alkylating agents, are prone to systemic toxicity and side effects. In addition, as patients with advanced TNBC are highly metastatic and aggressive, it is difficult to achieve good results with targeted therapy or hormone therapy alone (4).

TNBCs AND IMMUNOTHERAPY

In general, the immune system of healthy individuals is strong enough to shortly get rid of the mutated most cancers cells, while the immune function of most cancer patients can't successfully recognize and kill tumor cells (5). On the other hand, most tumor cells have many distinct mechanisms to defend them from being identified by means of immune cells (6). Different from the traditional therapies mentioned above, immunotherapy cannot efficaciously kill most tumor cells alone, however additionally decorate the immunity of patients, in particular in the removal of minimal residual lesions and drug-resistant tumor cells. It can keep away from many shortcomings of other therapies to the greatest extent (7). Cell immunotherapy, as a new technology with targeted killing effect on tumor cells, has achieved good results in clinical application in recent years.

Immune Checkpoint Inhibitors

ICIs are inhibitory molecules expressed on the cell surface, which are usually involved in regulating the activation of T cells. Basically, its most essential feature is comparable to the braking device of an auto-cell, which makes it "brake" in time when the immune system is activated, continues the activation of the immune system within normal limits, and avoids over-activation of the immune system. No matter whether overexpression or over-function of immune checkpoint molecules leads to suppression of immune function, resulting in low immunity and susceptibility to tumor and other diseases (8). Another way to think about it is that if the immunosuppressive function of checkpoint molecules is poor, the immune function of the body will be abnormal. Recent studies have shown that molecular pathways of immune checkpoints, such as programmed death ligand 1 (PD-L1) and programmed death ligand 2 (PD-L2), play a very important negative regulatory role in tumor immunity (9–11).

CTLA-4 and TNBC

It is conventional that CTLA-4 is a negative regulator, which is very vital for T cell-mediated immunity. In T cells, CTLA-4 and CD28 bind to the equal ligands (CD80 and CD86) on antigen imparting cells and have contrary effects. The interplay between CTLA-4 and its ligand inhibits T cell reaction, and when CD28 and its ligand bind, T cell reaction is activated. The affinity of CTLA-4 to CD80/CD86 is greater than that of CD28 (12). The upregulation of CTLA-4 in cancer patients is considered as an important mediator of immune escape. Studies have shown that tumor cells of TNBC patients express CTLA-4 in different cell compartments (13). Its foremost ligand, CD80/CD86, is expressed in TNBC cell lines and tumors. This means that blocking CTLA-4 with Ipilimumab (anti-CTLA-4 monoclonal antibody, which has been accredited as checkpoint inhibitor for melanoma treatment) can significantly activate the molecular cascade, which may help enhance the immune response to tumor cells (14). CTLA-4 expressed on the surface of tumor cells during the treatment of patients with TNBC may be the target of checkpoint inhibitors and a candidate biomarker for immunotherapy. In a word, we believe that the operation or chemotherapy of TNBC patients, not only can the combination of targeted immune checkpoint drug therapy play a synergistic role to a great extent, but also can increase the cure probability of cancer patients.

PD-1 and PD-L1

PD-1 antibody is a most researched and clinically developed immunotherapy. PD-1 is expressed in activated T cells, B cells, and myeloid cells. It has two ligands, PD-L1 and PD-L2. The binding of PD-1 and PD-L1 mediates the co-inhibitory signal of T cell activation, suppresses the killing function of T cells, and performs a negative regulatory role in human immune response (15–17). In a normal immune system, PD-1 is up to preserve the position of immune tolerance. Tumor cells can escape immune surveillance through immune escape. Targeted therapy based on immunosuppressive receptors and immunosuppressive checkpoint immunotherapy based on immune molecules are new hotspots in oncology research (18, 19). It is additionally discovered that PD-L1 binds to PD-1 receptor on activated T cells and weakens anti-tumor immunity by inhibiting T cell activation signal. PD-1^+ T cells can partially recover by blocking PD-1/PD-L1 signaling pathway (20–24). Some studies have proven that PD-L1 antibody combined with paclitaxel is effective in treating advanced in the treatment of advanced TNBCs (25). TNBC subtype research based on multi-group data shows that immunoglobulin subtype has high immune cell signal (2). Both clinical and economic characteristics indicate that immune recognition is activated in IM subtype, which shows that the mechanism of immune break out of these tumors may additionally contain the recruitment of immunosuppressive cells or the activation of immune checkpoint molecules. Based on what has been discussed above, we may conclude that high expression levels of immune checkpoint suppressor genes such as PD1, PD-L1, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), and IDO1 (Indoleamine 2,3-dioxygenase 1) may inhibit the activation of the immune system and lead to the occurrence of TNBC. A Phase III trials confirmed that Atezizumab (PD-L1 inhibitor) and nabo-paclitaxel in the treatment of advanced TNBC, compared with placebo + nabo-paclitaxel, atezizumab +

nabo- Paclitaxel can significantly improve the progression-free survival (PFS) (7.5 months vs 5.5 months, respectively) and overall survival (OS) (25.0 months vs 15.5 months, respectively) (26). The response rate of TNBC to ICIs is higher than that of hormone-receptor positive and HER2-positive breast cancers. Recently, the inhibitory effect of carbamazepine plus apatinib on PD-1 in advanced patients in Phase II trial was found, and carbamazepine plus apatinib had good tolerance and showed good ORR (43.3%) and PFS in advanced patients, regardless of lines of therapy and PD-L1 status [NCT03394287] (27). Furthermore, PD-1/PD-L1 inhibitors combined with chemotherapy are more successful in TNBC than single dose ICIs. These results indicate that combinations with chemotherapy could increase the response rate to immunotherapy compared to chemotherapy or immune checkpoint blockade alone.

We summarize the relevant clinical studies in the treatment of TNBC by ICIs in recent years (**Figures 1**–**3**) (28–43).

Adoptive Cellular Immunotherapy ($CD8^+$ T Cells)

The tumor immune microenvironment (TIME) plays a critical role in the progression, response to therapy and prognosis of most cancer patients. Tumor-infiltrating lymphocytes (TIL) are one of the predominant components of TIME, and the density and types of lymphocytes in the TIL fraction of a tumor have marked prognostic associations in breast cancer. This is especially actual of TNBC, which has the largest number of TILs. CD8 T cells are necessary immune cell in TIL (44). The infiltration of $CD8^+$ T lymphocytes into solid tumors is related to the good prognosis of various types of cancers including TNBC (45). The T cell antigen receptor (TCR) of $CD8^+$ T cells recognizes an antigenic peptide containing approximately 13–17 amino acids. It consists of major histocompatibility complex I molecules (MHC-I). Some research have observed that in TNBC transgenic mouse model, the levels of IFN-γ and TNF-α increased tumor-invasive $CD8^+$ T cells, and subsequently led to apoptosis (46). Inhibition of Tregs (Regulatory cells) amplification can enhance the anti-tumor response of $CD8^+$ T cells, thus affecting the growth of primary breast tumors or the metastasis of cancer cells to the lung (47, 48). Some studies have shown that LXR-inverse (Liver-X-Receptors) activation stimulates immune-mediated tumor destruction by means of improving CD8 T-cell activity in TNBC (49). It has currently been proved that PARP inhibitor olaparib induces $CD8^+$ T cell infiltration in TNBC model with a BRCA1-deficient (breast cancer 1) deficiency by activating STING (interferon gene) pathway. Similarly, the efficacy of PARP inhibitors depends on the recruitment of $CD8^+$ T cells in BRCA deficient TNBC model by activating intracavitary STING pathway (50, 51). STING-targeted immunotherapy enhances anti-tumor immunity mediated by natural killer cells and $CD8^+$ T cells. It provides a theoretical basis for combining PARP inhibitors with CAR-T (Chimeric antigen receptor T) cell remedy to deal with TNBC disease.

CAR-T and CAR-NK

CAR-T immunotherapy, which directly retargets the immune system od the patient to perceive and eradicate tumor cells with tumor-associated antigens (TAAs), and is presently being explored as a treatment for TNBCs (52, 53). However, immunotherapy is a new technology, and many bottlenecks remain to be overcome. For example, identifying specific goal tumor antigens and designing effective CAR is one of the many challenges of CAR-T therapy. Studies have shown that epidermal growth factor receptor-CAR (EGFR-CAR) lentivirus-infected T cells have a robust specific inhibitory effect on the growth of TNBC cells and tumor occurrence *in vitro* and *in vivo* (54). Some s find out about exhibit that ICAM1 (intercellular adhesion molecule-1)-specific CAR-T cells have been in a position to efficiently recognize ICAM1 expressing TNBC cells, and they can effectively minimize the growth of TNBC tumor inside and outside (55). Recently, the University of Pennsylvania completed a first phase scientific trial, which studied the security of injecting c-Met-CAR-T cell into TNBC patients [NCT01837602] (56). Results Inflammatory reaction was induced in TNBC tumor, and there was no evidence that drug-related side effects were greater than grade 1. Up to now, the research on MUC1 (Mucin1 glycoprotein)-CAR-T cell therapy has been the most investigated in clinical trials (57). The safety and efficacy of autologous MUC1-CAR-T cells are proposed to be evaluated in a phase I/II study in patients with relapsed or refractory TNBC [NCT02587689] (56). In addition, the inhibition of TGF-β-receptor signaling augments the anti-tumor function of ROR1 (receptor-tyrosine-kinase-like orphan receptor 1)-specific CAR T-cells against TNBC (58). Moreover,

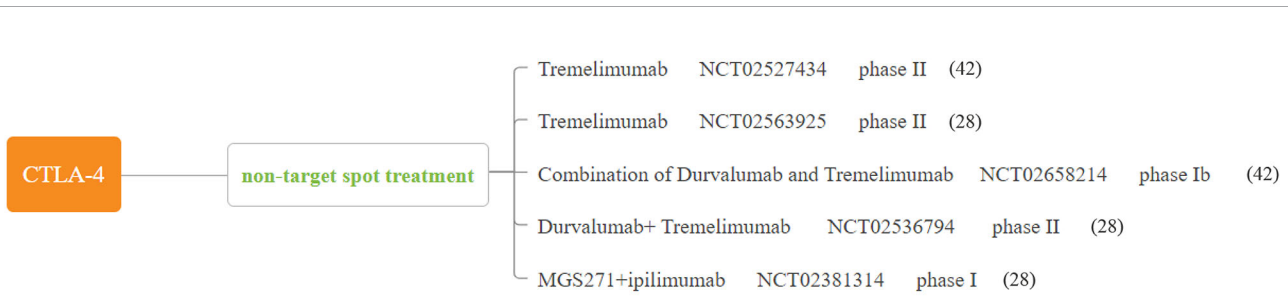

FIGURE 1 | Major published clinical trials using CTLA-4 inhibitors in TNBC.

FIGURE 2 | Major published clinical trials using PD-1 inhibitors in TNBC.

when recruiting participants, the safety and tolerance of allogeneic gamma delta (γδ) T cells transduced with CARs targeting NKG2D ligands on TNBC cells will be investigated in Phase I clinical trials [NCT04107142] (59, 60). The number of clinical trials of TNBC that CAR-T cell therapy is increasing, which may produce some exciting clinical effects. In addition, NK cells play a prominent role in the innate immune system because multiple receptors on the surface of the NK cells have been approved to kill cancer cells by interacting with their ligands of cancer cells, leading to apoptosis o cancer cells. Studies have shown that tissue factor as a new target for CAR-NK cell immunotherapy of TNBC (61). As EGFR is a potential therapeutic target for TNBC, EGFR-specific CAR NK cells (EGFR-CAR NK cells) is a promising strategy to inhibit tumor growth in breast cancer cell line-derived xenograft (CLDX) and patient-derived xenograft (PDX) mouse models (62).

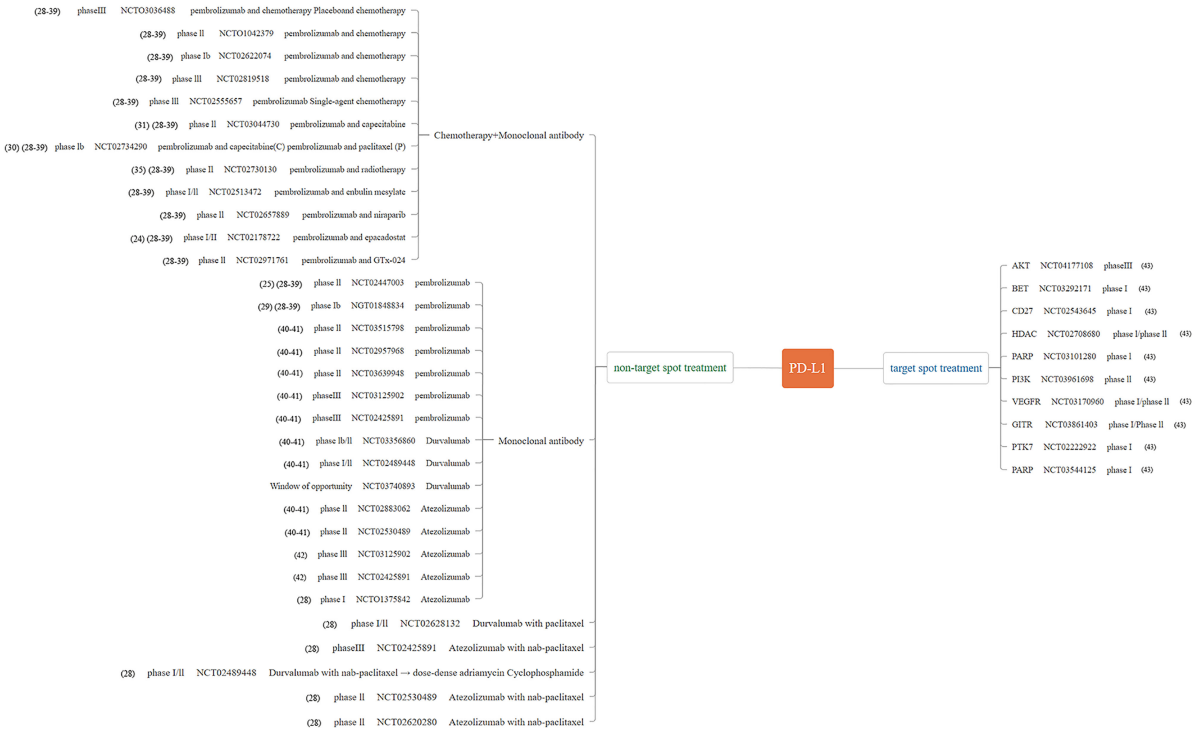

FIGURE 3 | Major published clinical trials using PD-L1 inhibitors in TNBC.

COMBINATION THERAPY

In targeted therapy of TNBC, there are some small molecule therapeutic targets, namely, PARP, DNA (cytosine-5)-methyltransferase 1(DNMT1), epidermal growth factor (EGF) and EGF receptor (EGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor (VEGF), and VEGF receptor (VEGFR) (63, 64). In TNBC subtype, basic helix-loop-helix (bHLH) transcription factors inhibitor of differentiation 1 (ID1) and inhibitor of differentiation 3 (ID3) (referred to as Id) play a vital role in maintaining cancer stem cell (CSC). Many molecules have been in preclinical trials. The application of ispinesib (a small molecule inhibitor in the ID1+ CSC results) to target the ID/Kif11 pathway, combined with chemotherapy, gave better response in TNBC subtype (65). This targeting ID1–Kif11 molecular pathway in the ID1+ CSCs, combined with chemotherapy and small molecular inhibitor, may reduce TNBC effect more effectively.

In addition, another promising strategy for combination therapy is to turn the "cold" tumors "hot" (66). Through a variety of methods, such as attracting T cell to the tumor through chemotherapy, radiation therapy, vaccines, and oncolytic viruses and bispecific antibodies (67). Other combination strategies include inhibition of other checkpoints or other immunosuppressive mechanisms, or enhancement of the activity of other checkpoint agonists, combination therapy to overcome T cell exhaustion, or conversion of immunosuppression [e.g., regulatory T cells (Tregs), myeloid-derived suppressor cells] into immunoreactive phenotypes (68, 69). In TNBC, however, chemotherapy combination of Atezolizumab enhanced the antitumor efficacy of Nab-paclitaxel only in patients with PD-L1 expression on tumor-infiltrating immune cells [NCT03371017] (26). On the other hand, chemotherapy combination of pembrolizumab paclitaxel protein-bound, or paclitaxel, or gemcitabine plus carboplatin also benefit patients with TNBC [NCT02819518] (70). Above all, tumors that respond to immune checkpoint inhibitors are typically so-called thermal or "hot" tumors with CD8 T cell infiltration, indicating that tumor cells are recognized by the immune system. CD8 positivity is often assessed as a predictor of response and a pharmacodynamic marker of response to combination therapies, which are hypothesized to enhance T cell infiltration and heat so-called "cold" tumors (71). Similarly, TNBC features immunological "cold" tumor, which with limited tumor infiltrating lymphocytes (72). To address this problem, we need to find a methodological strategy that actively recruits $CD8^+$ T cells into the tumor microenvironment (TME), reverses "cold" tumors into "hot" tumors, and significantly improves their reactivity to ICIs (73).

POTENTIAL THERAPEUTIC DIRECTIONS AND POSSIBLE STRATEGIES

Mesothelin and TNBC

Tumor-associated antigen-mesothelin (MSLN) is a glycoprotein that exists on the cell surface and is highly expressed in various

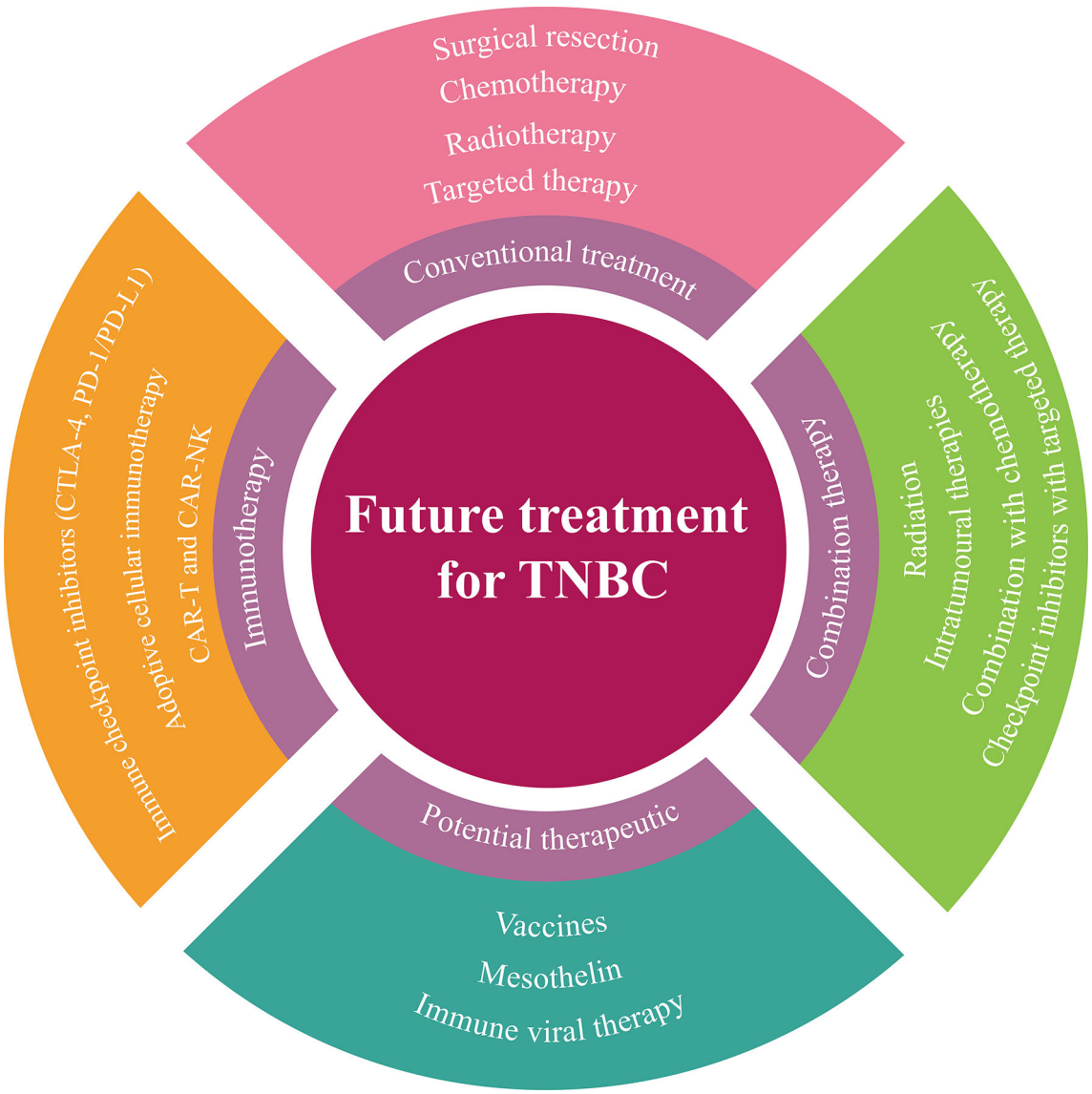

FIGURE 4 | A summary of future treatment strategies for TNBC.

tumor tissues such as mesothelioma, non-small cell lung cancer, pancreatic cancer, and metastatic triple negative breast cancer, while no longer expressed in normal tissues or is low expressed in mesothelial tissues (74). Due to the characteristic of MSLN, it has become the focus of specific targeting antigen of tumor cells. Recent lookup used to be discovery of MSLN, a carcinogenic glycosyl-phosphatidyl-inositol (GPI) is overexpressed in TNBC (75). Above all, MSLN additionally play an vital position in T cell cloning and expansion and effector function, including initiating T cell activation (76). MSLN immune-targeted therapy (mAbs, CAR-T, vaccine) has top notch potential, and many of them have entered clinical trials of pancreatic cancer and lung cancer (76, 77). As a new personalized therapy, MSLN targeted therapy may achieve positive clinical results in TNBC patients.

TNBC and Immune Viral Therapy

Recent trends in viral genetic engineering have allowed the development of oncolytic viruses with enhanced recognition capability to receptors overexpressed in tumor tissues, and viruses encoding or packaging suicide or pro-apoptotic genes or agents for delivery to cancer cells (78). Viruses can be manipulated to upregulate antigen presentation and T cell anti-tumor response. Talimogene laherparepvec (T-Vec, OncoVEXGM-CSF, Imlygic), an attenuated and genetically engineered herpes simplex virus (HSV) that overexpresses granulocyte-macrophage colony-stimulating factor (GM-CSF), is the only oncolytic virus approved for clinical use in the United States and Europe (ClinicalTrials.gov:NCT00769704) (79–81). Some studies have shown that cell vaccines primarily based on

oncolytic vesicular stomatitis virus can improve the prognosis of TNBC by enhancing the functions of natural killer cells and $CD8^+$ T cells (82). An oncolytic herpes simplex virus, which encodes the fundamental anti-tumor cytokine, interleukin 12 (IL-12), (designated G471-mIL12), can selectively kill cancer cells while inducing anti-tumor immunity (83), which is mainly manifested by the upregulation of $CD8^+$ T cells activation markers in tumor microenvironment and the inhibition of tumor angiogenesis (84). Immunovirotherapy may be a promising method to treat TNBC patients.

TNBC and Vaccines

Some studies have shown that mixed 19-peptide vaccine alone can achieve positive results in the treatment of refractory TNBC (85). The multi-epitope DNA and peptide vaccines is composed of the most immune dominant epitopes of SYCP1 (Synaptonemal Complex Protein 1) and ACRBP (Acrosin Binding Protein). As two conventional cancer/testis antigens, it can effectively activate the cellular and humoral immune response against 4T1 mouse breast tumor. In addition, this preventive combined immunization can drastically inhibit the growth of this mouse triple negative breast tumor (86). However, there are still some problems to be solved about vaccines, such as time, administration frequency and combination therapy strategy.

CONCLUSIONS

Recently, immunotherapy has delivered new hope to TNBC. The application of ICIs in TNBC will bring new light and advantage to patients. TNBC is currently exploring other new immunotherapy strategies, consisting of oncolytic virus and adoptive cell therapy, such as TIL metastasis and carcinoembryonic antigen T cells. Breast cancer vaccine constitutes another new therapeutic strategy to enhance anti-cancer immunity. Although the new preliminary immunotherapy still needs extensive clinical verification, these immunotherapies will promote the understanding of anti-cancer immunity of breast cancer and contribute to the development of effective strategies in the future. Further understanding of the mechanisms underlying immu-oncology are warranted to identify new immunotherapy-sensitive tumor types, combinations of different therapies will also become a promising strategy in the treatment of TNBC (**Figure 4**).

AUTHOR CONTRIBUTIONS

DQ: Writing - original draft, Writing—Review & editing. GZ, XY, and XX: Resources, Writing—Original draft. XM, SL, JW, XL, WW, JL, and YM: Participate in article writing. MM: Conceptualization, Writing—Original draft, Writing—Review & editing, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Multi-Omics Analyses Revealed GOLT1B as a Potential Prognostic Gene in Breast Cancer Probably Regulating the Immune Microenvironment

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As recently reported by The International Agency for Research on Cancer (IARC), breast cancer has the highest incidence of all cancers in 2020. Many studies have revealed that golgi apparatus is closely associated with the development of breast cancer. However, the role of golgi apparatus in immune microenvironment is still not clear. In this study, using RNA-Seq datasets of breast cancer patients from the public database (n = 1080), we revealed that GOLT1B, encoding a golgi vesicle transporter protein, was significantly higher expressed in human breast cancer tissues versus normal tissues. Besides, we verified GOLT1B expression in five breast cancer cell line using our original data and found GOLT1B was significantly up-regulated in MDA-MB-231, MCF-7, SKBR3. Subsequently, we identified GOLT1B as a potential independent prognostic factor for breast cancer. After a multi-omics analysis, we uncovered that the higher expression of GOLT1B in breast cancer tissues versus normal tissues might be due to the amplification of GOLT1B and altered phosphorylation of its potential transcriptional factors, including JUN and SIN3A. Subsequently, we discovered that GOLT1B potentially regulated the immune microenvironment basing on the finding that its expression was closely related to the tumor microenvironment score and infiltration of immune cells. Moreover, we revealed that GOLT1B might affect the overall survival rates of breast cancer through regulating the immune cell infiltration. Finally, we disclosed the potential pathways involved in the functions of GOLT1B in breast cancer, including metabolism and ECM-receptor interaction pathways. To sum up, we identified GOLT1B as a potential prognostic gene for breast cancer and disclosed its role in regulating the immune microenvironment.

Keywords: GOLT1B, Golgi apparatus, immune microenvironment, breast cancer, prognostic biomarker

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INTRODUCTION

The International Agency for Research on Cancer (IARC) has released "the latest data on the global burden of cancer in 2020" (1) showing that the incidence of breast cancer has replaced lung cancer in the first place in the world, accounting for 11.7% of new cancer cases. Nowadays, with the progress of diagnosis and treatment, although the 5-year survival rate of breast cancer has been improved, the mortality rate of patients with advanced breast cancer still reach to more than 70% (2). Nevertheless, distinguishing high-risk patients by prognostic biomarkers can frequently lead to an appropriate individualized treatment and thus reduce the mortality.

Recently, immunotherapy has attracted a lot of attention because of its long-lasting responses to different types of tumors, even advanced tumors (3, 4). However, the effects of immunotherapy in breast cancer are not satisfactory. Consequently, there is a necessary to further explore the mechanism underlying the development of breast cancer and search for key regulatory genes of immune microenvironment. Golgi apparatus is a secretory organelle composed of many flat vesicles in eukaryotic organisms (5). It's mainly involved in biological processes including protein processing, sorting, and transportation. Golgi apparatus is closely related to innate immune signal transduction and subsequent effect response (6). Although the essential role of the golgi apparatus in carcinogenesis has been well characterized, its functions in tumor immune microenvironment are still unclear.

GOLT1B encodes a golgi vesicle transporter that mediates vesicle transport between endoplasmic reticulum and golgi apparatus and is highly conserved in function. One current study has uncovered that the overexpression of GOLT1B can elevate the cell membrane level of DVL2, then activate Wnt/β-catenin pathway, increase the content of nuclear β-catenin, and subsequently induce the process named epithelial-mesenchymal transformation. On the other hand, GOLT1B also promotes the migration and invasion of colorectal cancer *via* inducing T lymphocyte apoptosis (7). Poorer prognosis has been observed in patients with GOLT1B amplifications in lung adenocarcinoma (8). However, little is known about the functions of GOLT1B in tumors. The role of GOLT1B in breast cancer and its functions in immune microenvironment have not been disclosed.

Here, using the multi-platform datasets from the public database, we described the expression, survival correlation, and potential prognostic values of GOLT1B in breast cancer, uncovered GOLT1B potential upstream regulators and relevant pathways, and demonstrated the probable functions of GOLT1B in immune microenvironment.

MATERIALS AND METHODS

Tumor Immune Estimation Resource (TIMER)

TIMER is a comprehensive web resource for systematical evaluations of immune cells in diverse cancers. Differential expression of GOLT1B in 27 tumor tissues versus adjacent normal tissues from the Cancer Genome Atlas (TCGA) was studied using the Diffexp module.

The Human Protein Atlas (HPA)

HPA provides the immunohistochemical results of protein expression in normal tissues and tumor tissues. The protein expression of GOLT1B in normal mammary tissue and breast carcinoma tissues was evaluated using the HPA.

UALCAN

UALCAN is a database for analysis and mining of transcriptome data from TCGA. The mRNA expression of GOLT1B and the relationship between GOLT1B and clinicopathological parameters of breast cancer (gender, tumor stage, lymph node metastatic status, age, ethnicity, and TP53 mutation status) was investigated using UALCAN. Besides, the protein and phosphorylation expression were also analyzed using UALCAN.

Kaplan-Meier Plotter

Kaplan-Meier Plotter database collects the datasets of gene chip and RNA-seq from European Genome-phenome Archive (EGA), TCGA, Gene Expression Omnibus(GEO), and other public databases. The overall survival rates (OS), relapse-free survival rates (RFS), and distant metastasis-free survival rates (DMFS) of GOLT1B in breast cancer were analyzed using Kaplan-Meier Plotter database. Besides, the relationship between GOLT1B expression and the OS of breast cancer patients with different immune infiltration was also investigated using Kaplan-Meier Plotter database.

Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA is a database analyzing gene expression based on datasets from genotype tissue expression (GTEx) and TCGA. In this study, the expression of GOLT1B in breast carcinoma tissues and mammary tissues was evaluated using the module "Expression DIY" of GEPIA.

cBioPortal

The cBioPortal database is a genomic database characterizing gene mutations in distinct tumors. More than 28,000 samples from different independent studies were included in this database. The type and frequency of GOLT1B mutations in invasive breast cancer were analyzed using the module named "Oncoprint" and "Cancer Types Summary" of cBioPortal.

Linkedomics

Linkedomics is a comprehensive database that contains multi-omics datasets within and across 32 cancer types. In our study, 5720 GOLT1B co-expressed proteins were obtained from the Linkedomics database, and the pathways that GOLT1B involved in were investigated using the module "Gene Set Enrichment Analysis (GSEA)" of Linkedomics.

Bioinformatics

The immune infiltration were investigated using CIBERSORT database following a standard protocol (9). The R package named survival v.2.4.2 was used to analyse the survival rates.

The potential prognostic value was calculated using the R package named Survival and RMS.

Cell Lines and Antibody

The immortalized normal mammary epithelial cell line MCF-10A and breast cancer cell lines MDA-MB-231, MDA-MB-231-Bone, SKBR3, MCF-7, and T47D were derived from the Key Laboratory of Clinical Laboratory diagnostics of Chongqing Medical University. The MCF-10A was cultured with MCF-10A cell-specific medium (Procell, CM-0525, China), MDA-MB-231, MDA-MB-231-Bone, SKBR3, MCF-7, and T47D were cultured with DMEM medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). Cells were placed in a humidified incubator at 37°C with 5% CO_2. The antibody of golt1b for western blotting was purchased from Invitrogen (PA5-103499) and the antibody of actin was purchased from Zoonbi (TE0303).

Statistical Analysis

The significance of differential expression was evaluated using Wilcoxon test. Logarithmic rank method was utilized to calculate the significance of survival analyses. A univariate Cox regression model was used to analyze the hazard ratio (HR) of GOLT1B, and a multivariate Cox regression model was used to determine potential independent prognostic factors. HR and confidence interval were set to 95%. The correlation coefficient between GOLT1B expression and immune infiltration was calculated using the Pearson tests. $p < 0.05$ was considered statistically significant. "*" indicated $p < 0.05$, "**" indicated $p < 0.01$, "***" indicated $p < 0.001$, and "****" indicated $p < 0.0001$.

RESULTS

The Expression of GOLT1B Is Increased in Breast Cancer Patients

To explore the functions of GOLT1B in tumorigenesis, we primarily analyzed the mRNA expression of GOLT1B in 27 human tumors using the datasets from TCGA and TIMER database. The results showed that GOLT1B was up-regulated in 25 types of tumors, such as breast invasive carcinoma (BRCA), adrenal cortical carcinoma (ACC), cholangiocarcinoma (CHOL), esophageal carcinoma (ESCA), clear cell carcinoma of the kidney (KIRC), squamous cell carcinoma of the head and neck (HNSC), liver hepatocellular carcinoma (LIHC), acute myeloid leukemia (LAML). Meanwhile, GOLT1B was significantly down-regulated in endometrial cancer (UCEC) and acute myeloid leukemia (LAML) (**Supplementary Figures S1A, B**). Focusing on breast cancer, we validated GOLT1B expression in breast cancer using GEPIA and UALCAN databases. Shown in **Figures 1A, B**, the mRNA expression of GOLT1B was higher in BRCA tissues than normal breast tissues. Besides, we also investigated the expression of GOLT1B in BRCA tissues and para-cancerous tissues using the datasets from TCGA, and the results showed that GOLT1B mRNA was significantly elevated in BRCA tissues (**Figure 1C**). Moreover, we disclosed that the expression of GOLT1B in BRCA was significantly increased using pair analysis of 112 BRCA tissues versus para-cancerous tissues (**Figure 1D**).

Furthermore, we evaluated the expression of GOLT1B protein in breast cancer tissues and normal tissues using The Human Protein Atlas database. As a result, GOLT1B protein was elevated in breast carcinima versus normal mammary tissues (**Figure 1E**). Next, we further confirm the protein expression level of GOLT1B in breast cancer cell lines using western blotting. The results showed that GOLT1B was higher expressed in MDA-MB-231 ($P = 0.0011$), SKBR3 ($P < 0.0001$) and MCF-7 ($P < 0.0001$) versus the normal mammary epithelial cell line MCF-10A (**Figure 1F**). These results indicated that the expression of GOLT1B was up-regulated in human breast cancer and implied a potentially important role of GOLT1B in cancer progression.

The Correlation Between GOLT1B Expression and Clinical Features of Breast Cancer

To clarify the correlation between the GOLT1B expression and clinical features of breast cancer, we studied GOLT1B mRNA expression in different groups using the UALCAN database. In terms of age, breast cancer patients aged 21-40, 41-60, and 61-80 years expressed higher levels of GOLT1B than healthy people ($P < 0.05$) (**Supplementary Figure S2A**). For gender, tumor stage and race, there was no significant difference between distinct groups (**Supplementary Figures S2B–D**). In terms of tumor subtypes, compared with luminal breast cancers, triple-negative type showed higher expression of GOLT1B ($P < 0.0001$), indicating that GOLT1B might be correlated with tumor malignancy (**Supplementary Figure S2E**). In terms of lymph node metastases, GOLT1B was lower expressed in patients classified as N3 than in N0, N1, and N2 patients ($P = 0.032$, $P = 0.033$, $P = 0.026$) (**Supplementary Figure S2F**). Finally, GOLT1B was up-expressed in patients with TP53 mutant than TP53 wild type ($P < 0.0001$) (**Supplementary Figure S2G**), implying that the high expression of GOLT1B might have a potential association with TP53 mutation.

Increased Expression of GOLT1B Predicts Poor Prognosis in Breast Cancer Patients

Since GOLT1B is potentially associated with the initiation and progression of breast cancer, we explored the relationship between GOLT1B mRNA expression and patient survival using the RNA-Seq datasets from TCGA. Analysis of breast cancer patients ($n = 1084$) showed that the OS, progression-free survival rate (PFI), and disease-specific survival rate (DSS) were lower in breast cancer patients expressing higher GOLT1B (**Figure 2A**). After analyzing the outcomes using Kaplan Meier database, we also revealed that breast cancer patients ($n = 1089$) with high expression of GOLT1B exhibited poorer OS, DMFS, and RFS (**Figure 2B**). Furthermore, we investigated the correlation between GOLT1B protein expression and patient survival and revealed that GOLT1B expression was extremely negatively related to patient OS, DMFS, and RFS (**Figure 2C**). Subsequently, to further clarify whether GOLT1B is one potential prognostic gene in human breast cancer, we analyzed other cohorts from prognoScan database and GEO (**Figure 2D**). Consequently, the corrected p values of OS (GSE1456), DSS

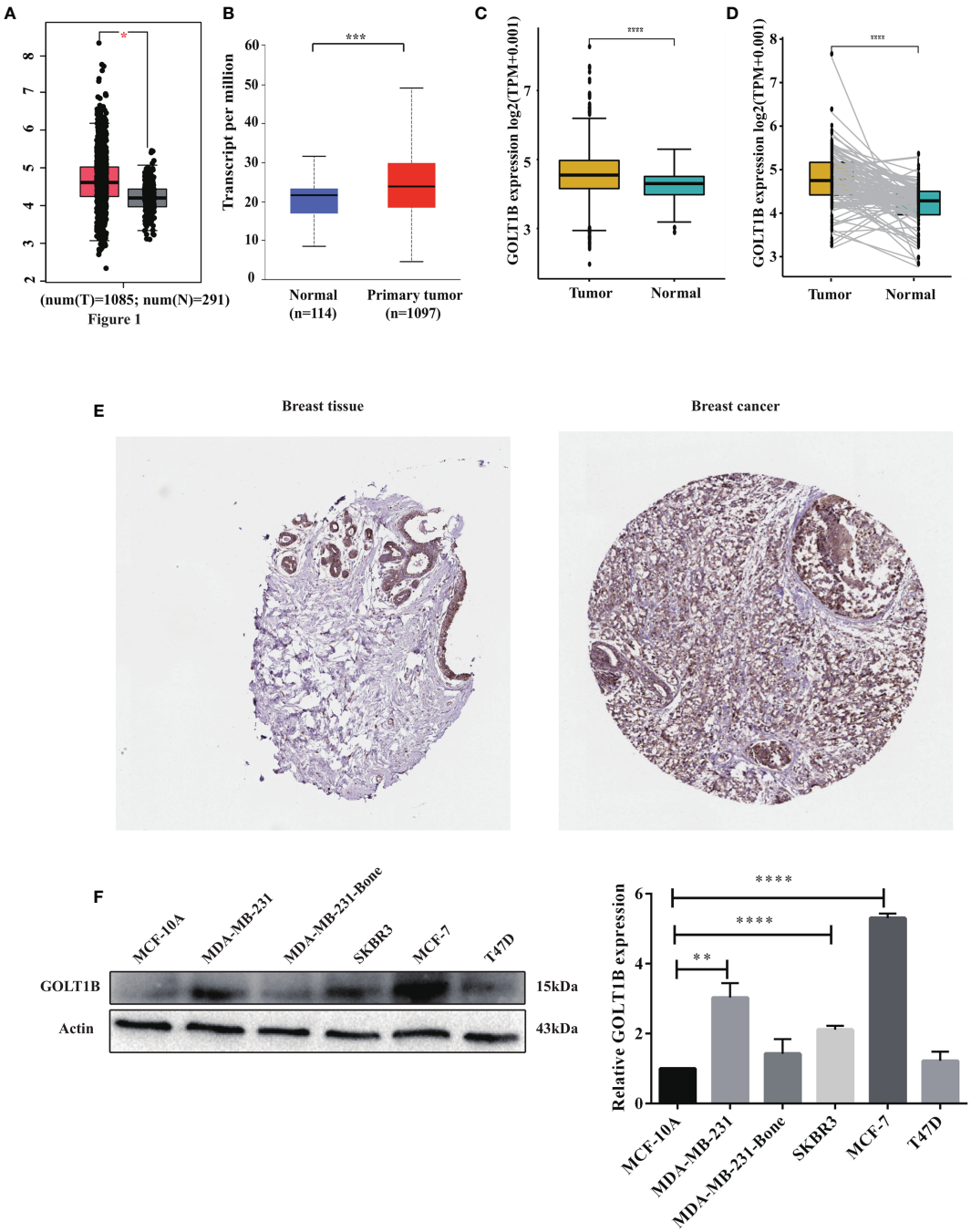

FIGURE 1 | The mRNA and protein expression of GOLT1B was up-regulated in human breast cancer. The expression of GOLT1B in breast cancer tissues versus normal tissues was investigated using the datasets from **(A)** the GEPIA database, **(B)** the UALCAN database, and **(C)** the TCGA database. **(D)** The GOLT1B expression in 112 paired breast cancer tissues and adjacent normal tissues using the datasets from TCGA database. **(E)** The protein expression of GOLT1B in breast cancer tissues and normal breast tissues was evaluated through immunohistochemical tests from The Human Protein Atlas database. **(F)** Western blotting detecting GOLT1B expression in normal mammary epithelial cell line and breast cancer cell lines. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(GSE1456) and RFS (GSE12276) were all less than 0.05, which additionally provided the possibility of GOLT1B as one prognostic gene in breast cancer. Based on the above results, it is suggested that the mRNA and protein expression of GOLT1B are both closely related to the outcomes of breast cancer. Furthermore, to evaluate the predictive value of GOLT1B, we performed Cox regression on various clinical features and GOLT1B expression. As a result, the univariate and multivariate Cox analyses identified GOLT1B as a

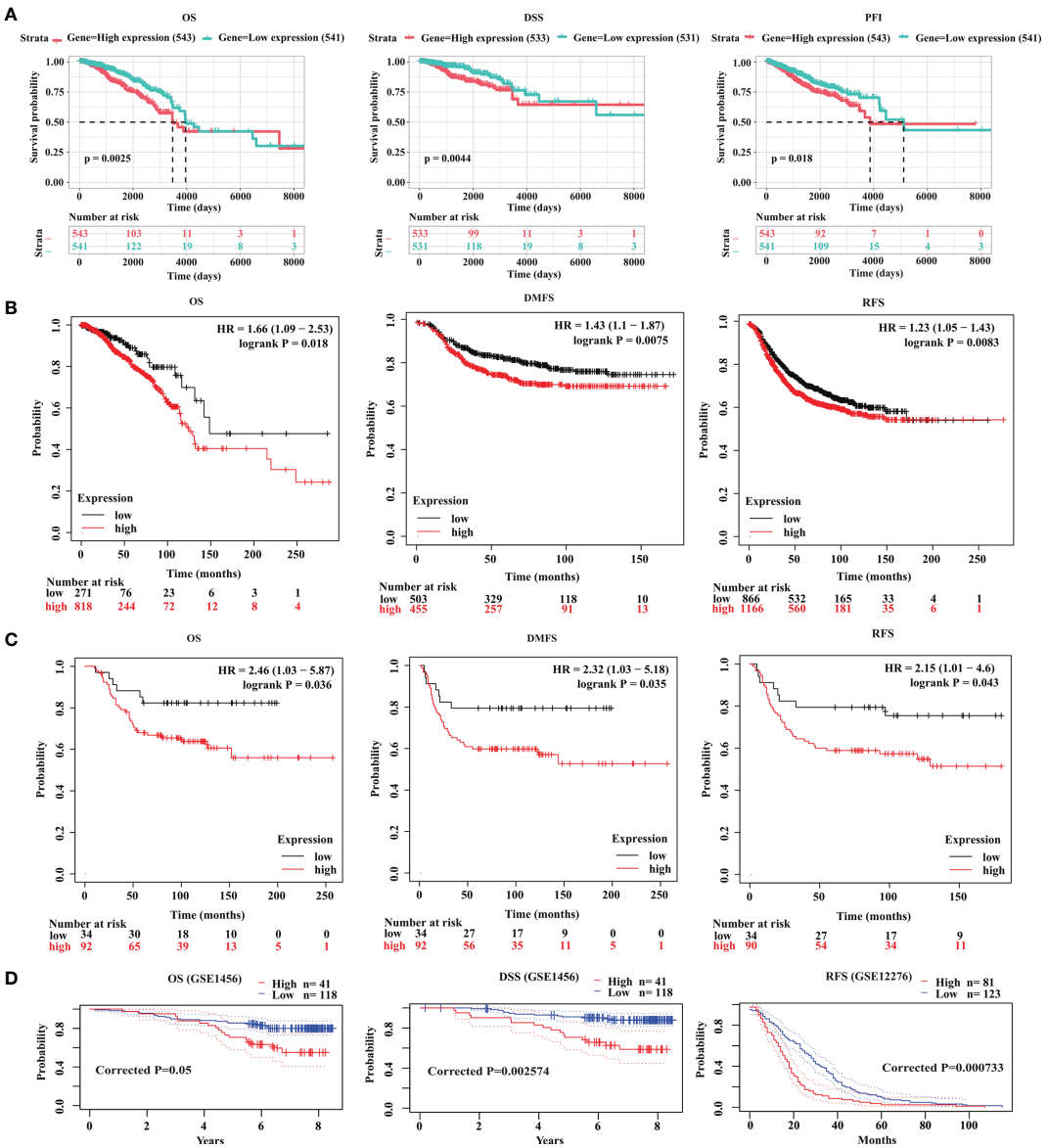

FIGURE 2 | GOLT1B is a potential prognostic factor for breast cancer. **(A)** The correlation between GOLT1B expression and OS, DSS, PFI of breast cancer patients using the datasets downloaded from TCGA database. The correlation between GOLT1B **(B)** mRNA expression and **(C)** protein expression with OS, DMFS, RFS of breast cancer patients as analyzed using the Kaplan-Meier plotter database. **(D)** The correlation between GOLT1B expression and OS, DSS, RFS of breast cancer patients using the datasets from prognoScan and GEO.

potential independent prognostic gene for breast cancer (**Figures 3A, B**), and it could be used in combination with other clinical diagnosis indicators to predict the prognosis of breast cancer (**Figures 3C, D**).

The Potential Mechanisms for the Up-Regulation of GOLT1B in Human Breast Cancer

It's well-known that the expression and activity of transcription factors have a close association with the expression of downstream genes. Therefore, to explore the potential mechanism underlying the up-regulation of GOLT1B in breast cancer, we performed a multi-omics analysis of the upstream transcriptional factors of GOLT1B. Firstly, we obtained 24 experimentally confirmed transcription factors of GOLT1B in breast tissues using the hTFtarget database (**Supplementary Table S1**). Meanwhile, we analyzed all co-expressed proteins of GOLT1B in breast tumor using the Linkomics database. As a result, 5721 proteins were found to be co-expressed with GOLT1B (**Supplementary Table S1**). The transcriptional factors of GOLT1B, including BRD4, JUN, MAX, and SIN3A, were discovered having a close expression correlation with GOLT1B (correlation coefficient > 0.5

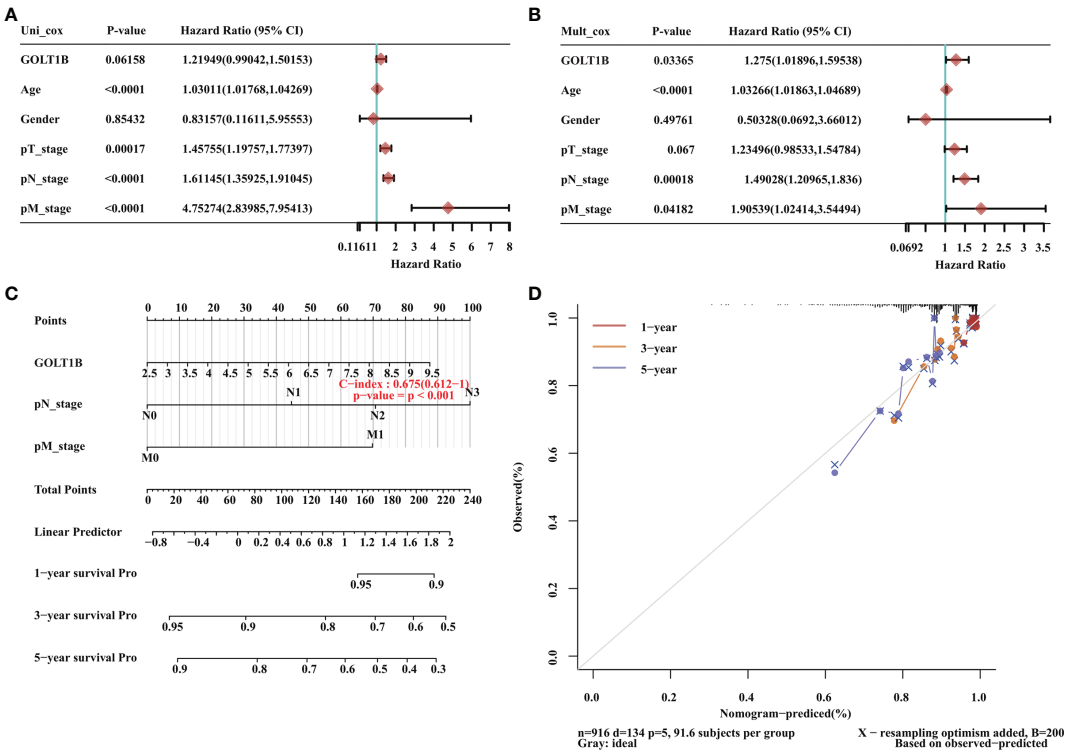

FIGURE 3 | GOLT1B is one potential independent prognostic gene for breast cancer. **(A)** Univariate Cox analysis revealed that GOLT1B was associated with the risk of breast cancer. **(B)** Multivariate Cox analysis identified GOLT1B as a potential independent prognostic gene of breast cancer. **(C, D)** The nomogram showing the function of GOLT1B in the scoring of prognostic risks of breast cancer.

or < -0.5, false discovery rate (FDR) < 0.05). Subsequently, we examined the protein and phosphorylation amount of the four transcriptional factors in breast cancer tissues versus the normal tissues. We observed that the four genes had no significant changes on their protein amount, but the phosphorylation of JUN on Thr 239 and Ser 243, and SIN3A on Ser 940, Ser 1112, and Thr 848 had significant alterations (**Figures 4A–D**). These results suggest that the up-regulation of GOLT1B in breast cancer may be related to the activity alteration of SIN3A and JUN.

On the other hand, we know that mutations may also lead to increased gene expression. Therefore, we investigated the mutation types and frequency of GOLT1B in breast cancer using Cbioportal database (n = 9555). As a result, more than 75% of the patients with GOLT1B mutations were diagnosed with amplifications (**Figures 4E, F**). Besides, we found that the GOLT1B expression was higher in patients with amplifications than the median expression of all mutations (**Figure 4G**). This result indicates that GOLT1B amplifications may be another reason responsible for the increase of GOLT1B expression.

The Functions of GOLT1B in Breast Cancer Are Potentially Associated With the Axis of "Ribosome-Proteasome-Lysosome"

To explore the potential mechanism of GOLT1B in breast cancer, we investigated the influence of GOLT1B on the BRCA signatures. The results showed that GOLT1B was related to tumor microenvironment (TME) scores, CD8+ T effector cells, immune-checkpoint, antigen-processing-machinery, TME-score-A, mismatch-repair, nucleotide-excision-repair, DNA-damage-response, DNA-replication, base-excision-repair, pan-fibroblast TGFβ response signature (Pan-F-TBRs), epithelial-to-mesenchymal transition 1 (EMT1), EMT2, EMT3, and TME-score-B (**Figure 5A**). Next, to understand the pathways that GOLT1B potentially regulated in breast cancer, we analyzed all co-expressed proteins of GOLT1B using the datasets from the Linkomics database. We obtained 5720 co-expressed proteins, and the top 50 positively-correlated and negatively-correlated genes were shown in **Figures 5B, C**. After analyzing the co-expressed proteins using GSEA, we discovered that GOLT1B was positively correlated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, such as proteasome, lysosome, tryptophan and drug metabolism, and pentose phosphate pathway; GOLT1B was negatively correlated with pathways including the ECM-receptor interaction, protein export, and ribosome (**Figure 5D**). It's well-known that ribosomes are responsible for protein synthesis, while proteasomes and lysosomes are related to protein degradation (10–12). Therefore, GOLT1B may be involved in regulating and monitoring protein production in breast cancer, promoting protein synthesis and inhibiting protein degradation.

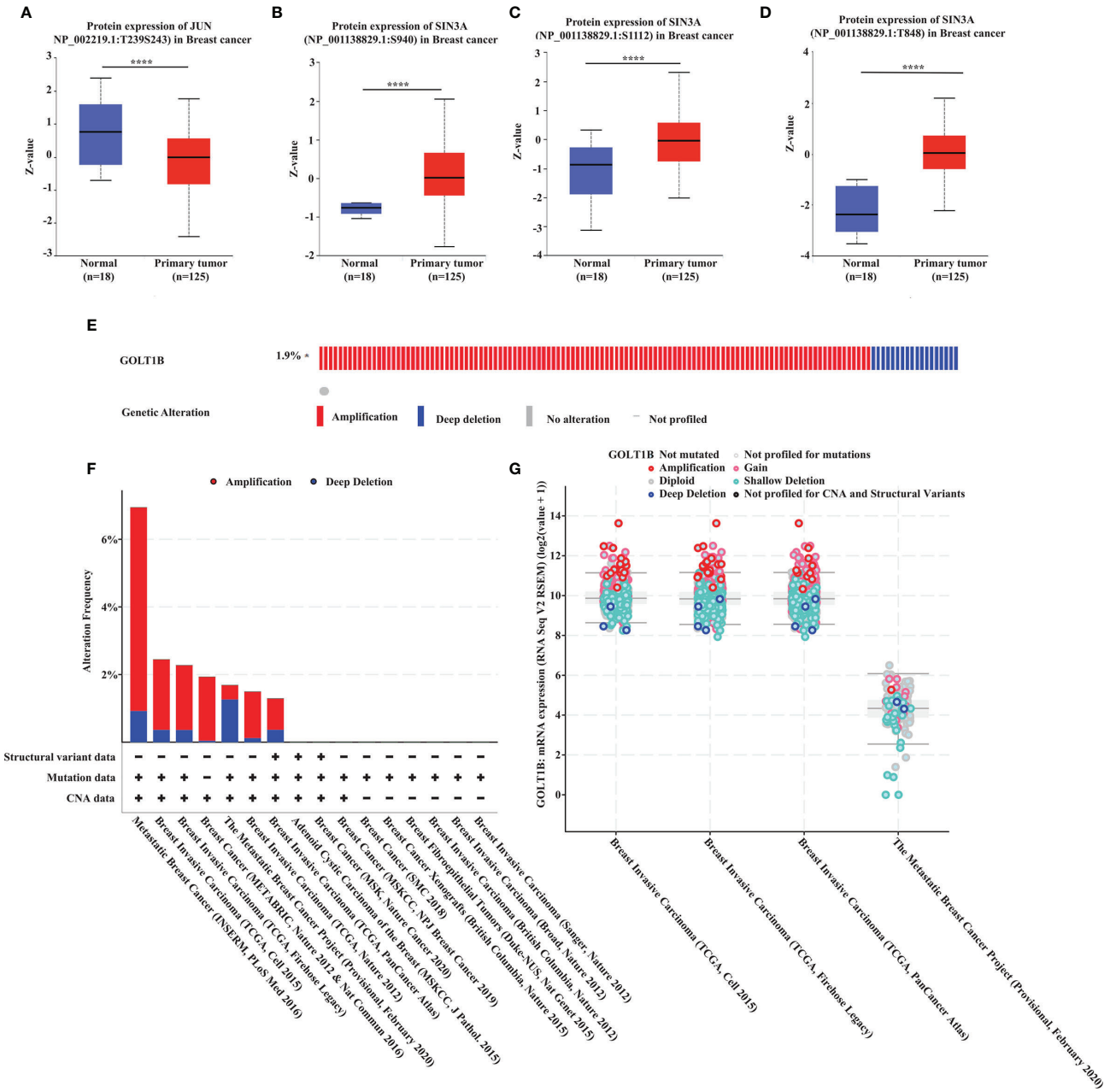

FIGURE 4 | The potential mechanism underlying the up-regulation of GOLT1B in breast cancer patients. **(A–D)** The phosphorylation of JUN and SIN3A in breast cancer tissues versus normal tissues. **(E)** The frequency of GOLT1B mutations in breast cancer patients. **(F)** The mutation type and frequency of GOLT1B in distinct independent studies of breast cancer. **(G)** The GOLT1B expression in breast cancer patients with different GOLT1B mutations. ****$p < 0.0001$.

GOLT1B Potentially Regulates Immune Microenvironment in Breast Cancer

In the above study, we found that GOLT1B was significantly correlated with TME scores and immune-related signatures including immune-checkpoint, CD8+ T effector cells, and antigen-processing-machinery. To further clarify the correlation between GOLT1B and immune microenvironment in breast cancer, we divided breast cancer patients into GOLT1B high-expressed and low-expressed groups. We then investigated the correlation between GOLT1B expression and the infiltration of twelve kinds of immune cells, including monocytes, CD8+T cells, CD4+T cells, regulatory T cells, helper T cells, plasma cells, natural killer (NK) cells, neutrophils, macrophages, M0 macrophages, M2 macrophages, and lymphocytes. As a result, the expression of GOLT1B was significantly positively correlated with the infiltration level of four kinds of immune cells, including

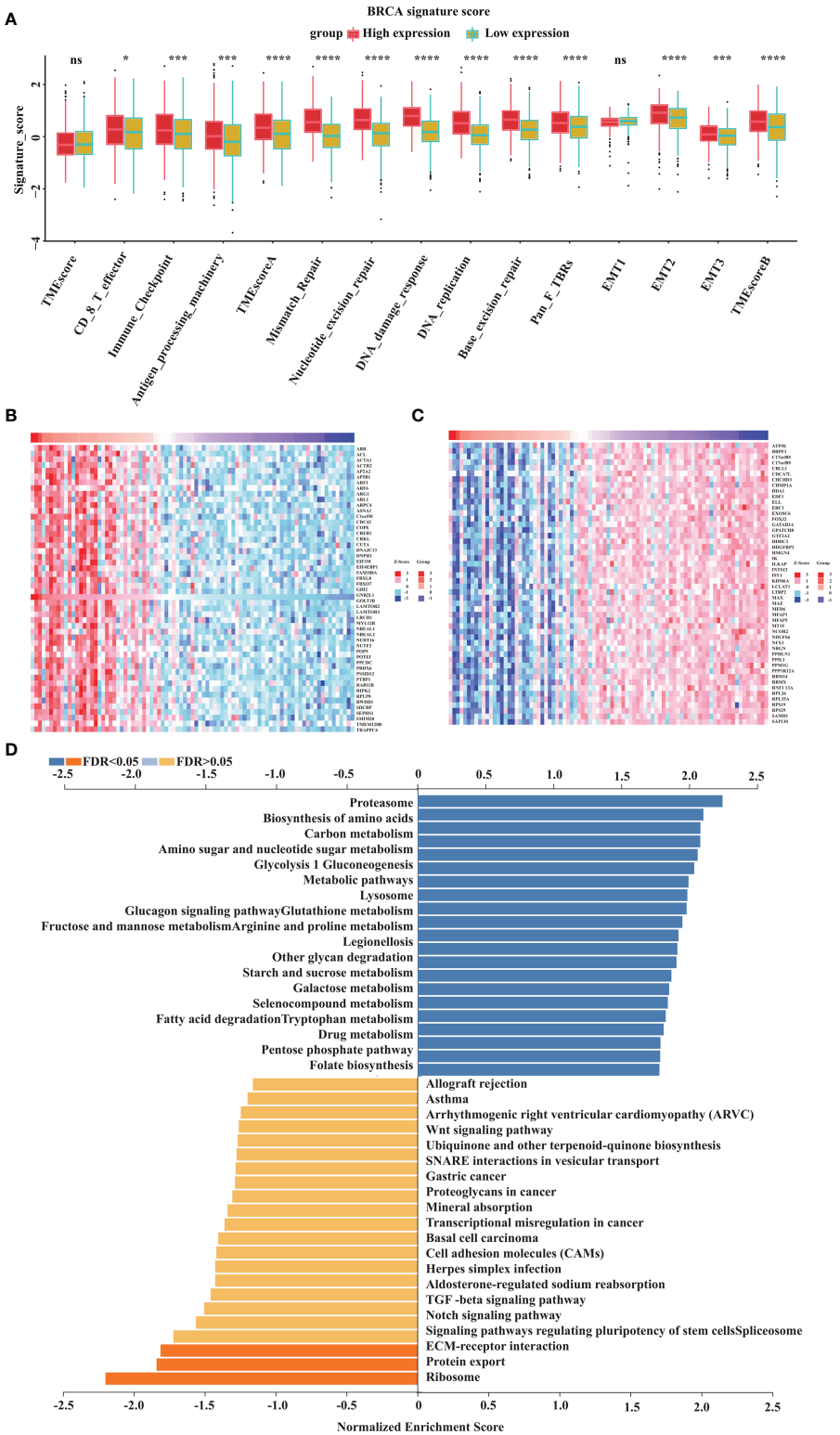

FIGURE 5 | GOLT1B potentially regulates the functions of ribosome, proteasome and lysosome in human breast cancer. **(A)** The correlation between GOLT1B expression and gene signatures. In each group, the scattered dots represent the mean of the signature genes, and the thick lines represent the median value. **(B)** Heat maps of the top 50 genes positively correlated with GOLT1B expression. **(C)** Heat maps of the top 50 genes negatively correlated with GOLT1B expression. **(D)** GSEA analysis showing the positively and negatively correlated pathways of GOLT1B expression. "ns" indicated no statistic significance, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

macrophages, M0 macrophages, M2 macrophages, and neutrophils but negatively correlated to monocytes, CD8+T cells, CD4+T cells, regulatory T cells, helper T cells, plasma cells, NK cells, neutrophils (**Figure 6A**). Furthermore, we confirmed the association between GOLT1B and immune infiltration using datasets from CIBERSORT database. The results showed that the expression of GOLT1B was positively correlated with the infiltration of eight kinds of immune cells in breast cancer, including induced regulatory T (iTreg), natural regulatory T (nTreg), macrophages, monocytes, dendritic cells, central memory T cells, regulatory T cells, and type 1 helper T cells, and a significant negative correlation with CD4+T cells, gamma delta T (Tgd), helper follicular T (TFH), mucosal associated invariant T (MAIT), natural killer T (NKT), NK, and CD8+T cells (**Figure 6B**). Subsequently, we revealed that the expression of GOLT1B was positively correlated with some major immune checkpoints, including CD274, TIGIT, and CTLA4 (**Figure 6C**). Furthermore, to confirm that GOLT1B affects tumor progression by regulating the immune microenvironment, we studied the effect of GOLT1B expression on the OS of patients with high/low immune cell infiltration using the Kaplan-Meier Plotter database. As a result, the GOLT1B expression was negatively correlated with the OS in breast cancer patients with decreased infiltration of type 2 helper T cells, but not affect patients with enriched infiltration. Besides, the GOLT1B expression was negatively correlated with the OS in breast cancer patients with enriched infiltration of regulatory T cells, but not affect patients with decreased infiltration (**Figure 6D**). These results demonstrated that GOLT1B was potentially a regulatory factor for the immune infiltration of breast cancer and possibly influences the tumor progression by regulating the immune microenvironment.

DISCUSSION

As one of the most common cancers globally, breast cancer is frequently diagnosed at an advanced stage with poor prognosis, being prone to visceral and bone metastasis (13). Nowadays, the role of golgi apparatus in breast cancer has attracted increasing attention. Some studies demonstrated that golgi somal membrane protein 1 (GOLM1) promoted the proliferation and metastasis of breast cancer cells by regulating matrix metalloproteinase-13 (MMP13) (14). Phosphatidylinositol 4-phosphate in golgi apparatus regulated cell adhesion and invasiveness of breast cancer (15). These studies reveal that golgi apparatus may be a promising target for clinical treatment of breast cancer.

GOLT1B, encoding protein of a golgi transporter, plays an important role in regulating vesicle transport between endoplasmic reticulum and golgi apparatus. One piece of research proclaimed that such vesicle transporters induced cell proliferation in breast cancer (16). Studies have shown that golgi vesicle transporter 1A (GOLT1A) (17, 18), another member of golgi vesicle transporter family, affects tamoxifen sensitivity in breast cancer and promotes cell proliferation in lung cancer. Besides, high expression of GOLT1B suggests poor prognosis of colorectal cancer, and induces immunosuppression by promoting PD-L2 membrane localization (7). Consistent with the previous report, our study revealed that the expression of GOLT1B was higher in breast cancer tissues than normal tissues (**Figure 1**). Breast cancer patients with high GOLT1B expression had significantly lower survival rates than those patients with low GOLT1B expression (**Figure 2**). GOLT1B was also identified as a potential independent prognostic gene in breast cancer (**Figure 3**). These results all demonstrate that GOLT1B is potentially an oncogene in breast cancer.

The initiation and development of breast cancer are closely related to the interaction between tumor and microenvironment. Breast cancer has a unique immune microenvironment in which vascular endothelial factors are highly expressed, and lymphocytes and tumor-associated macrophages are more infiltrated (19). Based on this, immunotherapy targeting the immune microenvironment has been emerging recently. Nowadays, the immunocheckpoint-targeted therapy, especially targeting programmed death receptor 1/programmed death ligand (PD-1/PD-L1), appears to be a promising treatment for cancer. However, the inhibition efficiency of PD-1/PD-L1 inhibitors on solid tumors is only 10-40%, which means that a large proportion of patients cannot benefit from the treatment (20). Therefore, it is of great significance to search for new immunotherapeutic targets and potential prognostic biomarkers. In our study, we found that GOLT1B was potentially a regulatory gene for the immune microenvironment of breast cancer patients and was closely related to the survival of patients. All these results reveal the potentials of GOLT1B as a drug target or prognostic biomarkers for immunotherapy.

Our study proclaimed that the altered phosphorylation of two potential transcription factors of GOLT1B, JUN and SIN3A, might be responsible for the increased GOLT1B expression in breast cancer. As potential upstream regulators of GOLT1B, JUN and SIN3A have been gained increasing attention in diagnosis and treatment of breast cancer. Many studies have declaimed that JUN is a biomarker and regulatory gene in breast cancer (21–23). In patients with short survival time (< 5 years), the expression of JUN in breast cancer tissue is down-regulated and the risk of recurrence of breast cancer is increased (24). Furthermore, JUN mediates the functions of some important cytokines in breast cancer, such as IL-34, IL-33, and IL-1β (22, 23, 25). On the other hand, SIN3A is a transcriptional suppressor promoting osteolytic destruction in ERα-positive breast cancer (26). In mammary adenocarcinoma cells, SIN3A interacts with STAT3 to silence tumor suppressor gene and inhibit cell survival (27). Besides, SIN3A is not only necessary for the survival and proliferation of breast cancer cells but also essential for maintaining epithelial stability and chemical sensitivity, and is one promising drug target for breast cancer treatment (28).

In the survival and correlation analysis, multiple testing strategies have been employed to ensure the accuracy of the results in our study. We analyzed the FDR of the survival results (**Figure 2**), but found that the FDRs based on the datasets from

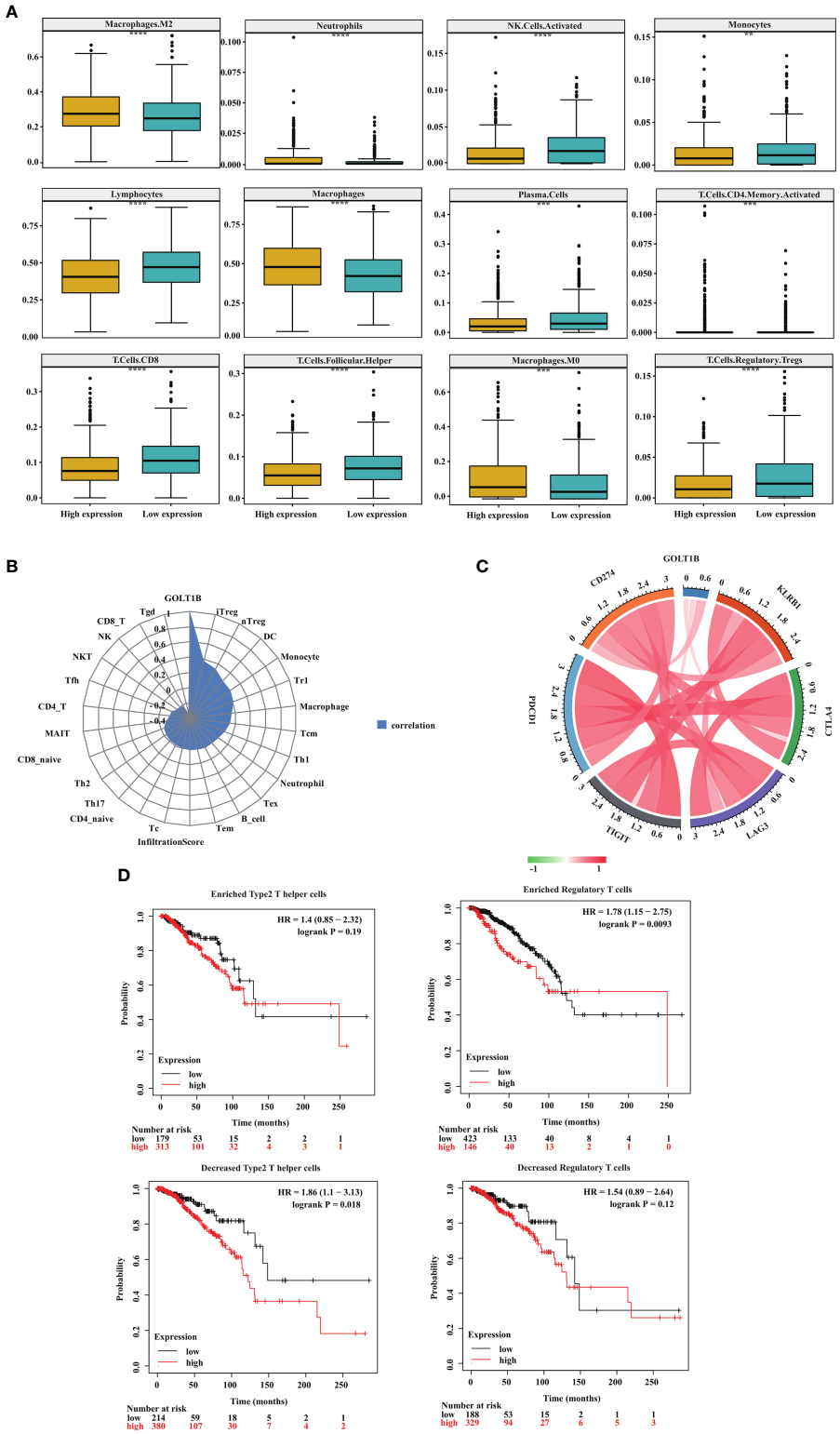

FIGURE 6 | GOLT1B expression is potentially associated with immune infiltration in breast cancer patients. **(A)** The correlation between the GOLT1B expression and infiltration of immune cells in breast cancer was analyzed using the TIMER database. **(B)** The correlation between the GOLT1B expression and infiltration of immune cells in breast cancer was analyzed using the CIBERSORT database. **(C)** The correlation between the expression of GOLT1B and immune checkpoints in human breast cancer. **(D)** The correlation between the expression of GOLT1B and OS of breast cancer patients with different infiltration of immune cells.

TCGA and Kaplan-Meier Plotter database were all greater than 0.5. In fact, we did the FDR correction for all potentially overall-survival-relevant genes (p values < 0.05, n = 2085) in breast cancer, and only found four genes with a FDR < 0.05. Subsequently, to clarify whether GOLT1B is one potential prognostic gene in human breast cancer, we further analyzed other cohorts from prognoScan database and GEO (**Figure 2D**). Consequently, the corrected p values of OS, DSS and RFS were all less than 0.05, supporting a possibility of GOLT1B as one prognostic gene in breast cancer.

Taken together, our study identified GOLT1B as a potential prognostic gene for breast cancer and demonstrated the functions of GOLT1B in immune microenvironment. Our findings in this study proposed a novel potential prognostic biomarker for breast cancer, improved our understanding of the functions of golgi apparatus in tumor immune microenvironment, and provided new opportunities for clinical diagnosis and treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Study concept and design: WZ and DT. Acquisition of data: WZ and YC. Analysis and interpretation of data: JL. Statistical analysis: WZ, JL, and WC. Drafting of the manuscript: WZ and JL. Critical revision and final approval of the manuscript: all authors. Obtained funding: MT, YD, and WZ. Study supervision: XC and MT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.805273/full#supplementary-material

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A Novel IGLC2 Gene Linked With Prognosis of Triple-Negative Breast Cancer

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Background: Immunoglobulin-related genes are associated with the favorable prognosis of triple-negative breast cancer (TNBC) patients. We aimed to analyze the function and prognostic value of immunoglobulin lambda constant 2 (IGLC2) in TNBC patients.

Methods: We knocked down the gene expression of IGLC2 (IGLC2-KD) in MDA-MB-231 cells to evaluate the proliferation, migration, and invasion of tumors *via* 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, wound healing, and transwell cell migration assay respectively. Relapse-free survival (RFS) and distant metastasis-free survival (DMFS) analyses were conducted using the KM plotter online tool. The GSE76275 data set was used to analyze the association of IGLC2 and clinical characteristics. A pathway enrichment analysis was conducted using the next-generation sequencing data of wild-type and IGLC2-KD MDA-MB-231 cells.

Results: The low gene expression of IGLC2 was related to unfavorable RFS, DMFS. The high expression of IGLC2 was exhibited in the basal-like immune-activated (BLIA) TNBC molecular subtype, which was immune-activated and showed excellent response to immune therapy. IGLC2 was positively correlated with programmed death-ligand 1 (PD-L1) as shown by Spearman correlation ($r = 0.25$, $p < 0.0001$). IGLC2 had a strong prognostic effect on lymph node-negative TNBC (RFS range: 0.31, q value= 8.2e-05; DMFS = 0.16,

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q value = 8.2e-05) but had no significance on lymph node-positive ones. The shRNA-mediated silencing of IGLC2 increased the proliferation, migration, and invasion of MDA-MB-231 cells. The results of pathway enrichment analysis showed that IGLC2 is related to the PI3K-Akt signaling pathway, MAPK signaling pathway, and extracellular matrix–receptor interaction. We confirmed that MDA-MB-231 tumor cells expressed IGLC2, subverting the traditional finding of generation by immune cells.

Conclusions: IGLC2 linked with the proliferation, migration, and invasion of MDA-MB-231 cells. A high expression of IGLC2 was related to favorable prognosis for TNBC patients. IGLC2 may serve as a biomarker for the identification of TNBC patients who can benefit the most from immune checkpoint blockade treatment.

Keywords: immunoglobulin, breast cancer, triple-negative breast cancer (TNBC), MDA-MB-231, prognosis, next-generation sequencing, relapse-free survival, distant metastasis-free survival

HIGHLIGHTS

- ►Immunoglobulin lambda constant 2 (IGLC2) is a novel prognostic biomarker for TNBC patients.
- ►The high expression of IGLC2 is related to favorable relapse-free survival, distant metastasis-free survival, tumor size, and TNBC molecular subtypes.
- ►IGLC2 has a strong prognostic value for lymph node-negative TNBC.
- ►Silencing of IGLC2 linked with the proliferation, migration, and invasion of MDA-MB-231 cell lines.
- ►IGLC2 influences TNBC possibly through the pathways of PI3K-Akt signaling, MAPK signaling, and extracellular matrix–receptor interaction.
- ►IGLC2 is positively associated with programmed death-ligand 1 (PD-L1).

INTRODUCTION

Breast cancer (BC) is the leading cause of cancer and the most common cancer in women worldwide, affecting approximately 12% of females during their lifetimes (1). BC is very heterogenic and classified into distinct molecular subtypes based on hormone receptors, namely, estrogen receptor (ER)/progesterone receptor (PR), and growth factors, including human epidermal growth factor receptor 2 (HER2) and Ki-67 as a proliferation marker (2). Triple-negative BC (TNBC) is a subtype of BC that lacks the expression of ER, PR, and HER-2, is generally aggressive, has high rates of relapse, and results in a decreased overall survival (3, 4). TNBC accounts for 10%–20% of all BCs (5). Given that TNBC lacks the expression of ER/PR/HER2, the application of endocrine therapy or targeted therapy against HER2 is difficult. Chemotherapy has become the main treatment mode, but it generally presents a poor efficacy. Standardized TNBC treatment regimens are still lacking. Therefore, the development of new TNBC treatment strategies has become an urgent clinical need (4). Understanding the molecular profiles of TNBC is critical for the development of new therapeutic options to prevent the progression of metastatic illness and eventually improve the survival of this patient population (6).

TNBC can be categorized into various molecular subtypes, including by the six subtypes by Lehmann (7) or the four subtypes by Burstein (8), based on gene expression profiling of tumor samples (4). Burstein's four subtypes include luminal androgen receptor (LAR), mesenchymal (MES), basal-like immunosuppressed (BLIS), and basal-like immune-activated (BLIA) subtypes, with BLIA displaying the upregulation of genes controlling B-cell, T-cell, and natural killer cell functions with distinct prognoses (8). The efficacy of existing treatment regimens, therapeutic drugs, and targeted treatment regimens on TNBC subtypes varies (4). Immunotherapies [e.g., programmed cell death protein 1 (PD-1) or Programmed death ligand 1 (PD-L1) inhibitors] may be useful treatments for BLIA tumors, whereas VTCN1-immuno-regulator may be effective treatments for BLIS tumors (9).

Novel biomarkers have been successfully identified by genetic co-expression network (GCN) analysis in BCs (10–24). GCN is an undirected graph, where each node corresponds to a gene, and a pair of nodes is connected with an edge if there is a significant co-expression relationship between them (25). In our previous study (26), we combined the methods of GCN and gene expression profiling to identify six novel immunoglobulin-related gene modules (*IGHA1, IGHD, IGHG1, IGHG3*, immunoglobulin lambda constant 2 (*IGLC2*), and *IGLJ3*) associated with favorable prognosis for TNBC patients. The mRNA expression data sets of 920 BC tumor tissue samples were from the National Center for Biotechnology Information GEO data sets (recurrence n = 354, no recurrence n = 566, and average follow-up time of four data sets = 6–9 years).

IGLC2 had the most significant effects out of six genes; its hazard ratios (HRs) in relapse-free survival (RFS) and distant metastasis-free survival (DMFS) were 0.64 (p = 0.038) and 0.13 (p = 0.025), respectively. These six immunoglobulin genes were

Abbreviations: TNBC, triple-negative breast cancer; BC, breast cancer; GCNA, genetic co-expression network analysis; RFS, relapse-free survival; DMFS, distant metastasis-free survival; DEGs, differentially expressed genes.

involved in the tumor microenvironment of B lymphocytes, which play important roles in BC prognosis, especially in TNBC (27). Growing evidence indicates that variants of immunoglobulin segments are associated with the prognosis of BC (including TNBC) (28, 29). The stromal immunoglobulin kappa chain (IGKC) serves as an immunologic biomarker of initiation, prognosis (30, 31), and treatment response of BCs and other cancers (32). Among the six discovered immunoglobulin genes, IGLC2 has the most significant effect on prognosis, but only its association with lymphoid neoplasia (33) and amyloidosis has been discussed (34). To the best of our knowledge, no study has evaluated the prognostic effect and function of IGLC2 in TNBC. Therefore, we aimed to analyze the function and prognostic value of IGLC2 in TNBC patients.

METHODS

Kaplan–Meier (KM) Survival Analysis

We used the KM plotter online cancer survival analysis tool (35) to conduct the Kaplan–Meier plot and survival analysis of IGLC2. The data of breast cancers comprised 55 independent datasets from National Center of Biotechnology Information Gene Expression Omnibus database. The total number of breast cancer arrays was 9,423 and 7,830 unique samples (36). All the available data sets of TNBC were basal-like in the KM plotter online cancer survival analysis tool. The case numbers of RFS and DMFS were 392 and 306, respectively (**Table 1**).

GSE Data Set

We used GSE76275 (37) to analyze the association of IGLC2 mRNA expression and clinical characteristics using linear regression. We included all the TNBC tissues (n = 198) and variables of age, body mass index, menopause, TNBC molecular subtypes, tumor size, stage, grade, the number of positive nodes, and metastasis. There were 115 TNBC tissues left for analysis after missing data was removed. The pairwise scatter plots and boxplots of IGLC2 with other variables were shown in **Figure S1**. **Table 2** shows the description of GSE76275. The gene expression of whole-genome was converted to the log base 2 of the value before the statistical analysis. After transformation, the distributions of whole-genome expression were close to normal distribution and in a similar range (**Figure S2**).

Correction for Multiple Comparisons

The Benjamini–Hochberg method was applied to control the False Discovery Rate (FDR) for multiple hypothesis testing (38) and q values were calculated using the R function "stats" of built-in package "stats" (39). The p values and q values were displayed in the tables and figures.

Next Generation Sequencing (NGS)

One wild type (WT) and two IGLC2 knockdown (IGLC2-KD) cell lines were collected. We used poly-T oligo-attached beads to purify mRNA, which was also fragmented primed for cDNA synthesis, and used a reverse transcriptase and random primer to synthesize the first-strand cDNA and dUTP in place of dTTP to generate a double-stranded (ds) cDNA. A single "A" nucleotide was added to the 3′ end of the ds cDNA. Then, multiple indexing adapters were ligated to the 5′ and 3′ of the ends of the ds cDNA. Polymerase chain reaction (PCR) was used to selectively amplify the DNA fragments that had adapters on both ends. The library was validated on an Agilent 2100 Bio-analyzer and Real-Time PCR System. We conducted NGS following the protocol of Illumina NextSeq sequencing and calculated the gene expression (RSEM, http://deweylab.github.io/RSEM/), differential gene expression (EBSeq, https://www.biostat.wisc.edu/~kendzior/EBSEQ/), pathway enrichment, and Gene Ontology (GO) enrichment.

Pathway Enrichment Analysis

Pathway enrichment analysis helps researchers gain mechanistic insights into gene lists generated from genome-scale (omics) experiments and identifies biological pathways that are enriched in a gene list more than that would be expected by chance (40). We adopted the method of Gene Set Enrichment Analysis (GSEA) which is a computational method that determines whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states (41, 42). Kyoto Encyclopedia of Genes and Genomes (KEGG) and GO pathway enrichment analyses were conducted using differentially expressed genes (DEGs) from the NGS results of IGLC2-KD and WT cells. KEGG is a database resource of high-level functions and utilities of the biological system from large-scale molecular datasets (43). The GO knowledgebase is the world's largest source of information on the functions of genes, in three aspects of cellular component (CC), biological process (BP), and molecular function (MF) using

TABLE 1 | Description of validation data set from KM plotter for RFS and DMFS analysis of IGLC2.

	TNBC tissues for RFS survival analysis (n = 392) n (%)	TNBC tissues for DMFS survival analysis (n = 306) n (%)
Molecular subtypes		
Basal like	392 (1)	306 (1)
Lymph node status		
Positive	189 (49)	117 (53)
Negative	197 (51)	102 (47)
Grade		
1	15 (5)	2 (1)
2	34 (12)	24 (13)
3	240 (83)	153 (85)

TABLE 2 | Association of IGLC2 mRNA expression and clinical characteristics of TNBC tissues from GSE76275 data set.

	IGLC2 log2 mRNA expression					q values*
	Mn	SD	%	n	p value[#]	
Age (years)	56	13	100.00%	115	0.17	0.54
BMI (kg/m^2)	28	6	100.00%	115	0.69	0.76
Race						
Asian or Pacific islander	12.83	3.56	3.5%	4	ref	
Caucasian	12.46	1.99	93.0%	107	0.72	0.76
Missing	12.51	1.74	3.5%	4		
Female	12.47	2.03	100.0%	115		
Menopause						
Pre-menopause	12.45	1.81	26.1%	30	ref	
Menopause	13.27	1.36	3.5%	4	0.43	0.74
Post-menopause	12.56	2.04	51.3%	59	0.81	0.81
Missing	12.13	2.39	19.1%	22		
Molecular subtype						
Basal-Like Immune-Activated (BLIA)	13.04	1.78	27.8%	32	ref	
Basal-Like Immune-Suppressed (BLIS)	11.93	2.16	31.3%	36	**0.02**	0.20
Luminal-AR (LAR)	11.69	1.79	20.0%	23	**0.01**	0.20
Mesenchymal (MES)	13.27	1.93	20.9%	24	0.66	0.76
Tumor size (cm)						
≤2cm	12.71	1.77	18.3%	21	ref	
2-5cm	12.51	2.10	70.4%	81	0.69	0.76
>5cm	12.11	2.07	6.1%	7	0.22	0.54
Any size with direct extension	11.54	1.93	5.2%	6	0.50	0.74
Stage						
I	12.71	1.77	18.3%	21	ref	
II	12.51	2.10	70.4%	81	0.69	0.76
IIIA	12.11	2.07	6.1%	7	0.50	0.74
IIIB	11.54	1.93	5.2%	6	0.22	0.54
Grade						
Well Differentiated	14.89		0.9%	1	ref	
Moderately Differentiated	11.78	1.88	27.0%	31	0.11	0.54
Poorly Differentiated	12.75	1.89	57.4%	66	0.26	0.54
Number of positive nodes						
0	12.14	2.22	51.3%	59	ref	
1-3	12.84	1.86	32.2%	37	0.10	0.54
4-9	12.88	1.39	9.6%	11	0.27	0.54
≥10	12.63	1.88	7.0%	8	0.52	0.74
Metastasis						
No	12.51	2.00	98.3%	113	ref	
Yes	10.55	3.43	1.7%	2	0.18	0.54

[#]p values of univariable linear regression. ref, reference group; Mn, mean; SD, standard deviation. P < 0.05 was marked in bold.
**q values were calculated using Benjamini-Hochberg method. Missing values were not included for statistical analysis.*

computational analysis of large-scale molecular biology and genetics experiments (44).

Cell Culture and Reagents

The human breast carcinoma cell line MDA-MB-231 (RRID: CVCL_0062) was maintained in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C in humidified air containing 5% carbon dioxide.

Knockdown of IGLC2 Expression in MDA-MB-231 Cells

The knockdown of IGLC2 gene in MDA-MB-231 cells was generated using IGLC2-specific shRNA. IGLC2-shRNA-containing lentiviral vectors were purchased from Applied Biological Materials Inc. (#246730910296) and prepared in accordance with standard protocols. The target sequences of IGLC2 were 37 CGCCCTCCTCTGAGGAGCTTCAAGCCAAC, 158 GGAGACCACCACACCCTCCAAACAAAGCA, 197 CGCGGCCAGCAGCTATCTGAGCCTGACGC and 255 AGCTGCCAGGTCACGCATGAAGGGAGCAC. The human breast carcinoma cell line MDA-MB-231 was transinfected with lentiviruses in the selection medium containing 2 μg/ml polybrene. At 48 h after infection, the cells were treated with 10 mg/mL puromycin to select a pool of puromycin-resistant clones. We measured the IGLC2 knock-down efficacy of multiple clones and selected the best one for further experiment (**Figure S3**).

Immunoblot Analysis

The cells were harvested in lysis buffer (50 mM Tris pH 8.0, 5 mM NaCl, 0.5% NP-40, and 1X protease inhibitor). The protein concentration was determined using the Bradford method (Bio-

Rad, Hercules, CA). Samples with an equivalent amount of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide gel and electrophoresed. The separated proteins were transferred to a nitrocellulose membrane. Then, the membrane was probed anti-IGLC2 antibody (IGLC2 monoclonal antibody (5E12B9), ThermoFisher Scientific, MA5-31776), followed by a secondary antibody in phosphate-buffered saline (PBS)/Tween 20 with 5% Carnation nonfat milk. Proteins were detected using an enhanced chemiluminescence reagent (ECL Plus, GE).

MTT Assay

3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate cell proliferation. Cells were seeded at a density of $1x10^4$ cells/well in 24-well plates. Subsequently, the MTT reagent (Sigma) was added to each well, and the plates were incubated for 1 h at 37°C. The remaining crystals were dissolved in a mixture medium consisting of 100 µl dimethyl sulfoxide and 100 µl 95% alcohol (1:1). The crystals were shaken on a shaker for 10–15 min until dissolution. The absorbance was evaluated at $OD_{540-570}$ using an enzyme-linked immunosorbent assay reader. The assays were performed in triplicate. The significance was calculated using Student's t-test.

Wound-Healing Assay

Wound-healing assay was used to evaluate cell migration. Cells were seeded in six-well plates at $1x10^5$ cells per well in a growth medium. Confluent monolayers were starved overnight in assay medium, and a single scratch was created. The cells were washed with PBS to remove cell debris, supplemented with assay medium, and monitored. Images were captured under a microscope at 0 and 27 h post-wounding.

Transwell Cell Migration Assay

The migratory ability was evaluated in a BD Falcon cell culture insert (BD Biosciences). Aliquots of 1×10^5 cells suspended in 500 µl serum-free media were seeded into the upper part of each chamber, and the lower compartments were filled with media containing 10% FCS. After incubation for 24–72 h, nonmigrating cells were physically removed from the upper surface of the membrane. The MDA-MB-231 cells were stained using 0.2% crystal violet. The MDA-MB-231 cells were counted in at least 10 randomly fields per insert at 100x magnification.

RESULTS

IGLC2 Had a Beneficial Effect on TNBC Patients

We conducted the survival analysis of IGLC2 mRNA expression of TNBC tissue samples with 392 RFS and 306 DMFS (**Table 1**) *via* the KM plotter online tool (35). The TNBC molecular subtypes of this data were all basal like. The proportion of TNBC patients with negative or positive lymph node was similar in the analysis data of RFS and DMFS (**Table 1**). Over 80% of the TNBC tissues were Grade 3. In the sensitivity analysis of prognosis, the TNBC tissues were analyzed as a whole and divided into three subgroups by Grade 3 and lymph node status (negative and positive). The cut-off point of IGLC2 gene expression was set to be the lower quartile rather than the median (**Figure S4**) for better prediction results. A high mRNA expression of IGLC2 was associated with improved RFS and DMFS in TNBC patients (**Figures 1A, B**). IGLC2 is a great prognostic gene, especially for TNBC patients developing lymph node-negative (**Figures 1C, D**) and lymph node-negative with Grade 3 (**Figures 1E, F**), compared with all TNBC patients (**Figures 1A, B**), lymph node-positive TNBC patients (**Figures 1G, H**) and Grade 3 TNBC patients (**Figures 1I, J**). Meanwhile, IGLC2 was not a significant prognostic predictor of RFS and DMFS for lymph node-positive TNBC (**Figures 1G, H**). For Grade 3 TNBC (**Figures 1G, H** and **Figure 2**), IGLC2 was not a significant prognostic predictor of DMFS. IGLC2 presented a better prognostic value for DMFS than RFS in TNBC patients developing lymph node-negative, although no statistical significance was found (**Figure 2**). No survival analysis was conducted for patients developing Grades 1 and 2 TNBC due to the limited sample number (less than 35) (**Table 1**) for meaningful survival analysis.

In addition, we used TCGA-BRCA gene expression data of TNBC (n=131) to validate IGLC2 in our previous study (26). Because there was no corresponding gene IGLC2 in TCGA-BRCA microarrays, we validated all related immunoglobulin genes: IGLL3, IGLL1, IGSF9B, IGDCC3, IGDCC4, IGBP1, IGSF5, IGSF11, IGSF22, IGSF21, IGHMBP2, IGSF10, IGSF8, IGSF9, IGSF6, IGSF1, IGSF3, IGFN1, and IGJ. The results showed that IGDCC3 and IGSF3 were significantly associated with RFS.

Association of *IGLC2* and Clinical Characteristics

We analyzed the association of IGLC2 and clinical characteristics using the mRNA expression data set of 115 TNBC tissues from GSE76275 (37). The IGLC2 mRNA expression was associated with the TNBC molecular subtypes but not significant after the adjustment of multiple comparisons (**Table 2**). The mRNA expression of IGLC2 was higher in BLIA and MES molecular subtypes of TNBC compared with BLIS and LAR (**Figure 3A**).

A low IGLC2 mRNA expression was exhibited in large tumor-size tissues; especially in tumors of any size with direct extension (**Figure 3B**). The expression of IGLC2 was lower in Stage IIIB patients compared with that in Stage I patients (**Table 2**). IGLC2 expression decreased from TNBC patients with positive lymph nodes 1-3 to ≥ 10 (**Table 2** and **Figure 3C**). The IGLC2 expression was lower in TNBC patients with metastasis than those without metastasis (**Figure 3D**).

Association of *IGLC2*, PD-1, and Programmed Death-Ligand 1 (PD-L1)

Immune checkpoint blockade is a promising treatment for TNBC. However, the selection of patients who will benefit the most remains a challenge. PD-L1 expression is widely used as a

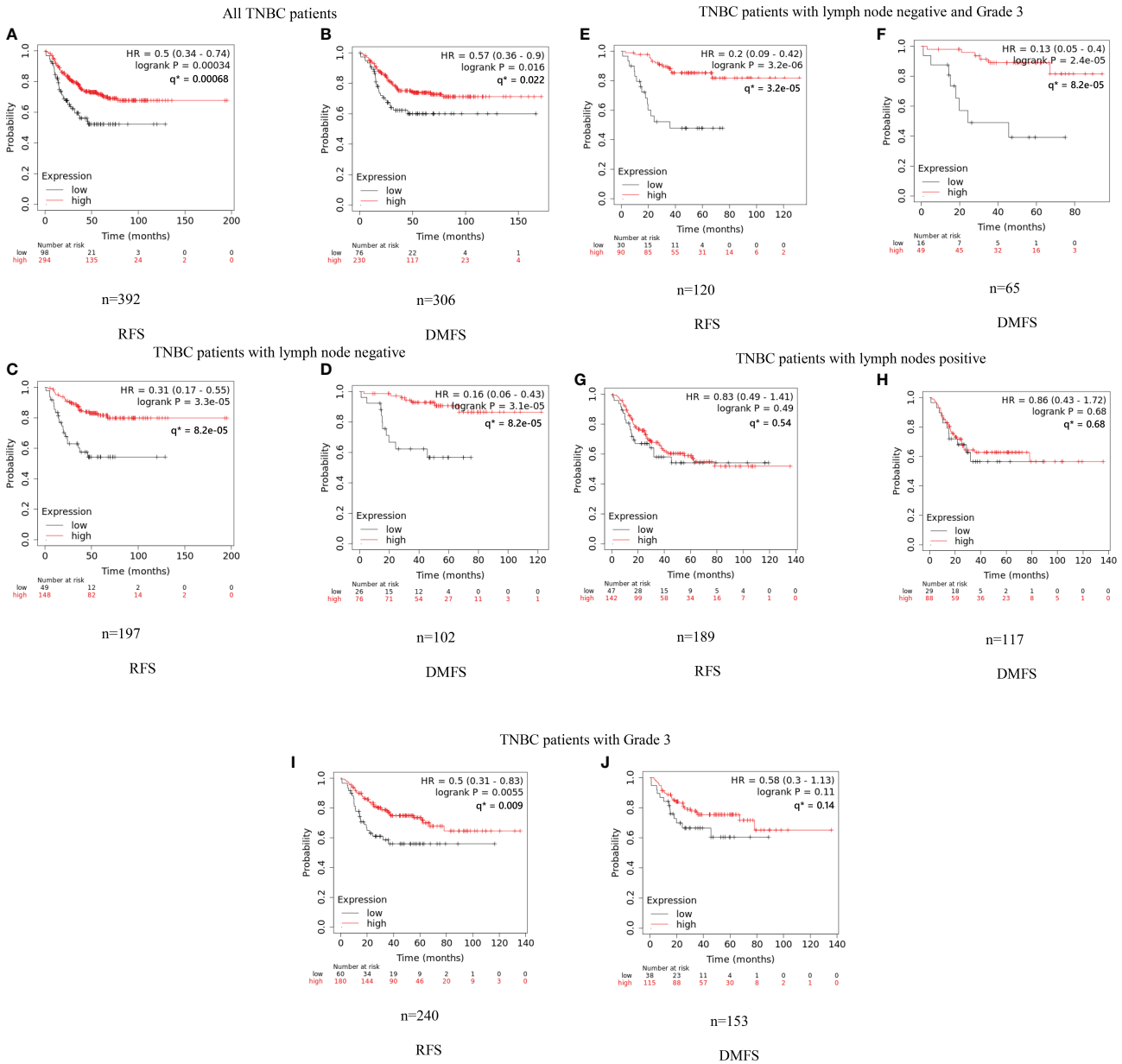

FIGURE 1 | Kaplan–Meier analysis of RFS and DMFS of IGLC2 mRNA expression for TNBC subgroups. We used the KM plotter online cancer survival analysis tool (http://kmplot.com/analysis/) to evaluate the RFS **(A, C, E, G, I)** and DMFS **(B, D, F, H)** in TNBC subgroups grouped by grade and lymph node status. The panels **(A, B)** are all from TNBC patients; **(C, D)** are TNBC patients developing negative lymph nodes; **(E, F)** are TNBC patients developing negative lymph nodes and Grade 3; **(G, H)** are TNBC patients developing positive lymph nodes; **(I, J)** are TNBC patients developing Grade 3. The lower quartile was set to be the cut-off point of IGLC2 gene expression. Grade 1 and 2 subgroups were not analyzed given the limited sample size (n < 35) for meaningful analysis. Y axis denotes the probability of RFS or DMFS. * q values were calculated using Benjamini-Hochberg method.

predictive biomarker due to its association with desirable response rates to PD1/PD-L1 blockade for TNBC patients (45). Therefore, we analyzed the association of IGLC2, PD-1, and PD-L1 to unveil the potential of IGLC2 as a biomarker for identifying TNBC patients who can benefit from immune checkpoint blockade. IGLC2 and PD-L1 were positively correlated, with r = 0.25 (p value < 0.0001) in Spearman correlation (**Figure S5**).

Figure 4 shows the heatmap of the mRNA expressions of IGLC2, other highly co-expressed immunoglobulin genes of IGLC2 found in our previous study (26), PD-1, and PD-L1. The IGLC2 expression was more variable in TNBC samples than those of co-expressed immunoglobulin genes and PD-1, PD-L1. In addition, IGLC2 was more relevant to the RFS and DMFS of TNBC patients than PD-L1 (**Figure 2**, **Figure S6** and **Table S1**).

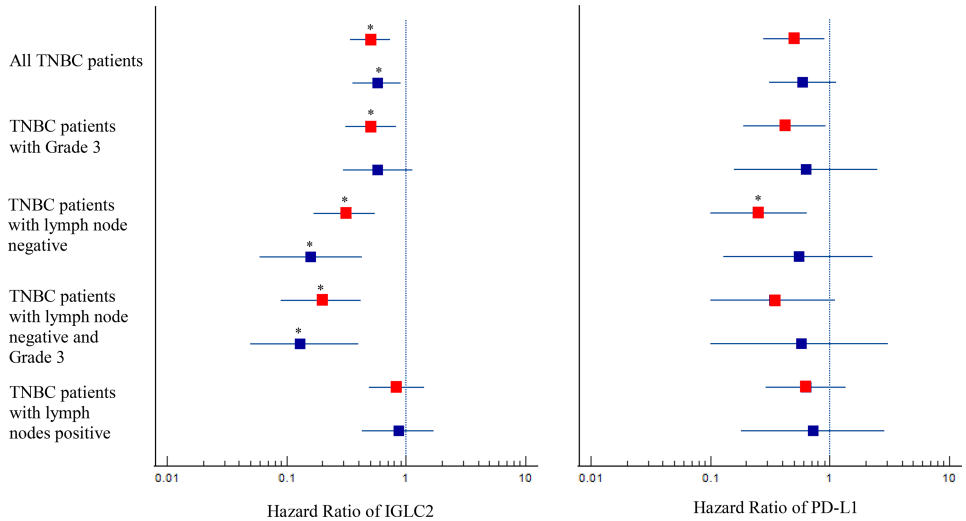

FIGURE 2 | Forest plot with hazard ratio (HR) for IGLC2 and PD-L1 of Kaplan–Meier (KM) survival analysis of RFS and DMFS. The HRs were obtained from the KM plotter online tool. The analysis data were grouped as a whole, by lymph node status, and by Grade 3. The boxplot was the 95% CI of HR. A 95% CI of HR not equal to 1 denotes statistical significance before multiple comparison adjustment. The HR results of RFS were marked in red, and those of DMFS were marked in blue. The correction of multiple comparisons was using Benjamini-Hochberg method and q values less than 0.05 were marked with an asterisk (*).

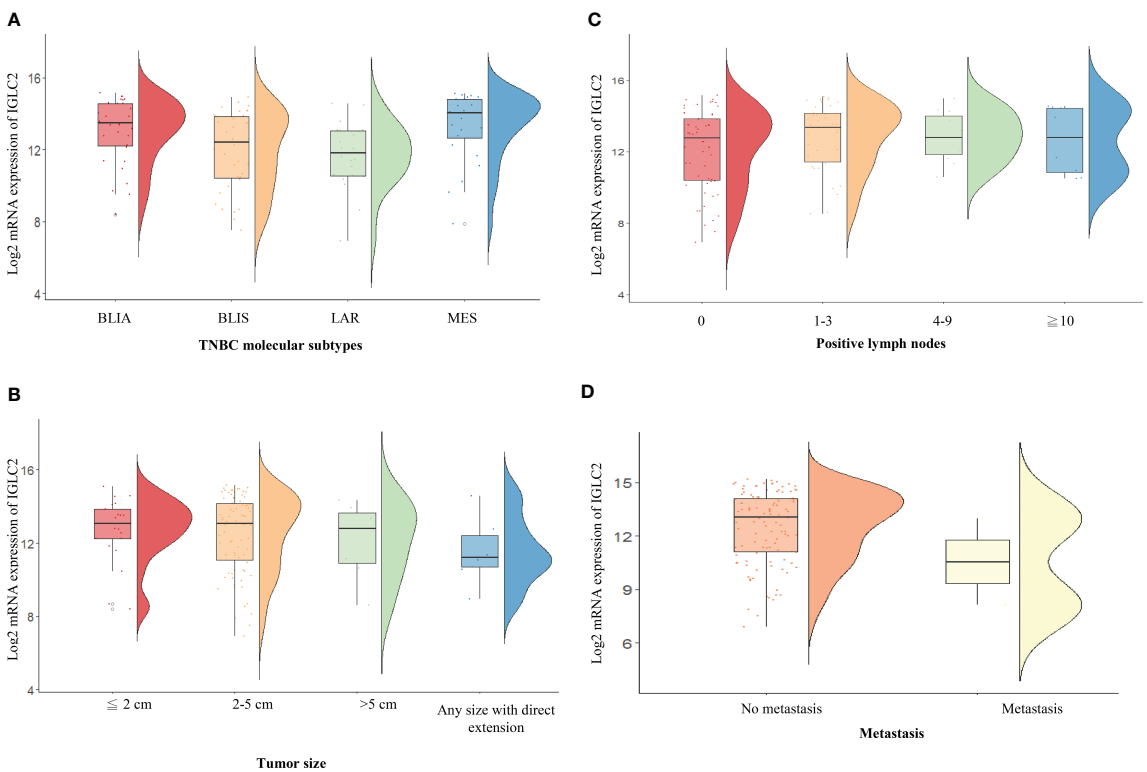

FIGURE 3 | RainCloud plots of IGLC2 mRNA expression grouped by **(A)** TNBC molecular subtypes [BLIA, BLIS, LAR, and MES], **(B)** tumor size (≤2, 2–5, and >5 cm), **(C)** the number of positive lymph nodes (0, 1–3, 4–9, and ≥10), and **(D)** metastasis (no and yes) using the GSE76275 data set.

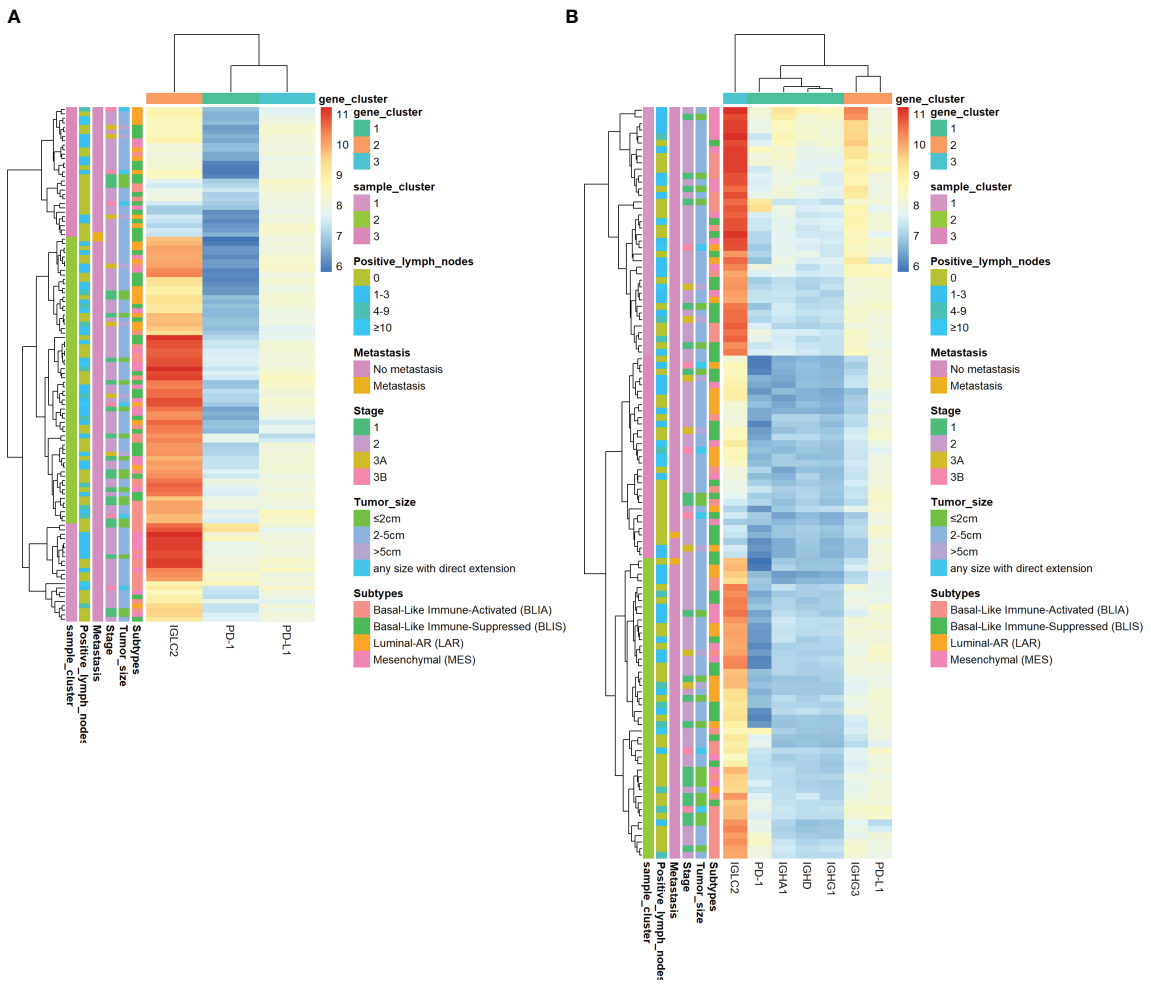

FIGURE 4 | Heatmap of log2 mRNA expression of IGLC2, PD-1, and PD-L1 of TNBC tissues using GSE 76275. **(A)**The heatmap of IGLC2, PD-1, and PD-L1, **(B)** The heatmap of IGLC2, other highly co-expressed immunoglobulin genes found in our previous study (26), PD-1, and PD-L1.

The lower expression of IGLC2 was associated with unfavorable tumor size and metastasis although there was no statistical significance (**Figure 3**). Since IGLC2 was related to PD-L1 and more specific to the clinical phenotypes of TNBC patients compared with PD-L1, IGLC2 may be a potential biomarker for the identification of TNBC patients who can benefit the most from immune checkpoint blockade treatment as well as a prognostic biomarker.

Role of *IGLC2* in Tumor Cell Proliferation

We knocked down the gene expression of IGLC2 (IGLC2-KD) in MDA-MB-231 cell lines using short hairpin RNA (shRNA) and stably transfected cells. The protein expression of IGLC2 decreased and was confirmed in the immunoblot analysis (**Figure 5A**). MTT assay was used to ascertain the role of *IGLC2* in tumor proliferation. The silencing of *IGLC2* significantly increased the proliferation of MDA-MB-231 cell lines at 48 and 72 h ($p < 0.01$) (**Figure 5B**).

Silencing *IGLC2* Influenced the Migratory and Invasive Abilities of MDA-MB-231 Cell Lines

Cell mobility is a key indicator of malignant tumor progression. Metastasis is also an important issue in clinical therapeutics. The wound-healing ability of *IGLC2*-KD cells increased compared with that of scrambled control cells in the MDA-MB-231 cell line (**Figure 5C**). We analyzed the role of *IGLC2* in tumor migration using the transwell cell migration assay. As shown in **Figure 5D**, the migratory behavior of cells significantly increased ($p < 0.01$) after the knockdown of IGLC2 in MDA-MB-231 cell lines. These results revealed that *IGLC2* mediates the migratory ability of MDA-MB-231 cell lines. Concerning the potential association of cell death and IGLC2, we analyzed the correlation of IGLC2 and well-known cell cycle genes (CDK2, CDK4, CDK6, CCNA1, CCNB1 and CCND1) (46) using GSE76275 data set (37) (**Figure S7**). IGLC2 was significantly associated with CDK6 and CCNA1 with the correlation coefficients r -0.19 and 0.17 ($p<0.05$)

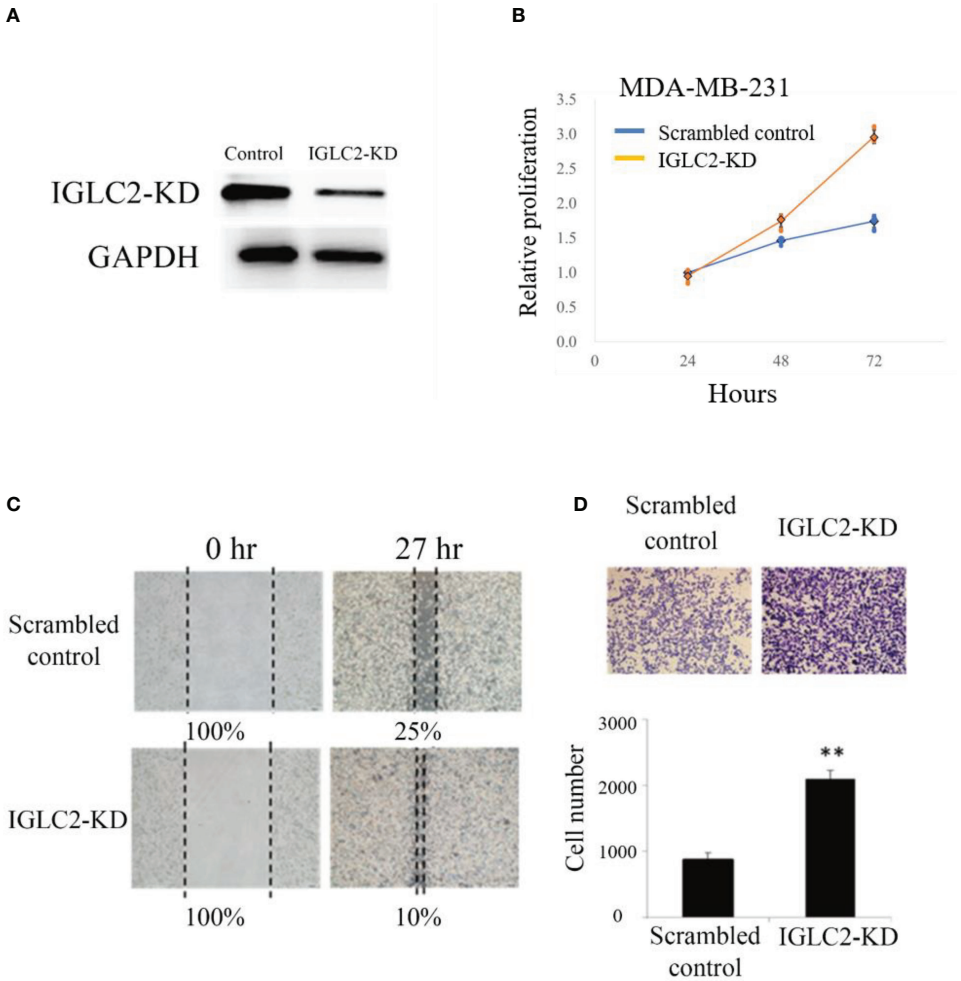

FIGURE 5 | Knockdown of expression of IGLC2 increased proliferation, migration, and healing of MDA-MB-231 cells. **(A)** Immunoblot analysis of IGLC2-KD in MDA-MB-231 cells. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. **(B)** The proliferation ability of IGLC2-KD MDA-MB-231 cells increased compared with that of the scrambled control in the MTT assay. **(C)** In the wound-healing migratory assay, IGLC2-KD MDA-MB-231 cells showed a faster healing ability than the scrambled control cells. **(D)** The migratory ability of IGLC2-KD MDA-MB-231 cells increased compared with that of the scrambled control in the transwell cell migration assay. The MDA-MB-231 cells were dyed by crystal violet staining. Statistical analysis was performed using Mann–Whitney U test. $^{**}P < 0.01$ was considered significantly different.

using Spearman correlation (**Figure S8**). The mechanism of cell death induced by IGLC2 warrants further study.

Pathway Enrichment Analysis

To understand the possible pathways related to IGLC2, we conducted NGS of three MDA-MB-231 cell lines, which included one wild-type (WT) and two IGLC2-KD MDA-MB-231 cell lines (IGLC2-KD#2 and IGLC2-KD#8) with various knockdown degrees. We observed 341 and 191 differentially expressed genes (DEGs) in IGLC2-KD#2 and IGLC2-KD#8 cells compared with WT cells, respectively. We computed the pathway enrichment analysis of the abovementioned DEGs using KEGG and GO annotations. Six enriched KEGG pathways (**Figure 6A** and **Table S2**) were recorded: phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, arrhythmogenic right ventricular cardiomyopathy (ARVC), hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and extracellular matrix (ECM)–receptor interaction. The GO-enriched pathways included urogenital system development, ECM organization, and renal system development in the BP database (**Figure 6B** and **Table S3**); proteinaceous ECM and ECM components in the CC database (**Figure 6C** and **Table S4**); glycosaminoglycan binding, growth factor binding, heparin binding, insulin-like growth factor binding, and sulfur compound binding in the MF database (**Figure 6D** and **Table S5**). Overall, IGLC2 may influence the metastasis of TNBC through the pathways related to ECM organization and cell binding, including glycosaminoglycan, growth factors, heparin, insulin-like growth factor, and sulfur compound binding.

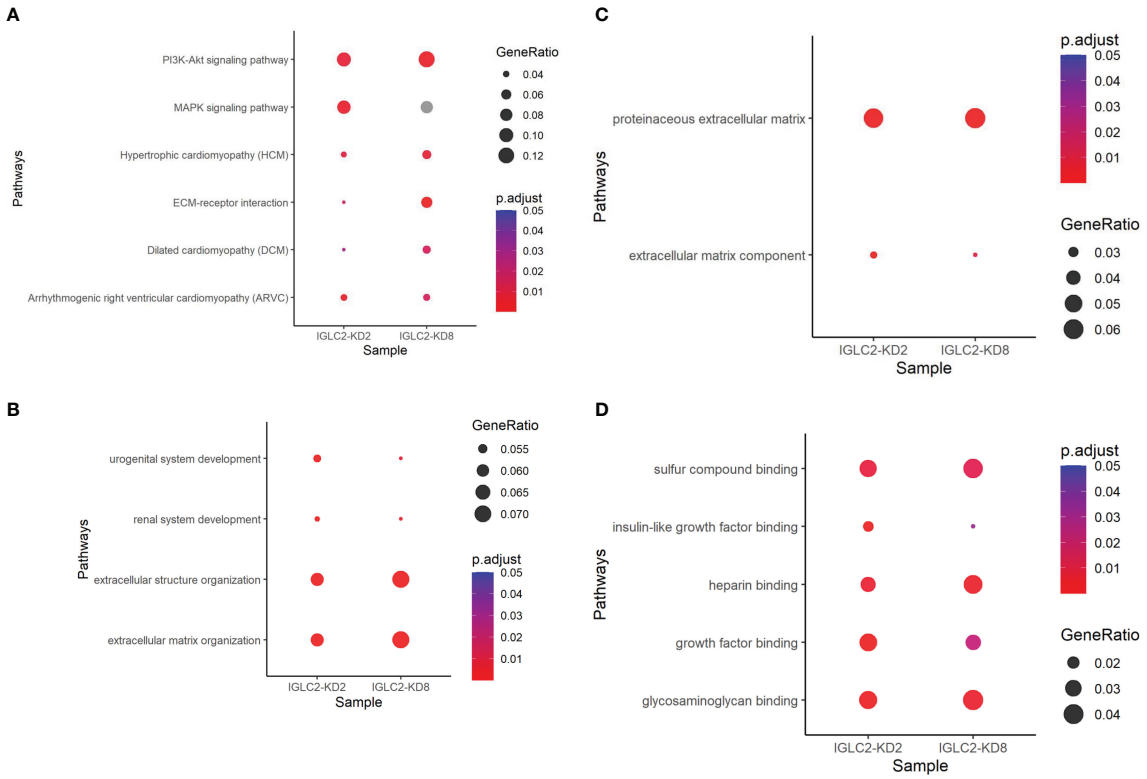

FIGURE 6 | Dot plots of pathway enrichment analysis using annotations of **(A)** KEGG, **(B)** GO-BP, **(C)** GO-CC, and **(D)** GO-MF. P.adjust means adjusted p value of pathway enrichment analysis. GeneRatio denotes the percentage of target genes existing in the enriched pathway.

DISCUSSION

To our knowledge, this research is the first study to confirm the function and beneficial prognostic effect of IGLC2 on the progression of TNBC. The knockdown of IGLC2 expression increased the proliferation, migration, and invasion of MDA-MB-231 cells. A high IGLC2 gene expression increased the RFS and DMFS in TNBC patients. The prognostic value of IGLC2 was stronger in predicting DMFS than RFS. IGLC2 had a strong prognostic value for lymph node-negative TNBC patients but had no significant effect on lymph node-positive and Grade 3 TNBC ones. IGLC2 was expressed highly in BLIA molecular subtype which was significant with unadjusted p value but became insignificant after correction for multiple comparisons. The results of pathway enrichment analysis indicated that IGLC2 may influence the proliferation and metastasis of TNBC through the PI3K-Akt, MAPK, and ECM–receptor interaction pathways.

The mRNA expressions of IGLC2 and PD-L1 were positively correlated. IGLC2 was more specific to TNBC prognosis, molecular subtypes, and clinical phenotypes than PD-1/PD-L1. Thus, IGLC2 has potential as a prognostic biomarker for the identification of TNBC patients who can benefit the most from immune therapy of PD-1/PD-L1 inhibitor agents. In addition, we confirmed that IGLC2 was expressed in MDA-MB-231 cells rather than simply generated by immune cells, as mentioned in previous studies (26).

IGLC2 is a protein-coding gene. Its protein constitutes the constant region of the immunoglobulin heavy chain. IGLC2 shows a potentially high mutation burden in a pan-cancer context (24, 47). Evidence indicates that the overexpression of immunoglobulin gene signatures (48) leads to a better prognosis of overall survival and disease-free survival in the ER–/HER2– subgroup and TNBCs (49). Other studies showed that the overexpression of a seven-gene module (*C1QA, XCL2, SPP1, TNFRSF17, LY9, IGLC2*, and *HLA-F*) has a better prognosis in ER-negative BCs. The downregulation of this module confers a high risk of distant metastasis (HR: 2.02, p = 0.009) that is independent of lymph node status and lymphocytic infiltration (50). Bianchini's team (51) discovered that B-cell/plasma metagene dominated by immunoglobulin *(IGL, IGKC, IGHC3 IGHA1*, and *IGHG3*) has an independently prognostic value in ER-negative BCs, indicating that a high B-cell/plasma metagene score is correlated to favorable DMFS. We observed that IGLC2 and IGKC were highly correlated with each other with a Pearson correlation r close to 1 (**Figure S9**). IGKC is an independent prognostic biomarker for TNBC patients and a marker of the humoral immune system (31). Tumor-infiltrating plasmablasts and plasma cells were identified as sources of IGKC expression (52). Therefore, we speculated that IGLC2 is a biomarker of the humoral immune system and associated with tumor-infiltrating lymphocytes (TILs). High expressions of IGLC2 reflect the activated humoral immune system.

Immunoglobulin genes are generally regarded to be produced by plasma or B cells rather than tumor cells (53, 54). In this study, IGLC2 was also expressed in tumor cells and played important roles in the prognosis of TNBC. This finding may explain the controversial effects of immunoglobulins on TNBC; Yang (55) stated that high immunoglobulin expression in BCs is correlated with the malignancy and American Joint Committee on Cancer stages of cancers (55), which are contradictory to our findings. This situation implies that various types and origins of immunoglobulins may exert different roles in cancers and thus warrants further study to understand the mechanisms of tumor cells. Growing evidence has indicated that immunoglobulins are not only produced by mature B lymphocytes or plasma cells but also by various normal cell types at immune privileged sites and neoplasms, including non-hematopoietic human cancer cells (55–57) and BC (55). Babbage's team (53) found rearranged V_H transcripts in the most commonly used BC cell lines. They guessed that BC tumor cells *in vivo* acquired extraneous genes from neighboring cells and kept them in their genome, or malignant epithelial cells may have initiated the required cascade of complex molecular events to rearrange the VH genes (53).

Rakha's team observed that tumor size, lymph node stage, and androgen receptor are clinical prognostic markers in TNBC (58). In the lymph node-positive subgroup, the size and androgen receptor retained their prognostic significance. However, in the lymph node-negative tumor subgroup, basal phenotype is the sole prognostic marker identified (58). Thus, the prognostic factors of TNBC varied with lymph node status. Molecular subtypes played major roles in the prognosis of lymph node-negative TNBC patients. We observed that IGLC2 had a strong beneficial prognostic effect on lymph node-negative TNBCs but had no significant influence on lymph node-positive ones. In addition, the IGLC2 expression was associated with TNBC molecular subtypes. Our finding echoed Rakha's results.

We also observed the high expression levels of PD-1 in lymph node-positive TNBC (**Figure S10**). PD-1 plays a vital role in inhibiting immune responses. The PD-1/PD-L1 axis inhibits T-cell activation, proliferation, and survival and cytotoxic secretion within cancer cells (59). We speculated that the immune response was suppressed in lymph node-positive TNCB. Thus, IGLC2 no longer reflected the effective prognostic effect of immune response against tumors. Therefore, no significant prognostic value was observed with IGLC2 in lymph node-positive TNBC.

The tumor microenvironment is a complex system formed by distinct and interacting cell populations, and its composition is related to cancer prognosis and response to clinical treatment (60). Immune modules were predictive of the pathological complete response to neoadjuvant chemotherapy in ER-/HER2-BCs (48, 61–63). B cells are present and activated in approximately one quarter of BCs and represent up to 40% of the TIL population in several BCs (49). The success of monoclonal antibody-based immunotherapy indicates the potential for harnessing the humoral immune response in BC treatment (64–67). Immunotherapy has become a promising treatment for TNBC. However, the immune checkpoint inhibitor monotherapy targeting PD-1/PD-L1 shows a mild response for TNBC patients (68). Nevertheless, promising results were found in the combination treatment of immune checkpoint inhibitors and chemotherapy in clinical trials (69). PD-L1 is the only biomarker applied in clinical practice for the selection of patients who are likely to respond to PD-1/PD-L1 immune checkpoint inhibitors. The elevated PD-L1 expression of TNBC tumors predicted an improved response to PD-1/PD-L1 immune checkpoint inhibitor treatment (45). However, the definition of "PD-L1-positive" population in clinical practice remains challenging (69). In addition, assessing whether a cancer is "immune activated" or "immune inactivated" is difficult (70). Therefore, finding new molecular biomarkers for the prediction immunotherapy response for TNBC is an urgent issue.

TNBC is the subtype of BCs most related to TIL infiltration and PD-1/PD-L1 expression (70). Among the TNBC subtypes, BLIA is enriched in immune-response genes, immune activated, and can benefit the most from immune checkpoint inhibitor treatment (69). We observed that the IGLC2 expression was significantly associated with PD-L1 expression and expressed in higher levels in BLIA subtypes.

He's team identified three TNBC subtypes, namely, Immunity High (Immunity_H), Immunity Medium (Immunity_M), and Immunity Low (Immunity_L) subtypes. Immunity_H is characterized by greater immune cell infiltration and anti-tumor immune activities and better survival prognosis compared with the other subtypes. A high immunity is positivity associated with PD-L1 levels (71). We speculated that IGLC2 is a biomarker of immune cell infiltration and anti-tumor immune activities. Therefore, we observed the positive correlation between IGLC2 and PD-L1. In addition, IGLC2 and PD-1/PD-L1 belong to the immunoglobulin superfamily, and IGLC2 is more specific to TNBC. Compared with PD-1/PD-L1, IGLC2 was more correlated with the prognosis of TNBC patients and more variable with TNCB clinical phenotypes. Thus, IGLC2 may be a potential biomarker for the identification of TNBC patients who are immune activated and will benefit from the current immune therapy.

The results of GO pathway enrichment analysis of IGLC2 indicated that IGLC2 may influence the migration of TNBC *via* the pathways of receptor binding and ECM organization. Insulin-like growth factor binding was one of the notable pathways identified. Insulin-like growth factor binding protein-3 (IGFBP-3) drives an oncogenic pathway in human TNBC cell lines (7) involving the activation of tyrosine kinase receptor epidermal growth factor receptor (EGFR) and lipid kinase sphingosine kinase (SphK) (72) and is associated with poor prognosis (73). IGFBP-3 promotes the growth of TNBC cells by increasing the EGFR signaling, which is mediated by SphK1, and the combined inhibition of EGFR and SphK1 has potential as an anticancer therapy in TNBC in which EGFR and IGFBP-3 expression is high (74).

In the results of KEGG pathway enrichment analysis, IGLC2 may influence the progression of TNBC *via* the PI3K-Akt signaling pathway, MAPK signaling pathway, ECM–receptor interaction, HCM, DCM, and ARVC. The findings of MAPK signaling and PI3K-Akt signaling were the same with He's study, that is, these

pathways are hyperactivated in TNBC subtype with high immunity (71). The PI3K/AKT/mammalian target of rapamycin pathway is the most frequently altered pathway in BC (75) and TNBC (76, 77). This pathway has been studied to identify promising new targets for the treatment of TNBC (76, 78–80). Ras-MAPK pathway activation promotes immune evasion and is related to the resistance to conventional chemotherapy in TNBC (81). Several studies have analyzed the targeted inhibitors of the Ras/MAPK pathway to identify potential treatment targets in TNBC (81–85). Increasing evidence emphasizes the crucial role of the ECM in BC progression, invasion, and metastasis (86). The ECM–receptor interaction pathway is associated with a poor prognosis, high metastatic risk (87, 88), and high incidence of chemotherapy resistance of BCs (89). Cardiomyopathy is a common adverse effect of chemotherapeutic agents (i.e., trastuzumab and doxorubicin) for BC (90–92), which may partially explain why IGLC2 is related to HCM, DCM, and ARVC. IGLC2 may serve as a potential biomarker to monitor or reduce cardiomyopathy in BC chemotherapy.

There were some limitations in the study. We only used one TNBC cell line MDA-MB-231 to validate IGLC2. The biological processes related with cell death involved by IGLC2 should be analyzed. Thus, we used multiple independent mRNA data sets from TNBC tissues to support our findings. Our team is still working on the functional analysis of IGLC2. The abovementioned limitations will be included in our future work.

CONCLUSIONS

The suppression of IGLC2 gene expression increases the proliferation and migration of MDA-MB-231 cell lines. The function pathways may be involved in PI3K-Akt, MAPK, and ECM–receptor interaction. The high expression of IGLC2 mRNA was related to favorable RFS and DMFS of TNBC patients. IGLC2 exhibited a strong beneficial prognostic effect on lymph node-negative TNBC patients but had no prognostic value for lymph node-positive TNBC patients. The combination evaluation of IGLC2 and clinical lymph node status can provide a precise prognosis prediction of TNBC patients. IGLC2 is positively correlated with PD-L1 and specific to TNBC. IGLC2 may be a potential biomarker for identifying TNBC patients who can benefit the most from immune checkpoint blockade treatment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The validation data from the mRNA microarray and survival information are available in the online tool KM plotter (http://kmplot.com/analysis/). GSE76275 was from NCBI GEO database available at https://www.ncbi.nlm.nih.gov/geo.

AUTHOR CONTRIBUTIONS

Data curation, Y-TC; Formal analysis, Y-TC, Y-JC and W-ZL; Funding acquisition, C-YY, M-CL and J-MH; Investigation, Y-TC and Y-JC; Methodology, Y-TC and W-ZL; Resources, G-SL, C-YY, J-MH, C-MC, M-CL, H-MH and V-ST; Supervision, C-MC; Validation, Y-TC; Writing – original draft, Y-TC; Writing – review & editing, Y-TC, W-CT, G-SL, Y-JC, M-CL, J-MH, C-CW, G-SL, J-CY, C-MC and H-MH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.759952/full#supplementary-material

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Human Microbiota and Immunotherapy in Breast Cancer - A Review of Recent Developments

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Breast cancer (BC) is the most common malignancy and the second cause of cancer-specific death in women from high-income countries. Infectious agents are the third most important risk factor for cancer incidence after tobacco and obesity. Dysbiosis emerged as a key player that may influence cancer development, treatment, and prognosis through diverse biological processes. Metastatic BC has a highly variable clinical course, and more recently, immune checkpoint inhibitors (ICIs) have become an emerging therapy in BC. Even with standardised treatment protocols, patients do not respond similarly, reflecting each individual´s heterogeneity, unique BC features, and tumour microenvironment. However, there is insufficient data regarding predictive factors of response to available treatments for BC. The microbiota could be a crucial piece of the puzzle to anticipate better individual BC risk and prognosis, pharmacokinetics, pharmacodynamics, and clinical efficacy. In recent years, it has been shown that gut microbiota may modulate cancer treatments' efficacy and adverse effects, and it is also apparent that both cancer itself and anticancer therapies interact with gut microbiota bidirectionally. Moreover, it has been proposed that certain gut microbes may protect the host against inappropriate inflammation and modulate the immune response. Future clinical research will determine if microbiota may be a prognostic and predictive factor of response to ICI and/or its side effects. Also, modulation of microbiota can be used to improve outcomes in BC patients. In this review, we discuss the potential implications of metabolomics and pharmacomicrobiomics that might impact BC immunotherapy treatment.

Keywords: breast cancer, microbiota, microbiome, dysbiosis, pharmacomicrobiomics, treatment, immunotherapy

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INTRODUCTION

The human gut microbiota contains ~$3x10^{13}$ bacteria, most commensals (1). Microbiota plays a crucial role in balancing inflammation, infection and tolerance towards the commensal microbes and food antigens (2, 3). Furthermore, new evidence indicates that the microbiota influences oncogenesis and anticancer treatment outcomes by regulating local and systemic antitumour immunity (4).

Immunotherapy is a major emerging treatment for some haematological and solid tumours, including breast cancer (BC). Several BC clinical trials showed better outcomes with immune checkpoint inhibitors (ICI) than conventional chemotherapy. However, despite the promising data, the patients do not respond equally to immunotherapy treatments. Besides programmed death ligand-1 (PD-L1) expression, tumour-infiltrating lymphocytes (TILs), microsatellite instability (MMRd), and high tumour mutation burden (TMB), additional biomarkers for BC immunotherapy are still a significant unmet medical need (5–7).

Among these factors, the human microbiota could be a crucial piece of the puzzle to anticipate better individual BC predictive responses to ICI. More recently, studies have been showing the role of gut microbiota in modulating response and toxicity to ICI (8–10). This review highlights the relationship within the microbiota-host-breast cancer triad, exploring the potential implication of metabolomics and pharmacomicrobiomics that might impact BC immunotherapy treatment.

HUMAN MICROBIOTA AND IMMUNE SYSTEM

In a specific biosphere, the set composed of microorganisms, including bacteria, viruses, fungi, archaea, and protists, is designated by microbiota. The collective genome of these biological agents is called the microbiome. There are different microbiota ecosystems in the human body, such as the gastrointestinal tract, skin, vaginal mucosa, or the oral cavity, which account for trillions of microorganisms. The relationship between these ecological communities and the human body is ancient and evolved over time to benefit both parties simultaneously, thus achieving a symbiotic balance (11, 12).

The link between the host's immune system and microbiota allows tolerance for commensal bacteria and the recognition of potentially infectious pathogenic microorganisms. The intestinal mucosa, below lamina propria, is composed of a layer that, among conjunctive tissue, possesses Peyer plates and immune cells, such as T and B lymphocytes and antigen-presenting cells (APC). This set of lymphoid tissue is named gut-associated lymphoid tissue (GALT), and it influences local and system immune responses (13). The communication of host and microbes is in charge of sensors, known as pattern recognition receptors (PRRs), like Toll-like receptors (TLR), expressed by intestinal epithelial cells and innate immune cells. These PRRs recognize microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). The microbiota recognition *via* these PRRs influences immune responses, both locally and systemically, and may induce the memory response, mediated by the transcriptional changes in genes or a specific locus and epigenetic rewiring of these cells upon the primary exposure (12, 14).

The bacterial metabolites directly interfere with the immune local cell's actions, namely in the secretion of immunoglobulins (such as IgA), in the stimulation of lymphocytes differentiation into regulatory T-lymphocytes (Treg) and T helper 17 (Th17), in the production of immunomodulatory cytokines and even in the epigenetic regulation of histone deacetylase enzymes. The production of IgA by plasma cells improve immunity by blocking bacterial adherence to epithelial cells. In addition, the PAMPs derived from microbes promote the maturation of dendritic cells. These cells travel from the gut to mesenteric lymph nodes, where induce naïve CD4 T cells to differentiate into effector T cells (Tregs, Th17 cells). After maturation of these cells in the mesenteric lymph nodes, they can migrate back to the gut or enter systemic circulation and influence immunity in different sites. Circulating Th17 cells enhance antitumour immunity, protecting against bacterial and fungal infections, whereas circulating Tregs secrete anti-inflammatory cytokines. Activated by APC, these T cells can circulate systemically and allow an immune response against the same organism (12). The relationship between the microbiota and CD8 T cells remains poorly characterized, although recent studies showed that microbiota-mediated activation of these cells has implications in immunity and the response to cancer therapies (**Figure 1**). Some bacterial metabolites, like lipopolysaccharide (LPS), activate innate immune response by TLR pathway stimulation and then boosted antitumour CD8 T cells that migrate from the gut to the periphery (11, 15, 16).

Microbiota's deregulation, with modifications in its functional composition and metabolic activity, is designated by dysbiosis and is linked to the development of inflammatory, auto-immune and malignant diseases. The changes in microbiota homeostasis leading to an imbalance in the symbiosis between the host and its organic habitat facilitate the loss of beneficial bacteria, overgrowth of potentially pathogenic microorganisms and loss of overall bacterial diversity. A break in the intestinal mucosa's immunological barrier causes bacterial translocation, increased pro-inflammatory cytokines, and the recruitment of effector T-cells and neutrophils, generating a local and systemic inflammatory state (11, 17).

Gut Microbiota and the Breast-Gut-Axis

The impairment of the normal functioning of gut microbiota in maintaining host wellness may deregulate the microbial-derived products or metabolites, causing several other disorders on local or distant organs, including in the tissue breast (10, 18). In this context, some microorganisms seem to interfere with host cell proliferation and apoptosis, tissue inflammation, cell invasion, immune system function, gene expression, oncogenic signalling, mutagenesis, angiogenesis, and hormonal and detoxification pathways (10, 19, 20). In addition, human microbiota's composition also influences drug disposition, action and toxicity, including of ICI (10, 21–23).

Concerning the links between human microbiota and BC, some risk modulating metabolites are already known, such as oestrogens, active phytoestrogens, short-chain fatty acid (SCFA), lithocholic acid (LCA) and cadaverine. Oestrogen formation in gut microbiota is mainly due to β-glucuronidase (BGUS) activity, which is a part of the enzymatic complex of specific intestinal bacteria. The metabolism of these BGUS-producing bacteria leads to deconjugation of xenobiotics and sexual hormone oestrogens and to an increase of oestrogens reabsorption into the systemic circulation that may increase the risk of hormone-dependent BC in women (10, 20, 24). Furthermore,

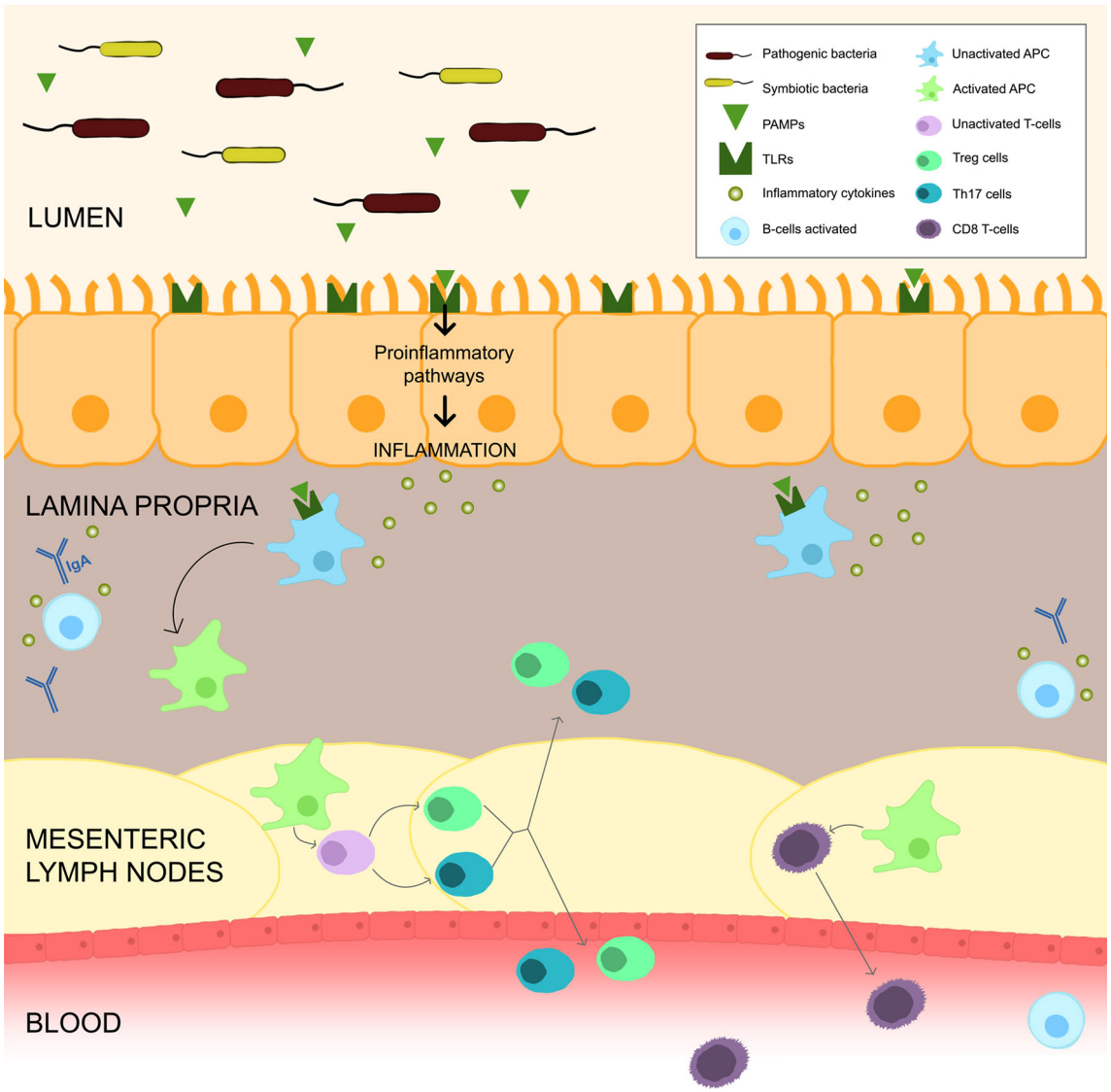

FIGURE 1 | Gut Microbiota and Immune System. Gut bacteria, through PAMPs, can upregulate the TLRs and activate inflammatory pathways, which causes a release of cytokines leading to an inflammation milieu. PAMPs can also activate APC which migrate to the mesenteric lymph nodes to stimulate T and B cells. Activation of B cells to plasma cells allows the release of IgA into the lumen. APC activate CD4 T cells to differentiate into Tregs and Th17 cells, that can migrate back to the gut or enter systemic circulation and influence immunity in different sites. APC may also stimulate CD8 T cells into effector cells that migrate from the gut to periphery.

several studies have shown differences in local and gut microbiota between BC patients and healthy controls (10, 25). On the other hand, other metabolites are linked to a protective or risk-reducing factor for BC development, including phytoestrogens, LCA and cadaverine (10). The manipulation of microbiota to select certain types of microorganisms, with the support of specific diets, prebiotics, probiotics or symbiotics, postbiotics, antimicrobial agents or even through fecal microbiota transplantation (FMT), is being studied and pondered, either as a prophylactic approach or as a therapeutic use for BC (10, 24).

The mechanism by which gut bacteria can promote BC is also through chronic inflammation, which is associated with tumour development. Gut bacteria, through PAMPs, can upregulate the TLR and activate NF-kB, which is an important inflammation regulator associated with cancer. The activation of NF-kB causes the release of several cytokines, like IL-6, IL-12, IL-17 and IL-18 and the tumour necrosis factor-alpha (TNF-alpha), leading to persistent inflammation in the tumour microenvironment. The PAMPs are recognized by innate-immune system cells and are essential components for pathogens such as the bacterial LPS, flagellin, lipoteic acid, peptidoglycans and unmethylated CpG oligodeoxynucleotides (26). In addition, secondary metabolites released by intestinal bacteria along with pro-inflammatory molecules that reach the liver *via* portal vein may promote

carcinogenesis. Butyrate, an intestinal microbial metabolite, can directly enhance the antitumour cytotoxic CD8 T cell response by modulating the ID2-dependent manner of the IL-12 signalling pathway (27).

The gut microbiome also contributes to epigenetic deregulation, which can interact with the tumour. The microorganisms can produce low molecular weight bioactive substances such as folates, short-chain fatty acids and biotin, which can participate in epigenetic processes, including altering substrates used for methylation or synthesising the complexes that change the action of epigenetic enzymes (28).

BREAST CANCER, MICROBIOTA AND IMMUNOTHERAPY

BC is the most common malignancy, and the incidence and the number of survivors continues to increase, with most developed countries reporting 85-90% five-year survival rates. However, patients with BC show different outcomes, according to different molecular profiles. Currently, four molecular subtypes of BC with prognostic and therapeutic relevance are well established: luminal A-like subtype, with high expression of oestrogen (ER) and progesterone (PR) receptors and low cell proliferation index; luminal B-like subtype with high expression of ER and PR and high cell proliferation index; HER2 overexpressing subtype and triple negative (TNBC) subtype (ER/PR and HER2 negative) (29, 30). Furthermore, depending on histological subtype and stage at diagnosis, the prognoses are different, with the luminal A-like and TNBC subtypes having the best and worst prognoses, respectively.

The most relevant BC risk factors are advanced age, exposure to endogenous and exogenous oestrogens, high breast density, history of atypical hyperplasia, personal or family history of breast disease, genetic predisposition and environmental factors (31). In addition, current evidence points to other clues for a complementary mechanism of non-hereditary risk of BC. Infectious agents are known to be the third most important risk factor after tobacco and obesity, contributing to 15–20% of cancer incidence. Gut microbiota is, as mentioned previously, an emerging field of research that is being associated with cancer through direct and indirect interference in diverse biological processes: host cell proliferation and death, immune system function, chronic inflammation, oncogenic signalling, hormonal and detoxification pathways (10, 32, 33).

Most BC patients are diagnosed in initial stages when the goal of treatment is to cure. In early and locally advanced BC, a multimodal approach is frequently used, incorporating surgery, radiotherapy and systemic therapy. The primary goals of treatment are to prolong survival and ameliorate the quality of life (10, 31).

Immunotherapy has become a forefront treatment of patients with specific malignancies. ICI utilise the immune system to exert an antitumour effect, suppressing the interaction of T-lymphocyte inhibitory receptors with their ligands on malignant cells, thereby re-stimulating the T-lymphocyte-mediated immune response against tumour-associated antigens (5, 7). BC is not traditionally considered a highly immunogenic tumour compared with other malignancies, such as lung cancer or melanoma, which have the highest rate of TMB. Although, recent data have shown immunotherapy benefits, mainly in the TNBC subtype. Usually, this BC subgroup of patients has a dismal prognosis, with worse survival and early relapse rates (34). KEYNOTE-012, phase Ib trial, investigated pembrolizumab monotherapy in previously treated TNBC patients and revealed an overall response rate (ORR) of 18.5% and a median time to response of 17.9 weeks (35). A phase II study using pembrolizumab (KEYNOTE-086) as first-line therapy for metastatic TNBC showed a safety profile and antitumour efficacy with an ORR of 23% (36). Other phases I trials, NCT01375842 and JAVELIN, evaluated the use of atezolizumab and avelumab and observed ORR of 10% and 5.2%, respectively (37, 38). A combination of immunotherapy with chemotherapy was also intensively investigated. Atezolizumab combined with nab-paclitaxel was tested in patients with metastatic TNBC, and the ORR was 67% in the first line, 25% in the second line, and 29% in the third or further lines (39). The phase III trial IMpassion 130 investigated the combination of atezolizumab plus nab-paclitaxel in untreated metastatic TNBC patients. In the intention-to-treat population (ITT) analysis, there was a progression-free survival (PFS) benefit in the combination arm (chemotherapy with atezolizumab), with 7.2 months vs 5.5 months. This benefit was most prominent in the PD-L1 positive population analysis, with 7.5 months vs 5.0 months. In the ITT population analysis of overall survival (OS), the benefit in the experimental arm was not statistically significant (21.3 months vs 17.6 months HR 0.84, 95% CI 0.69 to 1.02; P=0.08), but in the PD-L1 positive population, there was an increase in OS (25.0 months vs 15.5 months, HR 0.62; 95% CI, 0.45 to 0.86) (40). The ENHANCE-1/KEYNOTE-150 phase Ib/II trial evaluated eribulin combined with pembrolizumab, in which the ORR was higher in PD-L1-positive BC patients (30.6% vs 22.4%) (41). The KEYNOTE-355 trial evaluated the combination of pembrolizumab with chemotherapy, and patients were stratified according to PD-L1 value (combined positive score (CPS) ≥ 1, CPS≥ 10). In the CPS≥10 population, there was a significant PFS benefit in the pembrolizumab arm, 5.6 months vs 9.7 months (HR for progression or death, 0-65, 95% CI 0-49-0-86; one-sided p=0-0012) (42).

Links Between Microbiota and Immunotherapy

Treatment with immunotherapy has revolutionised cancer treatment over the past few years. However, not all patients will experience a favourable response to treatment. Thereby, predictive markers are of utmost importance for the physician to know whether the ICIs will benefit the patient.

Even with standardised treatment protocols, patients do not respond similarly, reflecting each individual´s heterogeneity, unique BC features, and tumour microenvironment (10). There is insufficient data regarding predictive factors of response to immunotherapy treatments for BC. HER-2+ and

TNBC are also more likely to express PD-L1 in the tumour microenvironment than luminal BC (43, 44). Higher levels of TILs and CD8+ T-cell/Treg ratio at diagnosis predict benefit from adjuvant and neoadjuvant chemotherapy (45, 46). Some tumours that harbour TILs and express PD-L1 are more likely to respond to ICI, suggesting this may also be the case for BC (47). In recent years, it has been shown that gut microbiota may modulate cancer treatments' efficacy and toxicity. On the other hand, it is also apparent that both cancer itself and anticancer therapies interact with gut microbiota bidirectionally. The pharmacomicrobiomics studies may support the potential use of gut microbiota analysis to predict patients' response to treatments, allowing a more personalised approach based on the microbiota-host-cancer triad (48–50).

The response and toxicity to ICI can be affected by gut microbiota (**Table 1**). In studies with mouse models, it was shown that specific microbes influence responses to this type of treatment differently, and a cause-effect relationship was established between the presence of a certain bacterial species within the intestinal microbiota and a favourable therapeutic outcome for the immune-based treatments (8, 9). A better response to anti-PD-(L)1 therapy was observed in mice with specific species of microbiota (e.g., *Akkermansia muciniphila*, *Bifidobacterium longem*, *Collinsella aerofaciens*, *Faecalibacterium prausnitzii*) (9, 10). In addition, recent data reported that OS and PFS rates were significantly higher in patients who had not received antibiotics before and during ICI treatment compared to those who had received (10, 56). Germ-free or antibiotic-treated mice received FMTs from patients' responders to ICIs and were inoculated with tumour cell lines two weeks after FMT and treated with ICIs targeting PD-1 or PD-L1. FMT from responders were enriched in *Akkermansia muciniphila*, *Bifidobacterium longum*, *Collinsela aerofaciens* and/or *Faecalibacterium* spp. The efficacy observed in mice undergoing FMT with responder faeces was associated with enhanced priming of CD45+ and CD8+ T cells in the intestine. Thus, antibiotics may pose some risk for dysbiosis due to the lack of specificity in the type of bacteria eliminated by their repeated use (54).

On the other hand, gut microbiota may also influence ICI toxicity (**Table 1**). Some studies have shown that patients with specific bacteria (e.g., *Bacteroidaceae*, *Barnesiellaceae*, *Rikenellaceae*) have a higher risk of immune-mediated toxicity. Evidence suggests that most colitis-associated phylotypes were related to Firmicutes (relatives of *Faecalibacterium prausnitzii* and *Gemmiger formicilis*), whereas no colitis was assigned to Bacteroidetes (52). In 2016 a prospective study with 34 patients

TABLE 1 | Clinical studies with association between gut microbiota and efficacy/toxicity of immune checkpoint inhibitors.

Reference	Study population	Results	
		Favourable microbiota	**Unfavourable microbiota**
Dubin et al. (51) Nature Communications 2016	Metastatic melanoma patients who received ipilimumab	Lower risk of anti-CTLA-4-induced colitis: • *Bacteroidaceae*, *Barnesiellaceae, Rikenellaceae*	–
Chaput et al. (52) Annals of Oncology 2017	Metastatic melanoma patients who received ipilimumab	Lower risk of anti-CTLA-4-induced colitis: • *Bacteroides* spp. associated with less anti-CTLA-4-induced colitis	Higher risk of anti-CTLA-4-induced colitis: • *Faecalibacterium prausnitzii*, *Gemmiger formicilis*, butyrate producing bacterium L2-21
Gopalakrishnan et al. (11) Science 2018	Metastatic melanoma who received PD-1 inhibitors	Higher clinical response: • > gut bacterial diversity • *Faecalibacterium prausnitzii*	Lower clinical response: • < gut bacterial diversity • *Anaerotruncus colihominis, Bacteroides thetaiotaomicron, Escherichia coli*
Matson et al. (53) Science 2018	Metastatic melanoma who received PD-1 inhibitor	Higher clinical response: • *Akkermansia muciniphila, Bifidobacterium adolescentis, Bifidobacterium longum, Collinsella aerofaciens, Enterococcus faecium, Klebsiella pneumoniae, Lactobacillus* spp., *Parabacteroides merdae, Veillonella parvula*	Lower clinical response: • *Roseburia intestinalis, Ruminococcus obeum*
Routy et al. (54) Science 2018	Metastatic urothelial carcinoma, NSCLC, and RCC who received PD-1/ PD-L1 inhibitors	Higher clinical response: • ↑ *Akkermansia muciniphila, Alistipes* spp., *Eubacterium* spp., *Ruminococcus* spp. • ↓ *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Parabacteroides distasonis*	–
Vetizou et al. (8) Science 2015	Advanced melanoma and NSCLC who received ipilimumab	Higher clinical response: • *B. fragilis, B. thetaiotaomicron*	–
Frankel et al. (55) Neoplasia 2017	Metastatic melanoma patients who received ICI	Higher clinical response: • *Bacteroides caccae*, *Bacteroides thetaiotamicro, Dorea formicogenerans, Faecalibacterium prausnitzii*, *Holdemania filiformis*	–

CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; ICI, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; RCC, renal cell carcinoma.

analysed the intestinal microbiota with the subsequent development of ICI-induced colitis. Bacteroidetes phylum is enriched in colitis-resistant patients and is consistent with an immunomodulatory role of these commensal bacteria (51).

CONCLUSION

A plethora of immunotherapy options is now part of treatment armamentarium of several malignancies, including BC. Unfortunately, despite this remarkable success, only a minority of BC patients respond to ICI and there is insufficient data regarding predictive factors of response.

In recent years, it has been shown that both cancer itself and anticancer therapies interact with gut microbiota bidirectionally. Thus, the pharmacomicrobiomics studies may support the potential use of gut microbiota analysis to predict patients' response to ICI, allowing a more personalised and precision medicine in oncology. Also, microbiota manipulation can be used to improve treatment outcomes in BC patients. However, further studies are necessary to validate microbiota analysis and modulation as part of the 'real world' BC clinical practice.

AUTHOR CONTRIBUTIONS

MV, SB, and DA contributed to the conception and design of the review. MV and SB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version

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Effect of Early-Stage Human Breast Carcinoma on Monocyte Programming

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Circulating monocytes are a major source of tumor-associated macrophages (TAMs). TAMs in human breast cancer (BC) support primary tumor growth and metastasis. Neoadjuvant chemotherapy (NAC) is a commonly used treatment for BC patients. The absence of the response to NAC has major negative consequences for the patient: increase of tumor mass, delayed surgery, and unnecessary toxicity. We aimed to identify the effect of BC on the subpopulation content and transcriptome of circulating monocytes. We examined how monocyte phenotypes correlate with the response to NAC. The percentage of CD14-, CD16-, CD163-, and HLA-DR-expressing monocytes was quantified by flow cytometry for patients with T1-4N0-3M0 before NAC. The clinical efficacy of NAC was assessed by RECIST criteria of RECIST 1.1 and by the pathological complete response (pCR). The percentage of CD14+ and CD16+ monocytes did not differ between healthy women and BC patients and did not differ between NAC responders and non-responders. The percentage of CD163-expressing $CD14^{low}CD16+$ and CD14+CD16+ monocytes was increased in BC patients compared to healthy women (99.08% vs. 60.00%, p = 0.039, and 98.08% vs. 86.96%, p = 0.046, respectively). Quantitative immunohistology and confocal microscopy demonstrated that increased levels of CD163+ monocytes are recruited in the tumor after NAC. The percentage of $CD14^{low}CD16+$ in the total monocyte population positively correlated with the response to NAC assessed by pCR: 8.3% patients with pCR versus 2.5% without pCR (p = 0.018). Search for the specific monocyte surface markers correlating with NAC response evaluated by RECIST 1.1 revealed that patients with no response to NAC had a significantly lower amount of $CD14^{low}CD16+HLA\text{-}DR+$ cells compared to the patients

with clinical response to NAC (55.12% vs. 84.62%, p = 0.005). NGS identified significant changes in the whole transcriptome of monocytes of BC patients. Regulators of inflammation and monocyte migration were upregulated, and genes responsible for the chromatin remodeling were suppressed in monocyte BC patients. In summary, our study demonstrated that presence of BC before distant metastasis is detectable, significantly effects on both monocyte phenotype and transcriptome. The most striking surface markers were CD163 for the presence of BC, and HLA-DR ($CD14^{low}$CD16+HLA-DR+) for the response to NAC.

Keywords: monocytes, HLA-DR, CD163, RNA-seq, breast cancer

INTRODUCTION

Breast cancer (BC) is the most common cancer among women and the second most common overall (1). State-of-the art breast cancer treatment is a multimodal approach integrating surgery, radiation, and systemic treatment, where surgery is the most effective BC treatment. Neoadjuvant chemotherapy (NAC) is commonly used as therapy for breast cancer patients, who receive chemotherapy before surgery to reduce tumor size to preserve healthy breast tissue. Efficient response to NAC correlates well with more prolonged overall survival (2, 3). However, the absence of the response to NAC has significant negative consequences for the patient: increase in tumor mass, delayed surgery, and unnecessary intoxication.

The innate immune system controls primary tumor development, growth, angiogenesis, and metastatic spread (4). Innate immune systems, especially tumor-associated macrophages (TAMs), can both cooperate with chemotherapy and block its effects (5). Circulating monocytes are precursors for the majority of TAMs that control tumor growth and metastasis (5–8). Potentially, circulating monocytes can differentiate to tumor-killing macrophages. However, intratumoral microenvironments, including hypoxia, cancer cell-produced cytokines, and growth factors, promote both the recruitment of monocytes into tumor tissue and their differentiation toward tumor-supporting M2-like macrophages (9, 10). Tumor-associated macrophages (TAM) are the most common and functionally active innate immune cells in the tumor microenvironment (6–9). There is a high correlation of proliferating TAMs with low patient survival due to the high malignancy of the tumor (4, 11, 12). The functions of TAMs are controlled on the transcriptional, epigenetic, and metabolic levels (4). The differentiation of monocytes after their migration into tissues affects the TAM function and significantly affects intramural immune status, level of angiogenesis and lymphangiogenesis, proliferation of cancer cells, and efficiency of adaptive immune response (5, 13–15). In majority of cancers, including breast, lung, prostate, and ovarian cancer, TAM substantially support tumor progression (12).

The total increase in circulating monocytes correlates with a poor clinical outcome in oral, breast, gastric, and rectal cancer (16–19). Also, in breast cancer, a high level of monocyte chemoattractant protein 1 (MCP-1, CCL2) in the tumor tissue and in the circulating blood correlates with a poor prognosis (9, 20–22). Different subsets of monocytes can act as precursors of tumor-associated macrophages (TAMs), which have pro-tumor activity and are involved in stimulating the secretion of mediators by the tumor and recruiting other blood monocytes into the tumor tissue with their subsequent differentiation into TAMs (10–13). Systemic regulation of monocytes is possible through blood cytokines and chemokines, mediators of inflammation, exosomes, and lipid and carbohydrate metabolites produced by tumor (15, 23). Therefore, tumor has a potential to affect the content and phenotype of circulating monocyte subtypes before monocytes are recruited into tumor mass. Since the population of blood monocytes is heterogeneous, different subpopulations can react to the tumor presence and correlate with tumor characteristics and treatment efficacy (24–26). Despite extensively accumulating knowledge about the mechanism by which TAM decrease the efficiency of chemotherapy, information about the role of monocytes as regulators of tumor response to chemotherapeutic agents is extremely limited (27).

In our study, we checked the hypothesis that content and activation of circulating monocytes can be affected by the presence of a breast carcinoma, and monocytes can have determinants that predict tumor sensitivity to chemotherapy. We provide the evidence that the monocyte subpopulation marked by CD163 and the whole transcriptome of circulating monocytes is affected by the presence of tumor. We found that HLA-DR+ minor monocyte subsets are indicative for the chemotherapy outcome.

MATERIALS AND METHODS

Patients

The study population of the discovery cohort consisted of breast cancer patients who were treated in the Cancer Research Institute, Tomsk National Research Medical Centre (Tomsk, Russia), from 2014 to 2021. All patients had an invasive breast carcinoma of no special type. The flow cytometry study cohort included 38 patients (**Table S1**). Patients received 4–8 courses of neoadjuvant chemotherapy (NAC) in accordance with the primary breast cancer: "ESMO Clinical Practice Guidelines for diagnosis, treatment, and follow-up 2015" (28) (**Table S1**). All patients were undergoing surgical treatment, radiotherapy, and an

adjuvant chemotherapy after NAC. The RNA sequencing study included patients with breast cancer (n = 9) and healthy females (n = 7) (**Table S2**). Real-time PCR analysis enrolled independent from RNA sequencing a research cohort of 20 patients with breast cancer and 15 healthy females (**Table S2**). An immunohistochemistry (IHC) analysis included an independent group of 122 female patients with invasive breast carcinoma (**Table S3**). For the IHC analysis, patients were divided into two groups according to the neoadjuvant treatment: 1) patients who did not receive NAC (N = 26) and 2) patients who underwent NAC (N = 96). Patients with NAC received 6–8 courses of chemotherapy in accordance with the recommendation described above (28). Chemotherapeutic regimens included FAX (fluorouracil, adriamycin, and capecitabine), CAX (cyclophosphamide, adriamycin, and capecitabine), CMX (cyclophosphamide, methotrexate, and fluorouracil), CP (cisplatin plus cyclophosphamide), CAP (cyclophosphamide, adriamycin, and platinum), and taxotere.

All patients were assessed using the RECIST 1.1 criteria after all courses of NAC based on the results of clinical examination, breast ultrasound, and/or mammography. Complete response (CR) (100% of tumor reduction), partial response (PR) (decreasing in tumor volume by more than 50%), stable disease (SD) (decreasing in volume by less than 50% or no more than 25% of increasing), and progression disease (PD) (increasing in tumor volume by more than 25%) were registered. According to the international recommendations, patients with complete and partial response composed the group with objective response, and patients with stabilization or progression compiled the group with the absence of response to NAC (29). Histological components of the "Residual Cancer Burden" were retrieved for calculating the score as described by Symmans (30). The RCB index enables the classification of residual disease into four categories: RCB-0 (complete pathologic response = pCR), RCB-I (minimal residual disease), RCB-II (moderate residual disease), and RCB-III (extensive residual disease). RCB has been calculated through the web-based calculator that is freely available on the internet (www.mdanderson.org/breastcancer_RCB).

Healthy female volunteers were enrolled in this study as a control group (17 for flow cytometry analysis, 5 for bulk RNA sequencing, and 15 for real-time qPCR). The inclusion criteria for the healthy women cohort were as follows: (a) age from 36 to 70 years, (b) no active medical conditions, (c) not taking immunomodulatory medication (over the counter or prescription) within 30 days of study, (d) willing and able to provide an informed consent, and (e) no current or past history of an oncology disease.

Peripheral Blood Mononuclear Cell Isolation and Multicolor Flow Cytometry Analysis

Whole-blood samples were obtained from the 17 healthy volunteers and 38 patients before any treatment procedures. The peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation using Lymphoset, Lymphozyte Separation Media (Biowest, France), density 1.077 g/ml. The PBMCs were washed and lysed using VersaLyse buffer (Beckman Coulter, USA). After red blood cell lysing, PBMCs were incubated with fluorescence-labeled antibody cocktail: CD45-APC-Cy7, CD14-FITC, CD16-APC, CD163-PE, HLA-DR-PE-Cy5 (**Table S4**), and 7-aminoactinomycin D (7-AAD, BD Biosciences) for dead cell discrimination. Cells were incubated for 15 min in the dark at room temperature and analyzed within 30 min. For each sample, a minimum of 200,000 events were collected. The compensation procedure was performed using VersaComp antibody capture beads (Beckman Coulter, USA). Sample acquisition was performed on a NovoCyte 3000 cytometer (ACEA Biosciences, USA) and following the gating strategy shown in **Figure S1**. Data analyses were performed with NovoExpress software (ACEA Biosciences, USA).

Monocyte Isolation for RNA Sequencing and RT-PCR Validation

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation using Lymphoset, Lymphozyte Separation Media (Biowest, France), density 1.077 g/ml. After that, monocytes from the PBMC fraction were obtained by FACS. Cells were resuspended in 150 µl of staining buffer (Cell Staining Buffer, Sony, Japan). Monocytes were defined as CD45+CD56-CD14+7-AAD- population. Conjugated monoclonal antibodies to CD45, CD56, CD14, and 7-AAD were added to the cell suspension (online **Table S4**). Samples were analyzed on a MoFlo XDP cell sorter (Beckman Coulter, USA). Sorting of monocytes was carried out in the Purify 1–2 mode, the sorting efficiency was 70%, and the purity of the target population was 96%–99% (**Figure S2**). Monocytes for real-time PCR analysis were isolated from peripheral blood by density gradients followed by positive magnetic selection using CD14+ MACS beads (no. 130-050-201, Miltenyi Biotec, Germany), resulting in 90%–98% monocyte purity as confirmed by flow cytometry.

RNA Extraction

RNA extraction total RNA was extracted from the lysed FACS-purified samples using the RNeasy Plus Micro Kit (Qiagen, USA). The quality of RNA was assessed by TapeStation 4150 automated electrophoresis system (Agilent Technology, USA). The RNA integrity index (RIN) was 9.0–9.9. The quantity of RNA was assessed by a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). The amount of obtained RNA was 0.4–2.8 ng/µl.

Whole-Transcriptome RNA Sequencing

RNA libraries were prepared with NEXTflex Rapid Directional qRNA-Seq Kit using indexed barcodes NEXTflex-qRNA-8nt-Barcodes (NOVA-5198-02, Bioo Scientific, PerkinElmer Applied Genomics, USA) according to the manufacturer's protocols. Ribosomal RNA depletion was performed with NEBNext® rRNA Depletion Kit (Human/Mouse/Rat) (NEB #E7400, New England Biolabs Inc., USA).

Whole-transcriptome sequencing was performed on a total of 9 samples of monocytes isolated from breast cancer patients and 7 healthy volunteers. Prepared libraries were then pooled

and sequenced on an Illumina NextSeq 500 instrument (Illumina, USA) with NextSeq 500/550 High-Output v2.5 Kit (75 cycles) (cat #20024906). Raw data quality control was performed using FastQC (FastQC, RRID : SCR_014583) and visualized by MultiQC (MultiQC, RRID : SCR_014982) (https://pubmed.ncbi.nlm.nih.gov/27312411/). Read alignment was performed using a STAR aligner (STAR, RRID : SCR_004463) with GRCh38 genome and GENCODE annotations (https://pubmed.ncbi.nlm.nih.gov/23104886/). The numbers of reads assigned to genomic features were calculated using QoRTs software (QoRTs, RRID : SCR_018665) (https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-015-0670-5). Subsequent analysis steps were performed using DESeq2 software (DESeq2, RRID : SCR_015687) (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0550-8). Differential expression data were visualized with pheatmap (pheatmap, RRID : SCR_016418), EnhancedVolcano (EnhancedVolcano, RRID : SCR_018931), ggplot2 (ggplot2, RRID : SCR_014601), and Phantasus software (https://genome.ifmo.ru/phantasus). Fgsea (fgsea, RRID : SCR_020938) (https://www.biorxiv.org/content/early/2016/06/20/060012) and clusterProfiler (clusterProfiler, RRID : SCR_016884) (https://www.sciencedirect.com/science/article/pii/S2666675821000667) were used for gene set enrichment analysis of biochemical and regulatory pathways using gene lists ranked by expression level and p-value. GSEA results were visualized using ggpubr (ggpubr, RRID : SCR_021139) and GOplot (https://pubmed.ncbi.nlm.nih.gov/25964631/).

Quantitative Real-Time PCR

The gene expression was quantified by quantitative real-time PCR using the TaqMan technology and was normalized to the expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were designed using the Vector NTI Advance 11.5.4 program and NCBI base. Primer synthesis was carried out by the DNA-synthesis company (Moscow, Russia). The complete sequences of used primers are listed in online **Table S5**. qRT-PCR was performed using the AriaMx Real-Time PCR thermocycler (Agilent Technologies).

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were obtained from breast cancer patients. The antigen unmasking was performed using the PT Link module (Dako, Denmark) in T/E buffer (pH 9.0). Immunohistochemical staining was performed using monoclonal rabbit anti-CD163 (1:500, ab182422, Abcam) and visualized using the Polymer-HRP detection system (ab236466, Abcam, USA). The staining results were acquired by a Carl Zeiss Axio Lab.A1 light microscope (Jenamed, Carl Zeiss, Germany) and assessed as the percentage of area occupied by positive stromal cells over the total intratumoral stromal area (according to Salgado et al.) (31). Cells outside of the tumor border and around DCIS and normal lobules, as well as in tumor zones with crush artifacts, necrosis, and regressive hyalinization, were excluded.

Immunofluorescence and Confocal Microscopy

FFPE tissue sections were obtained from 10 breast cancer patients. The antigen unmasking was performed using the PT Link module (Dako, Denmark) in T/E buffer (pH 9.0). For immunofluorescence (IF) staining, tumor FFPE clinical samples were treated with xylol solution and blocked with 3% BSA in PBS for 45 min, incubated with a combination of primary antibodies for 1.5 h; washed; and incubated with a combination of appropriate secondary antibodies for 45 min. Anti-CD163 rabbit monoclonal antibody (1:500, ab182422, Abcam), anti-CD68 monoclonal mouse antibody (1:100, NBP2-44539, clone KP1, Novus Biologicals), and anti-CD14 polyclonal sheep antibody (1:50, #BAF383, R&D Systems) were used. A combination of secondary antibodies was applied: Cy3-conjugated anti-rabbit, Alexa Fluor 488-conjugated anti-mouse (all donkey, dianova, Germany, dilution 1:400) and donkey Alexa Fluor 647-conjugated anti-sheep antibody (1:500, #A-21448, Thermo Fisher Scientific, USA). Samples were mounted with Fluoroshield Mounting Medium with DAPI (ab104135 Abcam, USA) and analyzed by confocal microscopy. Confocal laser scanning microscopy was performed with a Carl Zeiss LSM 780 NLO laser scanning spectral confocal microscope (Carl Zeiss, Germany), equipped with a ×40 objective. Data were acquired and analyzed with Black Zen software (RRID : SCR_018163). All four-color images were acquired using a sequential scan mode.

Statistical Analysis

Statistical analysis was performed using SAS software, release 9.4 (SAS Institute Inc., Cary, NC, USA). Variable distribution was presented as median [Q1–Q3]. In order to compare monocyte expressions between 2 groups, Wilcoxon 2-sample tests were used. Furthermore, simple and multiple logistic regression analyses were performed in order to investigate the binary outcome "health status". For each logistic regression analysis, the AUC (area under the curve) was assessed as a measure of goodness of the corresponding statistical model. A test with a p-value < 0.05 was considered statistically significant.

RESULTS

CD14 and CD16 Do Not Reflect Effect of Breast Carcinoma on Monocytes

The baseline characteristics of patients are presented in **Table S1**. All patients were divided into two groups depending on age (less 45 years old and more 45 years old). There are different stages of BC which were included in this study depending on the tumor size and locoregional metastasis status (**Table 1**). All patients did not have distant metastasis. The study cohort consisted of 38 BC patients with three different molecular subtypes: Luminal B (n = 17), Her2+ (n = 7), and triple-negative (n = 14). The clinical response was detected in 35 patients after NAC, where 64% of the group had an objective response (n = 24); 36% in this group had

TABLE 1 | Flow cytometry analysis of CD14+16-, CD14+16+, and $CD14^{low}16+$ in healthy female and cancer patients' group.

	CD14+16-, %Median (Q1–Q3)	CD14+16+, %Median (Q1–Q3)	$CD14^{low}16+$, %Median (Q1–Q3)
Healthy	86.12 (83.64–91.25)	3.68 (2.72–4.8)	2.70 (1.54–11.64)
Breast cancer	92.78 (83.60–98.87)	2.56 (1.44–5.60)	5.61 (1.23–8.1)

no clinical response for NAC (n = 11); and for three patients the NAC course was abrogated due to poor drug tolerance.

First, circulating monocyte subpopulations of breast cancer patients and healthy women were analyzed by flow cytometry. Monocyte subsets were identified according to the CD14 and CD16 expression into classical subpopulation (CD14+16-), intermediate subpopulation (CD14+16+), and non-classical subpopulation ($CD14^{low}16+$).

Breast cancer patients and healthy women had a similar distribution of CD14 and CD16 markers in monocyte subpopulations, indicating that these two monocyte surface markers do not reflect the effect of breast cancer on monocytes, and a deeper analysis of the subpopulations and whole transcriptome is needed (**Table 1**).

Elevated Levels of CD163 on Non-Classical Monocyte Subpopulation Are Indicative for the Presence of Breast Cancer

Next, we analyzed the median proportion of cells with the expression of CD163 and HLA-DR on the CD14+16-, CD14+16+, and $CD14^{low}16+$ subsets of monocytes in the study and control groups. Plots with gating strategies and expression histogram and gating strategy are demonstrated on the **Figure S1**. Analysis of the median proportion of HLA-DR+ classical, intermediate, and non-classical monocytes demonstrated similar parameters in the cancer group and healthy females (**Figure 1**).

We found higher median proportions of CD163-positive cells in the cancer group in CD14+16+ (98.08(86.40–100.00)%) and $CD14^{low}16+$ (99.08(83.47–99.99)%) subsets compared to healthy women: 86.96(77.33–93.02)% for CD14+16+ (p = 0.049) and 60.00 (41.06–91.3)% for $CD14^{low}16+$ (p = 0.004) cells (**Figure 2**). Moreover, using multiple logistic regression analysis with the binary outcome "health status," we found that $CD14^{low}16+163+$ monocytes were revealed to be the only significant variable for separating the two groups (odds ratio = 1.022, p-value = 0.015, AUC (area under the curve) = 0.745).

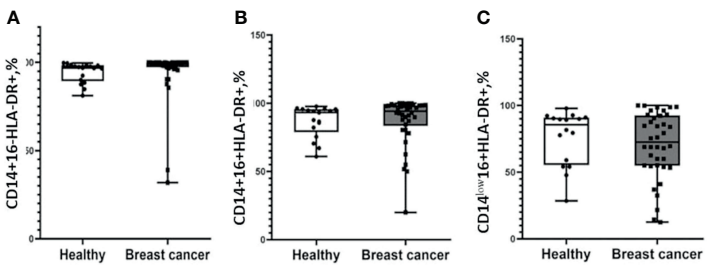

FIGURE 1 | Patients with breast cancer and healthy female individuals have a similar distribution of HLA-DR-positive monocytes. Flow cytometry analysis of CD14+16-HLA-DR+ **(A)**, CD14+16+HLA-DR+ **(B)**, and $CD14^{low}16+$HLA-DR+ **(C)**. Patients with breast cancer n = 38; healthy female individuals n = 17. Statistical analysis was performed by the Wilcoxon test.

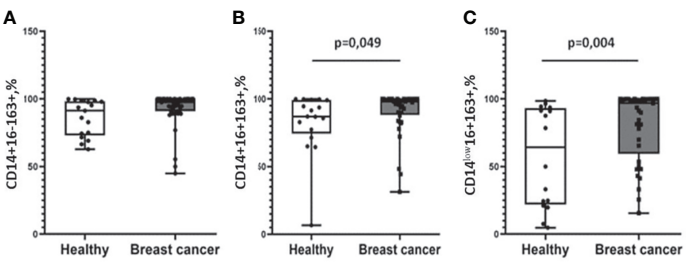

FIGURE 2 | Differential expression of CD163 on monocyte subpopulations in patients with breast cancer patients and healthy female individuals. No differences in CD14+16-163+ monocyte subset distribution **(A)**. Patients with breast cancer were characterized by a significantly higher percentage of CD14+16+163 **(B)** and $CD14^{low}16+163+$ **(C)** subpopulations compared with healthy women. Patients with breast cancer n = 38; healthy female individuals n = 17. Statistical analysis was performed by the Wilcoxon test.

Breast Cancer Alters Whole Transcriptome of Circulating Monocytes

In order to examine the effect of the presence of breast carcinoma on the transcriptional programming of circulating monocytes, we compared the whole transcriptome of CD14+ monocytes from 9 patients with BC and 7 healthy female individuals by NGS (RNA-seq). On average, 14 million filtered and aligned reads were generated for each sample. Differential expression analysis (DEA) of monocytes from patients with breast cancer (BC) versus monocytes from healthy female individuals revealed 235 upregulated and 121 downregulated genes in BC monocytes (false discovery rate (FDR) < 0.1). Principal component analysis (PCA) and hierarchical clustering separated the transcriptome of BC monocytes from the transcriptome of healthy monocytes **Figure 3A**. Although there are outliers, principal component analysis (PCA) and hierarchical clustering segregated the transcriptomic profiles of normal monocytes and monocytes from breast cancer patients differently (**Figures 3A, B**). The top significant genes are demonstrated by heatmap **Figure 3C**. A volcano plot shows genes (Log2FC > 0.58, FDR < 0.1) whose expression was significantly deregulated in breast cancer monocytes (**Figure 3D**).

The gene expression of *CD163* was upregulated in the breast cancer group with lg2FC = 0.54 and p-adj = 0.036 (**Figure 3E**) and correlated with flow cytometry analysis result (**Figure 2**). The top 20 upregulated genes such as *DDIT4*, *THBD*, *PLIN2*, *JUN*, *MAFB*, *SIGLEC1 ABCA1*, *CXCR4*, and *MX1* and other and the top 20 downregulated genes for monocytes from breast cancer patients, log2FC ≥ 0.58, FDR ≤ 0.05, were found (**Figures 3C, D**). However, CD163 expression is not at the top 20 in BC monocytes, which can be explained by the elevation of CD163 only on the minor $CD14^{low}16+$ monocyte subset, and for sequencing, we used the total pool of CD14+ monocytes (**Figure S2**). Validation of NGS data by qRT-PCR on monocytes isolated out of patients in the independent breast cancer cohort confirmed a significantly increased expression of the ABCA1 gene (**Figure 3F**). GSEA analysis reported enriched GSEA terms, such as an upregulated inflammatory response and migration in BC monocytes. Interestingly, downregulated were chromatin-remodeling pathways (**Figure S3**). The GOChord plot showed pathway enrichment of selected DEGs in BC monocytes such as inflammatory pathways (inflammatory, INFy, INFa, and INFb responses) and hypoxia pathway (**Figure S4**).

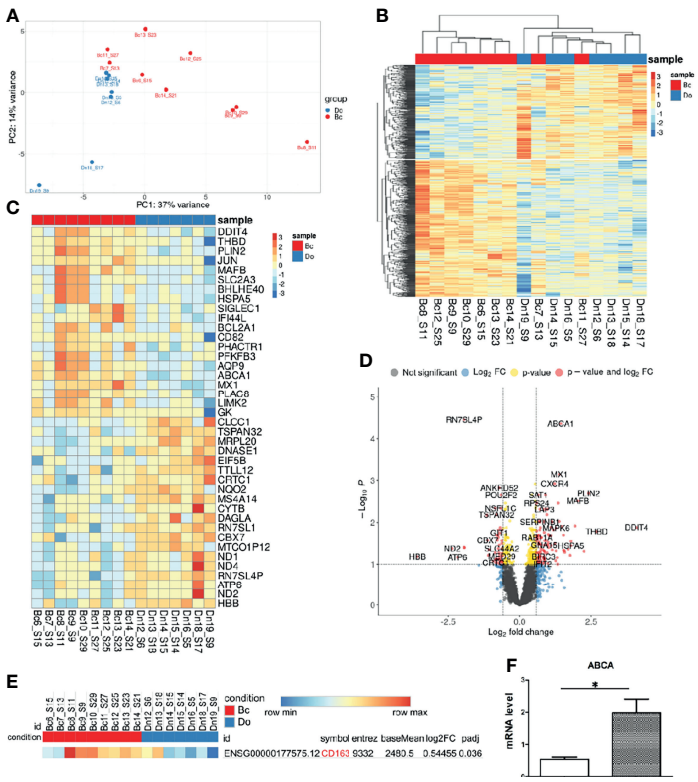

FIGURE 3 | Breast cancer alters transcriptome of circulating monocytes. **(A)** Principal-component analysis (PCA) plot of genes expressed in monocytes from healthy female donors (Do), n = 7, and from breast cancer patients (Bc), n = 9. **(B)** Hierarchical clustering of all differentially expressed genes (DEGs) between BC and healthy monocytes. Expression values are *Z* score transformed. Samples were clustered using complete linkage and Euclidean distance. **(C)** Top 20 DEG log2FC genes in healthy individuals and breast cancer patients' monocytes. **(D)** Volcano plot of RNA-Seq data breast cancer patients and healthy female monocytes. **(E)** *CD163* DEG in breast cancer and healthy female groups. **(F)** Expression of ABCA1 mRNA in breast cancer patient (n = 20) and healthy female (n = 15) monocytes (independent from the RNA-seq cohort), *p-value = 0.0006.

CD163+ Monocyte-Derived Macrophages Are Accumulated in Breast Cancer Tissue Before and After Chemotherapy

We compared the expression level of CD163+ macrophages in tumor tissue of patients without NAC and those who received NAC. The expression was assessed semiquantitatively similar to the recommendation of Salgado et al. (31). Stromal TAMs were scored as a percentage of the stromal areas alone excluding carcinoma cells. Examples of percent of area filled by CD163+ cells are presented in **Figure 4**. There, a score of 60% stromal cells means that 60% of the stromal surface area is occupied by CD163+ cells. We found that the percentage of area with CD163+ cells was higher in NAC-treated patients compared to untreated ones (10.0(5.0–20.0)%, mean = 14.06, N = 96 vs. 1.0 (1.0–10.0)%, mean = 8.92, N = 26, p = 0.014) (**Figure 4**).

Then, we questioned whether NAC affects the accumulation of CD163-positive monocytes into breast cancer tissue. We performed IF/confocal microscopy analysis in tumor tissues taken after NAC. It was demonstrated that CD163 is predominantly expressed on CD14+CD68+ monocyte-derived macrophages, which infiltrate tumor mass (**Figure 4**), indicating that NAC can induce the recruitment of CD163+ monocytes into breast cancer tumor. The next question was to identify whether an additional marker on circulating monocytes can be indicative for the NAC efficiency.

$CD14^{low}16+$ and HLA-DR+ Monocytes and Response to Neoadjuvant Chemotherapy

We addressed the question, whether monocyte subtypes before NAC can correlate with clinical response to NAC. The differences between NAC non-responders and responders at CD14+16+ (1.33(0.52–3.10) vs. 2.24(1.34–5.31), p = 0.082) and $CD14^{low}16+$ (5.45(2.01–10.23) vs. 2.24(1.17–4.67), p = 0.099) subsets before NAC slightly failed to reach statistical significance (**Table 2**). We found non-changed CD163+ cell proportions before treatment in the group with an objective response to NAC and the group without response to NAC in the CD14+16-, CD14+16+, and $CD14^{low}16+$ subsets (**Table 2**).

Before NAC, for HLA-DR we identified a higher proportion median of HLA-DR+ cells in the CD14+16+ subset: (97.72 (91.28–98.87)%, p = 0.005) and in the $CD14^{low}16+$ subset (84.62(63.98–93.16)%, p = 0.0447) for responders (**Figure 5A**). Accordingly, the non-responders' group had a lower level of CD14+16+HLA-DR+ (84.51(51.77–92.59)%) and $CD14^{low}16+$ HLA-DR+ (55.12(21.70–79.32)%) (**Figure 5A**). The tendency for the increased expression of HLA-DR on CD14+16- monocytes

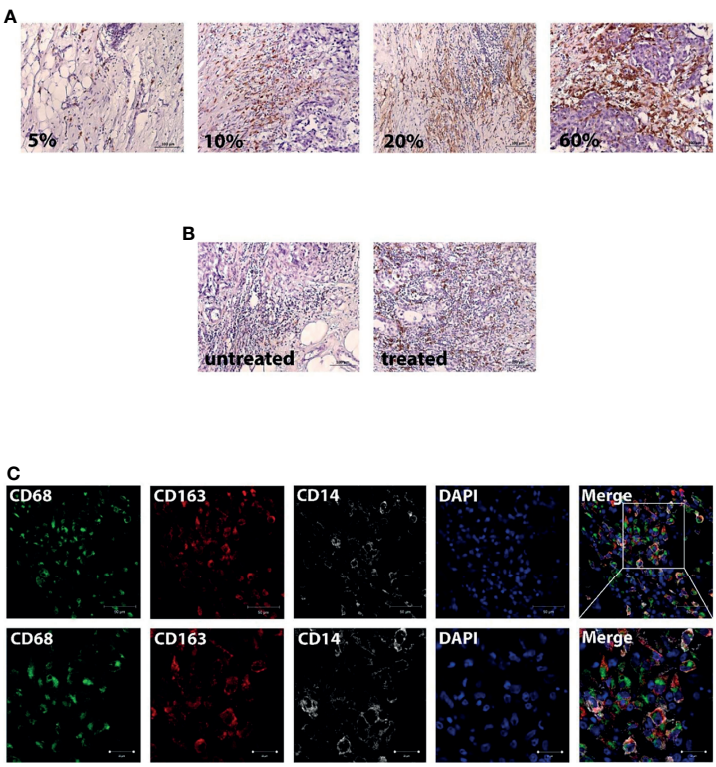

FIGURE 4 | Breast cancer tissue is infiltrated by CD163-positive monocyte. **(A)** Examples of the percent content of the stromal surface area which was occupied by CD163+ cells. Scale bars correspond to 100 μm (×200). **(B)** Representative images from untreated and NAC-treated breast tumor tissue. Scale bars correspond to 100 μm (×200). **(C)** IF/confocal microscopy analysis was performed for breast tumor tissues. The infiltration of CD14+CD68+CD163+ cells was found in all samples. Representative images are demonstrated. Scale bar corresponds to 50 μm in the main image and 20 μm in the zoom image.

TABLE 2 | Flow cytometry analysis of CD14, CD16, and CD163 markers on monocytes from BC patients depending on clinical response to NAC.

Subset	BC without response, %, Median (Q1–Q3)	BC with response,%, Median (Q1–Q3)	Wilcoxon test, p-value
CD14+16-	92.4 (88.07–98.00)	93.14 (87.67–95.64)	0.986
CD14+16+	1.33 (0.52–3.10)	2.24 (1.34–5.31)	0.082
CD14^{low}16+	5.45 (2.01–10.23)	2.24 (1.17–4.67)	0.099
CD14+16-163+	94.63 (90.74–97.49)	96.66 (90.05–99.86)	0.510
CD14+16+163+	96.30 (89.79–99.18)	98.78 (83.29–100)	0.590
CD14^{low}16+163+	84.28 (33.11–99.02)	98.58 (61.64–99.99)	0.112

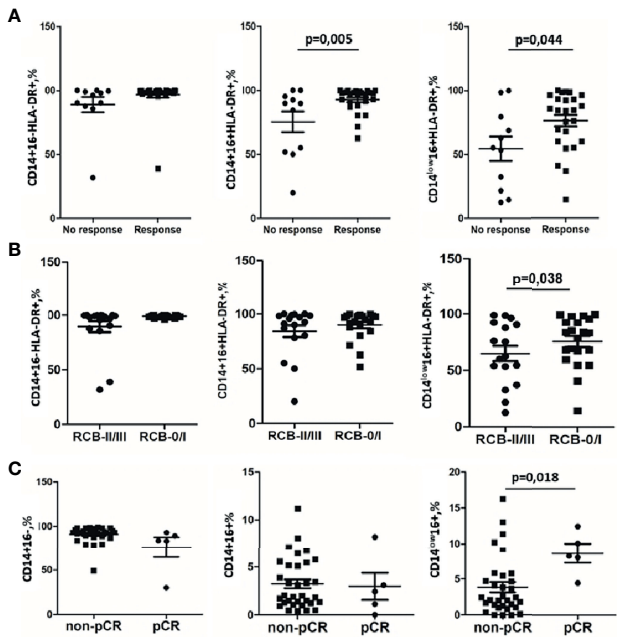

FIGURE 5 | Monocyte subpopulations before treatment are sensitive indicators for NAC response. **(A)** Strong significant difference between the group with clinical response to NAC and the group without response to NAC detected by the RECIST 1.1 scale was found for CD14+16+HLA-DR+ and CD14^{low}16+HLA-DR+. **(B)** CD14^{low}16+HLA-DR+ subset decrease in the group of patients with RCB II/III vs. RCB 0/I. **(C)** CD14^{low}16+ monocytes correlate with pCR. Statistical analysis was performed by the Wilcoxon test.

was detected for NAC responders compared to non-responders. The percentage of CD14+16-HLA-DR+ out of all CD14+16- was 98.75(98.1–99.01)%) the responders, and 91.86(74.62–95.67)% for non-responders (p = 0.077) (**Figure 5A**). A multivariable logistic regression analysis with the binary outcome "response" provided a statistical model including CD14+16-HLA-DR+ with odds ratio = 0.88 (p = 0.019). Also, the statistical model includes CD14+16- (odds ratio = 2.1, p = 0.032) and CD14^{low}16+ (odds ratio = 2.74, p = 0.019) either. The AUC of this model was 0.943.

The pathological complete response (pCR) is a clinically significant parameter for prediction of the long-term outcome in individual patients with early-stage breast cancer treated with preoperative systemic therapy (32, 33). We analyzed the correlation between pCR and monocyte subsets. The CD14^{low}16+ subpopulation before NAC had a significant correlation with pCR (**Figure 5C**). Patients without pCR had 2.5 (1.3–5.45)% of CD14^{low}16+ out of total monocytes, while patients with pCR had a significantly increased percentage of CD14^{low}16+: 8.3(8.1–12.4)% (p = 0.018). The percentage of CD14+16- was similar in non-pCR 92.52 (88.07–94.58)% and pCR 83.78(83.48–89.29)% (**Figure 5C**). Similar data were obtained for the CD14+16+ subset: 2.03(1.39–5.59)% in the non-pCR group vs. 2.42 (1.13–3.10) in the pCR group (**Figure 5C**). Next, we evaluated the response to NAC by analysis of the tumor size in BC in patients using residual cancer burden (RCB) as a clinical parameter. Based on RCB grade, we have compared 2 patients' groups, RCB-0/I group and RCB-II/III, and analyzed the percentage of CD14^{low}16+HLA-DR + monocytes in the CD14^{low}16+ monocyte subpopulation. We found that in the RCB-0/I group CD14^{low}16+HLA-DR+ constituted 84.28 (63.98–94.82)% and in the RCB-II/III group CD14^{low}16+HLA-DR+ constituted 60.03 (32.5–82.73)% (p = 0.038; **Figure 5B**). These data corresponded to the data obtained for the monocytes subtypes' correlation with NAC efficacy evaluated by the RECIST 1.1 scale (**Figure 5A**).

DISCUSSION

Systemic changes in the health status related to metabolic conditions and local processes characterized by inflammation result in change in the content of subpopulations and appearance of a non-typical biomarker on the circulating monocytes (23). In this study for the first time, we have identified the monocyte biomarkers indicative not only for the presence of breast cancer but also predicting the response of breast cancer patients to neoadjuvant chemotherapy, a broadly used approach to suppress the activity of primary tumor before the surgical intervention.

Chronic inflammation underlies the development of the most dangerous diseases, including malignant transformations (34–36). Monocytes can potentially sense the presence of tumor, and their clinical value was suggested (23). Up to date, the increased percentage of monocytes in the circulating mononuclear cells was found to be indicative for worse prognosis in cancer patients (16–19).

Isolated studies reported the correlations between main subsets of monocytes and clinical manifestation of cholangiocarcinoma (37), colorectal (38), and lung cancer (39). However, the data are still controversial, due to a lack of validation of the large samples, so there is no significant value for clinical use. Out study demonstrated that the main monocyte subsets (CD14+16-, CD14+16+, and CD14^{low}16+) do not change in patients with breast cancer controlled by healthy individuals. Similar observations were made by other research groups who

also did not find quantitative differences in the proportions of classical, intermediate, or non-classical subsets in breast cancer patients compared with healthy volunteers (40, 41). CD16 has been proposed to be a differentiation marker for monocytes, suggesting that $CD14^{low}16+$ monocytes are more mature than CD14+16– monocytes (42). Therefore, breast cancer presence seems not to affect the monocyte differentiation or maturation in the circulation.

Searching for the informative biomarker for the systemic cross-talk between the growing tumor and the innate immune system, we found a high percentage of HLA-DR-positive cells within the CD14+16- subpopulation of monocytes. MHC class II surface protein HLA-DR is a key mediator of antigen presentation which is highly expressed in monocytes of healthy individuals. Only two patients had a decreased percentage of CD14+16-HLA-DR+ monocytes, but 38 women had similar data compared with the healthy group. The non-classical subset had a lower median of HLA-DR+ compared with the classical subset in the study and control groups. Interestingly, the $CD14^{low}16$ +HLA-DR+ percentage varied from 12.5% to 100% in BC patients and from 35.3% to 97.7% in healthy women. The statistical significance for the differences between breast cancer patients and healthy individuals was not achieved by analyzing the expression of HLA-DR on monocytes; however, we cannot exclude that statistical significance can be potentially achieved if larger patient cohorts are available. As we did not have a clear vision of the relevant effect sizes, we refrained from performing a statistical power analysis. Nevertheless, despite of the rather small sample sizes we obtained statistically significant results which may be clinically relevant. We suggest that studies with higher sample sizes should be performed in order to verify these results.

CD163 is a scavenger receptor for the hemoglobin–haptoglobin (Hb–Hp) complexes. In general, the cellular expression of CD163 is upregulated by anti-inflammatory factors, whereas pro-inflammatory signals downregulate its expression (43, 44). In healthy conditions, scavenging of Hb–Hp complex-mediated CD163 is silent and does not induce an inflammatory response in monocytes. Data regarding CD163 expression in the classical, intermediate, and non-classical monocytes are controversial. In colorectal cancer patients, CD163 expression was found to be decreased in the classical and total subpopulations (45). On the other hand, the CD163+14+ cell frequency in malignant pleural effusion was higher than that in non-malignant pleural effusion (46). BC patients demonstrated a higher level of CD14+163+ and CD14+CD163+CD204+ in a cohort of 56 women from Shanghai Sixth People's Hospital (25). However, the authors did not analyze the distribution of CD163+ in classical, intermediate, or non-classical subsets. For the first time, we demonstrated that CD14+16+ and $CD14^{low}CD16+$ (but not CD14+16-) had a significantly higher percentage of CD163+ positivity than the same monocyte subpopulations in healthy volunteers.

Considering that CD16 is indicative for the maturation of monocytes in circulation, we can hypothesize that CD163 expression is stimulated by the circulating factors produced by the tumor. According to multiple logistic regression analysis, the CD14+CD16++CD163+ subset was statistically significantly increased in patients with breast cancer. The role of CD163 as a marker of the M2 phenotype is highly questionable due to its expression of the macrophages in mixed chronic inflammatory conditions; however, CD163 is frequently used to identify tumor-supporting TAM in various types of cancer (12, 46–48). We proposed that CD163+ cells are a functional biomarker which does not strictly define the M2 direction of TAM (12). We found an increase in CD163 expression on overall monocyte pull by whole-transcriptome RNA sequencing. The skew of circulating monocytes to the scavenging direction in patients with breast, colorectal, and lung tumors indicate the appearance of the previously described tumor-educated monocytes (40, 49, 50).

In this study, we found the evidence that CD163 is elevated on the circulating monocytes in patients with breast cancer and is intensively recruited to the tumor site, suggesting that CD163 can be used as a marker for monocyte-derived TAMs. CD163+ TAMs are associated with poor histological grade, larger tumor size, Ki67 positivity, and LN metastasis in patients (51–53). A lot of studies from different cohorts of BC patients showed that CD163+ macrophages can be predictors of poor survival (54–58). Frequently, higher infiltration of TAMs expressing CD163 correlated with unfavorable clinic-pathological features and reduced survival in patients with breast cancer. Their polarization and localization in different tumor compartments should be taken into account for determining the prognostic and/or predictive role of TAMs. It was shown that CD163+ macrophages can have a positive effect depending on the local microenvironment in LN (58).

Predicting the response to standard NAC in advance, before the treatment start, is a highly beneficial strategy for the personalized optimization of cancer treatment and has a good potential to improve therapy outcomes and patient survival. Our results demonstrate a statistically significant correlation between the percentage of CD14+16+HLA-DR+ and $CD14^{low}16+$HLA-DR+ and NAC efficacy. Patients who responded to NAC showed a higher level of HLA-DR+ monocytes in these subsets. Moreover, our statistical model included a CD14+16-HLA-DR+ variable with an odds ratio of less than one and a relatively high AUC value of 0.43. We suggest that an increased presence of CD14+16-HLA-DR+ is a good predictive marker because a higher percentage of this subset correlates with a small risk of non-response NAC.

In the last decade, CD14+$HLA\text{-}DR^{low}$ monocytes were found in the blood of patients with B-cell lymphomas (59, 60) and glioblastoma (61), renal (62), and prostate (63) cancers. A low expression of HLA-DR on the CD14+ cells was associated with impaired immune function in many inflammatory diseases (64, 65). Therefore, a lower percentage of HLA+ monocytes correlates with immunosuppression.

CONCLUSIONS

Monocytes are universal innate immune sensors for the non-self and unwanted-self circulating factors, including factors produced

by a growing tumor. Based on our data, we can hypothesize that the systemically suppressed antigen-presenting ability of the innate immune system diminishes also the effect of NAC, and patients with a lower percentage of CD14+HLA-DR+ have a higher risk of unsuccessful response to NAC and should be subjected to radical surgery as soon as possible. However, this hypothesis needs further verification on the large patient cohorts. Overall, our study showed that human breast cancer on the stages before hematogenous, distant metastasis is detectable, growth tumor has a systemic effect on the innate immunity, and monocytes are circulating innate immune sensors for tumor presence.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was approved by the Local Committee for Medical Ethics of Cancer Research Institute of TNRMC (Russian Federation) and performed according to the guidelines of the Declaration of Helsinki and the International Conference on Harmonisation's Good Clinical Practice Guidelines (ICH GCP) with written informed consent from all subjects. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JKz, MP, MS, and NC desined the project. MP, IL, MS, EG, AF, EP, MR, JKa and NT performed the experiments. JKz, MP, PY, CW, and LZ analyzed the data. JKz, MP, NC, and CW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.800235/full#supplementary-material

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REVIEW
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Immunotherapy in Breast Cancer and the Potential Role of Liquid Biopsy

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Liquid biopsy biomarkers, such as circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), are noninvasive diagnostics that could complement predictive and prognostic tools currently used in the clinic. Recent trials of immunotherapy have shown promise in improving outcomes in a subset of breast cancer patients. Biomarkers could improve the efficacy of immune checkpoint inhibitors by identifying patients whose cancers are more likely to respond to immunotherapy. In this review, we discuss the current applications of liquid biopsy and emerging technologies for evaluation of immunotherapy response and outcomes in breast cancer. We also provide an overview of the status of immunotherapy in breast cancer.

Keywords: breast cancer, circulating tumor cells, circulating tumor DNA, liquid biopsy, immunotherapy, biomarkers

1 INTRODUCTION

Predictive and prognostic biomarkers in oncology have played an important role in guiding treatment to improve patient outcomes (1, 2). The recent emergence of liquid biopsy-based biomarkers from blood—e.g., circulating tumor cells (CTCs) (3–5) and circulating tumor DNA (ctDNA) (6–10)—has offered minimally invasive approaches to assess tumor response and survival in early-stage and metastatic breast cancer (11, 12). Blood-based biomarkers have addressed the limitations poised by tissue-based biomarkers because they are more readily accessible than tissue (13). For example, blood markers offer several advantages over tissue assessment because of the ease of serial analysis *via* blood draws and the feasibility of monitoring of recurrence after surgical resection, when no clinically measurable disease is present (i.e., minimal residual disease) (14).

Breast cancer is the most common cancer in women and represents the leading cause of cancer-related deaths in women worldwide (15). A significant unmet need is effective treatment for triple negative breast cancer (TNBC), a particularly aggressive subtype of this disease. TNBC, defined by a lack of estrogen, progesterone, and human epidermal growth factor receptor 2 (HER2) receptors, accounts for 15% to 20% of all breast cancers and typically has a poor prognosis (16). Immunotherapy has revolutionized the management of multiple solid tumors. For TNBC, immune checkpoint inhibitor (ICI) agents targeting programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) and combined with chemotherapy have demonstrated

significant clinical activity in early-stage and metastatic TNBC, leading to regulatory approval in the U.S. (17–20). However, in the metastatic setting, only a subgroup of patients responds to these agents, and in the early-stage setting it is important to identify those who do not need ICI for optimal outcome. Therefore, it is important to discover predictive biomarkers to identify breast cancer patients who will benefit from immunotherapy.

Currently, the only predictive test for first-line immunotherapy in patients with metastatic TNBC is immunohistochemical (IHC) testing for PD-L1 expression (17, 21, 22). PD-L1 testing of tumor tissue currently lacks standardization to encompass the heterogeneity in the assays, the diversity of antibodies for testing and the assessment platforms (instrumentation), and the thresholds for scoring PD-L1 status. Additionally, there is diversity in the tumor microenvironment compartments that are analyzed (tumor cells, immune cells, or both). In addition to prediction, it is important to detect resistance to immunotherapy and identify biomarkers to monitor breast cancer patients during immunotherapy. Evaluating patient immunotherapy response by imaging presents another challenge, as standard radiologic criteria for assessing response to ICI therapy could miss progression. One of the obstacles is pseudoprogression, described as radiologic enlargement of the tumor mass due to infiltration of leukocytes (23). There is an unmet need to identify sensitive and specific predictive biomarkers to select patients who will benefit from ICI therapy and to avoid unnecessary toxicities and cost. Liquid biopsies could be a potential approach to identify more robust biomarkers associated with ICI. Recent studies have shown that CTCs frequently express PD-L1 and are associated with worse prognosis, and thus, could serve as a useful non-invasive biomarker for real-time assessment of PD-L1 status and estimation of risk of disease relapse and progression (24–30).

In this review, we discuss liquid biopsy applications to guide immunotherapy to treat breast cancer. We highlight the promises and challenges of liquid biopsy biomarkers for breast cancer immunotherapy. Here, we focus our discussion on two liquid biopsy biomarkers, CTCs and ctDNA, and the clinical studies that examined their utility (**Figure 1**).

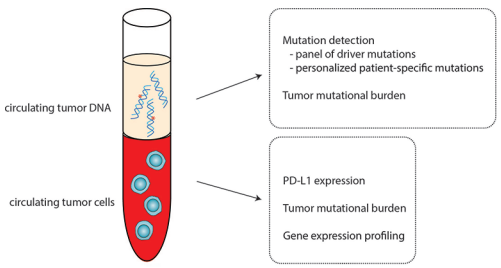

FIGURE 1 | Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) as biomarkers for immunotherapy. CTCs and ctDNA can serve as a noninvasive alternative for solid tissue assessment of candidate biomarkers to predict immunotherapy response and outcomes.

2 LIQUID BIOPSY BIOMARKERS: CHARACTERISTICS AND TECHNOLOGY PLATFORMS FOR ANALYSIS

The most established biomarkers for liquid biopsy assessment include CTCs (31) and ctDNA (6). Over the past decade or more, questions regarding the prognostic and predictive significance of these biomarkers have been actively studied (32–34). Below, we describe CTCs and ctDNA and discuss the detection platforms for each biomarker.

2.1 Circulating Tumor Cells

CTCs, defined as rare cells shed by primary tumors into the blood, are hypothesized to be precursors of distant metastases (35). Numerous studies have unequivocally demonstrated the prognostic value of these cells both in early-stage (3, 4) and metastatic breast cancer (5, 36). However, the clinical utility of these cells for guiding treatment to improve patient outcomes has yet to be fully established (37).

The many technologies for the detection and enumeration of CTCs have been reviewed in detail in recent articles (14, 38). To date, the only CTC detection platform to have received clearance from the US Food and Drug Administration (FDA) for enumeration of CTCs in breast cancer is the CellSearch™ system (31). CellSearch™ is a two-step method that involves: (1) immunomagnetic enrichment of cells expressing the epithelial cell adhesion marker (EPCAM), and (2) fluorescence microscopy detection of nucleated cells that are positive for cytokeratin (epithelial marker) and negative for CD45 (leukocyte marker) expression. The detection of 5 or more CTCs per 7.5 mL of blood has been demonstrated to be strongly prognostic for progression-free survival (PFS) and overall survival (OS) in patients with metastatic breast cancer (5, 31, 36). The prognostic value of CTCs in early-stage breast cancer, particularly in the neoadjuvant setting has been recently examined (4). Patients with one or more CTCs identified before neoadjuvant therapy have increased risk of local and distant recurrence as compared to those with no detectable CTCs (4).

Modifications to the standard CellSearch™ protocol for CTC enumeration has allowed for the reliable assessment of PD-L1 expression in CTCs (24, 25, 29, 30, 39). Researchers have added a fluorophore-conjugated antibody to PD-L1 (e.g., B7-H1) to the antibody cocktail (anti-cytokeratin and anti-CD45) for semi-quantitative analysis of PD-L1 expression in CTCs, using cancer cell lines with known PD-L1 expression levels as references. Strati and colleagues used RT-PCR to measure PD-L1 expression in CTC-enriched fractions after immunomagnetic enrichment using CellSearch (25). Others have used filter-based methods to enrich for CTCs prior to immunofluorescence staining to examine PD-L1 expression (26–28).

2.2 Circulating Tumor DNA and Cell-Free DNA

ctDNA are short fragments of DNA derived from a primary tumor, metastatic foci and/or circulating tumor cells. ctDNA can be detected in plasma and are present in an admixture of DNA

derived mainly from normal blood cells. Collectively, this admixture is known as cell-free DNA (cfDNA). Examination of the size distribution of cfDNA reveals a predominant length of 166 bp with a series of peaks every 10 bp (40). The size and periodicity indicate an association with nucleosomes and suggest that cfDNA is released into circulation *via* apoptosis or necrosis of cells (41). It is unknown whether the mechanisms involved in the release of cfDNA are the same as those of ctDNA (41, 42).

Detection of ctDNA can be performed using several methods, including deep next generation sequencing (**Figure 2**) (14). The primary goal of deep sequencing is to detect rare mutated DNA copies shed by tumors (ctDNA) and differentiate them from wildtype copies that are simultaneously released from normal hematopoietic cells undergoing apoptosis. Comparative sequencing studies have shown that specific mutations in ctDNA *vs*. matched primary tumor tissue are generally concordant (43, 44), however, temporal spacing (e.g., timing of sample collection) and tumor heterogeneity could also lead to discrepancies (45). Overall, these data suggest that ctDNA can complement tissue sequencing to find actionable biomarkers. Initial approaches to detection of ctDNA involved digital droplet polymerase chain reaction (ddPCR) (46). However, ddPCR has become less favored (over sequencing) because of its limitations, particularly the restricted number of mutations that can be assessed in one experiment. Sequencing, on the other hand, can interrogate whole genomes, or a panel of genes that include driver mutations frequently observed in cancer, or a personalized list of mutations identified from a patient's solid tumor (7, 10).

The presence of ctDNA in the blood of patients with early-stage breast cancer is associated with aggressive disease and portends poor clinical outcomes (10, 47). Failure to clear ctDNA during neoadjuvant or adjuvant therapy reflects treatment resistance and increased risk of metastatic recurrence (10, 47).

In the metastatic breast cancer setting, ctDNA testing is becoming a part of routine clinical practice because of the high prevalence of actionable mutations and its potential utility as a surrogate for tumor burden (48). A recently defined clinical use of ctDNA in metastatic breast cancer involves the detection of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA)* mutations, which is already used to guide treatment and is now cleared by the Food and Drug Administration (FDA) (49). Studies are also evaluating the use of ctDNA to detect new mutations during treatment that might represent an early indication of resistance (50), e.g., the emergence of *ESR1* mutations in metastatic breast cancer patients treated with CDK4/6 inhibitor in the PADA-1 Trial (51).

3 IMMUNOTHERAPY IN BREAST CANCER

Immune checkpoint blockade, which helps the immune system recognize and attack tumor cells, is used to treat various cancers with durable responses compared to most chemotherapy and targeted agents. Inhibiting the PD-L1/PD-1 axis with monoclonal antibodies is a breast cancer treatment strategy that provides cell-mediated antitumor activity. The binding of PD-L1 to its receptor on T cells, PD-1, inhibits adaptive immune responses in the tumor microenvironment, enabling malignant cells to escape immunosurveillance. Immunotherapy drugs approved for the treatment of multiple tumor types include anti-PD-1 (pembrolizumab, nivolumab and cemiplimab), anti-PD-L1 (atezolizumab, durvalumab and avelumab), and the cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) (ipilimumab and tremelimumab) (52). In the U.S., only pembrolizumab is approved for the treatment of early-stage TNBC in the neoadjuvant setting combined with chemotherapy, followed by adjuvant single agent treatment, and in combination with chemotherapy for PD-L1+ metastatic breast cancer. Atezolizumab in combination with chemotherapy is approved in other countries in PD-L1+ metastatic disease. Multiple ongoing studies are evaluating ICI in all subtypes of breast cancer. Key trials that examined the efficacy of ICI are summarized in **Table 1**.

3.1 Unresectable Locally Advanced and Metastatic Breast Cancer

Some breast cancers are immunogenic with their tumor microenvironment (TME) enriched with tumor infiltrating lymphocytes (TILs). Increasing evidence suggests that triple negative and HER-2 positive subtypes are often associated with substantial infiltration of immune cells with a prognostic and predictive value (69).

3.1.1 Metastatic TNBC

The primary treatment for metastatic TNBC has been chemotherapy, with a median OS of 12 to 18 months (70). However, growing evidence suggests that immunotherapy is an effective treatment strategy for PD-L1-positive TNBC. Several key factors make TNBC more likely to respond to ICI than other subtypes of breast cancer, including higher levels of TILs, a greater number of nonsynonymous mutations, and higher levels of PD-L1 expression on both tumor and immune cells. High TIL levels are associated with PD-L1 expression on tumor and tumor immune cells (IC), and PD-L1+ tumors with high TILs have better outcomes (54, 71). The emergence of immunotherapy in breast cancer requires robust, sensitive, and specific predictive and prognostic biomarkers for clinical practice. Liquid biopsy could be a valuable tool to provide baseline information on the tumor and to monitor response to ICI therapy.

Although response is higher in TNBC than in hormone receptor positive (HR+) and HER2+ breast cancers, the efficacy of ICI monotherapy, while correlated with tumor and/or immune cell PD-L1 positivity, remains low. The response rates to atezolizumab and pembrolizumab monotherapy were about 5% in patients with pre-treated disease, and ~21% in untreated patients with metastatic TNBC (53, 54). Low response rates with ICI monotherapy led to the investigation of the efficacy of combination therapy with immunotherapy and chemotherapy.

The IMpassion130 trial was the first phase III trial to report positive data with ICI and chemotherapy for breast cancer, investigated the safety and efficacy of nab-paclitaxel +/- atezolizumab as first-line treatment. In this trial, in patients with PD-L1-positive disease, both PFS and OS were significantly

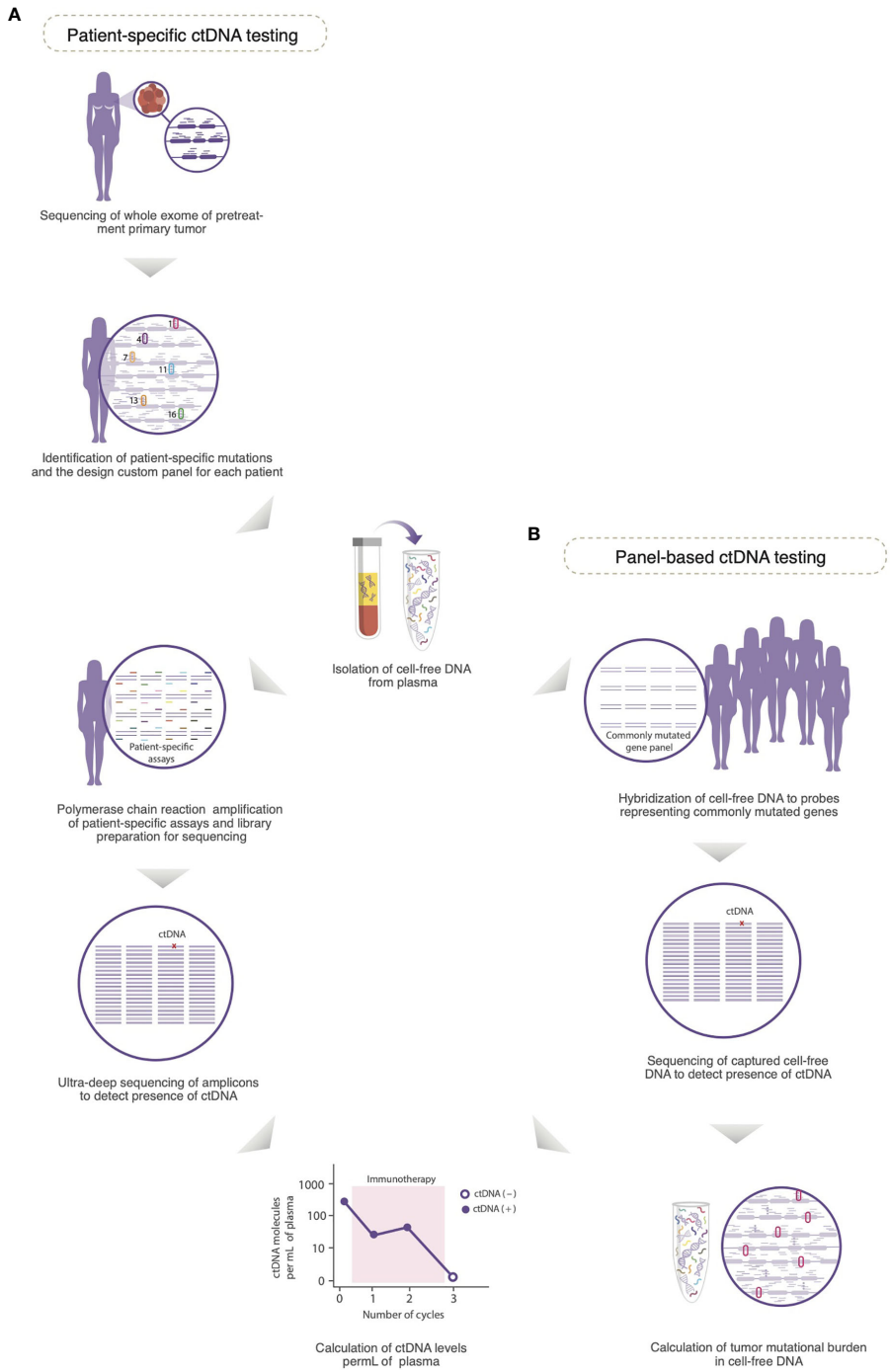

FIGURE 2 | Detection of circulating tumor DNA (ctDNA) in plasma. **(A)** A customized panel containing multiplexed assays is designed to detect patient-specific mutations in cell-free DNA. The personalized panel is created from a list of mutations detected from whole exome sequencing of the untreated primary tumor. Matched germline DNA is also sequenced to exclude non-somatic mutations due to clonal hematopoiesis of indeterminate potential. Amplicons produced by polymerase chain reaction amplification of genomic regions that contain the selected mutations are subjected to ultra-deep sequencing to detect the presence of ctDNA. **(B)** In a panel-based approach, cell-free DNA is hybridized to probes that represent a panel of frequently mutated genes (e.g., *PIK3CA* and *TP53*), and therefore, the mutational profile of the corresponding solid tumor is not required for testing. The captured cell-free DNA molecules are then subjected to next generation sequencing to detect the presence of ctDNA. Because the panel of genes used for testing is consistent across all samples, and includes highly mutated genes, the tumor mutational burden in cell-free DNA can be calculated. In both approaches for testing of ctDNA, serial plasma can be prospectively collected to monitor the levels of ctDNA as a potential biomarker of response to immunotherapy [Modified with permissions from (10)].

TABLE 1 | Summary of immunotherapy trials in breast cancer.

Trial	Subtype	Ph	ICI arm	Control arm	ORR%	PFS (mo), HR	OS (mo), HR	pCR%
Metastatic (Single agent ICI)								
KEYNOTE-086 Coh A (53)	TNBC	II	Pembro	/	5.3	2	9	NA
KEYNOTE-086 Coh B (54)	TNBC	II	Pembro	/	21.4	2.1	18	NA
KEYNOTE-119 (55)	TNBC	III	Pembro	TPC	9.6 *vs* 10.6	2.1 *vs* 3.3	9.9 *vs* 10.8	NA
NCT01375842 (56)	TNBC	I	Atezo	/	10	1.4	8.9	NA
JAVELIN (57)	TNBC	Ib	Avelumab	/	5.2	1.5	9.2	NA
JAVELIN (57)	HR+, HER2-	Ib	Avelumab	/	2.8	NA	NA	NA
KEYNOTE-28 (58)	HR+, HER2-	Ib	Pembro	/	12	I.8	8.6	NA
Metastatic (ICI+Chemo)								
IMpassionl30 (ITT) (21, 59)	TNBC	III	Atezo+Nab-pac	PBO+Nab-pac	56.0 *vs* 45.9	7.2 *vs* 5.5 HR=0.80	21.0 *vs* 18.7 HR=0.87	NA
IMpassionl30 (PD-L1 +) (21S;59)	TNBC	III	Atezo+Nab-pac	PBO+Nab-pac	58.9 *vs* 42.6	7.5 *vs* 5.0 HR=0.62	25.4 *vs* 17.9 HR=0.67	NA
KEYNOTE-355 (PD-L1 CPS≥ 10) (17, 60)	TNBC	III	Pembro+ Nab-pac /Pac/ Gem-Carbo	PBO+ Nab-pac /Pac/Gem-Carbo	53.2 *vs* 39.8	9.7 *vs* 5.6 HR=0.65	23.0 *vs* 16.1 HR=0.73	NA
IMpassionl31 (PD-L1+) (61)	TNBC	III	Atezo+Pac	PBO+Pac	63.4 *vs* 55.4	6.0 *vs* 5.7 HR=0.82	22.1 vs28.3 HR=1.11	NA
NCT03051659 (62)	HR+, HER2-	IIR	Pembro+Eribulin	Eribulin	27.0 *vs* 34.0	4.1 *vs* 4.2 HR=0.80	13.4 *vs* 12.5 HR=0.87	NA
KELLY (63)	HR+, HER2-	II	Pembro+Eribulin	/	40.9	6.0	1-year OS 59.1%	NA
Early-Stage (ICI+NAC)								
KEYNOTE-522 (20, 64)	TNBC	III	Pembro+Carbo+Pac	PBO+Carbo+Pac	NA	NA	NA	64.8 *vs* 51.2
IMpassion031 (65)	TNBC	III	Atezo+Nab-pac Atezo+AC Postop Atczo xl 1	PBO+Nab-pac PBO-AC Postop observation	NA	NA	NA	57.6 vs 41.1
I-SPY2 (66)	TNBC	II-R	Pembro+Pac	Pac	NA	NA	NA	60 *vs* 20 (est)
I-SPY2 (66)	HR+, HER2-	II-R	Pembro+Pac	Pac	NA	NA	NA	30 *vs* 13 (est)
GeparNuevo (67)	TNBC	II-R	Durvalumab+Nab-pac	PBO+Nab-pac	NA	NA	NA	53.4 vs 44.2
NeoTRIP (68)	TNBC	III	Atezo+Carbo+Nab-pac	Carbo+Nab-pac	NA	NA	NA	43.5 *vs* 40.8

AC, doxorubicin plus cyclophosphamide; Atezo, Atezolizumab; Carbo, carboplatin; Chemo, chemotherapy; Cis, cisplatin; Coh, cohort; Cyclo, cyclophosphamide; DOR, duration of response; Doxo, doxorubucin; est, estimated; gBRCAm, germline BRCA-mutated; Gem, gemcitabine; Gem-Carbo, Gemcitabine-Carboplatin; HER2-, human epidermal growth factor receptor 2 negative; HR, hazard ratio; HR+, hormone receptor positive; ICI, immune checkpoint inhibitor; ITT, intention-to-treat population; mo, months; NA, not available; Nab-pac, nab-paclitaxel; NAC, neoadjuvant chemotherapy; Nivo, Nivolumab; NR, not reached; ORR, objective response rate; OS, overall-survival; Pac, paclitaxel; PBO, placebo; pCR, pathologic complete response rate; PD-L1+, programmed death-ligand 1-positive; Pembro, Pembrolizumab; PFS, progression-free survival; Ph, phase; postop, postoperative; TNBC, triple negative breast cancer; TPC, treatment of physician's choice; II-R, phase II randomized.

improved with the addition of atezolizumab to nab-paclitaxel, by 2.5 (7.5 *vs* 5.0 months, Hazard Ratio (HR) 0.62; p<0.001) and 7.5 (25.4 *vs* 17.9 months, HR 0.67) months, respectively (21, 59). On March 18, 2019, the FDA granted accelerated approval for atezolizumab plus nab-paclitaxel to treat patients with unresectable, locally advanced or metastatic TNBC, whose tumor immune cells express PD-L1 at 1% or higher using the Ventana SP142 assay (72).

In a subsequent trial (IMpassion131), the addition of atezolizumab to paclitaxel in a similar setting failed to improve outcome in patients with PD-L1+ metastatic TNBC (61). Due to the inability to provide confirmatory data for IMpassion130, U.S. approval was withdrawn by the manufacturer (Roche) in August of 2021. The reason for the inconsistency in the results of IMpassion130 and IMpassion131 is not yet understood. A possible

explanation for such inconsistency is that patients in the PD-L1-positive control arm in IMpassion131 have a non-stratified pathologic factor that predicts chemotherapy sensitivity (73). Additionally, more patients in IMpassion131 had received prior taxanes than those in IMpassion130, whereas more patients in IMpassion130 had *de novo* metastatic disease than those in IMpassion131 (18, 61). Although exposure to steroids had been considered a potential confounding factor, the use of steroids in the KEYNOTE-355 trial described below makes this unlikely (17, 19).

KEYNOTE-355 randomized patients with metastatic TNBC in the first-line setting to receive pembrolizumab or placebo in combination with physician's choice of chemotherapy (paclitaxel, nab-paclitaxel, or gemcitabine and carboplatin). The success of this phase III trial resulted in the full regulatory approval of pembrolizumab in combination with chemotherapy in patients with PD-L1-positive TNBC, defined as a combined positive score (CPS) ≥10, and representing about 38% of patients with metastatic TNBC (74). Treatment with pembrolizumab compared to placebo resulted in a statistically significant improvement in PFS (9.7 *vs* 5.6 months, HR 0.65, p=0.0012 and OS (23 *vs* 16.1 months, HR 0.73, p=0.0093), as well as improving objective response rate (ORR, 53.2% *vs* 39.8%) and duration of response (19.3 *vs* 7.3 months) in patients whose tumors expressed PD-L1 (CPS ≥10) (17, 19).

Other chemotherapy agents have been combined with ICI in metastatic TNBC. The ENHANCE phase Ib/II trial evaluated eribulin mesylate (a microtubule-depolymerizing drug) in combination with pembrolizumab in 167 patients with metastatic TNBC, reporting an ORR of 23.4%, with median PFS of 4.1 months and median OS of 16.1 months (62, 75). A small study evaluated the combination of capecitabine with pembrolizumab. Thirty patients were enrolled (16 TNBC, 14 HR+, HER2 negative), reporting a median PFS of 4 months, similar to historic controls with capecitabine alone. Interestingly, the one-year PFS rate was 20.7%, suggesting durable responses in a subset of patients (76).

Preclinical data showed potential synergy with the combination of poly adenosine diphosphate-ribose polymerase (PARP) inhibition and ICI therapy. The phase Ib/II MEDIOLA trial evaluated the safety and efficacy of olaparib with durvalumab in patients with solid tumors, including 34 patients with germline BRCA1/2-mutated HER2 negative metastatic breast cancer. The median PFS was longer in patients who were treatment-naïve than in those with 2 prior lines of chemotherapy (11.7 *vs* 6.5 months; not clearly different than what has been seen with PARP inhibition alone in similar patient populations), and treatment was well tolerated (77, 78). Several other combinations of ICI with PARP inhibitors, AKT inhibitors, MEK inhibitors, antibody drug conjugates, and immunomodulatory drugs, among other drug classes, are under investigation to enhance the host immune response and broaden the subset of patients who could benefit from ICI in the metastatic setting (NCT03167619, NCT04191135). In addition, ICI are being actively studied in various combinations in patients with high-risk HR+ and HER2+ disease.

3.1.2 Tumor Mutational Burden

Tumor mutational burden (TMB) is a promising tool to identify patients with TNBC who could benefit from ICI therapies. In 2020, the FDA granted accelerated approval to pembrolizumab monotherapy in previously treated, unresectable/metastatic solid tumors with high TMB, defined as ≥ 10 mutations per megabase, based upon the results of KEYNOTE-158, which showed an ORR of 29% among 102 patients with 27 tumor types (79). The phase III KEYNOTE-119 study randomized patients with 1-2 lines of prior therapy for metastatic TNBC to receive pembrolizumab *vs* chemotherapy of physician choice, with a primary endpoint of OS. Pembrolizumab did not improve OS, but an intriguing subset analysis demonstrated improved OS in the PD-L1 enriched population (CPS ≥20) (80). A further exploratory analysis suggested a potential positive association between TMB and clinical benefit with pembrolizumab but not with chemotherapy, particularly in patients whose TMB ≥10 mutations per megabase. High TMB is uncommon in breast cancer, representing up to 8% of patients with invasive lobular cancer (81).

3.1.3 Metastatic HR+, HER2 Negative Breast Cancer

HR+, HER2 negative breast cancers have lower TILs and PD-L1 expression levels, so these are traditionally considered immunologically cold tumors (82, 83). However, a minority of patients with cold tumors could have meaningful responses to immunotherapy. The phase Ib KEYNOTE-028 trial evaluated pembrolizumab monotherapy in heavily pretreated patients with HR+, HER2 negative metastatic breast cancer. PD-L1 positivity was defined with a tumor CPS ≥ 1, and among 261 patients, 48 (19.5%) had PD-L1-positive tumors. Of these, 25 patients were enrolled and treated with pembrolizumab. The ORR was 12%, but the median duration of response was 12 months (58). In the phase I JAVELIN trial, 168 patients with pretreated metastatic breast cancer of all subtypes received avelumab monotherapy, including 72 patients (42.9%) with HR+, HER2 metastatic breast cancer, regardless of PD-L1 status. The ORR for the entire cohort was only 3.0% (five patients), including three with TNBC and two with HR+, HER2 negative disease (57). Tolaney et al. conducted a phase II trial evaluating the addition of pembrolizumab to eribulin in HR+, HER2 negative metastatic breast cancer. The addition of pembrolizumab did not improve PFS, ORR, or OS compared to eribulin alone in both the ITT and PD-L1-positive (positivity was defined as modified proportion score ≥1) (84). A multicohort phase Ib study evaluated the efficacy and safety of the combination of pembrolizumab and abemaciclib in patients with HR+, HER2 negative metastatic breast cancer. Early data from 28 patients in the pembrolizumab and abemaciclib arm, all with tumors which had progressed on endocrine therapy, demonstrated an ORR of 29%, with partial response (PR) in 8 patients. Median PFS and OS were 8.9 months and 26.3 months, respectively (84). One arm of this study evaluating the safety and preliminary anti-tumor activity of abemaciclib plus pembrolizumab and anastrozole demonstrated a numerically higher rate of transaminase elevations and pneumonitis which were considered immunotherapy related toxicity (84). Ongoing trials will illuminate the role of immunotherapy in HR+, HER2 negative disease in the coming years (NCT03147287, NCT04895358).

3.1.4 Metastatic HER2 Positive Breast Cancer

Higher levels of TIL infiltration and PD-L1 expression have generated interest in the possible value of ICI in the treatment of HER2+ breast cancer (85). In the phase Ib/II PANACEA trial, pembrolizumab plus trastuzumab had modest efficacy; 6 of 40

(15%) patients with PD-L1-positive disease progressing on prior anti-HER2 targeted therapy achieved an objective response whereas no patients responded in the PD-L1-negative cohort (86). In the KATE2 phase II randomized trial, 202 patients with previously treated HER2+ metastatic breast cancer were randomized to receive atezolizumab or placebo with trastuzumab emtansine. The trial met its futility endpoint due to toxicity in the combination arm, and PFS was not improved with the addition of atezolizumab (87). There are ongoing trials evaluating ICI agents in patients with metastatic HER2+ breast cancer (NCT03199885, NCT02849496).

3.2 Early-Stage Breast Cancer

In the early-stage setting, neoadjuvant chemotherapy has resulted in significant improvements in the management of stage II and III TNBC and HER2+ breast cancer (88). Improvements in pathologic complete response (pCR) are associated with excellent outcome, and post-surgical treatment for patients without pCR has reduced the likelihood of recurrence in this high-risk patient population (89, 90). Use of ICI in early TNBC was driven by encouraging results in the phase II I-SPY2 trial (66), and the association of PD-L1 positivity and TILs with pCR (91).

Two phase III trials have evaluated the addition of ICI to neoadjuvant chemotherapy, then continued post-surgery. The largest trial is KEYNOTE-522, leading to the first regulatory approval of a checkpoint inhibitor in early-stage breast cancer. This phase III trial randomized 1174 patients with stage II or III breast cancer in a 2 to 1 ratio to receive neoadjuvant pembrolizumab or placebo in combination with paclitaxel/carboplatin followed by anthracycline/cyclophosphamide. Following surgery, patients continued with blinded pembrolizumab or placebo to complete one year of therapy. In the first 602 patients, the addition of pembrolizumab significantly improved pCR (from 51.2 to 64.8%, P=0.00055), independent of PD-L1 positivity. The trial was designed with dual primary endpoints, including both pCR and event free survival (EFS). At the 4th interim analysis, the addition of pembrolizumab improved EFS at three years (from 76.8% to 84.5%) (64, 80). Interestingly, EFS was improved with pembrolizumab, in the patients who did not achieve a pCR, whereas patients with a pCR had excellent outcome regardless of the post-neoadjuvant treatment arm. Immune-related adverse events (irAE) increased, with 3 deaths attributed to study therapy. Based on this data, the FDA approved pembrolizumab for high-risk, early-stage TNBC in combination with chemotherapy as neoadjuvant treatment, continued as a single agent as adjuvant treatment after surgery on July 26, 2021 (92, 93).

The second phase III trial, IMpassion031, randomized 333 patients with stage II or III TNBC to receive atezolizumab or placebo with neoadjuvant nab-paclitaxel followed by doxorubicin/cyclophosphamide. Following surgery, atezolizumab was continued in a non-blinded manner to complete one year of therapy. The addition of atezolizumab was associated with a significant increase in pCR (from 41.1% to 57.6%, p=0.0044) regardless of PD-L1 expression (65).

The phase II GeparNuevo trial evaluated the efficacy of durvalumab in combination with neoadjuvant chemotherapy in 174 patients. Although pCR was not significantly improved with the addition of durvalumab, invasive disease-free survival (iDFS), distant disease-free survival (DDFS) and OS were improved with long-term follow-up (67, 94). These results, although not definitive, have brought into question the optimal duration of ICI in the treatment of early-stage disease. Lastly, the NeoTRIP phase III trial evaluated the addition of atezolizumab to a non-anthracycline, nab-paclitaxel and carboplatin backbone in 280 patients did not show improvement in pCR although the primary endpoint is EFS, which is still pending (68).

Ongoing trials are evaluating the effectiveness of ICI in the adjuvant and post-neoadjuvant setting. NSABP B-59/GeparDouze is an ongoing phase III trial evaluating neoadjuvant administration of atezolizumab with neoadjuvant chemotherapy followed by adjuvant atezolizumab in patients with high-risk TNBC (NCT03281954). IMpassion030 is a phase III trial investigating the efficacy of and safety of atezolizumab in combination with standard anthracycline/taxane adjuvant chemotherapy in patients with early-stage TNBC (NCT03498716). The primary endpoint is iDFS. SWOG S1418/BR006 (NCT02954874) is a phase III trial that randomizes patients with TNBC and ± 1cm residual invasive breast cancer and/or positive lymph nodes after neoadjuvant chemotherapy to receive standard of care or pembrolizumab 1 year after surgery. The I-SPY2 trial, an adaptive, randomized phase II trial in the neoadjuvant setting also has immunotherapy arms including cemiplimab, cemiplimab plus REGN3767, triaciclibdostarlimab, dostarlimab plus oral paclitaxel/encequidar, and dostarlimab plus oral paclitaxel/encequidar±carboplatin (NCT01042379). Complementary approaches to enhance immunogenicity, including the addition of targeted therapies, novel agents, and induction therapies, have become the recent focus of various clinical trials in breast cancer.

Immune checkpoint blockade can lead to activation of autoreactive T cells, resulting in various irAEs. Although any organ system can be affected, irAEs most commonly involve the gastrointestinal tract, endocrine glands, skin and liver (95). Neurotoxicity, cardiotoxicity and pulmonary toxicity are relatively rare but can be fatal. Whether these adverse events are associated with the efficacy of immune checkpoint blockade remains controversial. The occurrence of irAEs is not required to obtain a benefit from ICI (96). However, specific adverse events may be related with treatment efficacy. For example, several studies including patients with melanoma have demonstrated an association between vitiligo and beneficial clinical outcomes (97, 98). Liquid biopsy biomarkers could also be developed to identify patients who are likely to experience irAEs.

4 PREDICTIVE AND PROGNOSTIC VALUE OF CTCS IN IMMUNOTHERAPY IN BREAST CANCER

Although the presence of PD-L1 has been shown to have good predictive value for ICI efficacy in metastatic TNBC, many challenges persist. First, PD-L1 immunohistochemistry assessment is not always possible due to the lack of available tissue or a low percentage of tumor cells in the tissue sample.

Secondly, some patients with PD-L1-positive tumors may not respond to ICI, demonstrating the complexity and our incomplete understanding of the immunopathology of cancer (92). Some challenges are the heterogeneity and dynamic changes of PD-L1 expression in the tumor microenvironment, PD-L1 expression may vary between primary tumors and metastases, and in breast cancer immunotherapy trials, there were multiple assays for each antibody, multiple scoring systems, and different cut-offs to define PD-L1 positivity (99). To address these complexities, PD-L1 expression on the CTCs of metastatic breast cancer patients is actively under investigation as a predictive biomarker for PD-1/PD-L1 inhibition, potentially complementing or replacing PD-L1 detection on tumor cells and/or TILs in tumor tissue.

Liquid biopsy can identify potentially predictive biomarkers for various solid tumors. This approach is appealing since it is minimally invasive, cost-effective, and rapidly provides information to the clinician to guide therapeutic decision-making strategies (100). Liquid biopsy can be repeated longitudinally over the course of the disease, providing follow-up data for the patient during ICI therapy and beyond, and could help detect resistance mechanisms. CTCs can be isolated and analyzed using approaches designed for solid tissue biopsy, and therefore, could be a dynamic and promising strategy. Immune checkpoint proteins can be influenced by multiple factors, including micro-environmental, inflammatory, and therapeutic factors (27). CTCs may be derived from more than one tumor site and give a better systemic representation of PD-L1 expression than the evaluation of localized cells in tissue samples. There are some questions about the evaluation of PD-L1 expression on CTCs. The first is whether PD-L1 is expressed on all CTCs or only in a subpopulation of CTCs. The second is whether there is any discordance in PD-L1 expression between CTCs and the matched tissue biopsies. Lastly, does the prognosis and the predictive response to immunotherapy correlate with PD-L1 expression on CTCs at baseline or during the follow-up of treated patients (101)?

The evaluation of PD-L1 expression on CTCs has been reported in different solid tumor types including breast, lung, head and neck, colon, bladder and prostatic carcinoma (101). Previous studies evaluating the predictive and prognostic value of PD-L1-positive CTCs in patients treated with ICI have revealed provocative results. Nicolazzo et al. monitored CTCs in non-small cell lung cancer (NSCLC) during nivolumab treatment to investigate the association of PD-L1-positive CTCs with response to ICI therapy. At baseline, 20/24 (83%) patients were positive for CTCs with a very high prevalence of PD-L1 expression (100%). At 6 months of treatment, patients with PD-L1-negative CTCs all showed clinical benefit, while patients with PD-L1-positive CTCs experienced disease progression (39). Strati et al. including patients with head and neck cancer (HNC) reported that patients with CTCs overexpressing PD-L1 at the end of treatment had shorter PFS and OS (25). Similar findings were found by Guibert et al. in NSCLC. In this study, 96 patients with metastatic NSCLC receiving chemotherapy followed by ICI were included. PD-L1 was more highly expressed on CTCs (83%) than in matched tissue samples (41%). They found that patients with PD-L1-positive CTCs had lower response rates to nivolumab than those with PD-L1-negative CTCs. All patients who experienced disease progression had detectable PD-L1-positive CTCs (26). In another study including 71 patients with metastatic NSCLC, PD-L1 expression on CTCs and matched tissue biopsies were well correlated (27). Kulasinghe et al. isolated CTCs in 23 patients with HNC and in 33 patients with NSCLC. Positive PD-L1 expression was detected in 6/11 (54.4%) HNC samples and 11/17 (64.7%) NSCLC samples, respectively. PD-L1-positive CTC patients with HNC had shorter PFS while no significant difference in PFS was observed in the NSCLC cohort when stratified by PD-L1 CTC status (28). Another prospective study in 54 patients with advanced NSCLC evaluated the correlation with clinicopathological variables and prognostic value of PD-L1-positive CTC. CTCs and PD-L1-positive CTCs were detected in 43.4% and 9.4% of patients with NSCLC. The concordance of PD-L1 expression between tumor tissue and CTCs was low (54%). This study suggested that the presence of PD-L1-positive CTCs was associated with poor prognosis in patients with advanced NSCLC (30). Taken together, these studies demonstrate the feasibility of PD-L1 testing in CTCs and provide evidence of the predictive and prognostic value of CTCs expressing PD-L1.

Studies on CTCs in breast cancer patients receiving immunotherapy are summarized in **Table 2**. Mazel et al. evaluated the frequency of PD-L1 expression in patients with HR+, HER2 negative breast cancer (24). PD-L1 expression on CTCs was evaluated in breast carcinoma patients using the EPCAM dependent CellSearch method as well as the B7-H1 PD-L1 monoclonal antibody (**Figure 3**). This study included 16 metastatic breast carcinomas with PD-L1-positive CTCs detected in 11 of 16 patients (68.8%), although the fraction of PD-L1-positive CTCs varied from 0.2 to 100% in individual patients. This study was the first report demonstrating the expression of PD-L1 on CTCs (24). The detection of CTCs expressing PD-L1 could be predictive of response to anti-PD-L1 therapy, and patients with a high percentage of PD-L1-positive CTCs could be potential candidates for anti-PD-L1 therapy. In a follow-up prospective study in 72 patients with metastatic breast cancer, CTCs and PD-L1-positive CTCs were detected in 57 (79.2%) and 26 (36.1%) patients before initiation of treatment (29). There was no statistically significant correlation between PD-L1 expression in tumors *vs.* that of CTCs. PD-L1-positive CTCs were significantly associated with PFS while tissue PD-L1 expression was not. Patients with metastatic breast cancer harboring PD-L1-positive CTCs had shorter PFS; however, this finding was not confirmed in multivariable analysis. Further studies are needed to investigate the predictive role of PD-L1 expression in tumor tissue and CTCs during ICI therapy (29).

Schott et al. examined PD-L1 and PD-L2 expression in CTCs of 72 patients with breast cancer (103). CTCs expressing PD-L1 were found in 94.5% of patients using the Maintrac® method. In patients expressing PD-L1 and PD-L2, the proportion of PD-L1-positive CTCs was significantly higher than that of PD-L2-positive CTCs (54.6% versus 28.7%; $p < 0.001$). Furthermore, PD-L1-positive CTCs were detected in patients without metastatic disease, a finding that could extend the use of PD-L1 testing of CTCs in the early-stage setting. Additionally, patients with metastatic breast cancer had significantly more

TABLE 2 | Studies on CTC in breast cancer patients receiving immunotherapy.

Setting	Liquid Biopsy Technology	Endpoints	Sample	Results	Reference
Metastatic	CellSearch System (Veridex-LLC, Warren, NJ)	To evaluate the clinicopathological correlations and prognostic value of PD-L1 positive CTCs	72	Baseline CTCs and PD-L1-positive CTCs were detected in 57 (79.2%) and 26 (36.1%) patients. PD-L1 positive CTCs was significantly associated with PFS while tissue PD-L1 expression was not.	(29)
Metastatic	Triple immunofluorescence staining	To evaluate the incidence and clinical relevance of CTC expressing CD47 and/or PD-L1	98	The detection of high CD47 and/or PD-L1 expression on CTC is associated with shorter PFS (5.8 *vs* 13.3 months, p=0.010), whereas the detection of PD-L1 high CTC only was correlated with reduced OS (23.8 *vs* 35.7 months, p=0.043).	(102)
Metastatic and early-stage BC	Maintrac® method	Real-time liquid biopsy to determine PD-Ll and PD-L2 expression	Total=l28 BC=72	PD-L1 expressing CTC were detected in 94.5% of BC patients. Patients with non-metastatic BC had significantly more PD-L1-positive CTC than patients without metastasis (median 75% versus 61.1%; p <0.05).	(103)
HR+, HER-2 negative metastatic BC	CellSearch System (Veridex-LLC, Warren, NJ)	The frequency of PD-Ll expression	16	PD-L1 expressing CTC were detected in 11/16 patients with BC (68.8%) at baseline. The proportion of PD-L1-positive CTC varied from 0.2 to 100% in individual patients.	(24)
HER2 positive, early-stage BC (node-positive)	CellSearch System (Veridex-LLC, Warren, NJ)	Enumerating CTC for monitoring the response to a preventive HER/neu E75 peptide vaccine	16	CTC were detected in 14 of 16 (88%) patients. A significant reduction in HER2/neu- expressing CTC was observed in patients vaccinated with HER2/neu protein derived immunogenic peptide.	(104)

BC, breast cancer; CETC, circulating epithelial tumor cells; CTC, circulating tumor cells; HER2, human epidermal growth factor receptor 2; HR+, hormone receptor positive; PD-L1, programmed death ligand 1; PD-L2, programmed cell death ligand 2.

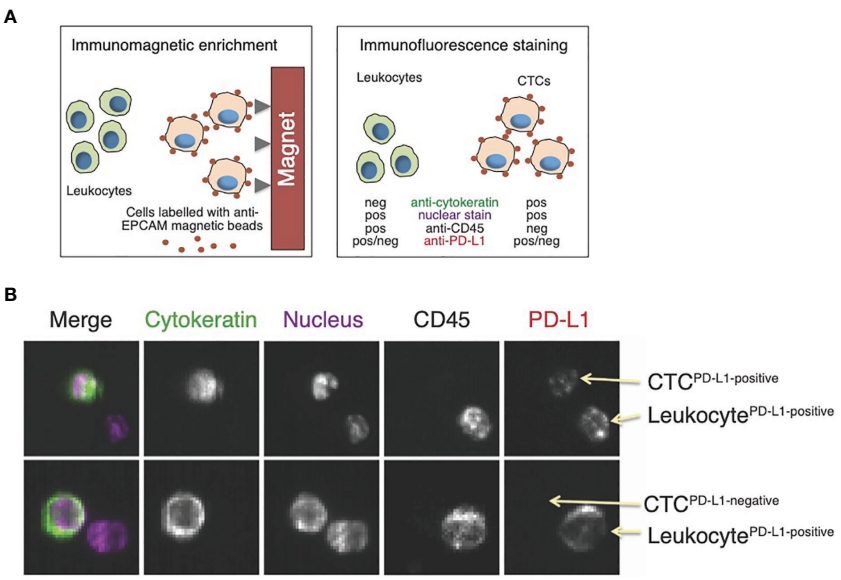

FIGURE 3 | Assessment of PD-L1 expression in circulating tumor cells (CTCs). **(A)** CellSearch is a semi-automated two-step system used for CTC detection. First, monoclonal antibodies against the epithelial cell adhesion marker (EPCAM)-conjugated to iron beads are added to the blood sample. Magnetic capture allows for the enrichment of tumor cells expressing EPCAM. This is followed by immunofluorescence staining to distinguish CTCs from leukocytes and to detect PD-L1 expression; **(B)** Examples of images from the CellSearch gallery to identify CTCs expressing PD-L1. Modified with permission from (24).

PD-L1-positive CTCs as compared to patients without metastasis (median 75% *vs* 61%; p<0.05). Dynamic monitoring of PD-L1 expression on CTCs during ICI therapy revealed that the number of CTCs and the percentage of the PD-L1-positive CTCs were reduced in patients that responded to ICI therapy. After discontinuing the ICI agent, the percentage of PD-L1-positive CTCs continuously increased. These findings demonstrated that the number of PD-L1-positive CTCs could be prognostic and correlates with tumor aggressiveness, as well as the potential response to immunotherapy (103).

CD47 is a key immune checkpoint which is highly expressed on a variety of cancer cells, making tumor cell resistant to host immune surveillance. Cell surface CD47 is a ligand for signal regulatory protein-α (SIRPα), a protein expressed on macrophages and dendritic cells, allowing cancer cells to send inhibitory signals to macrophages and impede phagocytosis and immune response (105, 106). Agelaki et al. evaluated the incidence and clinical relevance of CTCs expressing CD47 and/or PD-L1 in patients with metastatic breast cancer. Cytokeratin positive CTCs were detected in 22 of 98 patients (22.4%) with metastatic breast cancer. High CD47 and PD-L1 expression was identified in 41.9% and 11.6% of CTCs, respectively, with 9.1% of CTCs expressing high levels of both markers. High CD47 and/or high PD-L1 CTCs were associated with disease progression (27.8% *vs* 5.6%; p=0.005) and shorter PFS (5.8 *vs* 13.3 months; p=0.010), whereas the detection of high PD-L1 CTCs only was correlated with reduced OS (23.8 *vs* 35.7 months, p=0.043). The study showed that high CD47 and/or high PD-L1 CTCs were associated with increased risk of relapse and high PD-L1 CTCs were associated with high risk of death (HR 4.8; p=0.011). Patients with these CTC biomarker-positive populations could benefit from anti-CD47 and anti-PD-L1 immunotherapy strategies (102).

Quantification of CTCs to monitor response to the HER2/neu E75 peptide vaccine was evaluated in 16 patients with HER2+ breast cancer. Patients with node positive breast cancer were vaccinated monthly for six months after completion of standard therapy including surgery, chemotherapy, and radiotherapy. CTCs were detected in 14 of 16 (88%) patients at baseline. A significant reduction in HER2/neu- expressing CTCs was observed over the course of vaccination (104). This small pilot study suggested a potential role of CTCs enumeration in assessing response to vaccine-based therapy; however, these results were not validated in larger studies.

The persistence of PD-L1-positive CTCs in patients treated with ICI therapy in various cancer types has been associated with worse prognosis (39). However, there is no prospective data, and there are technical issues associated with the detection of CTCs: CTCs are rare and various methods might enrich CTCs populations differently, which could affect the PD-L1 assessment. However, liquid biopsy is a promising technique and a feasible strategy for dynamic assessment and sequential monitoring of PD-L1 expression in patients with breast cancer receiving ICI therapy. Given the small number of studies in patients with breast cancer, further studies are needed to understand the role of PD-L1 expression on CTCs during immunotherapy and to determine the relationship between the expression of PD-L1, CTCs, and tumor tissue.

5 PREDICTIVE AND PROGNOSTIC VALUE OF ctDNA IN IMMUNOTHERAPY IN BREAST CANCER

ctDNA detectable in blood has been demonstrated to reflect the mutational signatures of a primary tumor. ctDNA is emerging as a potential noninvasive biomarker to detect preclinical metastases and predict relapse following treatment for early-stage disease. ctDNA provides noninvasive access to cancer-specific somatic mutations and could be a technique used to identify specific mutations that are linked with therapeutic response (107, 108). However, ctDNA has not been used clinically for breast cancer patients treated with ICI.

Baseline ctDNA concentration and genomic instability number have been shown to predict response to ICI, and ctDNA monitoring could become a valuable tool for therapy guidance in the future. Genetic analysis of ctDNA is feasible and thus permits the assessment of TMB, which could be a novel biomarker for cancer immunotherapy. Araujo et al. demonstrated that high TMB could predict ICI efficacy in patients with metastatic breast cancer. Among the 16 patients with detectable mutations in both formalin-fixed paraffin-embedded (FFPE) tumor tissue and ctDNA, a statistically significant correlation between blood-based TMB and tissue-based TMB was found (p=0.002) (109). Tumors with high microsatellite instability (MSI) can also be detected using ctDNA based assays (110). Previous studies demonstrated that high MSI from ctDNA is associated with a good response to ICI across various cancers (111). Additionally, the detection of somatic mutations in cfDNA modulating tumor-specific immune response might be helpful to identify non-responding patients. However, genomic analysis to detect mutations and TMB in blood could contain some mutations associated with clonal hematopoiesis, so these non-tumor mutations should be filtered out to prevent misleading results (112).

Studies on ctDNA in breast cancer patients receiving immunotherapy are summarized in **Table 3**. INSPIRE, a multicohort phase 2 trial, was conducted to evaluate the performance of an amplicon-based bespoke (personalized) ctDNA detection to predict response in patients treated with pembrolizumab (113). This study aimed to investigate if baseline ctDNA levels would be prognostic and whether early changes in ctDNA levels would precede imaging response to an ICI. Five cohorts of patients with advanced solid tumors were included. A total of 106 patients were enrolled; of them, 18 patients were TNBC. Researchers analyzed ctDNA levels at baseline and the beginning of cycle 3 of pembrolizumab treatment. Patients who had a lower ctDNA level at cycle 3 than at baseline, had a higher clinical benefit rate (CBR) and a more favorable OS and PFS. They monitored dynamic levels of ctDNA during pembrolizumab treatment to evaluate the predictive value of ctDNA. Among patients with at least two ctDNA measurements, any rise in ctDNA levels above baseline (n=45) during surveillance was associated with rapid disease progression in most patients and with poor survival (median OS=13.7 months). Patients whose ctDNA cleared during treatment (undetectable for at least one on-treatment time point) had superior clinical outcomes. This study showed that serial ctDNA analysis using the bespoke assay could be a monitoring strategy for patients treated with ICI. Changes in ctDNA levels and Response Evaluation Criteria in Solid Tumors (RECIST) from baseline to cycle 3 were discordant in 23% of cases, but the combination of these two metrics was superior to RECIST alone for predicting OS. This study suggests broad clinical utility for ctDNA based surveillance in patients treated with ICI (113). This is a

TABLE 3 | Studies on ctDNA in breast cancer patients receiving immune-therapy.

Immunotherapy agent	Setting	Liquid Biopsy Technology	Endpoints	Sample	Results	Reference
Pembrolizumab	Metastatic		PFS, OS, CBR The change in genomics and immune landscapes, RNA expression correlates of treatment response.	316 serial plasma samples Total pts= 94 TNBC=11	Patients who had lower ctDNA level at cycle 3 than ctDNA level at baseline has higher CBR, favorable OS and favorable PFS. Patients whose ctDNA cleared during treatment had superior clinical outcomes.	(113)
Investigational Immunotherapy (ICI, vaccines, cytokines)	Metastatic	Next generation sequencing of a customized panel of genes	To evaluate ctDNA dynamics in responders.	Total=38 BC=5	Blood-based TMB correlated with tissue-based TMB High TMB was not associated with better survival An on-treatment decrease in VAF of mutations detected in ctDNA at baseline was observed in responders.	(109)
Pembrolizumab	Neoadjuvant	Personalized ctDNA test (Signatera™)	Association of ctDNA with with pCR and DRFS	511 serial samples from 138 patients (pembrolizumab arm n=2) HR+/HER2 negative=77 TNB=61	Early clearance of ctDNA during NAC treatment was significantly associated with increased likelihood of achieving pCR Residual ctDNA after neoadjuvant treatment was a significant predictor of metastatic recurrence and death.	(114)

BC, breast cancer; ctDNA, Circulating tumor DNA; DRFS, distant recurrence-free survival; pCR, pathologic complete response rate; pts, patients; TMB, tumor mutational burden; TNBC, triple negative breast cancer; VAF, somatic variant allele frequency.

noninvasive strategy to predict clinical benefit and long-term survival. Future large interventional studies are needed to confirm these results using ctDNA levels to guide ICI therapy.

In early-stage breast cancer, the addition of pembrolizumab to standard neoadjuvant chemotherapy improved pCR rates in patients with HR+, HER2 negative breast cancer and TNBC in the I-SPY2 trial (66). ctDNA levels were analyzed on 511 serial plasma samples during neoadjuvant treatment. The detection of ctDNA decreased over time in both the pembrolizumab arm and the control arm. All patients who achieved pCR (n=34) cleared their ctDNA prior to surgery. Among patients who failed to achieve pCR, the distant recurrence free survival (DRFS) rate was significantly better in patients who had ctDNA clearance prior to surgery compared to patients who were ctDNA positive (114).

6 PROMISES, PITFALLS, AND CHALLENGES OF CTCS AND CTDNA AS BIOMARKERS FOR BREAST CANCER IMMUNOTHERAPY

CTCs are extremely rare, with an estimated frequency of 1 CTC per one billion blood cells and are difficult to detect in circulation. Counting (enumeration) them requires special reagents (e.g., immunomagnetic beads) and equipment (e.g., automated fluorescent microscope). Current CTC detection technologies, such as that of the CellSearch™ system, have limited sensitivity. Given that CTCs are relatively more abundant in blood of metastatic breast cancer patients, the analysis of CTCs may be more robust in the metastatic setting than in early-stage breast cancer. Even so, only about 50% of metastatic breast cancer patients are positive for CTC (5, 36).

The detection of ctDNA, on the other hand, is less technically challenging than that of CTCs. The isolation of cfDNA, which serves as the input material for sequencing, can be easily performed using commercially available purification kits. The downstream analysis to detect ctDNA in cfDNA generally requires only a next generation sequencer, instrumentation that is available in academic research settings and fee-for-service commercial sequencing companies or clinical reference labs.

Because CTCs can be isolated as live cells, other substrates for biomarker detection and discovery (e.g., DNA, RNA, proteins, and other macromolecules) are available for interrogation. This is a significant advantage of CTCs over ctDNA, which is limited to DNA-based profiling due to the nature of the biomarker (**Table 4**).

7 EMERGING LIQUID BIOPSY TECHNOLOGIES

In addition to CTCs and ctDNA, other blood-based biomarkers have been recently developed (125, 126). In this review, we will focus on emerging cfDNA-based biomarkers beyond mutation profiling.

Other cfDNA-based biomarkers, in addition to the detection of tumor mutant DNA molecules (i.e., ctDNA) are being developed. Cristiano and colleagues described an approach to profile genome-wide fragmentation patterns of cfDNA, also referred to as "fragmentomics" (127). The authors showed that

TABLE 4 | Feasibility of assessment of candidate immunotherapy biomarkers in circulating tumor cells (CTC) and circulating tumor DNA (ctDNA).

Biomarker	CTC	Reference	ctDNA	Reference
DNA-based biomarker	TMB can be measured by DNA sequencing of single or small pools of CTC	(115)	TMB can be measured in cfDNA using a targeted panel or by whole exome sequencing of cfDNA.	(116–120)
			Genome-wide tumor-specific copy-number alterations can be profiled from cfDNA to monitor response to immunotherapy.	(121)
RNA-based biomarkers	Profiling of gene expression signatures associated with immunotherapy response in CTC is feasible.	(122)	n.a.	
Protein-based markers	PD-L1 expression can be assessed by staining of isolated CTC.	(120, 122–124)	n.a.	

cfDNA, cell-free DNA; CTC, circulating tumor cells; n.a., not applicable; PD-L1, Programmed death-ligand 1; TMB, tumor mutational burden.

TABLE 5 | Overview of ongoing clinical trials of liquid biopsy techniques in breast cancer undergoing immunotherapy.

Clinical Trial Number	Setting	#Patients	Assessments	Aim of Liquid Biopsy Analysis	Estimated Primary Completion Date
NCT03892096	Metastatic BC, NSCLC, CRC	750	ctDNA	The evaluation of ctDNA as a potential biomarker for early non-response to therapy	2022
NCT04591431	BC, GIC, NSCLC, other	384	ctDNA	Concordance between molecular profile on tumor tissue and ctDNA	2024
NCT02971761	Metastatic TNBC	29	ctDNA, CTC	To evaluate die effect of the combination therapy (Enobosarm and Pembrolizumab) on CTC and ctDNA.	2021
NCT04849364	Post-neoadjuvant residual TNBC	197	ctDNA	Patients wim residual TNBC assign to arms based on ctDNA positivity and genomic markers).	2024
NCT04837209	Metastatic TNBC	32	ctDNA	To evaluate changes in ctDNA in patients receiving the combination of niraparib, dostarlimab, and RT	2023
NCT04447651	Metastatic BC	60	ctDNA	To evaluate changes in ptDNA from baseline to 3 months in patients with spliceosome mutations receiving ICI	2022
NCT03515798	Inflammatory BC	81	CTC, ctDNA	To evaluate prognostic value of baseline CTC in IBC To purify ctDNA for disease monitoring	2025
NCT03145961	Early-stage TNBC	208	ctDNA	To assess whether ctDNA screening can be used to detect residual disease following standard primary treatment for TNBC To assess the safety and activity of pembrolizumab in patients widi positive ctDNA	2022
NCT03213041	HER2 negative metastatic BC	100	CTC, ctDNA	To evaluate the efficacy of carboplatin+ pembrolizumab in patients with CTC+ metastatic BC To measure ctDNA and correlate them with CTC enumeration and therapeutic benefit.	2022
NCT03818685	TNBC with residual disease	114	ctDNA	ctDNA detection at baseline and in case of disease relapse up to 2 years	2021
NCT03487666	TNBC with residual disease	45	ctDNA	Quantification of ctDNA at different time points during Nivolumab or capecitabine or combination therapy as adjuvant therapy for TNBC with residual disease following neoadjuvant chemotherapy	2021

BC, breast cancer; CRC, colorectal cancer; ctDNA, Circulating tumor DNA; GIC, gastrointestinal cancer; IBC, inflammatory breast cancer; ICI, immune checkpoint inhibitors; NSCLC, non-small cell lung cancer; PC, pancreas cancer; RT, Radiation Therapy.
Ongoing clinical trials were found at the website of https://www.Clinicaltrials.gov (accessed on 1 September 2021).

fragmentation profiling, combined with mutation-based analysis, can accurately discriminate between cancer patients and healthy individuals. Another approach involves methylation sequencing of cfDNA (128). For example, Liu and colleagues showed that evaluation of the methylation patterns in more than 900 CpG sites in cfDNA detected the presence of cancer and identified the cancer type in patients with advanced cancers. Chromatin state or nucleosome footprint analysis of the cfDNA is another approach that is currently under development (129, 130). The positions of nucleosomes on DNA determine chromatin structure which in turn affect gene expression (131). This approach involves generating genome-wide maps that show nucleosome occupancy and the evaluation of transcription factor binding in small fragments of cfDNA (129). Using this approach, Ulz and colleagues found patient- and tumor-specific nucleosome occupancy patterns and were able to accurately predict subtypes in prostate cancer (130).

Mutation detection in cfDNA is challenging because rare tumor-derived mutated DNA molecules are present in an overwhelming background of normal DNA from hematopoietic cells. Detection is particularly challenging in cancers with low or moderate tumor mutational burden, like breast cancer (132). These new emerging platforms offer the opportunity to interrogate genome-wide or significantly more genomic

loci than what is available for mutational profiling. For example, Jensen and colleagues describe the use of a genome-wide measure of genomic instability by low-coverage next generation sequencing of cfDNA, an assay that is validated for noninvasive prenatal testing, to detect tumor-specific copy number aberrations (13, 121, 133). Using this approach, the investigators developed a novel metric, genome instability number (GIN), that can be used to monitor response to immunotherapy drugs, including the differentiation of progression from pseudoprogression (121). The GIN assay and other novel technologies that interrogate the whole genome show promise in providing clinically relevant information above what ctDNA alone can provide. However, further testing to demonstrate their applications to guide immunotherapy, particularly in breast cancer, is warranted.

8 FUTURE DIRECTIONS AND SUMMARY

Immunotherapy has a defined role in the treatment of both early- and late-stage TNBC and is under active exploration in HER2+ as well as high-risk HR+ disease. Only a minority of patients in the metastatic setting are likely to benefit from adding ICI to standard chemotherapy, and outcome is particularly poor for patients with PD-L1-negative disease. In the early-stage setting, therapy is given with curative intent, so the balance of toxicity and efficacy is critical. In addition, ICI therapy is costly, and the duration of therapy has implications for both toxicity and patient quality of life. It is therefore of the utmost importance to identify better markers to predict efficacy. The analysis of PD-L1 expression on CTCs and the detection of ctDNA are actively under investigation. Confirming the predictive value of TMB in prospective trials and standardizing the assessment of TMB are critical next steps.

Further clinical studies are warranted to demonstrate the role of liquid biopsy in guiding immunotherapy in breast cancer. Blood biomarkers can monitor disease trajectory during and after therapy and have the potential to reveal mutational shifts and resistance mechanisms. These biomarkers reflect, in part, the changes in tumor burden during treatment. However, the correlation between tumor burden/response and the levels of CTCs and ctDNA is not perfect; therefore, additional biomarkers are needed to refine their predictive and prognostic value. Ongoing clinical trials involving the assessment of liquid biopsy technologies in patients with breast cancer receiving immunotherapy are listed in **Table 5**.

In conclusion, liquid biopsy applications to guide immunotherapy in breast cancer have not yet been implemented in clinical practice, but promising data and rapidly advancing technologies indicate that this approach has the potential to select patients who would benefit from immunotherapy.

AUTHOR CONTRIBUTIONS

The authors MM and OG contributed to the study conception, data collection and interpretation, and manuscript preparation. HR wrote sections of the manuscript. HR, RK, and LVV reviewed the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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A Comprehensive Survey of Genomic Mutations in Breast Cancer Reveals Recurrent Neoantigens as Potential Therapeutic Targets

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Neoantigens are mutated antigens specifically generated by cancer cells but absent in normal cells. With high specificity and immunogenicity, neoantigens are considered as an ideal target for immunotherapy. This study was aimed to investigate the signature of neoantigens in breast cancer. Somatic mutations, including SNVs and indels, were obtained from cBioPortal of 5991 breast cancer patients. 738 non-silent somatic variants present in at least 3 patients for neoantigen prediction were selected. *PIK3CA* (38%), the highly mutated gene in breast cancer, could produce the highest number of neoantigens per gene. Some pan-cancer hotspot mutations, such as *PIK3CA* E545K (6.93%), could be recognized by at least one HLA molecule. Since there are more SNVs than indels in breast cancer, SNVs are the major source of neoantigens. Patients with hormone receptor-positive or HER2 negative are more competent to produce neoantigens. Age, but not the clinical stage, is a significant contributory factor of neoantigen production. We believe a detailed description of breast cancer neoantigen signatures could contribute to neoantigen-based immunotherapy development.

Keywords: breast cancer, neoantigens, immunotherapy, *PIK3CA*, SNVs

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women worldwide (1). More than two million new breast cancer cases in 2018 contributed to one-fourth of women cancers (2). Breast cancer is a highly heterogeneous tumor that is currently classified by three molecular markers, including estrogen receptor (ER), progesterone receptor (PR) and HER2 (also called *ERBB2*). Treatment methods and prognosis of different breast cancer subtypes vary considerably (3, 4).

In recent years, cancer immunotherapy played an important role in a variety of solid tumors (5–7). The most representative immunotherapy approach is immune checkpoint blockade (ICB), but ICB therapy is only about 30 percent effective (8). Neoantigens exist specifically in tumor cells with better specificity and safety (9), and require major histocompatibility complexes (MHCs) to be recognized by immune cells to activate anti-tumor immune responses. Neoantigen-based immunotherapy can present a wide range of potential targets *via* MHC molecules presenting neoantigens (10), which is complementary to ICB therapy, such as neoantigen-based tumor-

infiltrating lymphocytes (TILs) therapy in metastatic breast cancer (11). However, Tumors have a variety of immune escape mechanisms and high heterogeneity, with differences in tumor variation between different subtypes and even between individual patients. The limitation of neoantigen-based immunotherapy is that there are fewer neoantigens shared among different patients, and neoantigen-based therapeutics may be affected by immune checkpoints. Combining neoantigen and immune checkpoint inhibition therapy or chemoradiotherapy may achieve better therapeutic effects (12). T-cell immunotherapy based on *KRAS* K12D mutation has been reported in colorectal cancer (13), but similar therapies have not been reported in breast cancer.

HLA (Human Leukocyte Antigen) is a 3.6Mb segment of the human genome at 6p21.3 (14). There are two classical types of HLA: HLA-I and HLA-II. HLA-I molecules are responsible for antigen recognition and presentation, making them vital in neoantigen-based immunotherapy. HLA-II molecules, which present extracellular antigens, are also crucial to the human immune system.

With the development of sequencing technology, more and more studies on the mutation characteristics of breast cancer based on second-generation sequencing technology have been published (15–18). Here we focus on common neoantigens derived from high frequency mutations to benefit as many patients as possible. By integrating clinical information and mutation data of the 8 previous breast cancer research cohorts, we obtained the mutational landscape of 5991 breast cancer patients (4, 15–19). Finally, combining the high-frequency HLA information and mutation data, we got the most common shared neoantigens in breast cancer patients, which provides a new road for neoantigen-based immunotherapy.

RESULTS

The Mutation Landscape of Breast Cancer Patients

The mutation status of all breast cancer samples was shown in **Figure 1** and **Supplementary Figure 1**. Missense mutation was the main variant classification. At base substitution level, C>T transition was the most common mutation event (**Supplementary Figures 2A–C**). Besides, the mutation load of each sample was

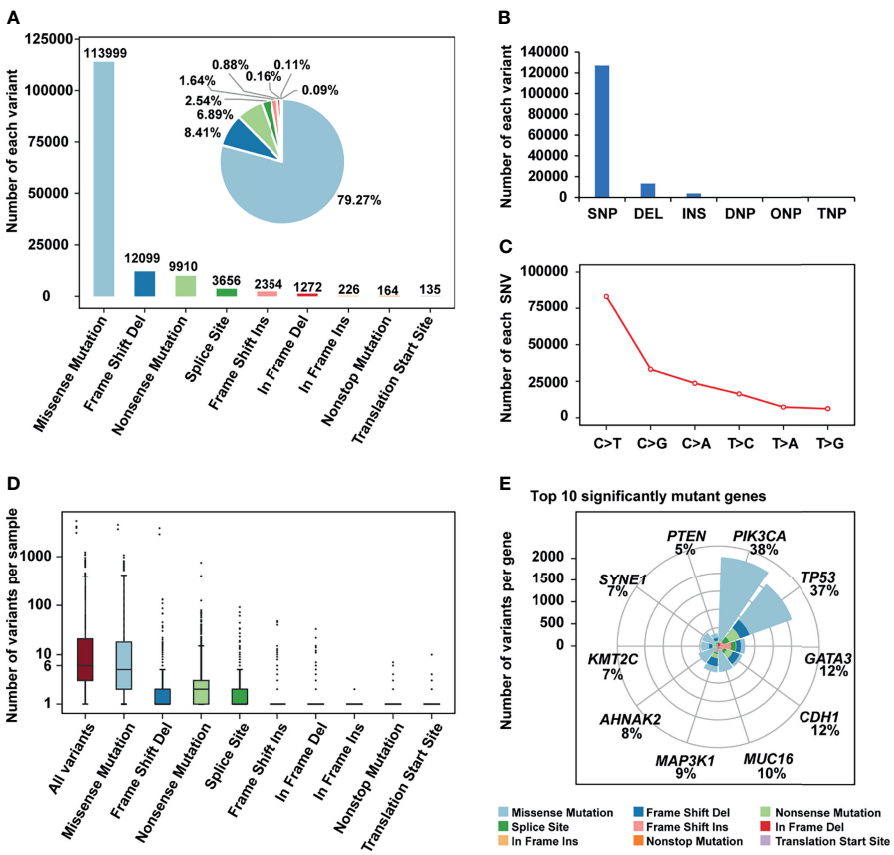

FIGURE 1 | The mutation landscape of breast cancer cohort. **(A)** Bar plot and pie plot showing the number of each variant; **(B)** Bar plot and pie plot showing the number of each variant type; **(C)** Line graph showing the number of each SNV class; **(D)** Boxplot showing the number of variants per sample, the median of mutations per patient is 6; **(E)** Top 10 significantly mutant genes and the composition of variants.

relatively low, with only 25.3 mutations per patient on average and 6 mutations for the median. *PIK3CA* (38%) and *TP53* (37%) were two significantly mutated genes, with frequencies higher than others, such as *GATA3* (12%) and *CDH1* (12%) (**Figure 1F**). Many cancer-causing genes are co-occurring or show strong exclusiveness. This kind of interaction was also observed in our breast cancer cohort (**Supplementary Figure 2D**). For instance, *PIK3CA* and *TP53* were mutually exclusive while *PIK3CA* and *CDH1* were co-occurrent.

Although *TP53* and *PIK3CA* mutations frequently occurred regardless of the HER2 status, the mutated rates differed. In $HER2^+$ patients, *TP53* (66%) mutated more frequently than *PIK3CA* (32%), while the mutated rates of *TP53* and *PIK3CA* were 33% and 40% in $HER2^-$ patients (**Supplementary Figure 3**). In further investigation, we identified 22 differentially mutated genes between these two subgroups (Fisher's exact test, $P < 0.01$, **Supplementary Figure 4**). The same analysis was also carried out in breast cancer patients with different HR (Hormone Receptor) statuses (**Supplementary Figure 5**).

Specially, we described the mutation status of triple-negative breast cancer (TNBC) patients. In our cohort, 70% of HR^- (ER^-/HR^-) patients were triple-negative breast cancer, leading to a high consistency of their mutation landscape (**Supplementary Figures 6A-F**). *TP53* mutations, which differentially happened between TNBC and non-TNBC patients, were observed in 79% of triple-negative breast cancer patients in our cohort (**Supplementary Figure 6G**).

Results of Neoantigen Prediction

Due to the difference in the frequency of HLA in different populations, high-frequency (> 5%) HLA genotypes were selected from Han Chinese (20) and Americans (21) to predict "public" neoantigens (**Supplementary Table 1**).

After filtering, there were 617 eligible SNVs and 121 eligible indels, producing 356 and 86 derived peptides respectively (**Supplementary Tables 2**, **3**). In terms of SNVs, mutations of *PIK3CA*, *AKT1*, *SF3B1*, and *ESR1* produced the top 10 neoantigens with the highest frequency (**Table 1**), especially for *PIK3CA*, occupying 6 of 10. As for indels (**Table 2**), although the mutation frequency was lower, the number of neoantigens per mutation was higher, 2.69 for each indel but only 1.34 for each SNV on average.

Comparison of Neoantigens in Different Subgroups

Patients were divided into different subgroups by several clinical characteristics to investigate the relations between clinical information and neoantigens. By comparing the fraction of neoantigen-carrying patients in the corresponding subgroup, we found a higher fraction of the elderly population carrying SNV-derived neoantigens than younger ones (Fisher's exact test, $P = 2.26e\text{-}5$, **Figure 2A**). As for the results of ER or PR status subgroups, the proportion of patients carrying SNV-derived neoantigens was higher in positive patients (**Figures 2C, D**). On the contrary, neoantigens of SNVs were more likely to be produced by HER2- patients (**Figure 2B**). No significant difference was observed in indel-derived neoantigens.

To evaluate the influence of SNV background within each subgroup, we compared the number of non-synonym SNVs in patients (**Supplementary Figure 7A**). For the age subgroup, the elderly population carried more SNVs (Wilcoxon test, $P = 0.021$). This may be why the elderly population is easier to produce neoantigens. As for ER or PR status, negative patients held a higher background. However, negative patients showed a lower non-synonym SNV load in the HER2 subgroup.

The clinical-stage was unlikely to be a critical factor in neoantigen production. Although we have observed the difference between patients in Stage I and Stage III (Fisher's exact test, $P = 0.005$, **Supplementary Figure 7B**), the difference in other stages was not statistically significant. Compared to indels, SNV-derived neoantigens could cover more patients no matter in which subgroup (Wilcoxon test, $P = 1.6e\text{-}5$, **Supplementary Figure 7C**).

Hotspot Mutations Derived Neoantigens May Serve as Targets of Immunotherapy in Breast Cancer and Pan-Cancer

H1047R (*PIK3CA*), E545K (*PIK3CA*), E17K (*AKT1*), and N345K (*PIK3CA*) produced recurrent neoantigens and had a higher mutation frequency in the breast cancer cohort (**Figure 3**). Thus, we focused on these mutations and corresponding neoantigens.

In this study, *PIK3CA* H1047R occurred in 14% of patients in our cohort, consistent with published research by Zehir *et al* (22). Besides, Meyer and colleagues reported that this mutation in the

TABLE 1 | Top 10 SNVs and corresponding neoantigens.

Chr	Location	Gene	AA change	Peptide	Frequency	HLA types
chr3	178952085	PIK3CA	H1047R	ARHGGWTTK	839	HLA-B27:05
chr3	178936091	PIK3CA	E545K	ITKQEKDFLW	415	HLA-B57:01
chr3	178936091	PIK3CA	E545K	STRDPLSEITK	415	HLA-A03:01; HLA-A11:01
chr14	105246551	AKT1	E17K	RGKYIKTWR	196	HLA-A31:01
chr3	178921553	PIK3CA	N345K	ATYVKVNIR	132	HLA-A31:01
chr2	198266834	SF3B1	K700E	QEVRTISAL	83	HLA-B40:01
chr2	198266834	SF3B1	K700E	GLVDEQQEV	83	HLA-A02:01
chr3	178938934	PIK3CA	E726K	KTQKVQMKF	64	HLA-A32:01; HLA-B57:01
chr6	152419926	ESR1	D538G	LYGLLLEML	59	HLA-A24:02
chr3	178927980	PIK3CA	C420R	KEEHRPLAW	48	HLA-B44:03

TABLE 2 | Top 10 indels and corresponding neoantigens.

Chr	Location	Gene	AA change	Peptide	Frequency	HLA types
chr3	178916938	PIK3CA	E110del	KVIEPVGNREK	11	HLA-A03:01; HLA-A11:01
chr5	56177011	MAP3K1	R763Cfs*35	LMFHKLSL	8	HLA-B08:01
chr5	56177011	MAP3K1	R763Cfs*35	FLLNFILIL	8	HLA-A02:01; HLA-A02:07
chr5	56177011	MAP3K1	R763Cfs*35	LILSVLMFH	8	HLA-A03:01
chr5	56177011	MAP3K1	R763Cfs*35	NFLLNFILI	8	HLA-A24:02
chr5	56155721	MAP3K1	R273Sfs*27	KSFPSAFSEW	7	HLA-B57:01
chr5	56155721	MAP3K1	R273Sfs*27	TTPKSPFTR	7	HLA-A11:01; HLA-A68:01
chr5	67591104	PIK3R1	K567_L570del	KRMNSIIQLR	7	HLA-B27:05
chr5	56155721	MAP3K1	R273Sfs*27	SPFTRWLL	7	HLA-B08:01
chr5	67591104	PIK3R1	K567_L570del	RMNSIIQLR	7	HLA-A31:01

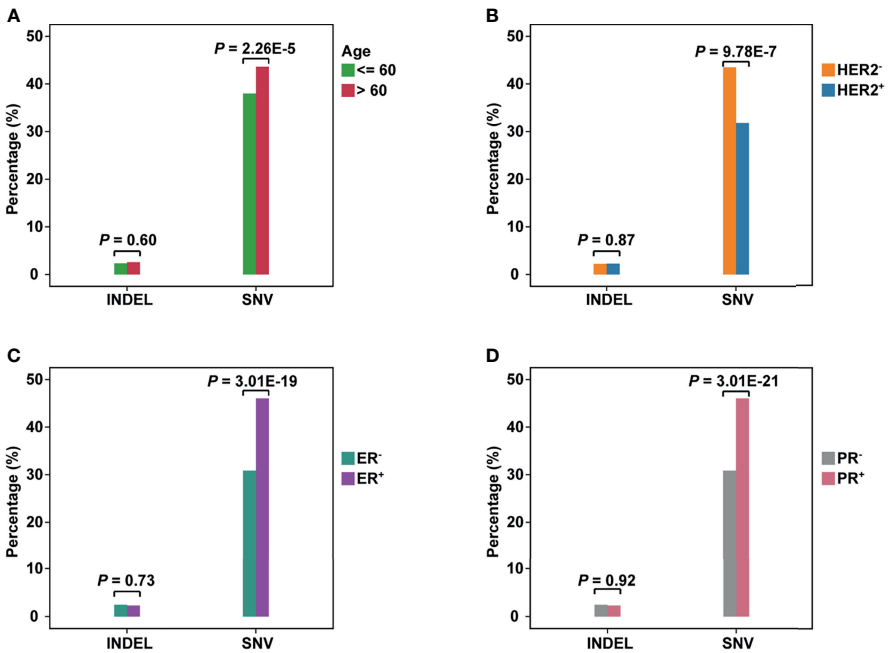

FIGURE 2 | The comparison of neoantigens between different subgroups of breast cancer. **(A–D)** The horizontal axis represents the neoantigens source, including INDELs and SNVs; the vertical axis represents the percentage of neoantigen-carrying patients in the corresponding subgroup. **(A)**: Group Age: <=60 *vs* >60; **(B)** Group HER2 status: $HER2^+$ *vs* $HER2^-$; **(C)** Group ER status: ER^+ *vs* ER^-; **(D)** Group PR status: PR^+ *vs* PR^-.

luminal mammary epithelium could induce tumorigenesis (23). In many other cancer types, this mutation also showed a pretty high frequency (**Figure 4A**).

PIK3CA E545K is a hotspot mutation with first-line drugs (24). This mutation holds a frequency of about 8% in breast cancer, second to bladder cancer (**Figure 4B**). As for *PIK3CA* N345K, its mutation frequency is relatively low across all cancers as shown in **Figure 4D**. A case report suggested this mutation might be associated with the sensitivity of Everolimus (25).

AKT1 E17K occurs in many solid tumors with a low frequency (**Figure 4C**). Compared to other *AKT1* mutations, E17K showed a higher occurrence (**Figure 3B**). In certain breast cancer patients, this mutation is most likely the driver mutation (26). Besides, a study has reported that *AKT1* E17K is a therapeutic target in many cancers (27).

DISCUSSION

In this study, we integrate 8 breast cancer research cohorts to depict the mutation panorama of breast cancer patients, which provide a reference for the genomics research of breast cancer and contributed to the in-depth study of clinical molecular typing of breast cancer patients. In addition, we predict a series of potential neoantigens based on the high-frequency mutation pairs after screening, which may serve as therapeutic targets for patients. *PIK3CA* and *TP53* are two highly mutated genes in breast cancer (28, 29). Our findings also showed this and further demonstrated they are mutually exclusive in mutations. Since TNBC is one of the most malignant breast cancers, we analyzed its mutation landscape and found *TP53* was a noteworthy gene with a very high frequency (79%). Besides, *PIK3CA* and *TP53*

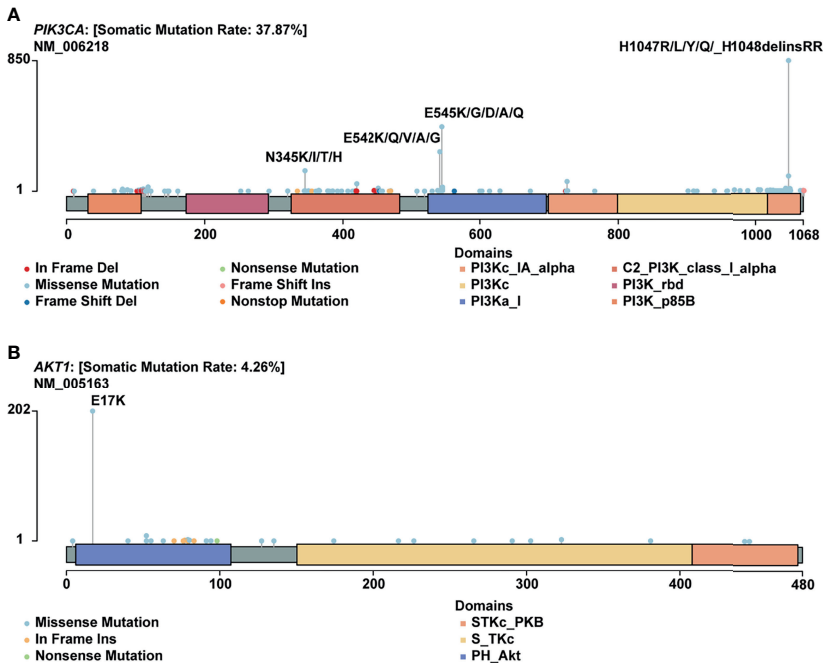

FIGURE 3 | Mutational spectrum of specific genes. **(A)** Mutations across the *PIK3CA* gene, no corresponding neoantigen for E542K; **(B)** Mutations across the *AKT1* gene.

were differentially mutated in whatever subtypes of breast cancer, indicating their importance in the heterogeneity and development of breast cancer.

Common mutations present in at least 3 patients were used for neoantigen prediction. Since previous studies have proved that the common neoantigens may serve as immunotherapy targets (30, 31), we try to find out whether there are common neoantigens in breast cancer populations in this way. Indels were more capable to produce neoantigens than SNVs and can be recognized by more HLA subtypes. However, there are more SNVs than indels in patients, making SNVs the primary source of neoantigens in breast cancer. No statistical difference of indel-derived neoantigens was observed among all subgroups. In terms of SNV-derived neoantigens, age and HER2/ER/PR status are the vital influence factors.

As age increases, tumor mutational burden (TMB) increases accordantly (32). In our breast cancer cohort, the elder population (age>60) also held a higher non-synonym SNV background and a higher fraction of SNV-derived neoantigens. ER^-, PR^- and HER^+ patients held a higher SNV background but a lower fraction of patients with neoantigen. Thus, we infer that the SNV background in the age group might affect neoantigen production, but not in other subgroups. ER, PR, and HER2 status could be used as predictors of neoantigens for breast cancer patients.

H1047R (*PIK3CA*), E545K (*PIK3CA*), E17K (*AKT1*), and N345K (*PIK3CA*) were four hotspot mutations with derived neoantigens. Especially for *PIK3CA* H1047R, a driver mutation in breast cancer (33), was also reported as a neoantigen source in gastric cancer (30). *PIK3CA* E545K produced two different peptides and could be recognized by multiple HLA molecules, including HLA-A03:01, HLA-A11:01, and HLA-B57:01. We can infer that these mutations may serve as therapeutic targets for other cancers owing to their wide range in many cancers and recognition by multiple HLA molecules.

Here we focus on common neoantigens derived from high frequency mutations to benefit as many patients as possible. In spite of these important advantages, this study has several limitations. Due to the limitation of sample sources, the samples in the current data are mainly from European and American populations, which may make it difficult for the results to accurately describe the mutation characteristics of other populations such as Asia. In addition, although we have adopted a variety of stable and feasible bioinformatics methods, the currently predicted neoantigens still need further experimental validation.

CONCLUSION

Based on the analysis of mutation data from eight breast cancer studies, we described the most complete mutation landscape of breast cancer so far. Forty-three HLA genotypes with high frequency in Chinese or TCGA cohort, and 738 non-silent somatic mutations were selected to predict the common neoantigens. The high-frequency mutations, including *PIK3CA* H1047R (14%), *PIK3CA* E545K (6.93%), *AKT1* E17K (3.27%) and *PIK3CA* N345K (2.20%), can be recognized by multiple HLA molecules, such as HLA-A11:01 and HLA-A03:01. These HLA genotypes are the dominant HLA subtypes in the Han Chinese and Americans, representing the commonality of neoantigens we identified among breast cancer patients. In conclusion, except for having constructed a

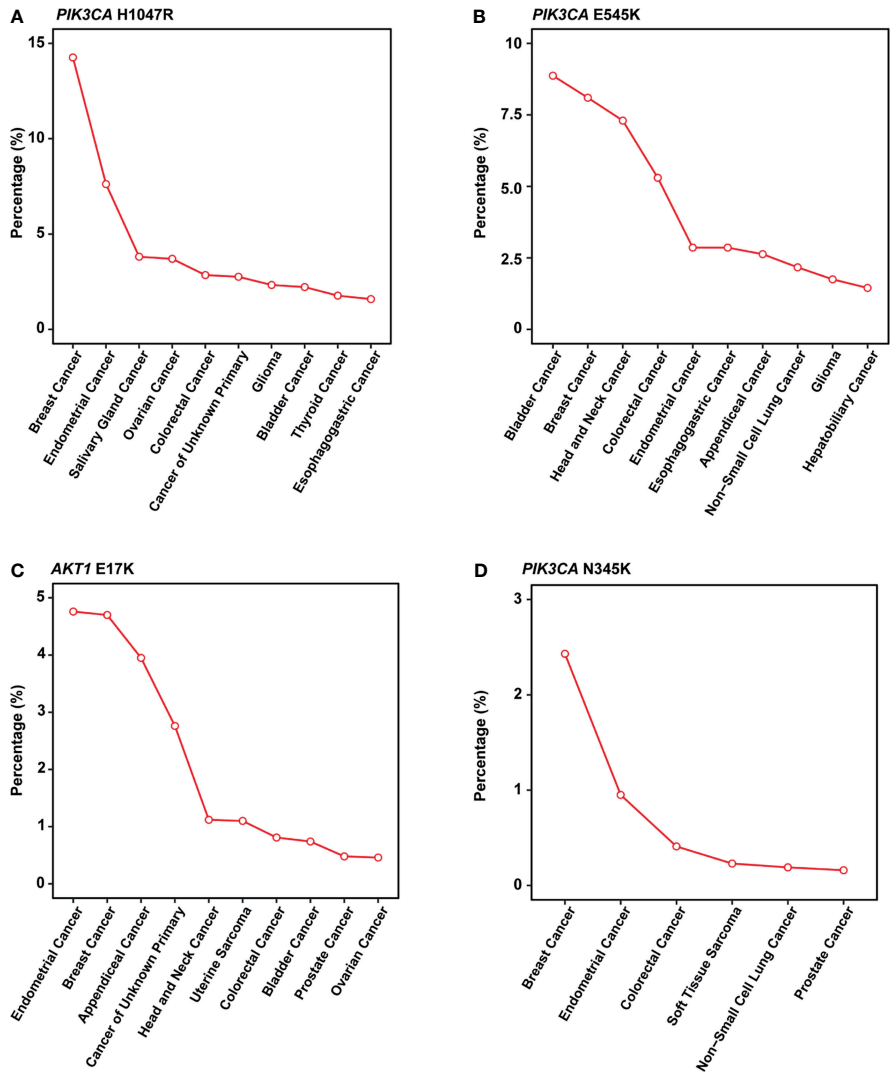

FIGURE 4 | Mutation frequency across multiple cancer types in MSK-IMPACT cohorts. **(A-D)** Line graph showing the percentage of cancer. **(A)** *PIK3CA* H1047R; **(B)** *PIK3CA* E545K; **(C)** *AKT1* E17K; **(D)** *PIK3CA* N345K. All cancer types shown above should meet the following criteria: 1) with a total number of patients equals or exceed 50 in MSK-IMPACT cohort; 2) mutated frequency of the corresponding mutation should exceed 0; 3) if there are over 10 cancer types, show only the top 10 results with the highest frequency.

comprehensive mutation landscape of breast cancer, we also have found a number of public neoantigens, which may contribute to the development of immunotherapy in breast cancer.

MATERIALS AND METHODS

Genomic Data for Breast Cancer Patients

All somatic mutations, including single nucleotide variants (SNVs) and short insertion/deletion (indels), were obtained from the published datasets. The data comprise 5991 breast cancer patients from eight studies, covering several important studies, such as The Cancer Genome Atlas Program (TCGA). Clinical information is shown in **Table 3** and **Supplementary Table 4**. There is no need for additional informed consent because all data were from public databases with informed consent provided in the original studies.

Neoantigen Prediction

Mutations should be non-silent somatic mutations and identified in at least 3 breast cancer patients. Subsequently, These mutations, combined with 43 high-frequency HLA genotypes in Chinese and TCGA cohorts, were used to predict neoantigens through NetMHC (34), NetMHCpan (35), PSSMHCpan (36), PickPocket (37) and SMM (38). Criteria for neoantigen screening refers to our previous research (30).

Statistical Analysis

We finished all statistical analyses in R-Studio (R version 3.6.0). The two R packages, maftools (39) and ggplot2 (40), were used

TABLE 3 | Summary of clinical information of 5991 patients from eight studies.

Characteristic	BCCRC (12) (n=65)	BROAD (4) (n=103)	IGR (16) (n=216)	MBCproject (n=180)	METABRIC (14) (n=2509)	MSK (15) (n=1746)	Sanger (13) (n=100)	TCGA (n=1072)	Total (n=5991)
Age (years)									
<=60	41 (63.1%)	86 (83.5%)	0 (0%)	107 (59.4%)	1163 (46.4%)	1333 (76.3%)	65 (65.0%)	593 (55.3%)	3388 (56.6%)
>60	22 (33.8%)	17 (16.5%)	0 (0%)	8 (4.4%)	1335 (53.2%)	413 (23.7%)	35 (35.0%)	479 (44.7%)	2309 (38.5%)
Unknown	2 (3.1%)	0 (0%)	216 (100%)	65 (36.1%)	11 (0.4%)	0 (0%)	0 (0%)	0 (0%)	294 (4.9%)
Stage									
0	0 (0%)	0 (0%)	0 (0%)	0 (0%)	24 (1.0%)	0 (0%)	0 (0%)	0 (0%)	24 (0.4%)
I	0 (0%)	11 (10.7%)	0 (0%)	11 (6.1%)	630 (25.1%)	469 (26.9%)	0 (0%)	181 (16.9%)	1302 (21.7%)
II	0 (0%)	73 (70.9%)	0 (0%)	27 (15.0%)	979 (39.0%)	512 (29.3%)	0 (0%)	608 (56.7%)	2199 (36.7%)
III	0 (0%)	19 (18.4%)	0 (0%)	21 (11.7%)	144 (5.7%)	370 (21.2%)	0 (0%)	246 (22.9%)	800 (13.4%)
IV	0 (0%)	0 (0%)	0 (0%)	51 (28.3%)	11 (0.4%)	381 (21.8%)	0 (0%)	18 (1.7%)	461 (7.7%)
Unknown	65 (100%)	0 (0%)	216 (100%)	70 (38.9%)	721 (28.7%)	14 (0.8%)	100 (100%)	19 (1.8%)	1205 (20.1%)
ER Status									
Positive	3 (4.6%)	44 (42.7%)	0 (0%)	94 (52.2%)	1825 (72.7%)	1372 (78.6%)	79 (79.0%)	0 (0%)	3417 (57.0%)
Negative	61 (93.8%)	28 (27.2%)	0 (0%)	19 (10.6%)	644 (25.7%)	329 (18.8%)	21 (21.0%)	0 (0%)	1102 (18.4%)
Unknown	1 (1.5%)	31 (30.1%)	216 (100%)	67 (37.2%)	40 (1.6%)	45 (2.6%)	0 (0%)	1072 (100%)	1472 (24.6%)
PR Status									
Positive	1 (1.5%)	40 (38.8%)	0 (0%)	83 (46.1%)	1040 (41.5%)	994 (56.9%)	60 (60.0%)	0 (0%)	2218 (37.0%)
Negative	63 (96.9%)	32 (31.1%)	0 (0%)	28 (15.6%)	940 (37.5%)	692 (39.6%)	40 (40.0%)	0 (0%)	1795 (30.0%)
Unknown	1 (1.5%)	31 (30.1%)	216 (100%)	69 (38.3%)	529 (21.1%)	60 (3.4%)	0 (0%)	1072 (100%)	1978 (33.0%)
HER2 Status									
Positive	0 (0%)	8 (7.8%)	14 (6.5%)	37 (20.6%)	247 (9.8%)	145 (8.3%)	30 (30.0%)	0 (0%)	481 (8.0%)
Negative	63 (96.9%)	47 (45.6%)	194 (89.8%)	71 (39.4%)	1733 (69.1%)	1248 (71.5%)	70 (70.0%)	0 (0%)	3426 (57.2%)
Unknown	2 (3.1%)	48 (46.6%)	8 (3.7%)	72 (40.0%)	529 (21.1%)	353 (20.2%)	0 (0%)	1072 (100%)	2084 (34.8%)

for mutations analysis and visualization, respectively. Unless special instruction was given, $P < 0.01$ was considered significant.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SZ, SL, LZ, and H-XS contributed to conception and design of the study. SZ and SL performed the statistical analysis. SZ, SL, LZ, and H-XS wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.786438/full#supplementary-material

Supplementary Figure 1 | Landscape of the top 10 significantly mutated genes in all the breast cancer patients.

Supplementary Figure 2 | The ratio of base conversion and transversion in mutations (including synonymous variants) and co-occurrence and exclusiveness of the top 20 mutated genes in breast cancer.

Supplementary Figure 3 | The Mutation landscape of breast cancer patients with $HER2^+$ and $HER2^-$.

Supplementary Figure 4 | Differentially mutated genes between $HER2^+$ and $HER2^-$ patients.

Supplementary Figure 5 | The Mutation landscape and the differentially mutated genes between HR^+ and HR^- patients.

Supplementary Figure 6 | The mutation landscape of TNBC patients and the differentially mutated genes between TNBC and non-TNBC patients.

Supplementary Figure 7 | Comparison of non-synonym SNV background within each subgroup, neoantigens among patients in different stages, and neoantigens derived from SNVs and indels.

Supplementary Table 1 | The list of high-frequency HLA genotypes in Chinese and TCGA cohorts.

Supplementary Table 2 | The list of SNV-derived neoantigens.

Supplementary Table 3 | The list of indel-derived neoantigens.

Supplementary Table 4 | The list of clinical information on samples in the previous eight studies of breast cancer.

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Cyclin-Dependent Kinase Inhibitors Function as Potential Immune Regulators *via* Inducing Pyroptosis in Triple Negative Breast Cancer

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Background: Immunotherapy is the most promising treatment in triple-negative breast cancer (TNBC), and its efficiency is largely dependent on the intra-tumoral immune cells infiltrations. Thus, novel ways to assist immunotherapy by increasing immune cell infiltrations were highly desirable.

Methods: To find key immune-related genes and discover novel immune-evoking molecules, gene expression profiles of TNBC were downloaded from Gene Expression Omnibus (GEO). Single-sample gene set enrichment analysis (ssGSEA) and Weighted Gene Co-expression Network Analysis (WGCNA) were conducted to identified hub genes. The CMap database was used subsequently to predicate potential drugs that can modulate the overall hub gene expression network. *In vitro* experiments were conducted to assess the anti-tumor activity and the pyroptosis phenotypes induced by GW-8510.

Results: Gene expression profiles of 198 TNBC patients were downloaded from GEO dataset GSE76124, and ssGSEA was used to divide them into Immune Cell Proficiency (ICP) group and Immune Cell Deficiency (ICD) group. Hub differential expressed gene modules between two groups were identified by WGCNA and then annotated by Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. A cyclin-dependent kinase (CDK) 2 inhibitor, GW-8510 was then identified by the CMap database and further investigated. Treatment with GW-8510 resulted in potent inhibition of TNBC cell lines. More importantly, *in vitro* and *in vivo* studies confirmed that GW-8510 and other CDK inhibitors (Dinaciclib, and Palbociclib) can induce pyroptosis by activating caspase-3 and GSDME, which might be the mechanism for their immune regulation potentials.

Conclusion: GW-8510, as well as other CDK inhibitors, might serve as potential immune regulators and pyroptosis promotors in TNBC.

Keywords: triple negative breast cancer, GW-8510, CDK inhibitor, pyroptosis, connectivity map database

INTRODUCTION

Representing 15% of breast cancers, Triple Negative Breast Cancer (TNBC) is the most aggressive breast malignancy (1, 2). The absence of hormone receptors and human epidermal growth factor receptor 2 (HER2) makes TNBC not respond to targeted therapies and exhibit a poor prognosis (3). Chemotherapy, although with relatively high clinical response rates, its clinical application is limited by unavoidable toxicities and growing prevalence of chemoresistance (4, 5). Thus, emerging novel efficient treatments such as immunotherapy are becoming highly desirable in TNBC (6).

In the past decades, tremendous efforts have been made to restore antitumor immunity. Among them, the most famous one is the broad application of immune checkpoint therapy, which prevent the effective T cells from dysfunction and normalized their anti-tumor activities, thus preventing immune escape, which is a hallmark of carcinogenesis and a major cause of cancer metastasis and progression, and ensuring prolonged remissions eventually (7–9). Despite the encouraging results achieved in various malignancies such as non-small-cell lung cancer and melanoma, the response rate of immune checkpoint therapy in TNBC is far from satisfaction (10). Favorable responses to immunotherapy are observed only in a small subset of TNBC patients (11), with an overall response rate ranged between 5% and 20% across different trials (5). What's more, compares to chemotherapy, pembrolizumab monotherapy did not produce significant long-term survival benefits (12). Therefore, it is important to find new ways to boost the immune treatment efficiency and ensure an advantageous long-term prognosis in patients with TNBC.

According to previous research, the efficiency of immunotherapies is largely dependent on the intra-tumoral immune cell infiltrations (13, 14). Recent studies have segregated the tumor immune microenvironment into three main phenotypes, namely "the immune-desert phenotype", "the immune–excluded phenotype" and "the inflamed phenotype" (15). And robust anti-PD-1 efficiency was established in the presence of pre-treatment tumor-infiltrating T lymphocytes, with immune-infiltrated tumors achieving better responses than immune-desert ones (16). The recruitment of peripheral T cells into tumor microenvironments has also been recognized as the functional fundamental for Immune Checkpoint Blocker-(ICB-) induced anti-tumor activities (17, 18). Thus, strategies to increase intra-tumoral immune cell infiltrations may assist anti-cancer immunotherapy.

Previous studies have revealed several ways to increase immune cell infiltrations, including modulating immune-related gene expressions. However, considering the complicity of immune system, the perturbation of single genes might have limited power in reshaping the entire tumor immune microenvironment (13). Therefore, we hypothesized that the global modification of genes whose expressions are related to immune cell infiltrations in TNBC may be more efficient. Small molecular agents have been reported to play an active role in modulating gene clusters globally in previous studies (19, 20), this study was thus conducted to identify and verify drugs with the potential to increase immune cell infiltration *via* the perturbation of related genes.

In this study, hub genes related to immune cell infiltrations in TNBC were revealed using comprehensive bioinformatics analysis. The tight associations among hub genes enabled them to be regulated globally by GW-8510, an inhibitor of cyclin-dependent kinase (CDK) 2. What's more, the anti-tumor activity and the ability to induce pyroptosis of CDK inhibitors were validated for the first time, and the latter is likely to be the major mechanism for increased immune cell infiltration.

METHODS

Gene Expression Data Acquisition and Patient Classification

The gene expression profiles and clinical information of 198 TNBC patients were downloaded from Gene Expression Omnibus (GEO, accession number: GSE76124) (21, 22).

TNBC samples were grouped by single-sample gene set enrichment analysis (ssGSEA) according to the gene expression signatures of immune cell types and immune pathway enrichment (23). An immune cell proficiency (ICP) and an immune cell deficiency (ICD) group were established accordingly. Distinct immune microenvironments between the two groups were further revealed by significant differences in Stromal Score, Immune Score, ESTIMATE Score, Tumor Purity Score, and the immune cell infiltration fractions calculated by the CIBERSORT algorithm. The above analysis was conducted on R software using the R package "GSVA" and "hclust".

Gene Co-Expression Network Construction

Weighted Gene Co-expression Network Analysis (WGCNA) was performed to assess the co-expression similarities among genes and their correlations with immune cell infiltration. Genes with similar expression peculiarities established the same module. Gene significance (GS, the correlation between the gene and the immune cell infiltration) and module membership (MM, the correlation between the gene and the gene modules) were used to quantify the configurations of modules and features. WGCNA was performed using R software (version 3.6.2).

Functional Enrichment and Protein-Protein Interaction Analysis

The Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for targeted genes were accomplished by "clusterProfiler" and "enrichplot" on R software. STRING (http://www.string-db.org/) database was used to calculate the associations among selected genes and construct protein-protein interaction (PPI) networks.

Connectivity Map Analysis

By documenting gene expression perturbations after pharmacologic interfering, the CMap database (https://www.broadinstitute.org/drug-repurposing-hub) can recommend compounds based on the given gene expression changes (24). In this study, hub gene expression differences between ICP and ICD groups were uploaded for the compound prediction. The enrichment scores are calculated (between 0 to 1), and molecules with enrichment scores close to 1 are supposed to be able to enhance the query gene expression pattern and have therapeutic potentials.

Cell Culture and Reagents

Mouse mammary carcinoma cell line (4T1), and human TNBC cell lines (BT549, MDA-MB-231) were purchased from ATCC. MDA-MB-231 were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum, 1‰ penicillin/streptomycin. 4T1 and BT549 were maintained in RPMI-1640 Medium (Gibco) with 10% fetal bovine serum, 1‰ penicillin/streptomycin. All cell lines were incubated at 37°C in a humidified incubator with 5% CO2. GW-8510 (CAS 222036-17-1) was pursued from Santa Cruz Biotechnology, Inc. Dinaciclib (HY-10492), and Palbociclib (HY-50767) were pursued from MedChemExpress.

Cytotoxic Assay

BT549, MDA-MB-231, and 4T1 cells were seeded in 96-well plates at a density of 2×10^3 cells per well and incubated overnight at 37°C with 5% CO2. Cells were then treated with different concentrations of GW-8510 or DMSO (vehicle control) for 24 h, 48 h, or 72 h, followed by Cell Counting Kit- (CCK-) 8 for another 2 h at 37°C. The absorbance at 450 nm was measured using a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA, United States).

Colony Formation Assay

A total of 1×10^3 cells per well were seeded evenly into six-well plates and incubated at 37°C overnight. After treatment with gradient concentrations (0, 1.25, and 2.5 μM) of GW-8510 for 24 hours. The medium was discharged and cells were cultured with fresh medium for another 10 days. Then, cells were washed with pre-warmed PBS, fixed with 4% PFA, and stained with Giemsa solution for 15 min.

LDH Release Assay

To determine the LDH release caused by GW-8510, BT549, MDA-MB-231, and 4T1 cells were seeded in 12-well plates and incubated grown to almost 50%-60%. Cells were then treated with different concentrations (0, 2.5, 5, and 10 μM) of GW-8510 or DMSO (vehicle control) for 24 h. Then the medium was collected and the LDH release was detected using Cytotoxicity Detection Kit (LDH) (11644793001, Sigma-Aldrich® Brand) according to the manufacturer's instructions.

Flow Cytometry Assay

For evaluation of apoptosis, cells were treated with gradient concentrations of GW-8510 for 24 h, and then labeled with the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA) following the manufacturer's protocol. Thereafter, cells were analyzed immediately using a flow cytometry FACS Calibur System (Beckman Coulter).

Western Blot Analysis

Cells were washed with ice-cold PBS and lysed in RIPA lysis buffer with protease inhibitors on ice for 20 min, followed by centrifugation at 13,000 rpm for 30 min at 4°C, and the supernatants were collected. Protein concentrations were then determined and 20 μg total protein was resolved in 10% SDS-PAGE gels followed by electrophoretic transfer onto PVDF membrane. Blots were blocked at room temperature for 1 h in 5% BSA Tris-buffered saline (TBS)–Tween (TBS-T) on a shaker and then incubated with the primary antibodies overnight at 4°C. The membrane was washed in TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin G at room temperature for 1 h. Immunoreactive proteins were then detected by ECL reagent according to the manufacturer's protocol. And the following antibodies were used: anti-GAPDH (1:2,000; catalog: 10494-1-AP; Proteintech), anti-Cleaved Caspase-3 (1:1,000; catalog: ab32042; Abcam), and anti-DFNA5/GSDME-N-terminal (1:1,000; catalog: ab215191; Abcam).

Xenograft Study

4-6-week-old female BALB/c mice were purchased from Beijing HFK Bioscience Co. Ltd. The mice were housed in a specific pathogen-free (SPF) environment at Laboratory Animal Care Center of Tongji Hospital, and allowed to recover and were monitored closely for one week before any treatment. Then 4T1 breast cancer cells (1×105) were subcutaneously injected into the right posterior limb. For treatment, mice were randomized into two groups (n=5 per group), vehicle and Dinaciclib, since tumor volumes reaches 50 mm3. Mice were treated 3 times per week with Dinaciclib 30mg/kg administered *via* i.p injection. The tumor size was monitored every 3 days. Tumor length and width were measured using electronic calipers. The tumor volume was calculated as follows: volume = 0.5 × length × width2. At sacrifice, portions of tumors were stored in liquid nitrogen for follow-up western blot test or were fixed in 4% Polyformaldehyde for routine histopathologic processing. And the following antibodies were used: anti- HMGB1 (1:400; catalog: ab79823; Abcam), anti-CD8 (1:2,000; catalog: ab209775; Abcam), and anti-Granzyme B (1:3,000; catalog: ab255598; Abcam). All animal procedures were performed in accordance with the approved Guide for the Care and

Treatment of Laboratory Animals of Tongji Hospital and approved by the Ethics Committees of Tongji Hospital.

Statistical Analysis

Data were expressed as the means ± standard deviation. The WGCNA method was analyzed by Pearson correlation analysis. Statistical analyses were performed using Student's t-test or one-way analysis of variance (ANOVA) followed by a *post-hoc* test. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

RESULTS

Establishment of Immune Groups

198 TNBC samples were obtained from Gene Expression Omnibus (GEO) and then grouped as the immune cell proficiency (ICP) (n = 114) or immune cell deficiency (ICD) (n = 84) group by single-sample gene set enrichment analysis (ssGSEA) (**Figure 1A**). Samples of the ICP group had enriched genes signatures in immune cell infiltrations (including NK cells, Macrophages, T helper cells, Dendritic cells, Mast cells, Treg cells, neutrophils, CD8+ T cells, and B cells) and immune pathways (Type I/II Interferon responses, Antigen presentation activities, Inflammations, T cell co-stimulation\inhibitions, and Cytolytic activities). Distinct immune microenvironments were established in two groups, evidenced by lower Tumor Purity but higher ESTIMATE Score, Immune Score, and Stromal Score in the ICP group (**Figure 1B**). Higher expression of HLA genes can also be observed in the ICP group (**Figure 1C**), along with the increased fraction of M1 macrophage, Dendritic cells, and CD4+ T memory cells calculated by CIBERSORT algorithm (**Figure 1D**). Interestingly, T gamma/delta cells, which have been revealed as the most favorable prognostic T cells (25), were also significantly increased in the ICP group.

Identification of Genes Related to High Immune Cell Infiltration by WGCNA

Differentially expressed gene modules between ICP and ICD group were identified by Weighted Gene Co-expression Network Analysis (WGCNA). The soft threshold β value equaled 15 to satisfy the scale-free topology for the co-expression network (**Figure 2A**). Five gene modules were identified (**Figure 2B**) and labeled with different colors (turquoise, brown, blue, green, and grey). Genes within the blue and brown modules were more likely to be overexpressed in the ICP group, while the upregulated gene expressions in the turquoise and grey module were more commonly seen in the ICD group (**Figure 2B**).

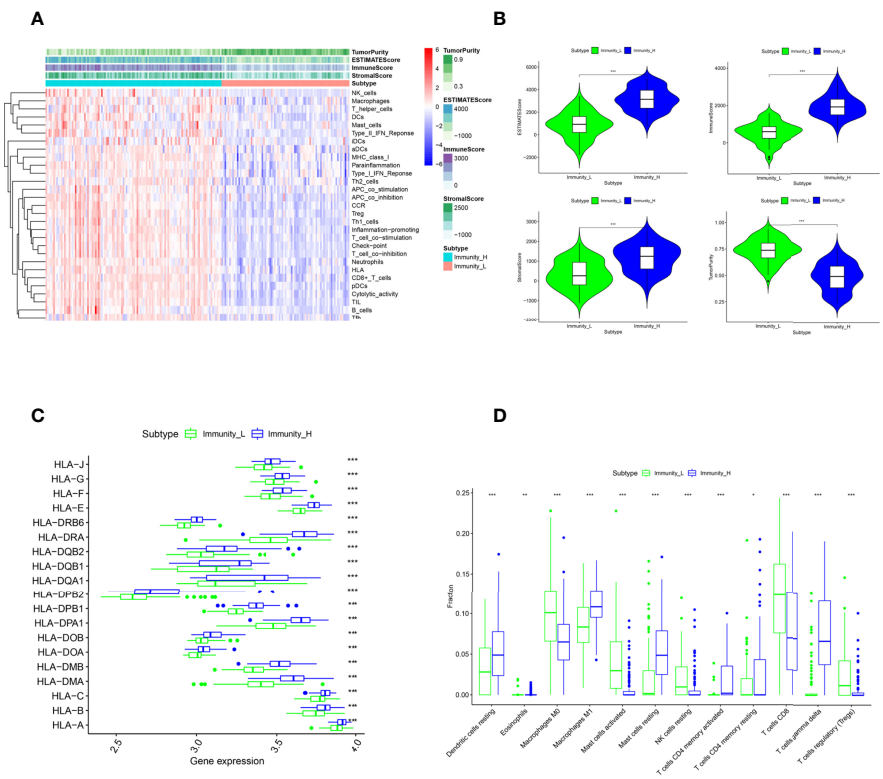

FIGURE 1 | Patient classification by immune cell infiltration. **(A)**. The ssGSEA divided patients into Immune Cell Proficiency (ICP) group and Immune Cell Deficiency (ICD) group. The Tumor Purity, ESTIMATE Score, Immune Score, and Stromal Score of each sample gene were also displayed. **(B)**. The difference in Tumor Purity, ESTIMATE Score, Immune Score, and Stromal Score between the two groups. **(C)** The expression of HLA family genes in the two groups. **(D)** The immune cell infiltration fractions in the two groups caculated by the CIBERSORT algorithm. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

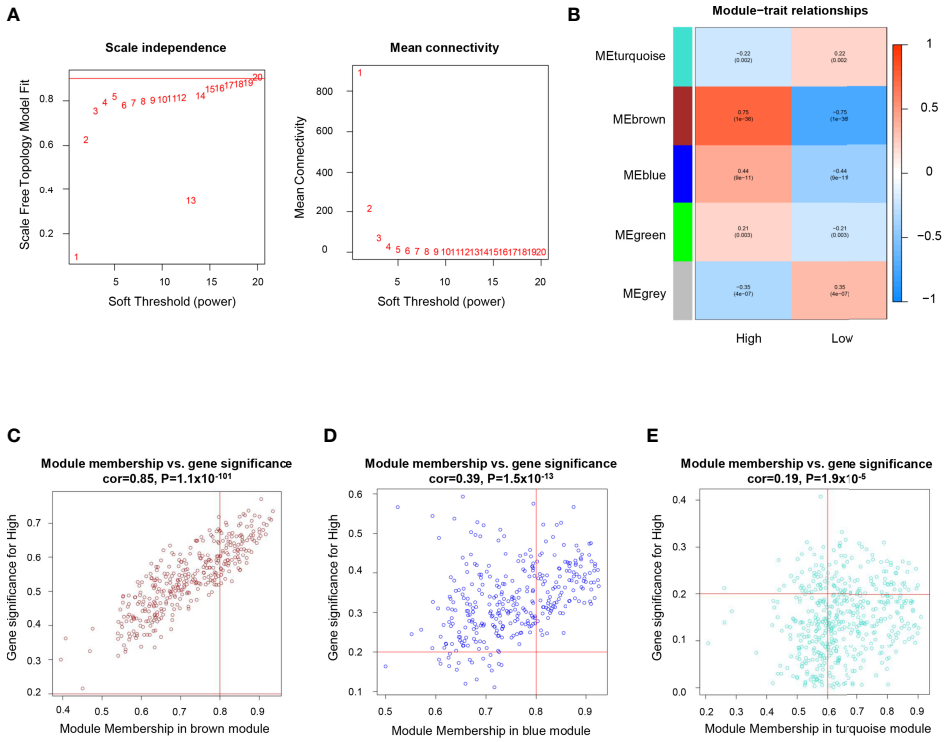

FIGURE 2 | Identification of genes related to high immune cell infiltration. **(A)** Determination of soft threshold by evaluating the scale-free topology fit index (left) and mean connectivity (right). **(B)** Heatmap for the correlations of gene modules to immune cell infiltration. Correlations between module membership and gene significance values were presented in scatterplots for the brown **(C)** blue **(D)**, and turquoise **(E)** gene modules.

In the immune positive-related gene modules (brown and blue module), the hub genes referred to those with module membership (MM) >0.8 and gene significance (GS) >0.2. As a result, 98 genes in the brown modules (**Figure 2C**), and 108 genes in the blue modules (**Figure 2D**) were revealed as the hub genes and included for further analysis. While in the grey and turquoise module, which is negatively related to immune infiltration, only 78 genes in the turquoise module with MM >0.6 and GS >0.2 were considered to be immune negative-related hub genes (**Figure 2E**).

Annotation of Hub Genes

The Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were then conducted to annotate the biological activity related to hub genes. For immune positive-related hub genes (genes in the brown and blue modules), the GO analyses revealed that the dominant biological functions included T cell and lymphocyte activation (**Figure S1A**). Both KEGG and GO analysis displayed that these hub genes took an active part in the biological process of cell adhesion (**Figures S1A, B**). In KEGG analysis, pathways concerning immune cell regulation (leukocyte transendothelial migration, natural killer cell-mediated cytotoxicity, Th1/Th2 cell differentiation, hematopoietic cell lineage, Th17 cell differentiation, and T cell receptor signaling pathway) were revealed to be closely related to the hub genes (**Figure S1B**). For genes negatively related to immune infiltration, the most involved biological process disclosed by KEGG and GO analysis was cell cycle (cell cycle checkpoint and the regulation of mitotic cell cycle phase transition in GO; cell cycle in KEGG) and cell division (nuclear division, organelle fission, chromosome segregation, and mitotic nuclear division in GO; DNA replication and Oocyte meiosis in KEGG) (**Figures S2A, B**).

PPI Network and Profiling of Co-Expressed Genes

The inherent associations among hub genes were demonstrated on the transcriptomic and proteomic levels using co-expression coefficients and protein-protein interaction (PPI)networks to see if these genes could be modulated globally. The strong intercorrelations for gene expressions within the immune positive- and negative- related gene modules were demonstrated and attested by the correlation plot (**Figures S3A, B**). Similar conclusions could be drawn on the protein level (**Figure 3**). In the brown and blue (immune positively related) modules, key immune regulatory molecules including PTPRC (26, 27) (also known as CD45, the key molecular in TCR activation and lymphocyte proliferation), CD8a (28, 29) (a glycoprotein that defines cytotoxic effector cells), LCK (30, 31) (a key signaling molecule in the selection

and maturation of developing T-cells), and CD247 (32, 33) (also known as CD3, presented on the T-lymphocyte cell surface that played an essential role in adaptive immune response) seemed to be the center of PPI network, with interaction number of 82, 52, 40 and 38 respectively (**Figures 3A, B**). In the turquoise (immune negatively related) module, proteins also demonstrated a relatively tight relationship, with the TOP2A (34, 35) (an enzyme that controls and alters the topologic states of DNA during transcription) having the most interactions of 80 (**Figures 3C, D**). Generally, there are strong interactions among hub genes which allowed them to be regulated together.

The Identification of GW-8510 and Its Anti-Tumor Activity in TNBC

To regulate the abovementioned hub genes globally and increase immune cell infiltration, the changes of hub genes were analyzed by Connectivity Map analysis (CMap), and promising molecules were discovered and shown in **Table 1**. Among these recommended drugs, GW-8510 exhibited the highest enrichment score, which indicated that it may be the best-fitting drug for the investigated purpose. As GW-8510 has little research on its anti-tumor effect in TNBC, we first tested its anti-tumor activity *in vitro*. Human (MDA-MB-231, BT549) and mouse (4T1) breast cancer cell lines were treated with different concentrations of GW-8510. The result showed that 2.5 μM of GW-8510 could decrease the viability of TNBC cells after 24-hour treatment, and higher doses of GW-8510 (5 and 10 μM) would result in more effective inhibition of cancer cells (**Figure S4A**). To further confirm the tumor-suppressive activity of GW-8510, colony formation assays were performed and found that the number of cells was significantly reduced upon 1.25 μM and 0.625 μM of GW-8510 24 h exposure (**Figure S4B**).

GW-8510 Induces Pyroptosis *via* Activated Caspase-3 and Cleaved GSDME

When treated with GW-8510, TNBC cells exhibited microscopic features of cell swelling and balloon-like bubbling, which are morphological features of pyroptotic cells (36, 37) (**Figure 4A**).

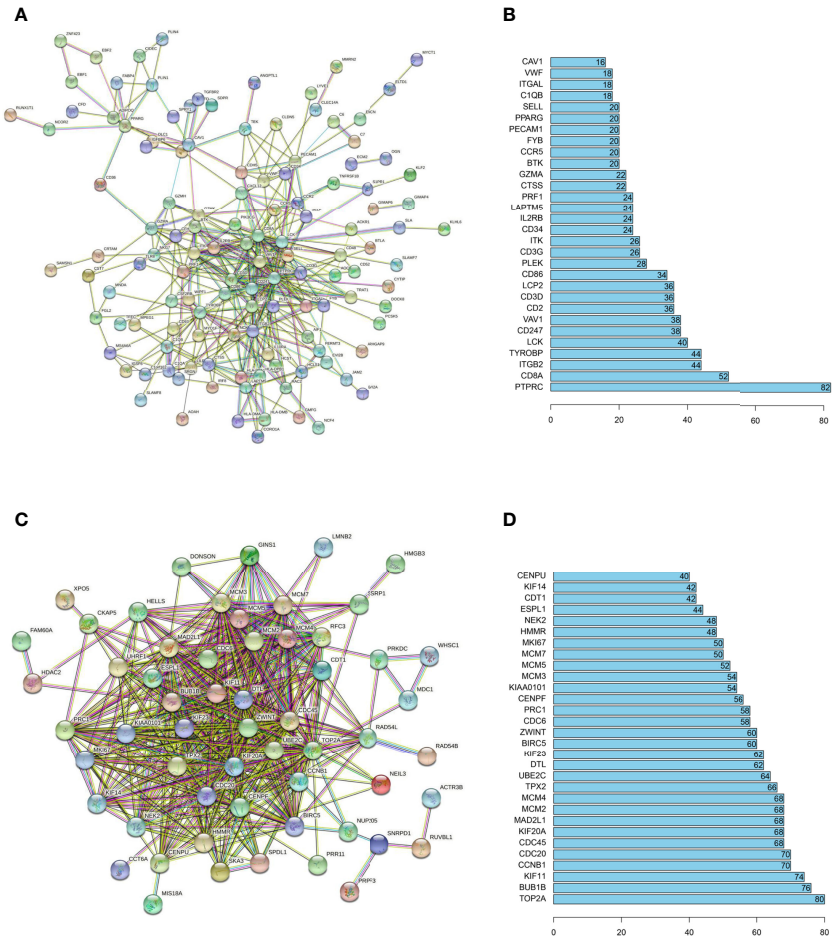

FIGURE 3 | PPI networks analysis for immune related hub genes. PPI networks were drawn by STRING (URL: https://string-db.org/) for hub proteins positive- **(A)** and negative- **(C)** related to immune infiltration. The minimum interaction score was set at 0.7. The disconnected nodes were not shown in the network. The number of connections was also listed for hub proteins of the immune positive- **(B)** and negative- **(D)** related hub genes.

TABLE 1 | Potential effective small-molecule agents predicted by CMap.

CMap name	n	Enrichment	p	Specificity	Percent non-null
GW-8510	4	0.952	0	0.0663	100
phenoxybenzamine	4	0.925	0.00004	0.1485	100
MS-275	2	0.921	0.01221	0.1255	100
daunorubicin	4	0.895	0.0001	0.0404	100
DL-thiorphan	2	0.893	0.02372	0.0882	100
menadione	2	0.876	0.03068	0.1733	100
rottlerin	3	0.868	0.00427	0.0985	100
blebbistatin	2	0.859	0.03986	0.0732	100
thioguanosine	4	0.849	0.00074	0.0294	100
medrysone	6	0.827	0.00004	0.0079	100

Considering that cancer cell pyroptosis would result in inflammation in the tumor microenvironment and increase immune cell infiltration (38), we further investigated whether GW-8510 could induce pyroptosis in TNBC. Since pyroptotic cells were positive for both Annexin V and PI (37, 39), we ran the flow cytometry analysis and observed an increase in Annexin V+/PI+ cells after 10 μM of GW-8510 treatment for 24 h (**Figure 4B**). Furthermore, the release of lactate dehydrogenase (LDH) was measured as an indication of pyroptotic cell cytotoxicity in previous studies (40), since pyroptosis could break the plasma membrane integrity and release cytosolic components. The results displayed that GW-8510 treatment significantly increased the LDH release of TNBC cells in a dose-dependent manner (**Figure 4C**). For the next step, we investigated whether caspase-3/GSDME was involved in GW-8510 induced pyroptosis since caspase-3 activation followed by clipping of GSDME within the N terminus plays a major part in switching apoptotic cell death to pyroptotic cell death in various cancers (41, 42). Results showed that GW-8510 treatment elevated the level of N-terminal fragments of GSDME with concomitant cleavage of caspase-3 in a dose-dependent manner (**Figure 5**). Taken together, these data suggest that

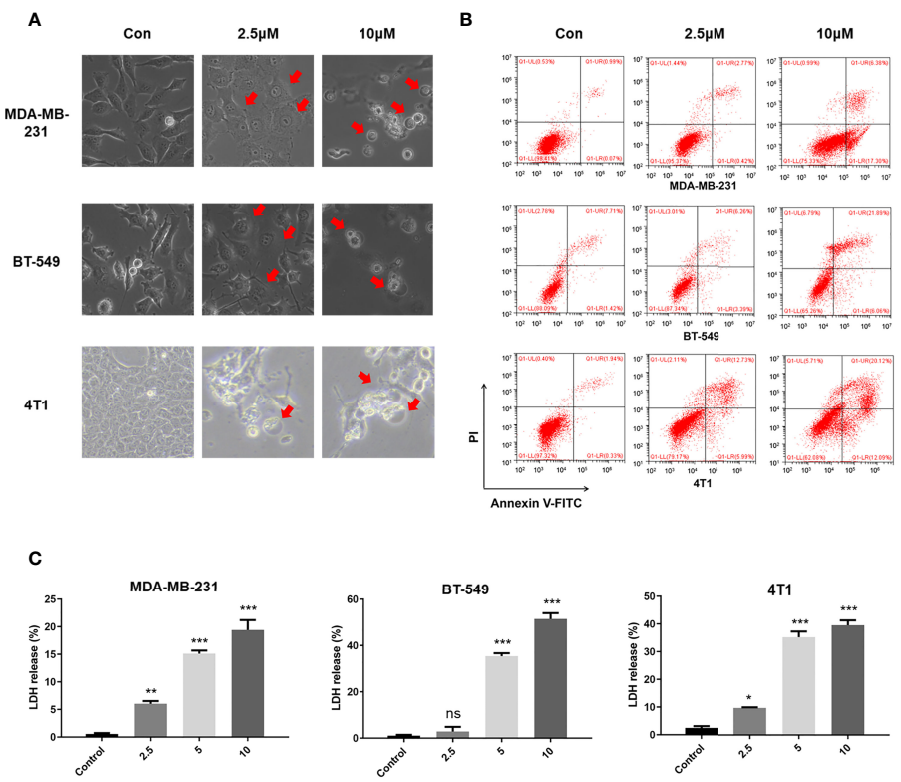

FIGURE 4 | GW-8510 induces pyroptotic cell death in TNBC cells. **(A)** Representative phase-contrast images of GW-8510 treated cells with 0, 2.5, and 10 μM for 24 h. Original magnification, ×400. **(B)** Flow cytometry analysis of GW-8510-treated TNBC cells stained by Annexin V-FITC and PI. **(C)** Release of LDH from TNBC cells treated with indicated concentration of GW-8510 for 24 h. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

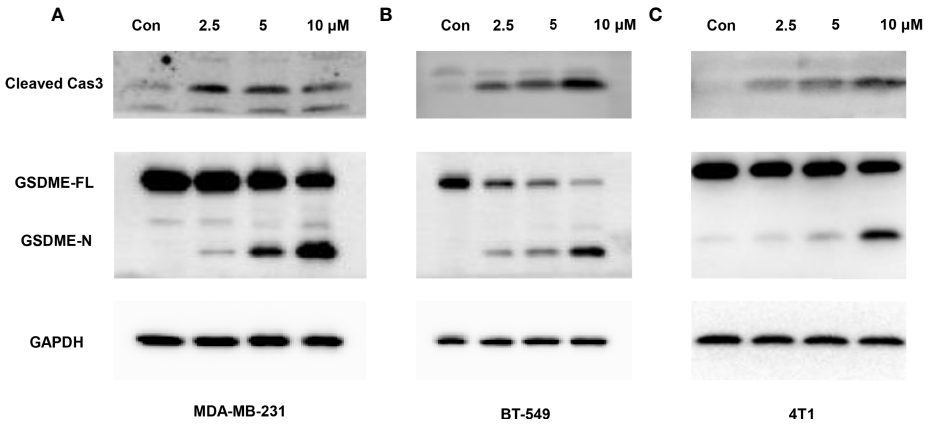

FIGURE 5 | Caspase-3-mediated cleavage of GSDME is involved in GW-8510-induced pyroptosis in TNBC cells. Representative immunoblot analysis of cleaved caspase-3 and N-terminal fragments of GSDME in **(A, B)** human TNBC cells (MDA-MB-231, BT549) cells and **(C)** mouse TNBC cells (4T1) treated with GW-8510 with 0, 2.5, 5, and 10 μM for 24 h. GAPDH was used as an internal control.

GW-8510 may significantly induce pyroptosis *via* caspase-3 and GSDME activation.

Moreover, considering that GW-8510 is an inhibitor of CDK2 and CDK inhibitors have demonstrated promising therapeutic potentials in TNBC, we further investigated the ability to promote pyroptosis in other CDK inhibitors including a broad-acting CDK inhibitor Dinaciclib and a highly selective CDK4/6 inhibitor Palbociclib. As we expected, both of them could induce pyroptosis-specific morphological features such as cell swelling and balloon-like bubbling (**Figure 6**) and increase the cleaved caspase 3 and N-terminal fragments of GSDME (**Figure 7**). Meanwhile, it has been reported that other members of the gasdermin family, like GSDMB, GSDMC also involved in the pyroptosis of cancer cells (36). To find out whether these molecules participated in the CDK inhibitor-induced pyroptosis, the expression of GSDMB and GSDMC was tested by western blot, and no N-terminal fragments were detected for GSDMB and GSDMC after CDK inhibitors treatments (**Figure S5**). Taken together, our *in vitro* studies provided evidence that CDK, inhibitors including GW-8510, Dinaciclib, and Palbociclib, exerted their anti-tumor effect possibility through pyroptosis, which could further ignite an anti-tumor immune response.

Dinaciclib Induces Pyroptosis of Cancer Cells *In Vivo* and Renders Infiltration of Immune Cells

In vivo studies were further conducted to better demonstrate the pyroptosis-inducing and immune-evoking ability of CDK inhibitors. Dinaciclib was chosen for subsequent experiments considering its accessibility and potential in clinical applications (43). Mouse breast cancer cells (4T1 cell line) were injected subcutaneously in immune-competent mice under general anesthesia. The treatment group were administrated with Dinaciclib (30mg/kg, 3 times per week, i.p), and mice in the control group were treated by the same volume of vehicle.

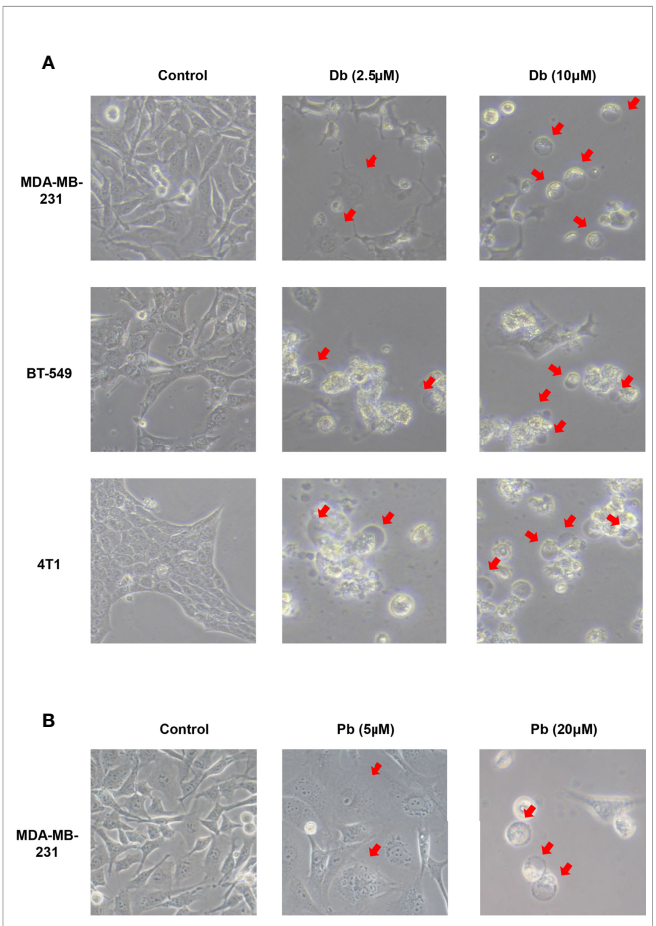

FIGURE 6 | Pyroptotic cell death induced by Dinaciclib and Palbociclib in TNBC cells. Representative phase-contrast images of Dinaciclib **(A)** and Palbociclib **(B)** treated cells with indicated concentration for 24 h. Original magnification, ×400.

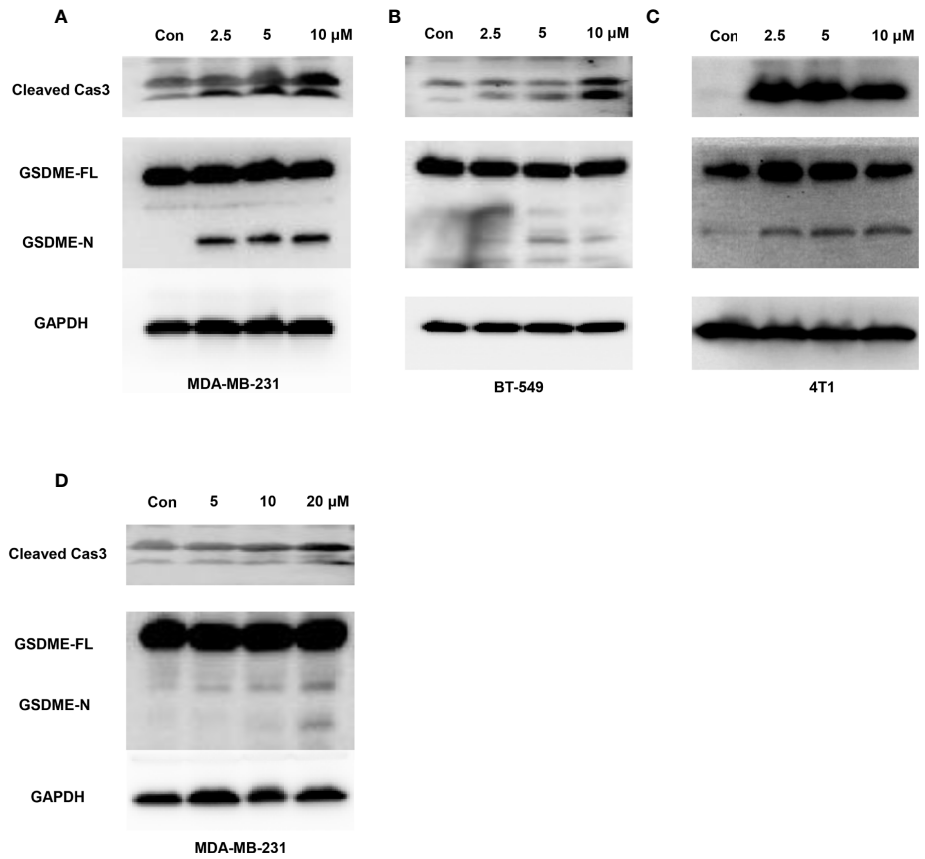

FIGURE 7 | Caspase-3-mediated cleavage of GSDME induced by Dinaciclib and Palbociclib in TNBC cells. Representative immunoblot analysis of cleaved caspase-3 and N-terminal fragments of GSDME in MDA-MB-231 **(A)**, BT549 **(B)**, and 4T1 **(C)** cells treated with Dinaciclib with 0, 2.5, 5, and 10 μM for 24 h. **(D)** Representative immunoblot analysis of cleaved caspase-3 and N-terminal fragments of GSDME in MDA-MB-231 cells treated with Palbociclib with 0, 5, 10, and 20 μM for 24 h. GAPDH was used as an internal control.

Consisting with *in vitro* results, Dinaciclib treatment significantly restricted the growth of xenograft tumors (**Figure 8A**). At the time of sacrifice, the tumor volume and weight were significantly lower in the Dinaciclib group than the controls (**Figures 8B, C**). Furthermore, western blots for tumor samples showed more cleaved GSDME-N after Dinaciclib treatment (**Figure 8D**). In the meantime, HMGB1, as one indicator of immunogenic cell death, was much higher in tumor samples collected from the Dinaciclib group (**Figure 8E**). Consistently, CD8 T cells and granzyme B were also increased after Dinaciclib treatment (**Figure 8E**).

DISCUSSION

In this study, hub genes with the potential to increase immune cell infiltration and enhance immunotherapy efficiency in TNBC were identified using bioinformatics analysis. What's more, GW-8510, a CDK2 inhibitor, was recommended by the CMap database to achieve the global modification of hub genes. *In vitro* and *in vivo* studies were performed and revealed the anti-tumor effect of GW-8510 and other CDK inhibitors. Meanwhile, GSDME-mediated pyroptotic phenotype was validated in these CDK inhibitors, highlighting their immune evoking abilities and the possibilities of combining immunotherapies with CDK inhibitors in TNBC.

According to the gene enrichment analysis, hub genes exhibited high involvements in immune-related biological processes, which could also be validated by the central role of serval vital immune molecules (CD45, CD8, LCK, and CD3) in the PPI network. The immunoregulatory function of hub genes was consisted with our study objectives and supported the validity of our *in silico* analysis. Meanwhile. the strong associations among hub genes provided reassurance for modulating the overall hub gene network and added significance for the CMap predicted drugs (44, 45). Among the top 10 fittest drugs revealed by CMap, apart from GW-8510, MS-275 (also known as Entinostat) was previously reported to help reprogram the tumor's innate and adaptive immune landscape and induce an anti-tumor response in multiple human tumor types (46–50), also indicating the good reliability of our research strategies.

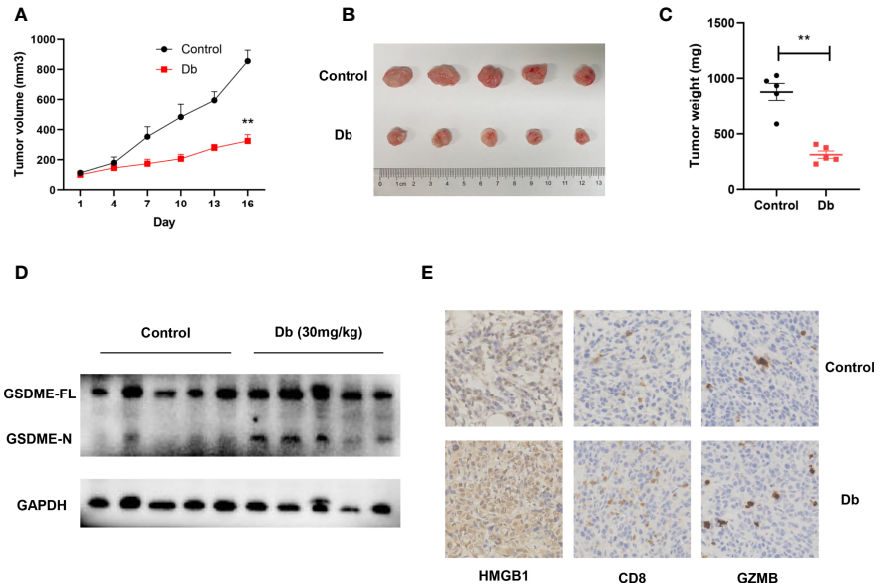

FIGURE 8 | Dinaciclib induces pyroptosis of tumor cells in immune-competent mice models, resulting in increased infiltration of immune cells. **(A–E)** Mice were subcutaneously injected with 4T1 cells. Intraperitoneal injections of Db (30mg/kg and 3 times per week, n = 5) or vehicle (n = 5) lasted for 2 weeks after 4T1 cell inoculation. **(A)** Tumor growth curve. **(B)** Representative image of tumors harvested from mice. **(C)** Tumor weight of mice. **(D)** Western blots showing indicated protein changes in tumor tissues after treatment with Dinaciclib or vehicle in 4T1 xenografts. **(E)** Representative histologic sections of xenografts from tumors of 4T1 were staining with HMGB1 (left), CD8 (middle), and granzyme B (right). **, p<0.01 calculated by the student t test.

CDK inhibitors, highlighted in this study, emerged as novel target therapies in breast cancer. The aberrant expression of cyclin-dependent kinases were common features among various malignancies including breast cancer (51, 52). CDK4/6 inhibitors such as palbociclib, ribociclib, and abemaciclib could induce cell cycle arrest and enable better control over tumor progression (53). Thus, CDK4/6 inhibitors achieved broad clinical applications in the current breast cancer treatments (54, 55). However, intrinsic or acquired resistance to clinically approved CDK4/6 inhibitors have emerged as a major obstacle that hinders their utility in breast cancers (56). Other CDK inhibitors including CDK2 inhibitors were therefore introduced (57). Previous studies reported that GW-8510 could suppress lung cancer cell proliferation and re-sensitize them to gemcitabine through autophagy induction (58). And its role in the anti-tumor activity and immune modulation in TNBC was revealed for the first time in this article, as well as the pro-pyroptosis effect of GW-8510 and other CDK inhibitors.

The role of CDK inhibitors in anti-cancer immune and its potential combined therapy with immune checkpoint blockers have been noticed in previous studies (59–61). Recently, researchers recognized that tumor regression mediated by CDK4/6 inhibition is partially dependent on the presence of cytotoxic T cells (61). Other CDK inhibitors such as CDK12/13 inhibitors were also reported to induce immune death in different cancers. In clinical trials, a Phase Ib trial (NCT02779751) aimed to assess the safety and antitumor activity of abemaciclib plus pembrolizumab in patients with endocrine-resistant, metastatic ER+ breast cancers reported an overall response rate (ORR) of 29%, a high disease control rate of 82%, and a clinical benefit rate of 46%, along with higher durations of PFS (8.9 months) and OS (26.3 months) compared to abemaciclib monotherapy. Another CDK4/6 inhibitor-based immunotherapy combination (palbociclib plus pembrolizumab) in a Phase II study in postmenopausal patients with metastatic ER+ breast cancer patients (NCT02778685) also demonstrated a prolonged median follow-up time of 13.7 months and increased partial response rate of 42.1% (62).

Despite the progress, underlying mechanisms for the immune modulation ability of CDK inhibitors remained poorly investigated. In this study, our results indicated that pyroptosis can be induced by different CDK inhibitors, which might provide serval new insights into this question. Pyroptosis is a lytic pro-inflammatory type of cell death depending on the formation of gasdermin pore on the plasma membrane and pore-induced membrane lysis (63). Pyroptotic cells would release “find me” and “eat me” molecular signals and thus boost antitumor immunity. Among gasdermins, GSDME was a potent executor that could be cleaved by the apoptotic caspase-3 and induce robust pyroptosis in different types of cancer cells (64–66). In this study, TNBC cancer cells treatment with GW-8510 and two other CDK inhibitors all showed an increased level of cleaved caspase-3 and N-terminal fragments of GSDME, which is an indication of GSDME-mediated pyroptosis.

There are several limitations to this study. First, the role of hub proteins obtained from bioinformatic analysis were not verified by clinical samples, which should be further investigated in the future. Second, only GEO dataset were

included in current analysis, which may limited the broader application of study results. Besides, whether the ability to induce pyroptosis of CDK inhibitors is specific to cancer cells or to both cancer cells and immune cells should be further estimated.

In conclusion, hub genes related to immune infiltrations were identified in TNBC. A CDK 2 inhibitor, GW-8510, was predicted to be able to improve anti-tumor immunity by globally modulating these genes. The *in vitro* and *in vivo* studies verified the potent anti-tumor activity of CDK inhibitors. More importantly, these CDK inhibitors could trigger pyroptosis *via* the activation of caspase 3 and GSDME, which could be the mechanism for their potential in boosting immune and enhancing immunotherapy efficiency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Care and Treatment of Laboratory Animals of Tongji Hospital and approved by the Ethics Committees of Tongji Hospital.

AUTHOR CONTRIBUTIONS

QG, YX, and XL designed the experiments and supervised the study. TX, ZW, and JL collected, analyzed, and interpreted the data. GW, DZ, and YD participated in revising the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.820696/full#supplementary-material

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