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Triple-negative breast cancer: Heterogeneity, tumor microenvironment and targeted therapy

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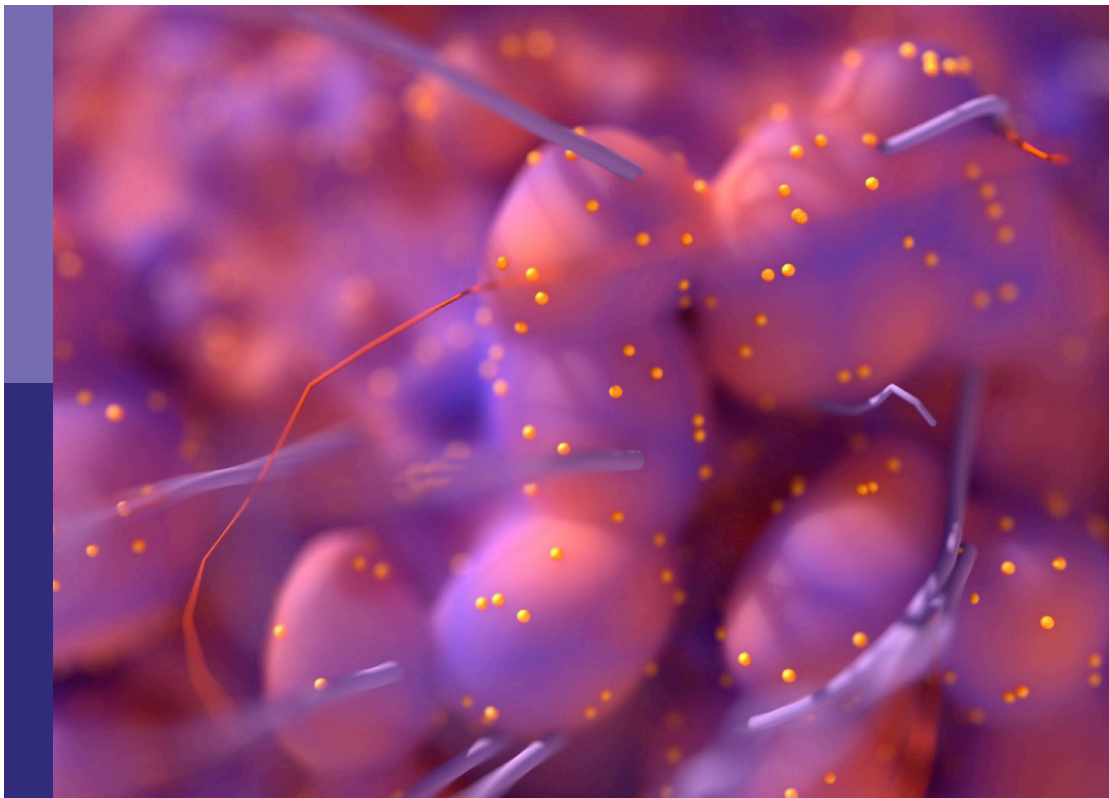

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Triple-negative breast cancer: Heterogeneity, tumor microenvironment and targeted therapy

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Editorial: Triple-negative breast cancer: Heterogeneity, tumor microenvironment and targeted therapy

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Editorial on the Research Topic
Triple-negative breast cancer: Heterogeneity, tumor microenvironment and targeted therapy

Introduction

Triple-negative breast cancer (TNBC), a complex subtype of breast cancer that lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), is characterized by aggressive behavior, high incidence of relapse, and unfavorable prognosis (1). Emerging targeted therapeutic strategies currently approved for the clinical treatment of TNBC include poly (ADP-ribose) polymerase (PARP) inhibitors (2), immune checkpoint inhibitors (ICIs) (3), and antibody-drug conjugates (ADCs) (4). Although some improvements have been observed in survival outcomes, the overall efficacy in unselected TNBC patients remains unsatisfactory. It is reported that the response rate of ICI monotherapy in TNBC ranges between 5% and 25% (5). The reasons for treatment refraction are many and are at least partly attributable to the heterogeneity of the tumor microenvironment (6, 7). Novel therapeutic options for different subtypes of TNBC, particularly those taking into consideration the unique biological features and the highly heterogeneous nature of the tumor microenvironment in different subtypes, are urgently needed and have become an area of active investigation in TNBC research.

In this Research Topic, we present the theme "*TNBC: Heterogeneity, Tumor Microenvironment and Targeted Therapy*" through 11 articles including 5 original research papers, 5 review (or mini-review) articles and 1 bibliometric analysis. The

original research papers include one that focuses on TNBC tumor microenvironment (Wang et al.), one that describes about a nomogram model for predicting distant metastasis of lymph node-negative TNBC (Peng et al.), one that presents potential novel therapeutic strategy targeting intracellular signaling pathway in TNBC (Cui et al.), and one that identifies cancer stem cells as a novel cellular target for TNBC (Zheng et al.). A bibliometric analysis of the research hotspots in TNBC is also included (Hao et al.). The 5 review (or mini-review) articles cover tumor subtyping (Ensenyat-Mendez et al.) and targeted therapies particularly focusing on targeting the tumor immune microenvironment (Li et al., Yi et al., Tan et al. and Clark and Yang).

Novel strategies of targeted therapies for TNBC

As mentioned above, although targeted therapeutic strategies have achieved clinical benefit in some patients, the overall responses in unselected TNBC patients are still limited. Therefore, there is an urgent need in developing more robust targeted approaches for improving the outcomes in TNBC. Signaling pathways that are under active investigation as potential targets for TNBC include intracellular signaling such as tyrosine kinases, as well as cell cycle regulation, DNA damage and cell death regulation, etc (8, 9). Protein tyrosine kinases (PTKs) are a group of enzymes that can transfer a phosphate group from ATP to the tyrosine residues of specific proteins inside a cell. Phosphorylation of proteins by PTKs is an important mechanism of intracellular signaling that regulates diverse cellular processes, e.g., cell division. Classic PTK inhibitors, such as imatinib and osimertinib, that have achieved excellent efficacy in other cancers have failed to meet the same expectations in TNBC. Cui et al. analyzed breast cancer tissues for the expression of PTK7, a member of the PTK superfamily, which plays a critical role in tumor development and progression. They found that high expression of PTK7 significantly correlated with high rates of metastasis and poor prognosis in TNBC patients (Cui et al.). Whether these novel signaling molecules can be explored as therapeutic targets for TNBC needs to be further evaluated.

Statins are well known for their lipid-lowering effects in patients with cardiovascular disease. The recently recognized anti-cancer activity of statins may be due to their pleiotropic effects, including targeting cancer stem cells, a small heterogeneous population of cancer cells that contributes to tumor initiation, metastasis, and recurrence. Through LC-MS/MS-based proteomics and lysine acylation profiling, researchers at Hunan Normal University demonstrated that lovastatin, a naturally occurring lipophilic statin, inhibits TNBC cancer stem cells by dysregulating the cytoskeleton, thus suppressing epithelial-to-mesenchymal transition (EMT) and metastasis (Zheng et al.). Other old drugs that have been shown to inhibit TNBC cancer stem cells include mifepristone (10), metformin (11), disulfiram (12), salinomycin (13), etc. Mifepristone and metformin have been examined in phase I clinical trials in solid tumors including TNBC (NCT02014337 for mifepristone and NCT01650506 for metformin). Although these trials have been completed 5 years ago, no results have been posted yet. Disulfiram has been evaluated examined in two phase II trials in metastatic breast cancer (NCT03323346, NCT04265274), pending release of trial results. These drugs should be examined in better designed clinical studies for their potential for repositioning as clinically beneficial drugs.

Biomarkers to predict the risk and therapeutic efficacy of TNBC

Prediction models can be an excellent tool to identify the patients at high risk. The poor prognosis of lymph node-negative TNBC has been well documented, but reliable biomarkers to predict those at increased risk of metastasis are still lacking. Researchers at Fudan University Shanghai Cancer Center generated a nomogram by incorporating a seven-gene signature with clinical parameters, including patient age and tumor size. This composite model shows improved prognostic accuracy and holds promise for individualized treatment by identifying lymph node-negative TNBC patients who are at a higher risk of distant metastasis (Peng et al.).

Clinical trials have demonstrated that PDL1-positive advanced TNBC patients benefit from atezolizumab-based ICI plus chemotherapy. Biomarkers for PD1/PDL1-targeted ICI therapy include PDL1 expression level, tumor mutational burden (TMB), and tumor-infiltrating lymphocytes (TILs) (Tan et al.,) (14). However, in patients with early TNBC, PDL1 cannot predict the efficacy of ICI plus chemotherapy. Advanced TNBC patients with TMB≥10 mutations/Mb can achieve clinical benefits from pembrolizumab-based ICIs. Higher levels of TILs (e.g., ≥5% in the stroma) have been shown to predict a better response to pembrolizumab-based ICIs in TNBC. In this Research Topic, Wang et al. demonstrated that Ki67, in combination with the TIL level, can be used as a biomarker to predict the outcomes of TNBC patients with residual disease after neoadjuvant chemotherapy. They found that in TNBC patients with residual disease, TIL levels were correlated with favorable survival outcomes in patients with no change in Ki67, but not in patients with decreased Ki67 (Wang et al.). Even so, PDL1 remains the best, though imperfect, predictive biomarker for ICI efficacy (Tan et al.). Other biomarkers with predictive values, such as plasma IL-8 levels (15) and signatures generated from platelet-derived genes (16), are also worth exploring.

Perspectives

Owing to the unique biological features and the aggressive clinical behavior of TNBC, more robust therapeutic approaches are urgently needed to improve patient outcomes. With the emergence of novel targeted therapeutic strategies, we are now seeing some improvements in clinical outcomes in TNBC. Unfortunately, the benefit of these novel therapies on the majority of TNBC patients remains subtle. To achieve improved outcomes, several issues need to be tackled with due attention. First, overcoming the limitations of immunotherapy through combination with other therapies such as cyclophosphamide, apatinib (inhibitor of VEGFR2), PARP inhibitors, oncolytic viruses, adoptive cell therapy, etc (17). Second, more personalized therapy should be implemented based on individual sequencing/multiomics profiling and/or drug efficacy test results (18). With more in-depth understanding of the molecular details behind the pathogenesis of TNBC and the utilization of the state-of-the-art technology, TNBC patients will expect better clinical outcomes in the future, hopefully not far from now.

Author contributions

XD and CZ: Reviewing the literature and writing the original manuscript. FT and TR: Revising and editing the manuscript. Z-MS: Revising and reviewing the manuscript. All authors have read and approved the final submitted manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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RNA-Sequencing Reveals Heat Shock 70-kDa Protein 6 (HSPA6) as a Novel Thymoquinone-Upregulated Gene That Inhibits Growth, Migration, and Invasion of Triple-Negative Breast Cancer Cells

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Objective: Breast cancer has become the first highest incidence which surpasses lung cancer as the most commonly diagnosed cancer, and the second highest mortality among women worldwide. Thymoquinone (TQ) is a key component from black seed oil and has anti-cancer properties in a variety of tumors, including triple-negative breast cancer (TNBC).

Methods: RNA-sequencing (RNA-seq) was conducted with and without TQ treatment in TNBC cell line BT-549. Gene Ontology (GO) function classification annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses for these genes were conducted. Western blot and semi-quantitative RT-PCR were used to verify the regulated gene. Functional assays by overexpression or knocking down were performed for HSPA6 and its mediator TQ for inhibiting growth, migration and invasion of TNBC cells. The regulatory mechanisms and prognosis for HSPA6 for breast cancer survival were conducted through bioinformatics and online databases.

Results: As a result, a total of 141 downregulated and 28 upregulated genes were identified and 18 differentially expressed genes, which might be related to carcinomas, were obtained. Interestingly, GO and KEGG pathway showed their roles on anti-cancer and anti-virus. Further analysis found that the *HSPA6* gene was the high significantly upregulated gene, and showed to inhibit TNBC cell growth, migration and invasion. High expression of *HSPA6* was positively correlated with long overall survival (OS) in patients with breast cancer, indicating the tumor-suppressive roles for HSPA6. But DNA methylation of *HSPA6* may not be the regulatory mechanism for *HSPA6* mRNA upregulation in breast cancer tissues, although the mRNA levels of *HSPA6* were increased in these cancer tissues compared with normal tissues. Moreover, TQ enhanced the inhibitory effect of migration and invasion when HSPA6 was

overexpressed; while HSPA6 was knocked down, TQ attenuated the effects of HSPA6-promoted migration and invasion, demonstrating a partially dependent manner through HSPA6 by TQ treatment.

Conclusion: We have successfully identified a novel TQ-targeted gene *HSPA6*, which shows the inhibitory effects on growth, migration and invasion in TNBC cells. Therefore, identification of HSPA6 not only reveals a new TQ regulatory mechanism, but also provides a novel candidate gene for clinical management and treatment of breast cancer, particularly for TNBC.

Keywords: triple-negative breast cancer, thymoquinone, HSPA6, migration, invasion, RNA-seq

INTRODUCTION

As the malignant tumor, female breast cancer has become the first highest incidence which surpasses lung cancer as the most commonly diagnosed cancer, and the second highest mortality among women worldwide (1). In this year, breast cancer was estimated to reach 2.3 million new cases (11.7%), followed by cancers of lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) (1). The incidence for breast cancer in China is increasing year by year (2). The treatment of breast cancer includes radiotherapy, endocrine therapy, chemotherapy, biological targeted therapy and traditional Chinese medicine adjuvant therapy; but the efficacy still needs to be further improved to benefit the patients.

Thymoquinone (TQ) is a key component from black seed oil from traditional herb medicine and has anti-cancer properties in a variety of tumors (3, 4). Previous studies in our laboratory and others demonstrated that TQ has significant inhibitory effects on the migration and invasion on breast cancer cells, including triple-negative breast cancer (TNBC) (5–9). TNBC is the most aggressive and chemoresistant subtype in breast cancer, with a typical characterization of lack of receptor expressions of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The management for TNBC imposes an economic burden on the society and family and represents a main challenge for both patients and clinicians. New molecular targets and therapeutic reagents are required for improving TNBC patient prognosis and survival. The global regulatory effects and its targets by TQ in TNBC cells are still unknown. Thus, it is necessary to identify novel TQ-targeted genes for breast cancer, including TNBC.

Heat shock 70-kDa protein 6 (HSPA6) (OMIM: 140555), which is cytogenetically located on human chromosome 1q23.3, encodes a 70-kDa protein. HSPA6 was first identified by Leung et al. in 1990 as a stress-induced heat-shock gene (10). *HSPA6* and *HSPA7* were reported to share more than ninety percent nucleotide identity through their coding regions; but HSPA7 showed no protein-coding potential (11). Although HSPA6 was discovered three decades ago, the functional roles in cancer progression are unclear (12–14). Recently, HSPA6 was discovered to be dispensable for Withaferin A-mediated apoptosis/autophagy or migration inhibition of breast cancer (15). In this study, RNA-sequencing (RNA-seq) was performed and TQ-targeted gene *HSPA6* was successfully identified for TNBC inhibition functionally.

MATERIALS AND METHODS

Reagents and Cell Culture

BT-549 and MDA-MB-231 cells, both are TNBC cell lines, and HeLa cells (cervical cancer cell line) were purchased from the American Type Culture Collection (Manassas, VA, USA). RPMI1640 and DMEM were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The fetal bovine serum (FBS) was purchased from Pan Biotech (Bavaria, Germany). TQ was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) (Corning, Manassas, VA, USA). For BT-549 cell culture, the RPMI1640 medium containing 10% of FBS, 0.023 U/ml of insulin was used. For MDA-MB-231 and HeLa culture, the DMEM medium containing 10% of FBS was used. Then we incubated the cells in an incubator at 37°C with a 5% CO_2 air atmosphere.

RNA Extraction, Library Preparation, and RNA-Sequencing

After BT549 cells were treated with TQ for 6 h, total RNA was extracted by TRIzol Reagent (Invitrogen, cat. No 15596026) as described previously (16, 17). DNA contamination should be removed by digestion with DNase I after RNA extraction. The concentration and quality of RNA was measured by detecting A260/A280 with Nanodrop™ spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) and the integrity of RNA was verified with 1.5% agarose gel electrophoresis. Then Qubit 3.0 with Qubit™ RNA Broad Range Assay Kit (Q10210, Life Technologies) was used to quantify the RNA. Preparation for stranded RNA-sequencing library was constructed with 2 μg of total RNA using KC-Digital™ Stranded mRNA Library Prep Kit from Illumina (Catalog # DR08502, Wuhan Seqhealth Co. Ltd., China). Then, we got enriched and quantified library products with 200 to 500 bps in length for RNA-seq on Novaseq 6000 sequencer (PE150 model, Illumina), according to the instruction of NovaSeq 5000/6000 S2 Reagent Kit (cat #: 20012861, Illumina). Briefly, we firstly thawed the preconfigured sequencing by synthesis (SBS) reagent cartridge and the cluster generation reagent cartridge. The library and the SBS reagent cartridge were then mixed and denatured. Then, the library tubes were put into the thawed cluster generation reagent cartridge. Subsequently, we put the cluster generation reagent cartridge into the flow tank for running. Finally, we selected "sequence" in the software, set parameters and started running.

RNA-Seq Data Analysis, GO and KEGG Analyses

After RNA-seq, we used Trimmomatic (version 0.36) to filter raw data, discarded the low-quality reads, and trimmed the reads contaminated by adaptor sequences to ensure the clean data were good enough to use for standard RNA-seq analysis (18). Then, they were mapped to the reference genome of *Homo_sapiens*. GRCh38 was from URL: ftp://ftp.ensembl.org/pub/release-87/fasta/homo_sapiens/dna/using STAR software. Reads mapped to the exon regions of each gene were counted by software of featureCounts (version 1.5.1, Bioconductor), and then Reads Per Kilobase per Million mapped reads (RPKM) was calculated. Using the edgeR package (version 3.12.1) (19), genes differentially expressed with and without TQ treatments were identified. To judge the significantly statistical significance of gene expression differences, a p value cutoff score of 0.05 and fold-change cutoff score of 2 were used. Gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were applied for differentially expressed genes, implemented with software for KOBAS (version: 2.1.1) with a p value cutoff score of 0.05 (20).

Analysis of mRNA Expression by Semi-Quantitative RT-PCR

After extraction, 1 μg of total RNA was used to generate cDNA. The total volume of cDNA synthesis reaction system (reverse transcriptase/RT-PCR) is 10 μl, including 1 μl of dNTPs, 2 μl of 5 × RT buffer, 0.5 μl of random primer, 0.5 μl of RevTra Ace enzyme (which was purchased from TOYOBO company, China), 0.25 μl of RT-enhancer, 0.25 μl of super RI, approximate amount volume of RNase free water and 1 μg of total RNA were also added. The reactions were carried out in a Mastercycler gradient thermocyler (Eppendorf, Germany) as follows: 15 min at 37°C, 5 min at 50°C, 5 min at 98°C, final holding at 16°C. The reaction products were used as templates for semi-quantitative PCR (21). Primers 5'-tggacaaggcccag attcat-3' and 5'-atcctctccacctcctcctt-3' were used to measure *HSPA6* mRNA levels. Meanwhile, the 5'-acagtcagccgcatcttctt-3' and 5'-ttgattttggagggatctcg-3' were used to measure *GAPDH* mRNA level, which served as an internal control to show the difference of *HSPA6* mRNA level among the experimental groups. The semi-quantitative RT-PCR experiments were repeated three times.

Western Blot Assays

The proteins were extracted with EBC lysis buffer, separated on polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (BioRad, USA) (22). The membrane was then kept in 5% skim milk (1 × TBST) at room temperature for 1~2 h, shaken gently in primary antibody solution at 4℃ for 8~12 h, washed thrice with 1 × TBST, and then incubated with secondary antibody (tagged with HRP) for 2~4 h at room temperature. Finally, the membrane was washed thrice with 1 × TBST buffer. After chemiluminiscence reaction, the protein bands on the membrane were visualized by using a digital imaging system from BioRad Lab (Universal Hood II, Italy). The primary antibodies were anti-HSPA6 (Santa Cruz Biotechnology, Inc., CA, USA), anti-β-actin (Cell Signaling Technology, Inc., MA, USA), and anti-Flag (Sigma-Aldrich, Inc., MO, USA). The secondary antibodies, corresponding to primary antibodies, were anti-rabbit or anti-mouse (Cell Signaling Technology, Inc., MA, USA).

HSPA6-Overexpressed and HSPA6-Knocking Down Cell Lines

To generate HSPA6-overexpressed cell lines, 500 ng of pcDNA3.1-C-(k)DYK-HSPA6 plasmid or pcDNA3.1-C-(k) DYK empty vector (Nanjing Genscript Inc., China) was transfected into HeLa cells, and 24 h after transfection, western blot was performed to test whether HSPA6 was successfully overexpressed. In BT-549 cells, knocking down of HSPA6 was achieved by transferring pHS-ASO-LW529, pHS-ASO-LW530 or pHS-ASO-LW531 (Beijing Syngentech Co., LTD., Beijing, China). Meanwhile, plasmid pHS-ASO-LW429 was transfected as a negative control. Three days after transfection, western blot was performed to test whether HSPA6 was successfully knocked down.

Assays for Real Time Cell Analysis (RTCA)

We used a real time cell analyzer (xCELLigence RTCA DP, Roche, Germany) to analyze cell migration, invasion and growth index, which was reported previously (5, 22). A CIM plate was used for cell invasion/migration assays. The matrigel (cat #: 354277, BD Biosciences) was diluted in 1 × PBS at 1:40, and then added to its upper chamber and solidified in cell incubator at 37°C. After the glue was solidified (about 1~2 h), 10% serum supplemented medium was added to the lower chamber wells to induce cell invasion, and 100 μl of cell suspensions (total number of cells 5×10^3) was added into the upper chamber. After installing the upper and lower boards, we started the experiment by setting up the program, and monitored the processes of cell invasion/migration every 15 min till the end of the experiments. About 7 h later, the experimental group was treated with TQ at a final concentration of 10 μmol/L. The cell migration test was similar to the invasion test, except that there was no matrigel in the superior chamber wells. The cell growth experiment was carried out with E-Plate. First, 50 μl of 10% serum supplemented medium was added to each well after the cells were digested and counted so that each 100 μl cell suspension containing 5×10^3 cells was added to each well, and the experiment began. The methods of TQ treatment were same as the invasion and migration experiments. All experiments were repeated three times.

Protein Expression Analysis

We utilized the data from Clinical Proteomic Tumor Analysis Consortium (CPTAC) in UALCAN (University of Alabama Cancer) database (23) (http://ualcan.path.uab.edu/cgi-bin/CPTAC-Result.pl?genenam=HSPA6&ctype=Breast) to analyze the HSPA6 protein expressions between normal tissues and breast invasive carcinoma (BRCA) tumor tissues.

Methylation Analysis for HSPA6 Promoter

The methylation status of *HSPA6* promoter region in the tissues of BRCA patients from The Cancer Genome Atlas (TCGA)-BRCA was explored through the UALCAN database and the database of DNA methylation interactive visualization database (DNMIVD). The associations between the *HSPA6* expression and promoter methylation of *HSPA6* in the normal and BRCA tissues were conducted by the database of DNMIVD (http://119.3.41.228/dnmivd/query_gene/?gene=HSPA6&panel=Summary&cancer=BRCA) (24–26).

Prognosis Analysis

The clinical data for breast cancers from GEO, EGA, or TCGA were used for an overall survival (OS) analysis (27). The two patient cohorts according to upper quantile expressions of *HSPA6* were compared using a Kaplan-Meier survival plot (https://kmplot.com/analysis/index.php?p=service) (27, 28). The gene name *HSPA6* was searched in the database website and the patients were split by median, with or without restriction to breast cancer subtypes.

RESULTS

Results for Genes That Are Differentially Expressed by TQ Treatment in Breast Cancer Cells BT-549

To identify globally affected target genes by TQ, RNA-seq was performed in TNBC cells BT-549 with or without TQ treatments. After RNA-seq, we have successfully identified a total of 141 downregulated and 28 upregulated genes (**Figure 1A** and **Supplementary Figure 1**, **Supplementary Table 1**, $p<0.05$). Then, GO enrichment and KEGG pathway analyses were performed to investigate the functions and pathways which are involved. Results for GO enrichment analysis of these differentially expressed genes in details are presented in **Supplementary Figure 2** and **Supplementary Tables 2, 3**, mainly in regulation of nucleotide-binding oligomerization domain containing 2 signaling pathway, positive regulation of tumor necrosis factor-mediated signaling pathway, protein refolding, cellular response to heat, viral life cycle, response to oxidative stress (GO up, **Supplementary Table 2**), negative regulation of myosin-light-chain-phosphatase activity, sister chromatid segregation, nuclear chromosome segregation, single-organism organelle organization, cytoskeleton, cell cycle (GO down, **Supplementary Table 3**), etc. Results for KEGG pathway analyses of differentially expressed genes are presented in **Supplementary Figure 3** and **Supplementary Tables 4, 5**, revealing that mainly in ribosome, longevity regulating pathway, legionellosis, estrogen signaling pathway, antigen processing and presentation (KEGG up, **Supplementary Table 4**), Fanconi anemia pathway, notch signaling pathway, Salmonella infection, pathways in cancer (KEGG down, **Supplementary Table 5**), etc.

The Expression of HSPA6 Is Increased by TQ Treatment in Triple-Negative Breast Cancer Cells

From above differentially expressed genes, we found 18 differentially expressed genes, which might be closely related to carcinomas, either as oncogenes or tumor suppressor genes. After carefully analyzing, the *HSPA6* gene, as the highly significantly upregulated gene (**Figure 1A**, right panel) and involved into multiple pathways (**Supplementary Tables 2, 4**)

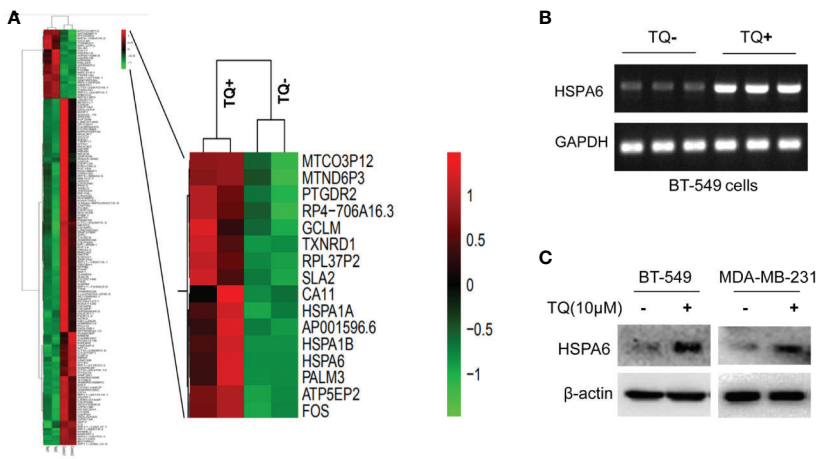

FIGURE 1 | HSPA6 is a novel target by TQ regulation. **(A)** Clustering of differential genes from RNA-sequencing (RNA-seq) data with and without TQ treatments in TNBC BT-549 cells. Left panel, the heatmap of RNA-seq shows all significantly upregulated and downregulated genes after treated by TQ; right panel, the heatmap of RNA-seq shows part of significantly upregulated genes after treated by TQ. Red indicates highly expressed genes whereas green indicates lower expressed genes. A horizontal (X) axis presents different samples whereas a vertical (Y) axis presents the name of gene. The mRNA levels **(B)** and protein levels **(C)** for HSPA6 are increased by treatment with TQ for 6 h in the indicated breast cancer cell lines. The *GAPDH* and β-actin were set as internal controls for mRNA and protein respectively.

by TQ treatment, was captured by us, and previous studies showed that this gene might be related to tumor repression (12). For further verification whether this gene had changes consistent with the results of RNA-seq in BT-549, we subsequently performed semi-quantitative RT-PCR and western blot. As expected, the obviously increased expression of mRNA level in BT-549 cells (**Figure 1B**) and protein level in both BT-549 and MDA-MB-231 cells (**Figure 1C**) were confirmed. Thus, *HSPA6* may be a novel TQ-targeted gene for our further study.

HSPA6 Inhibits Cancer Cell Growth, Migration, and Invasion

Based on the above experimental data, we identified HSPA6 as one of the target genes of TQ. In order to further verify the inhibitory effect of HSPA6 on cancer cell growth, we performed HSPA6 overexpression on HeLa cells with undetectable endogenous HSPA6. To do so, we transfected HSPA6 plasmid into HeLa cells and western blot was performed to check whether it was successfully expressed. **Figure 2A** shows that empty vector in HeLa cells did not express HSPA6, and the HSPA6 plasmid with Flag tag was successfully expressed. On the basis of this successful experiment, we further checked the effect of HSPA6 overexpression on cell growth, migration and invasion by RTCA assays. As presented in **Figures 2B–D**, HSPA6 did inhibit the cell growth, migration and invasion (**Figures 2B–D**). On the other hand, knocking down of HSPA6 in breast cancer cells BT-549 with highly endogenous expression was performed by using three shRNA plasmids. **Figure 3A** shows that HSPA6 was successfully silenced by all three shRNA plasmids, indicating plasmid 531 with more efficiency. Further RTCA assays revealed that the growth curve of BT-549 cells was significantly higher than that of the control group (**Figure 3B**). In addition, this inhibitory effect of HSPA6 may not be affected throughout cell cycle (**Supplementary Figure 4**).

Then, we'd like to further ask whether HSPA6 inhibits cancer cell migration and invasion, the results by RTCA assay found that HSPA6 inhibited the migration (**Figure 4A**, red line *vs.* green line) and invasion (**Figure 4B**, red line *vs.* green line) when HSPA6 was overexpressed; while knocking down of HSPA6 promoted the migration (**Figure 5A**, red line *vs.* green line) and invasion (**Figure 5B**, red line *vs.* green line) in TNBC BT-549 cells.

Taken together, these studies strongly demonstrated the inhibitory effects of HSPA6 on tumor cell growth, migration and invasion.

TQ Enhances the Inhibitory Effects of Cell Migration and Invasion When HSPA6 Was Overexpressed, While Knocking Down Attenuates the Effects

It has been reported that TQ inhibits breast cancer cell migration and invasion (5, 8), and further study here reveals that TQ upregulates HSPA6 expression. With these regards, by overexpression or knocking down of HSPA6 and then assays of cell migration and invasion were performed by RTCA. And the results found that TQ enhanced the inhibitory effect of cancer

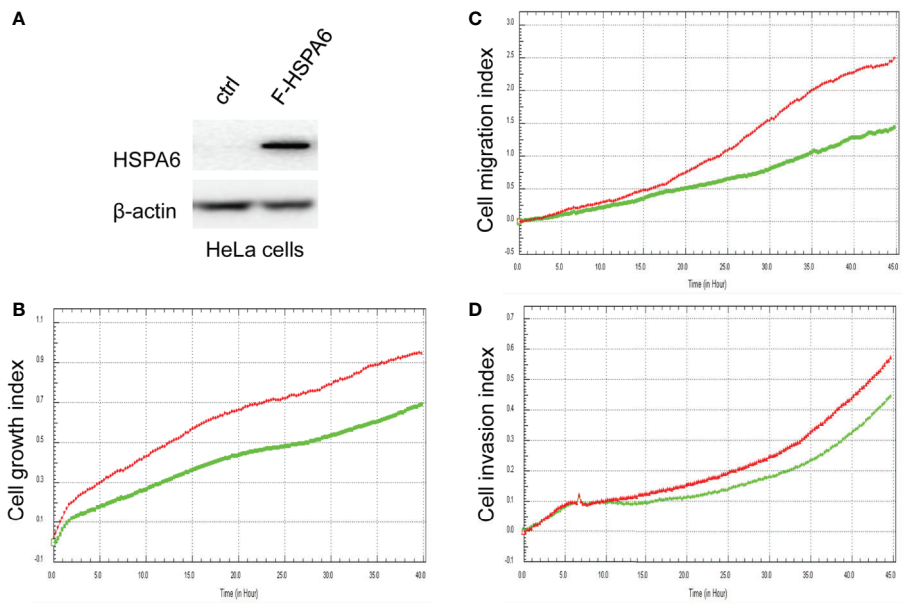

FIGURE 2 | Overexpression of HSPA6 inhibits cancer cell growth, migration and invasion. **(A)** Overexpression of HSPA6 in HeLa cancer cell line. Lane "ctrl" indicates empty vector without HSPA6 expression as a control, whereas lane "F-HSPA6" indicates overexpressions of HSPA6 protein with western blot detected by Flag antibody. β-actin was set as an internal control for total protein loading. **(B)** Cell growth. **(C)** Cell migration. **(D)** Cell invasion. Red lines, controls; green lines, overexpressions of HSPA6.

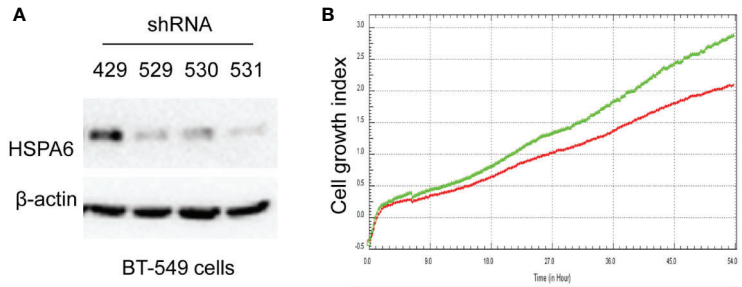

FIGURE 3 | Knocking down HSPA6 promotes cancer cell growth. **(A)** Knocking down HSPA6 in TNBC cell line BT-549. Clones 529, 530, and 531 show the efficiency for knocking down of HSPA6, and clone 531 shows more efficiency; whereas clone 429 shows the empty vector control without knocking down. **(B)** Cell growth. Red lines, controls; green lines, knocking down HSPA6.

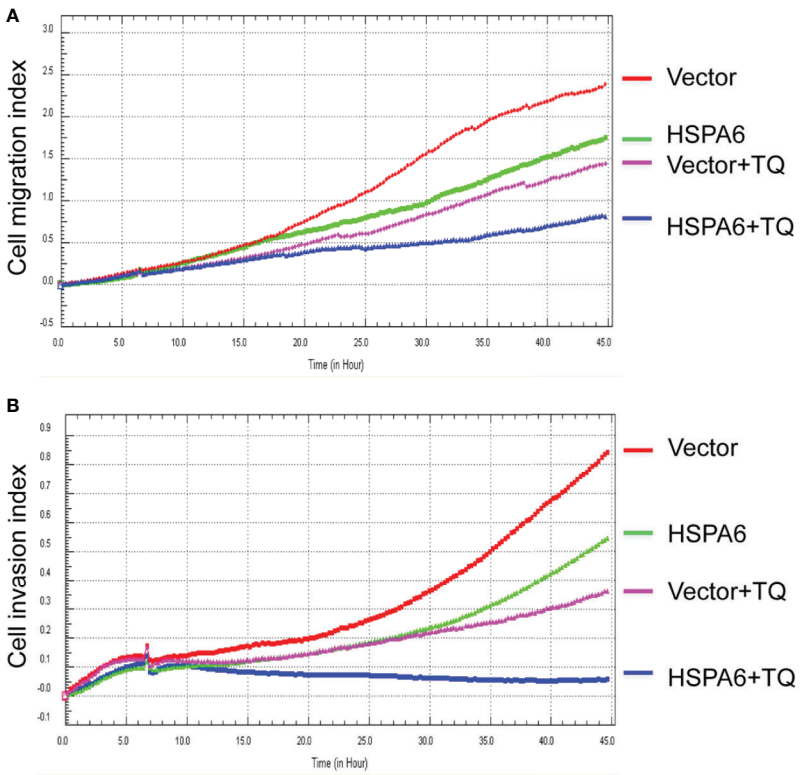

FIGURE 4 | TQ enhances the inhibitory effect of cell migration and invasion when overexpression of HSPA6, demonstrating a partially dependent manner on HSPA6. **(A)** Cell migration. **(B)** Cell invasion. The efficiency for overexpression of HSPA6 was shown in **Figure 2A**. "vector" indicates the empty vector without HSPA6 expression, whereas "HSPA6" indicates overexpression of HSPA6.

cell migration (**Figure 4A**, blue line *vs*. pink line) and invasion (**Figure 4B**, blue line *vs*. pink line) when HSPA6 was overexpressed; when knocking down HSPA6, TQ attenuated the inhibitory effects of cell migration (**Figure 5A**, red line *vs*. green line) and invasion (**Figure 5B**, red line *vs*. green line) of HSPA6-promoted, thus demonstrating a partially dependent manner through HSPA6 by TQ treatment.

The Mechanism for Regulation of HSPA6 Expression in Breast Cancer Tissues

To further investigate the HSPA6 expressions and its clinical significance in breast cancer patients, we thus utilized the data from CPTAC, and results showed that the HSPA6 protein expressions were decreased in breast cancer tissues compared with normal tissues (**Figure 6A**). However, the mRNA levels of

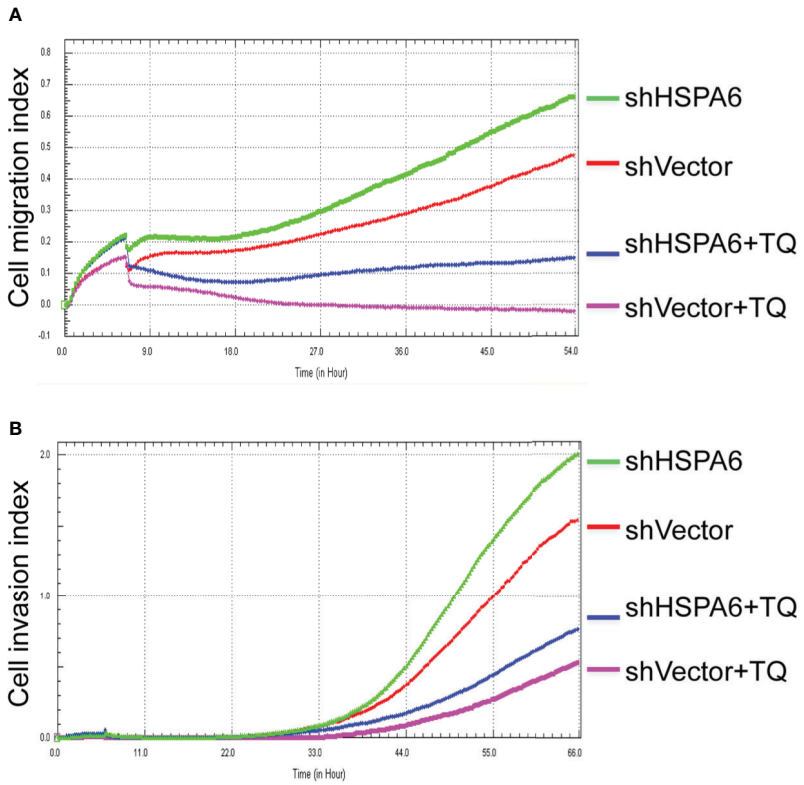

FIGURE 5 | TQ attenuates the inhibitory effect of cell migration and invasion for HSPA6 when knocking down of HSPA6. **(A)** Cell migration. **(B)** Cell invasion. The efficiency for knocking down of HSPA6 was shown in **Figure 3A**. "shHSPA6" indicates knocking down of HSPA6 for clone 531, and "shVector" indicates the empty vector as a control without knocking down.

HSPA6 were increased in breast cancer tissues compared with normal tissues (data not shown). The mechanistic study by *HSPA6* promoter analysis indicated that the promoter regions of *HSPA6* in BRCA samples were increased in cancer tissues compared with matched normal tissues (**Figure 6B**), indicating that DNA methylation of *HSPA6* may not be the regulatory mechanism for *HSPA6* mRNA upregulation in those breast cancer tissues. And promoter methylation and HSPA6 expression in BRCA were also positively correlated (**Figures 6C, D**).

High Expression of HSPA6 Is Positively Correlated With Long Overall Survival in Both All Subtypes of Breast Cancer Patients and TNBC Patients

Through analyzing the clinical data of breast cancer (samples 213418_at) from Kaplan-Meier Plotter database, we found that high expression of HSPA6 was positively correlated with long overall survival (OS) in patients with both all subtypes of breast cancer (low expression cohort *vs*. high expression cohort for upper quantile expressions of HSPA6 were 43 months *vs*. 57.3 months) (**Figure 7A**, HR=0.8, 95% CI: 0.72~0.9) and TNBC (low expression cohort *vs*. high expression cohort for upper quantile expressions of HSPA6 were 25 months *vs*. 36.04 months) (**Figure 7B**, HR=0.86, 95% CI: 0.57~1.32), indicating the tumor-suppressive roles for HSPA6 in breast cancer. In another set of samples (117_at) from Kaplan-Meier Plotter database, similar results were also obtained (data not shown). But we should point out, p value was large than 0.05 in TNBC patients, it may be due to small sample numbers. Nevertheless HSPA6 can serve as a prognostic marker for breast cancer.

DISCUSSION

In order to identify target genes/pathways globally affected by TQ, RNA-seq was performed in TNBC cells BT-549, a total of 141 downregulated and 28 upregulated genes were found. GO function classification annotation showed mainly in protein refolding, cellular response to heat, nuclear chromosome segregation, sister chromatid segregation, microtubule cytoskeleton, chromosome segregation, single-organism organelle organization, cell cycle, viral life cycle, response to oxidative stress, etc.; KEGG pathway revealed mainly in Fanconi anemia pathway, Salmonella infection, pathways in cancer, or ribosome, longevity regulating pathway, legionellosis, estrogen signaling pathway, antigen processing and presentation, etc. Genes demonstrating in pathways of cancer and in

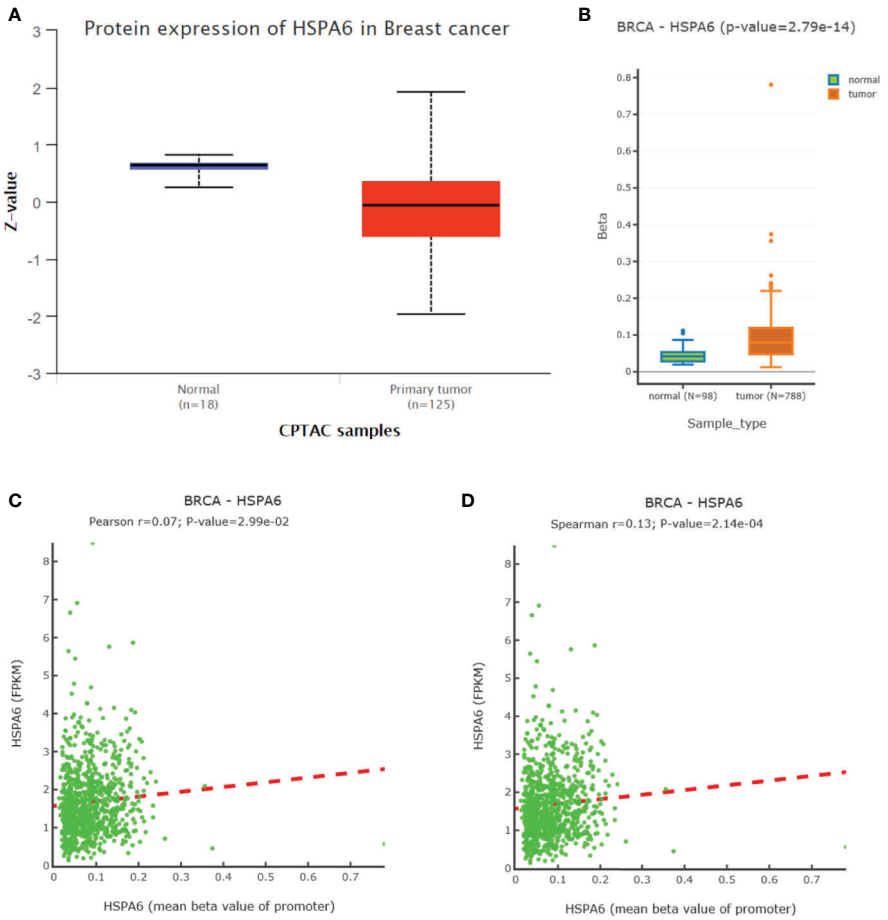

FIGURE 6 | Expression and DNA methylation of HSPA6 in breast cancer tumor tissues. **(A)** HSPA6 protein levels in normal samples and breast cancer tumors (CPTAC samples) ($p<0.01$). Z values represent standard deviations from the median across samples in BRCA. **(B)** Boxplots of DNA methylation for *HSPA6* in BRCA (promoter region) ($p<0.01$). **(C)** Scatter plots of methylation-expression Pearson correlation for *HSPA6* in BRCA. **(D)** Scatter plots of methylation-expression Spearman correlation for *HSPA6* in BRCA. CPTAC, Clinical Proteomic Tumor Analysis Consortium. BRCA, breast invasive carcinoma. Horizontal (X) axes present *HSPA6* promoter methylation values whereas vertical (Y) axes present the HSPA6 mRNA expression level (FPKM). FPKM, Fragments Per Kilobase of exon model per Million mapped fragments.

viral life cycle indicate that TQ has roles for both anti-cancers and anti-viruses. Interestingly, recent studies found that TQ might have inhibitory potential against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) protease (29), particularly for cancer patients (30). As we know, novel virus SARS-CoV-2 causes coronavirus disease 2019 (COVID-19), and the World Health Organization (WHO) declared COVID-19 as a global pandemic as earlier of March 11, 2020 (31–33). As of the March 22, 2021, the total confirmed cases are 123,719,955, and death cases are 2,724,465 worldwide from the report of Johns Hopkins University (https://coronavirus.jhu.edu/).

From differentially expressed genes, we found the *HSPA6* gene was the high significantly upregulated gene by TQ treatment in BT-549 TNBC cells, and showed that HSPA6 inhibited TNBC cell growth, migration and invasion *via* overexpression and knocking down assays. Through analyzing the clinical data of breast cancer by Kaplan-Meier Plotter, we found that high expression of HSPA6 was positively correlated with long OS in patients with both all subtypes of breast cancer and TNBC, indicating the tumor-suppressive roles for HSPA6. Thus, the data through bioinformatics analysis of multiple databases support the inhibitory effect of HSPA6 on breast cancer. Then, further mechanistic study showed that, although the mRNA levels of *HSPA6* were increased in breast cancer tissues compared with matched normal tissues, the promoter regions of *HSPA6* in BRCA samples were increased in cancer tissues compared with matched normal tissues, indicating that DNA methylation of *HSPA6* may not be the regulatory mechanism for *HSPA6* mRNA upregulation in those breast cancer tissues. And correlation for promoter methylation and *HSPA6* expression in BRCA was positively related. These data suggest that, in addition to heat stress, other mechanisms, such as small molecules for example TQ, should be

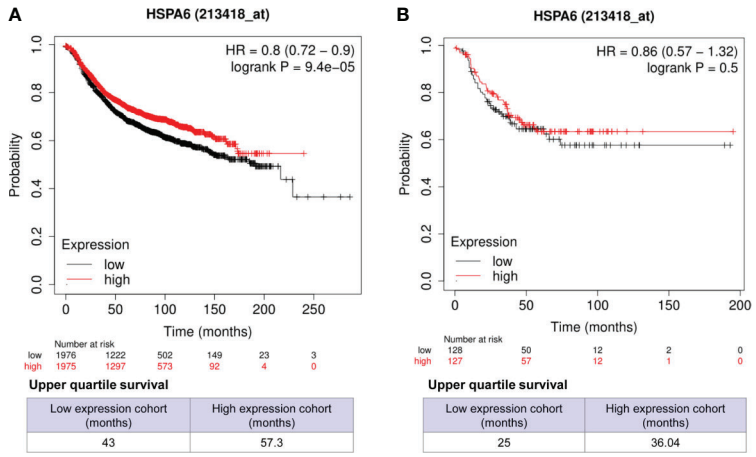

FIGURE 7 | High expression of *HSPA6* is correlated with long overall survival for breast cancer patients in clinic. **(A)** All subtypes of breast cancer patients. **(B)** TNBC patients. The breast cancer patient samples are split into two groups according to the indicated upper quantile expressions of *HSPA6* from dataset of 213418_at. The HR with 95% confidence intervals and logrank p value were calculated. The logrank p value <=0.5 was set as a difference and logrank p value <=0.01 was set as a significant difference. HR, hazard ratio.

involved in HSPA6 upregulation. Thus, these studies strongly demonstrated the inhibitory effects of HSPA6 on tumor cell growth, migration and invasion.

TQ has been reported to inhibit breast cancer cell migration and invasion and epithelial-mesenchymal transition (EMT) markers (5, 9, 34), and our RNA-seq data further revealed that TQ upregulates HSPA6 expression. With these regards, by overexpression or knocking down of HSPA6, the inhibitory roles of cell migration and invasion by TQ were performed, and we found that TQ enhanced the inhibitory effects of cancer cell migration and invasion when HSPA6 was overexpressed; while knocking down, TQ attenuated the inhibitory effect of growth, migration and invasion of HSPA6-promoted, thus demonstrating a partially dependent manner through HSPA6 by TQ. Altogether, identification of TQ-targeted HSPA6 not only reveals a new TQ regulatory mechanism, but also provides a novel candidate target for clinical management and treatment of breast cancer, particularly for TNBC upon TQ.

CONCLUSIONS

By RNA-seq, we have successfully identified a novel TQ-targeted gene *HSPA6*, which showed the inhibitory effects on growth, migration and invasion in TNBC cells. The *HSPA6* promoter DNA methylation may not be the cause for *HSPA6* mRNA upregulation; other mechanism should be involved. Overexpression or knocking down of HSPA6 demonstrates a partially dependent manner through HSPA6 by TQ for HSPA6 inhibitory effects on TNBC cell growth, migration and invasion. Altogether, identification of HSPA6 will provide a novel candidate target for clinical management and treatment of breast cancer, particularly for TNBC on TQ.

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

JF: designed and supervised the project. SS and CW: experimental studies. JF and SS: bioinformatics analysis. JF: wrote and edited the manuscript. All authors contributed, read 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.2021.667995/full#supplementary-material.

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REVIEW
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Immune Checkpoint Inhibition for Triple-Negative Breast Cancer: Current Landscape and Future Perspectives

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Triple-negative breast cancer (TNBC) is characterized by the lack of clinically significant levels of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Owing to the aggressive nature and the emergence of resistance to chemotherapeutic drugs, patients with TNBC have a worse prognosis than other subtypes of breast cancer. Currently, immunotherapy using checkpoint blockade has been shown to produce unprecedented rates of long-lasting responses in patients with a variety of cancers. Although breast tumors, in general, are not highly immunogenic, TNBC has a higher level of lymphocyte infiltration, suggesting that TNBC patients may be more responsive to immunotherapy. The identification/characterization of immune checkpoint molecules, i.e., programmed cell death protein 1 (PD1), programmed cell death ligand 1 (PDL1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA4), represents a major advancement in the field of cancer immunotherapy. These molecules function to suppress signals downstream of T cell receptor (TCR) activation, leading to elimination of cytotoxic T lymphocytes (CTLs) and suppression of anti-tumor immunity. For TNBC, which has not seen substantial advances in clinical management for decades, immune checkpoint inhibition offers the opportunity of durable response and potential long-term benefit. In clinical investigations, immune checkpoint inhibition has yielded promising results in patients with early-stage as well as advanced TNBC. This review summarizes the recent development of immune checkpoint inhibition in TNBC, focusing on humanized antibodies targeting the PD1/PDL1 and the CTLA4 pathways.

Keywords: triple-negative breast cancer, immune checkpoint, PD1/PDL1, CTLA4, Immunotherapy

INTRODUCTION

Triple-negative breast cancer (TNBC), accounting for about 10–20% of all breast cancer cases, is the most aggressive and fatal subtype of breast cancer (1, 2). Compared with other subtypes, TNBC cases are more prevalent in women of African ancestry and tend to be younger at diagnosis (3). Due to the lack of clinically significant levels of estrogen receptor (ER), progesterone receptor (PR), and

human epidermal growth factor receptor 2 (HER2), there is no effective targeted therapeutic agent currently available for TNBC. At present, chemotherapy remains the mainstay of systemic treatment in TNBC (4). Resulting from the emergence of resistance to chemotherapeutic drugs, TNBC patients have a worse prognosis than patients with receptor-positive breast cancer, with a median overall survival (OS) of ≤ 18 months (5, 6). Nowadays, inhibitors of poly (ADP-ribose) polymerases (PARPs) have been approved for a proportion of TNBC patients, i.e., those with BRCA mutation (7). Obviously, more effective treatment modalities are needed to improve the prognosis of this subtype of breast cancer.

Unlike other cancer types that respond well to immunotherapy, most breast cancers are not inherently immunogenic and typically have a low level of lymphocyte infiltration. However, as a special subtype with poorer prognosis, TNBC has greater tumor immune infiltrate, which is characterized by a higher number of tumor-infiltrating lymphocytes (TILs). Clinical investigations have shown that a higher percentage of $CD8^+$ cytotoxic T lymphocytes (CTLs) is a feature associated with higher response rates to immune checkpoint inhibition and can predict favorable survival outcomes in TNBC patients (8, 9). Based upon the findings of a phase III clinical trial IMpassion130 (10), the US FDA granted accelerated approval to the immune-chemotherapy combination of an anti-programmed cell death ligand 1 (anti-PDL1) antibody (atezolizumab) and chemotherapy for PDL1-positive metastatic TNBC (11).

IMMUNE CHECKPOINTS AS IMPORTANT TARGETS OF ANTI-CANCER THERAPY

Immune checkpoints refer to a plethora of inhibitory mechanisms hardwired into the surfaces of tumor cells and immune cells that are crucial for modulating the level and duration of anti-tumor immune responses. These checkpoints are composed of the ligands on the cancer cell and the respective receptors on the $CD8^+$ T cell. The ligands expressed on the cancer cell include PDL1, CD80/CD86, major histocompatibility complex class II (MHC II), CD155, and galectin-9 (GAL9), while their corresponding receptors on the $CD8^+$ T cell include programmed cell death protein 1 (PD1), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), lymphocyte activating gene 3 (LAG3), T-cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT), T cell immunoglobulin and mucin-3 (TIM3), etc. In addition, there is evidence that V-set domain containing T-cell activation inhibitor 1 (VTCN1) also has an important tumor immunosuppressive effect, but its corresponding ligand is not clear yet (12) (**Figure 1**). Activation of the immune checkpoints involves interactions of the inhibitory ligand-receptor molecules. The three most important checkpoint molecules currently used for drug development include PD1, PDL1, and CTLA4 (**Figure 2**).

Up to now, a total of seven antibodies including two anti-PD1 antibodies, three anti-PDL1 antibodies, and two anti-CTLA4 antibodies, have been approved by the FDA for medical use (**Table 1**). In recognition of the eminent contribution to the field of immune checkpoints, the 2018 Nobel Prize in Physiology or

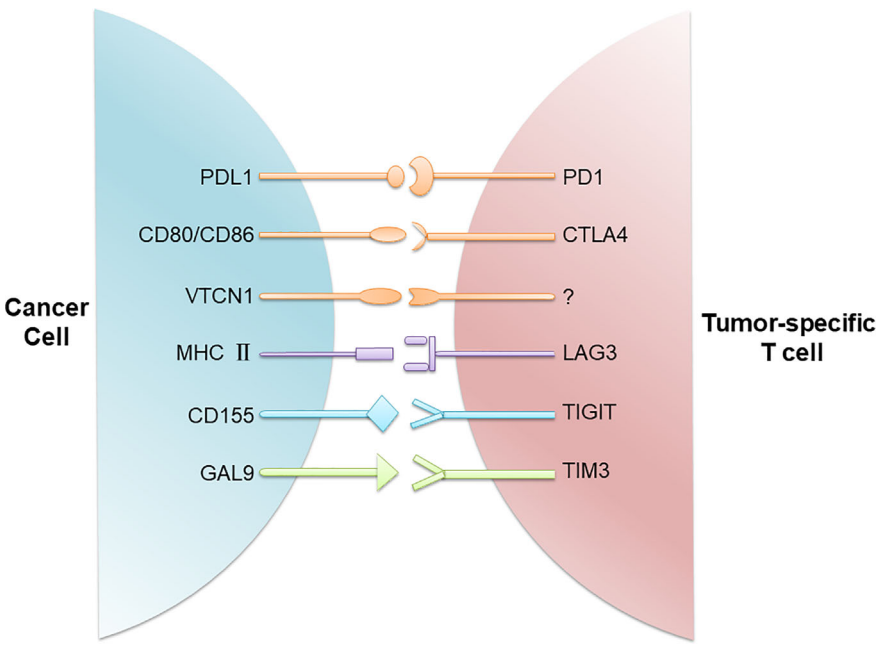

FIGURE 1 | Immune checkpoints involved in T cell inactivation. Cancer cells evade the host immune system through upregulation of immune checkpoints composed of the ligands on the cancer cell and the respective receptors on the $CD8^+$ T cell. These ligand/receptor pairs include PDL1/PD1, CD80/CD86/CTLA4, MHC II/LAG3, CD155/TIGIT, and GAL9/TIM3. In addition, VTCN1 is also found on TNBC cells, although its receptor on T cells is not known.

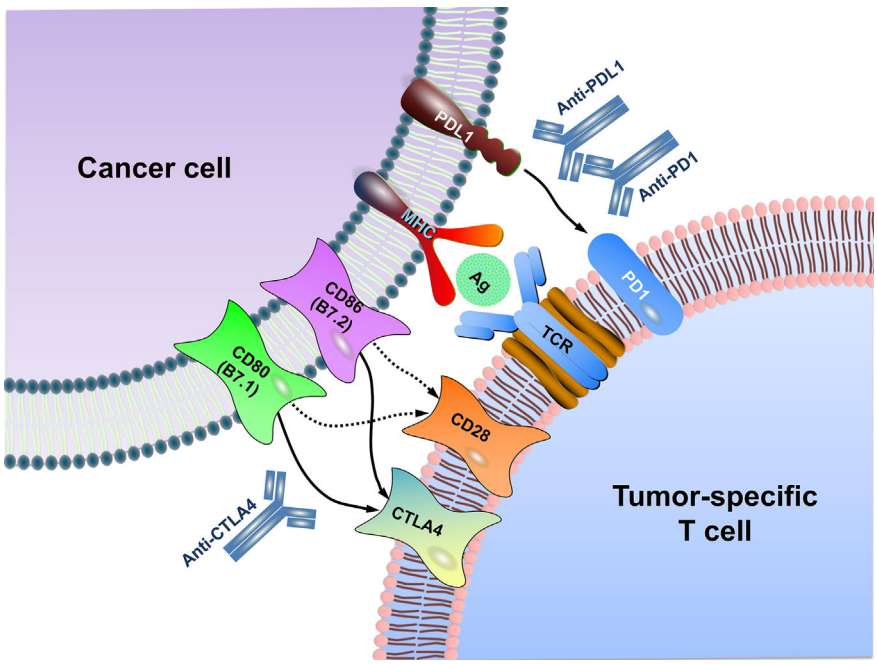

FIGURE 2 | Schematic diagram of immune checkpoint blockade. MHC generally presents antigen on the surface of cancer cells for recognition by $CD8^+$ T cells *via* their TCR. CTLA4, as a negative regulator, is homologous to the T cell co-stimulatory protein CD28, both of which bind to CD80 and CD86 on the surface of cancer cell but with different affinity. Overall, CTLA4 has a much higher affinity than CD28 to CD80/CD86. PD1 is expressed on T lymphocyte surface. The binding of PD1 on the T cell with PDL1 functions to suppress signals downstream of TCR activation, leading to apoptosis of the CTL. Antibodies (anti-CTLA4, anti-PD1, anti-PDL1) inhibit these checkpoint targeting proteins to restore the activity of T cells and kill cancer cells. MHC, major histocompatibility complex; TCR, T cell receptor; Ag, antigen.

TABLE 1 | Summary of immune checkpoint-targeting antibodies.

Target	Antibody	Trade name	Isotype	Initial approval time
PD1	Pembrolizumab	Keytruda	IgG4	Sep 05, 2014
	Nivolumab	Opdivo	IgG4	Jun 22, 2015
PDL1	Atezolizumab	Tecentriq	IgG1	May 18, 2016
	Avelumab	Bavencio	IgG1	Mar 23, 2017
	Durvalumab	Imfinzi	IgG1	May 1, 2017
CTLA4	Ipilimumab	Yervoy	IgG1	Mar 25, 2011
	Tremelimumab	\	IgG2	Apr 15, 2015

Medicine was awarded to James P. Allison at the University of Texas MD Anderson Cancer Center and Tasuku Honjo at Kyoto University. Their seminal work led to the development of antibody-based immune checkpoint inhibitors and the designing of the strategies for activating the anti-tumor immunity in cancer therapy (13).

TARGETING THE PD1/PDL1 PATHWAY IN TNBC

PD1 (also known as CD279), an inhibitory receptor expressed on the surface of CTLs, is emerging as a promising target of immune checkpoint inhibition (14). The primary role of PD1 is to limit T cell activity in peripheral tissues at the time of an inflammatory response to infection, thus limiting autoimmunity (15). The binding of PD1 on T cells with its ligand PDL1 (also known as B7-H1 or CD274) suppresses the signals downstream of T cell receptor (TCR) activation (16, 17). Expression of PDL1 has been found in 40–60% of all breast tumors and is associated with higher histologic grades, larger tumor sizes, and triple-negative status, all of which are independent indicators of poor prognosis in breast cancer (18–20).

Immune checkpoint inhibition using the antibodies against the PDL1/PD1 pathway has shed light on TNBC. The stages of development of anti-PD1 or anti-PDL1 antibodies and their respective combinatorial agents used in clinical trials of TNBC are summarized in **Table 2**. Particularly, the clinical benefit of TNBC has been derived from the combination of immunotherapy with radiotherapy or chemotherapy (21). Theoretically and practically,

TABLE 2 | PD1/PDL1 inhibitors in TNBC immunotherapy for clinical trials.

Antibody	Combinatorial agent	Clinical trial ID	Phase	Status
Pembrolizumab	\	NCT02981303	II	Completed
	\	NCT03197389	I	Completed
	\	NCT02447003	II	Completed
	Capecitabine; Eribulin; Gemcitabine; Vinorelbine	NCT02555657	III	Completed
	Nab-paclitaxel; Paclitaxel; Gemcitabine; Carboplatin	NCT02819518	III	Active, not recruiting
	Nab-paclitaxel; Doxorubicin; Cyclophosphamide; Carboplatin; Paclitaxel	NCT02622074	I	Completed
	Carboplatin; Doxorubicin; Cyclophosphamide; Epirubicin; Paclitaxel	NCT03036488	III	Active, not recruiting
	LTX-315	NCT01986426	I	Completed
	Lenvatinib	NCT03797326	II	Recruiting
Nivolumab	TAK-659	NCT02834247	I	Completed
	Doxorubicin; Cyclophosphamide; Cisplatin	NCT02499367	II	Active, not recruiting
Atezolizumab	\	NCT03281954	III	Recruiting
	Nab-Paclitaxel	NCT02425891	III	Active, not recruiting
	Pegylated liposomal doxorubicin; Cyclophosphamide	NCT03164993	II	Recruiting
	Paclitaxel; Doxorubicin or Epirubicin; Cyclophosphamide	NCT03498716	III	Recruiting
	Nab-paclitaxel; Doxorubicin; Cyclophosphamide; Filgrastim; Pegfilgrastim	NCT03197935	III	Active, not recruiting
	Nab-Paclitaxel	NCT01633970	I	Completed
	Gemcitabine; Capecitabine; Carboplatin	NCT03371017	III	Recruiting
Avelumab	\	NCT01772004	I	Completed
Durvalumab	\	NCT02489448	I/II	Active, not recruiting
	Nab-Paclitaxel; Epirubicin; Cyclophosphamide	NCT02685059	II	Completed
	Olaparib	NCT03801369	II	Recruiting
	Cediranib; Olaparib	NCT02484404	I/II	Recruiting
	Cyclophosphamide; Doxorubicin hydrochloride; Paclitaxel	NCT00856492	II	Completed
	Hiltonol	NCT02826434	I	Active, not recruiting

these combinations should increase the mutational load of tumors and optimize the microenvironment, thus priming the tumor for immunotherapy and improving progression-free survival (PFS) of the patients. Indeed, these combinations have significantly enhanced the curative effect on TNBC patients, which will be discussed in more detail below.

Anti-PD1 Antibodies

Pembrolizumab

As a humanized anti-PD1 antibody that received initial FDA approval for unresectable or metastatic melanoma in 2014, pembrolizumab is one of the best studied immune checkpoint inhibitors (22). In 2016, a phase Ib study (the KEYNOTE-012 trial) reported the efficacy with an acceptable safety profile when pembrolizumab was given to patients with heavily pretreated, advanced TNBC. Among the 27 patients evaluable for anti-tumor activity, the overall response rate was 18.5%, with a median response time of 17.9 weeks (23).

The combined immunotherapy of pembrolizumab and chemotherapy has been investigated in breast cancer. In the locally advanced breast cancer, the addition of pembrolizumab to standard neoadjuvant chemotherapy (paclitaxel followed by doxorubicin and cyclophosphamide) increased the rate of pathological complete response (pCR) by approximately three-fold (60% *vs.* 20%) (24). It is reported that pembrolizumab/chemo combination improves PFS in metastatic TNBC. Results showed that in the intention-to-treat analysis of the full cohort, regardless of PDL1 status, the median PFS was 7.5 months with pembrolizumab and 5.6 months with placebo. The 6-month PFS rates were 55.4% and 47.8%, respectively, and the 12-month PFS rates were 29.8% and 20.9%, respectively (25). Clinical trials of pembrolizumab alone or in combination with different chemotherapeutic agents, monoclonal antibodies, or small molecule inhibitors are now under active investigation in numerous clinical trials in TNBC (**Table 2**).

A strategy of combination of pembrolizumab with PARP inhibitor yielded an objective response rate of 45% compared to 16.7% in single-agent PARP inhibitor group (26). A clinical trial (NCT02555657) aimed to treat metastatic TNBC with pembrolizumab, in which 622 patients were randomly assigned to receive either pembrolizumab or chemotherapy. Median follow-up time was 31.4 months for the pembrolizumab group and 31.5 months for the chemotherapy group. Median OS in patients with PDL1 with combined positive score (CPS) of 10 or more was 12.7 months for the pembrolizumab group and 11.6 months for the chemotherapy group. In the overall population, median OS was 9.9 months for the pembrolizumab group and 10.8 months for the chemotherapy group (27). Another clinical trial funded by Merck Sharp & Dohme (NCT03036488) showed that among patients with early TNBC, the percentage of patients with a pCR was significantly higher among those who received pembrolizumab plus neoadjuvant chemotherapy than those who received placebo plus neoadjuvant chemotherapy (28).

Nivolumab

Nivolumab is another humanized anti-PD1 monoclonal antibody. Due to its significant clinical efficacy against several types of malignancies, nivolumab has become one of the most eye-catching checkpoint inhibitors. A clinical trial (NCT02834247) investigated TAK-659, a selective inhibitor of the Syk tyrosine kinase, in combination with nivolumab in patients with metastatic TNBC. The maximum tolerated dose and the overall response rate

were determined after the patients received TAK-659 at 60 mg/day in combination with nivolumab at 3 mg/kg. This study has been finished on November 30, 2018, and the specific grouping experiment results are available on ClinicalTrials.gov (13). Some scholars pointed out that previous research has shown that anti-PD (L)1 therapy can induce durable responses in patients with metastatic TNBC, but that the response rate is relatively low, about 5-10%. The TONIC study is a currently ongoing phase II trial for patients with metastatic TNBC. The objective response rate (ORR) per RECIST v1.1 with nivolumab for the whole cohort was 22% and 24% for iRECIST, which included 1 (2%) complete response (CR), and 11 (22%) partial responses (PR). Additionally, stable disease (SD) lasting more than 24 weeks was achieved in 1 (2%) patient, which resulted in a 26% clinical benefit rate. This is the first trial that has shown promising results using nivolumab after giving either radiation or chemotherapy. The completion of this study is estimated to be in August of 2022 (29). In a study published in 2019 (NCT02499367), 67 patients with metastatic TNBC were treated with the anti-PD1 antibody nivolumab after 2 weeks of either hypofractionated irradiation of a single tumor site, low-dose cyclophosphamide, cisplatin, or doxorubicin, or no induction therapy. Overall, the ORR was 20% and, although the median PFS was only 1.9 months, the median duration of response was 9 months (30). Trials of nivolumab alone or in combination with ipilimumab (anti-CTLA4 antibody), different chemotherapeutic agents, monoclonal antibodies, or vascular endothelial growth factor receptor (VEGFR) inhibitor on TNBC are ongoing (**Table 2**).

Anti-PDL1 Antibodies

Atezolizumab

Atezolizumab (MPDL3280A), a humanized monoclonal antibody against PDL1, was reported to significantly increase median OS and objective remission rate in lung cancer patients in a phase II trial (31). In May of 2016, the FDA granted accelerated approval to atezolizumab for the treatment of locally advanced and metastatic tumors (11). An initial phase I study demonstrated that of the nine patients with advanced TNBC evaluated for efficacy of atezolizumab, the overall response rate was 33% (32). Recently, a phase III clinical trial (NCT02425891) evaluating the effects of atezolizumab in combination with nab-paclitaxel as first-line treatment in metastatic TNBC patients yielded exciting results. Among the patients with PDL1-positive tumors, atezolizumab plus nab-paclitaxel significantly prolonged the median OS compared with placebo plus nab-paclitaxel (25.0 *vs.* 15.5 months) (10). It should be noted that as the benefit was observed in the patients with PDL1-expressing tumors (accounting for about 40-60% of all TNBC) (10, 23), the overall effect on TNBC patients as a whole is not satisfactory and still needs improvement. A phase Ib clinical trial (NCT01633970) examined the safety, tolerability, and clinical activity of atezolizumab (one or more doses) plus nab-paclitaxel in 33 patients with metastatic TNBC. All patients experienced at least 1 treatment-related adverse event (AE), 73% patients experienced grade 3/4 AEs, and 21% patients had grade 3/4 AEs of special interest. No death was noted in this study. The ORR was 39.4%, and median PFS and OS were 5.5 months and 14.7 months, respectively (33). As mentioned earlier, the FDA has granted accelerated approval to the combination of atezolizumab with nab-paclitaxel for the treatment of PDL1-positve metastatic TNBC. Ongoing trials in TNBC are using atezolizumab alone or in combination with different chemotherapeutic agents, monoclonal antibodies, or small molecule inhibitors (**Table 2**). These efforts are expected to lead to new treatment options for patients with TNBC in the near future.

Avelumab

Avelumab, another anti-PDL1 antibody, was investigated as adjuvant treatment for TNBC in a phase Ib randomized trial (NCT01772004). In this trial, 168 patients with metastatic breast cancer, including 58 patients with TNBC, were included. Patients refractory to or progressing after standard-of-care therapy received avelumab. 13.7% patients had higher than grade 3 AEs, including two deaths. The ORR was 3.0% in all subtypes of breast cancer and 5.2% in TNBC patients. A trend toward a higher ORR was seen in patients with PDL1-positive vs. PDL1-negative tumor-associated immune cells in the overall population (16.7% vs. 1.6%) and in the TNBC subgroup (22.2% vs. 2.6%) (34). Furthermore, avelumab alone or in combination with different chemotherapeutic agents, monoclonal antibodies, or lansoprazole, a proton-pump inhibitor, is currently under investigation in TNBC (**Table 2**).

Durvalumab

Several trials are also being performed with durvalumab for patients with metastatic TNBC in combination therapy (19) (**Table 2**). In the GeparNuevo trial, the positive rates of pCR in patients receiving durvalumab treatment 2 weeks before chemotherapy was significantly higher than that in the placebo group (61% *vs.* 41.4%). Less improved response rate of 48.4% was seen in patients receiving durvalumab in conjunction with neoadjuvant GeparNuevo (NCT02685059).

A phase Ib trial (NCT02826434) studied the immunotherapeutic effects with a peptide vaccine, PVX-410, and durvalumab as adjuvant setting in treating stage II or III TNBC. The dose-limiting toxicity of PVX-410 vaccine with durvalumab and the immune response of $CD8^+$ CTLs to vaccine-specific peptides were detected after patients received 6 injections of the PVX-410 vaccine with poly-ICLC (a dsRNA analog used as an agonist of Toll-like receptor 3 (TLR3)) every 2 weeks and 2 infusions of durvalumab with the 4^{th} and 6^{th} cycle. Currently, this study is still in progress and should be completed in August of 2022.

In a phase I/II trial (NCT02489448), stage I–III TNBC patients were evaluated in terms of whether they produced a higher pCR with adding durvalumab to nab-paclitaxel weekly and then with dose-dense doxorubicin and cyclophosphamide for 4 cycles compared with chemotherapy alone. Additionally, this trial will also demonstrate whether durvalumab is safe and can be given in the full dose when added to this chemoregimen, and the secondary object is to assess the safety and toxicity of adding durvalumab to nab-paclitaxel followed by adding it to dose-dense doxorubicin/cyclophosphamide. Results showed patients treated at the recommended phase II dose of 10 mg/kg of durvalumab achieved a pCR rate of 44%. Among PDL1 positive patients, the pCR rate was 59% and among PDL1

negative patients, the pCR rate was 32%. No significant difference was observed ($P = 0.26$) (35).

Another randomized phase II study (NCT02685059) was performed to evaluate the efficacy of addition of durvalumab to an anthracycline + taxane-based neoadjuvant therapy in early TNBC. A total of 174 patients were randomized, 117 of whom participated in the window-phase. The pCR rate was 53.4% in the durvalumab group and 44.2% in the placebo group. There was a trend for increased pCR rates in PDL1-positive tumors, which was significant for PDL1-tumor-cell in durvalumab group and for PDL1-immune cell in placebo group (36). Targeted mRNA sequencing was performed in samples from patients with early TNBC of the GeparNuevo trial. Signatures were evaluated to predict response to neoadjuvant PDL1 inhibition in combination with chemotherapy. Two mRNA signatures (G6-Sig and IFN-Sig) were predictive for treatment response in a multivariate model, while a simple metric of two key cytolytic effector transcripts (GZMA and PRF1) predicted pCR in the durvalumab arm, and the proliferation-associated gene signature in the placebo arm. Seven genes were identified highly expressed in responders in the durvalumab arm, but not in the placebo arm. These genes were associated with cellular antigen processing and presentation and IFN signaling (37).

TARGETING THE CTLA4 MOLECULE IN TNBC

CTLA4 (also known as CD152), the first co-inhibitory molecule identified and the first immune checkpoint receptor clinically targeted (38), is expressed exclusively on T cells where it primarily regulates the amplitude of early-stage T cell activation. The ligands of CTLA4, i.e., CD80 (also known as B7.1) and CD86 (also known as B7.2), are shared by the co-stimulatory receptor CD28 (39). Compared with CD28, CTLA4 has a much higher overall affinity for both CD80 and CD86 (40). Therefore, the expression of CTLA4 on T cell surface dampens the activation of T cells by outcompeting CD28's positive co-stimulatory signal. This dominance of negative signals from CTLA4-CD80/CD86 interaction results in reducing T cell proliferation and decreasing IL-2 production (41). The central role of CTLA4 in inhibiting T cell activity is demonstrated by the systemic immune lethal hyperactivation phenotype of CTLA4-knockout mice (42).

Preclinical Studies of CTLA4 Blockade

As an important strategy of cancer immunotherapy, CTLA4 blockade results in broad enhancement of immune responses that are dependent on helper T cells (43). The strategy of blocking CTLA4 was questioned because of lack of tumor specificity to the expression of CTLA4 ligands and because of the dramatic lethal autoimmune and hyperimmune phenotype of CTLA4-knockout mice. Initially, a high degree of immune toxicity associated with blockade of this receptor was predicted. However, Allison and colleagues used preclinical models to demonstrate that a therapeutic window was indeed achieved when CTLA4 was partially blocked with antibodies against CTLA4 (44). Subsequent studies demonstrated significant anti-tumor responses without overt immune toxicities, when the mice bearing partially immunogenic tumors were treated with CTLA4 antibodies. Poorly immunogenic tumors did not respond to anti-CTLA4 as a single agent but did respond when anti-CTLA4 antibody was combined with a granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced cellular vaccine (45). These preclinical investigations indicate that antibody-mediated CTLA4 blockage has the potential of clinical application in treating immune-related tumors.

Humanized Antibodies Against CTLA4

The above preclinical findings encouraged the development and testing of two fully humanized CTLA4 antibodies. Ipilimumab (trade name Yervoy), a monoclonal antibody able to effectively block CTLA4 binding to its ligand, is the first immune checkpoint inhibitor approved by the FDA for clinical use (46). Tremelimumab is another anti-CTLA4 monoclonal antibody. As with virtually all anti-cancer agents, initial testing was as a single agent in patients with advanced melanoma and ovarian cancer that were not responding to conventional therapy (47). Both antibodies produced objective clinical responses in ~10% of patients with melanoma, but immune-related toxicities involving various tissue sites were observed in 25–30% of patients, with colitis being a particularly common concern. The first randomized phase III clinical trial to be completed was for tremelimumab in patients with advanced melanoma. In this trial, 15 mg/kg tremelimumab was given every three months as a single agent and compared with dacarbazine, a standard melanoma chemotherapy treatment. The trial showed no survival benefit with this dose and schedule relative to dacarbazine (48). Currently, anti-CTLA4 immunotherapy is being tested in non-small cell lung cancer and melanoma, with a focus on brain metastases, either as monotherapy or in combination with other therapeutic agents (49, 50). The clinical trials of anti-CTLA4 antibodies in TNBC are still in progress, with no definite results published yet.

OTHER IMMUNE CHECKPOINT TARGETS IN TNBC

While immune checkpoint inhibition through the PD1/PDL1 axis and CTLA4 may still not be satisfactory in TNBC, other molecules such as TIM3, LAG3, and TIGIT are investigated in some studies (51, 52). LAG3, an immunological molecular marker expressed in activated T cells, NK cells, B cells, and plasma cell-like dendritic cells (DCs), is the only known ligand for major histocompatibility complex (MHC) molecules (53, 54). Strikingly, in a mouse model of TNBC, the dual blockade of LAG3 and PD1 was shown to achieve a better anti-tumor effect than either one alone (55). TIM3, a member of the TIM family, and expressed in regulatory T cells, DCs, other lymphocyte subsets, subpopulations of macrophages and monocytes (56). Moreover, tissue microarray showed that high TIM3 expression in TILs was significantly associated with better DFS and OS in TNBC patients (57). Surprisingly, it was found that co-blocking

TABLE 3 | Summary of adverse events in immune checkpoint inhibition.

Agent	Adverse events
Pembrolizumab	**Arthralgia**, **Pneumonitis**, **Hepatotoxicity**, Autoimmune hepatitis, Fatigue, Pruritus, Rash, Diarrhea, Colitis, Nausea, Vomiting, Hypothyroidism, Hyperthyroidism
Nivolumab	**Endocrine toxicities**, Pneumonitis, Hepatitis, Diarrhea, Colitis, Fatigue, Pruritus, Nausea
Atezolizumab	**Fatigue**, **Hypothyroidism**, **Nausea**, **Vomiting**, Pruritus, Rash, Diarrhea, Pneumonitis, Arthralgia
Ipilimumab	**Skin**, **Gastrointestinal toxicities**, **Renal toxicities**, Autoimmune hepatitis, Fatigue, Diarrhea, Colitis, Nausea, Vomiting, Pneumonitis, Hypothyroidism, Hyperthyroidism, Arthralgia

The major adverse events are depicted in boldface.

of PD1 and PDL1 can upregulate the co-expression of TIM3 and LAG3 on $CD4^+$ $CD25^+$ T cells, suggesting that resistance to PD1/PDL1 inhibition may develop through upregulation of other immune checkpoint molecules (58). Further investigation on these immune checkpoint molecules may provide alternative immunotherapeutic strategy for TNBC.

TREATMENT-RELATED ADVERSE EVENTS FOR IMMUNE CHECKPOINT INHIBITORS

Safety issue has always been an enormous concern for novel cancer therapeutics. Although the results of immune checkpoint blockade are promising so far in clinical trials, most patients do not show long-lasting remission and some cancers have even become completely refractory. Benefit of immune checkpoint blockade may be achieved at the cost of toxicities, in the form of immune-related AEs, which have been subject of discussion in recent publications (59, 60). In general, immune checkpoint inhibition can be continued in patients with most grade I toxicities. Treatment should be suspended, delayed, or discontinued for higher grade toxicities. In a systematic review and meta-analysis of data from 36 comparative phase II and III trials (n = 15,370), investigators compared the safety profiles of commonly used immune checkpoint inhibitors. Atezolizumab (anti-PDL1 antibody) had the best overall safety profile, followed by nivolumab (anti-PD1 antibody), pembrolizumab (anti-PD1 antibody) and ipilimumab (anti-CTLA4 antibody). The common AEs related to the clinical use of immune checkpoint inhibitors are summarized in **Table 3**.

CONCLUDING REMARKS

Immune checkpoint molecules can prevent the excessive activation of T cells caused by inflammation in order to maintain their own tolerance. Tumor cells are able to activate these checkpoint molecules to suppress host's immune response (61), thereby impairing immune surveillance and assault (62). However, early clinical trials have shown that tremelimumab monotherapy is inefficient and will lead to AEs such as skin rash, diarrhea, and endocrine abnormalities (63). Additionally, colitis may be caused by autoimmune-related mechanisms during treatment with CTLA4 blockade (64). Because of this and other shortcomings related with anti-CTLA4 inhibition, more studies are being focused on PD1/PDL1 inhibition. Hopefully, new CTLA4 inhibitors and/or combinations with better performance will be developed.

In addition, selection of those patients who would benefit from immunotherapy is of utmost importance and is a major challenge in considering immune checkpoint-based immunotherapy. Particularly, due consideration should be given to the different subtypes of TNBC. It is now well accepted that TNBC is a heterogeneous group of diseases comprising different subtypes with different histopathological and molecular makeups. TNBC can be grouped into six (21) or four subtypes (65, 66), depending on the classification system used. In general, the immunomodulatory (IM) subtype of TNBC possesses elevated infiltration of immune cells, and hence, is more likely to be responsive to immunotherapy (67). Additionally, basal-like TNBCs are deemed to have high frequency of BRCA1 and BRCA2 mutations and genetically unstable, which is another predictor of immunotherapy response (65, 68). Furthermore, since the immune checkpoint molecules (e.g., PDL1) is expressed in a portion of TNBC patients (69, 70), we need to be aware of the status of these molecules in TNBC patients in order to get optimized immunotherapeutic efficiency. Nevertheless, immune checkpoint-based therapies provide the opportunity of less toxicity and enhanced potency leading to durable and long-lasting responses for TNBC patients.

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Lovastatin Inhibits EMT and Metastasis of Triple-Negative Breast Cancer Stem Cells Through Dysregulation of Cytoskeleton-Associated Proteins

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Triple-negative breast cancer (TNBC) is more aggressive and has poorer prognosis compared to other subtypes of breast cancer. Epithelial-to-mesenchymal transition (EMT) is a process in which epithelial cells transform into mesenchymal-like cells capable of migration, invasion, and metastasis. Recently, we have demonstrated that lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor and a lipid-lowering drug, could inhibit stemness properties of cancer stem cells (CSCs) derived from TNBC cell *in vitro* and *in vivo*. This study is aimed at investigating whether lovastatin inhibits TNBC CSCs by inhibiting EMT and suppressing metastasis and the mechanism involved. In the present study, we found that lovastatin dysregulated lysine succinylation of cytoskeleton-associated proteins in CSCs derived from TNBC MDA-MB-231 cell. Lovastatin inhibited EMT as demonstrated by down-regulation of the protein levels of Vimentin and Twist in MDA-MB-231 CSCs *in vitro* and *vivo* and by reversal of TGF-β1-induced morphological change in MCF10A cells. Lovastatin also inhibited the migration of MDA-MB-231 CSCs. The disruption of cytoskeleton in TNBC CSCs by lovastatin was demonstrated by the reduction of the number of pseudopodia and the relocation of F-actin cytoskeleton. Combination of lovastatin with doxorubicin synergistically inhibited liver metastasis of MDA-MB-231 CSCs. Bioinformatics analysis revealed that higher expression levels of cytoskeleton-associated genes were characteristic of TNBC and predicted survival outcomes in breast cancer patients. These data suggested that lovastatin could inhibit the EMT and metastasis of TNBC CSCs *in vitro* and *in vivo* through dysregulation of cytoskeleton-associated proteins.

Keywords: lovastatin, triple-negative breast cancer, cancer stem cells, epithelial-to-mesenchymal transition, cytoskeleton

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INTRODUCTION

Breast cancer is by far the most common malignancy and the second leading cause of cancer-related mortality among women (1). It is a type of heterogeneous disease that differs in pathomorphology, biology, clinical manifestation, and treatment response (2). Triple-negative breast cancer (TNBC), as a subtype of breast cancer, accounts for 10-20% of them. Owing to the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and lack of expression or amplification of human epidermal growth factor receptor 2 (HER2), TNBC patients are insensitive to endocrine therapy or molecular targeted therapy, resulting in high recurrence and metastatic potential (3). Currently, new treatment strategies for TNBC, including poly (ADP-ribose) polymerase (PARP) inhibition and immune checkpoint inhibition, are being actively developed in preclinical and clinical studies (4, 5). Unfortunately, only a small proportion of TNBC patients could benefit from these treatments (6, 7). Therefore, it is an urgent task to explore promising targeted drugs so as to improve the efficacy for TNBC.

There is substantial evidence that breast cancer development is hierarchically organized and driven by a minute population of cancer cells known as cancer stem cells (CSCs) which contribute to tumor metastasis and relapse (8, 9). Targeting CSCs has become a popular goal for treating a wide range of tumor types, and may be especially important for TNBC patients (10). Numerous clinical trials have been conducted for targeting breast cancer CSCs (11), but limited data currently exist clinically for the treatment of TNBC (10). Therefore, discovery of drugs that target CSCs will have an enormous impact in TNBC therapeutics. In the process of cancer metastasis, CSCs undergo epithelial-to-mesenchymal transition (EMT), thereby acquiring mesenchymal features which have the ability to migrate and invade (12, 13). EMT involves the loss of intracellular cohesion, disruption of the extracellular matrix (ECM), modifications of the cytoskeleton, and increased cell motility and invasiveness (14, 15). Accumulating evidence showed that EMT-inducible factors also enhance or induce CSC-like features in cancer cells. Within breast cancer, the acquisition of tumor stem cell-like features, the formation of tumor spheres and the appearance of a breast cancer stem cell-specific phenotype ($CD44^+/CD24^-$) were all promoted by the occurrence of EMT (16).

Post translational modifications (PTMs) are one of the most efficient biological mechanisms for expanding the genetic code and for regulating cellular pathophysiology. Lysine succinylation (Ksucc), a newly identified form of PTMs, could cause significant changes in the structure and function of proteins (17). Several lines of evidence suggest that Ksucc has been involved in the initiation and development of numerous different types of tumors, such as gastric and breast cancer (18–20). However, Ksucc also exerts tumor-inhibitory effect in hepatocellular carcinoma and intestinal cancer (21, 22). Importantly, the mechanism of some anti-tumor drugs may also be related to Ksucc modification. For example, heat shock protein 90 (HSP90) inhibitor exhibits an anti-tumor activity against bladder cancer by affecting Ksucc modification (23).

Lovastatin is a natural statin derived from *Monascus*-fermented rice or dioscorea and occurs at a high content in *Oyster* mushroom (24). It has been widely used in prevention and treatment of hyperlipidemia (25). In the last two decades, the antitumor effect of lovastatin has gained increasing attention (26). *In vitro* studies have shown that lovastatin could inhibit the cell cycle progression (27), induce apoptosis (28), and suppress cell migration and invasion (29). *In vivo*, lovastatin could suppress the growth of transplanted tumor or prevent pulmonary metastasis derived from breast cancer (27). Recently, we have demonstrated that lovastatin could inhibit stemness properties of CSCs derived from TNBC cell lines *in vitro*, in a mouse model of orthotopic tumor growth, and in a patient-derived xenograft (PDX) model ((30–32) and manuscript in preparation). Our findings support the evidence that lovastatin may be a candidate drug for the treatment of TNBC.

Through global profiling of lysine acylation, we found that lovastatin preferentially targets CSCs derived from TNBC over non-TNBC cells through Ksucc of proteins involved in cytoskeleton. Our studies demonstrated that lovastatin could selectively inhibit the viability of TNBC CSCs *in vitro* and *in vivo*. Therefore, this study aims to further investigate whether lovastatin exerts its anticancer effect in TNBC CSCs through inhibiting the EMT program and metastasis *via* regulation of cytoskeleton-associated proteins.

MATERIALS AND METHODS

Key Reagents

Lovastatin (ab120614) was obtained from Abcam (Cambridge, UK) and dissolved in DMSO at a stock concentration of 20 or 30 mM and stored at -80°C before use. Doxorubicin was purchased from Selleck, dissolved in DMSO and stored as directed. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN) and was dissolved in an aqueous solvent (vehicle) containing 4 mM HCl and 1 mg/ml BSA.

Cell Lines

Breast cancer cell lines MDA-MB-231 (TNBC) and MDA-MB-453 (non-TNBC) were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, maintained in DMEM medium supplemented with 10% fetal bovine serum. The immortalized mammary epithelial cell line MCF10A was obtained from Kunming Institute of Zoology, Chinese Academy of Sciences. MCF10A cells were maintained in DMEM/F-12 medium supplemented with 5% horse serum, 20 ng/mL EGF, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin and 10 μg/L insulin. All cell lines were routinely cultured at 37°C with 21% O_2 and 5% CO_2 and were tested negative for mycoplasma contamination.

Enrichment and Characterization of Breast Cancer Stem Cells

MDA-MB-231 or MDA-MB-453 cells were trypsinized to single cells and seeded into 6-well ultra-low attachment plates (2,500 cells/mL) using breast cancer stem cell medium (DMEM/F12, 1× B27, 20 ng/mL EGF, 20 ng/mL bFGF, 0.4% BSA, 4 μg/mL insulin and 0.2% hydrocortisone) (33). $CD44^+/CD24^-$ cells were sorted by sequential magnetic sorting after addition of beads coated with anti-CD44 or anti-CD24 antibody according to our published protocol (30). These cells with CSC-like properties were designated sphere-forming cells (SFCs) to distinguish from their parental cells (PCs). The CSC phenotype was characterized by prolonged mammosphere formation in ultra-low attachment culture and by their enhanced tumorigenic ability as demonstrated by two orders of magnitude higher tumorigenicity of SFCs than PCs in nude mice (**Supplementary Figure 1** and **Supplementary Table 1**).

Immunofluorescence – Laser Scanning Confocal Microscopy

The cover glasses were put into 6-cm dishes, and MDA-MB-231 CSCs (1×10^5 cells/mL) were seeded and allowed to grow overnight at 37°C. The cells were treated with different concentrations of lovastatin for 48 hours. These cover glasses were fixed with 4% paraformaldehyde and collected for indirect immunofluorescence staining. The primary antibodies included those against Vimentin (ZSGB-BIO, Cat#ZM0260, Mouse, 1:100) or Twist (Abcam, Cat#ab50581, Rabbit, 1:100). The secondary antibodies were DyLight 488 anti-mouse IgG (H+L) (Vector, Cat#DI-2488, 1:100) and DyLight 594 anti-rabbit IgG (H+L) (Vector, Cat#DI-1594, 1:100), respectively.

Western Blot Analysis

Cultured cells were lysed using 1× cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with 1× protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) added. After centrifugation, the supernatants (whole cell lysates) were collected and quantified by the BCA protein quantification method. The lysates were mixed with the LDS sample buffer and reducing agent and denatured by boiling. The same quantity of protein from each sample was then separated on 10% denaturing PAGE gels followed by incubation with the respective primary antibodies (Vimentin, CST, Cat#5741, 1:1,000; Twist, Abcam, Cat#ab50581, 1:1,000; GAPDH, ZSGB-BIO, Cat#TA08, 1:10,000) and the HRP-conjugated secondary antibody followed by subsequent ECL development according to our standard procedure (34).

Nude Mouse Models

Balb/c-nu mice (female, 5 – 6 weeks old, weight 16 – 18 g) were purchased from Hunan SJA Laboratory Animals Co., Ltd. The mice were maintained on a regular sterile diet under SPF animal house conditions. For the model of orthotopic tumor growth and EMT phenotype (**Figure 3A**), CSCs resuspended in cold 1× PBS were injected into the fourth mammary fat pad. Two weeks later, the nude mice were randomly grouped based on tumor sizes (n = 10/group). Lovastatin (2 mg/kg) or vehicle (PBS) was administered twice weekly through oral gavage until the end of the experiment. The tumor growth was monitored by measuring the major (a) and minor (b) axes of the tumor using a caliper twice weekly. The tumor volume (V) was estimated by the equation $V = (a \times b^2)/2$ as described (35). Three weeks after drug treatment, the mice were sacrificed and the tumors were resected, weighed, and photographed. Part of the tumor tissue was fixed in 4% buffered formaldehyde and subjected to routine paraffin-embedding and microtome sectioning.

A model of tumor metastasis (**Figure 6A**) was generated by injecting the CSCs (5×10^3/100 μL/animal) into the tail vein of the nude mice. The mice were randomized into the following four groups (n = 8/group): saline control, doxorubicin (1 mg/kg), lovastatin (2 mg/kg), and doxorubicin (1 mg/kg) + lovastatin (2 mg/kg). Drug administration started the next day after tumor cell injection and continued twice weekly for 7 weeks. At the end of drug treatment, the mice were sacrificed, and the tumors and the livers resected, weighed, and photographed. Part of the tumor tissue was fixed in 4% buffered formaldehyde and subjected to routine paraffin-embedding, microtome sectioning, and H&E or immunohistochemical staining. Metastatic burden was evaluated by counting the metastatic nodules on the surface of each liver. Micrometastasis in the liver tissues were quantified based on the literature (36). All animal studies were approved by the Hunan Normal University Animal Care Committee.

Immunohistochemistry

Immunohistochemical staining was carried out using the PV-9000 plus poly-HRP anti-mouse/rabbit IgG detection system as described in our previous study (37).The details of primary antibodies were as follows: Vimentin, CST, Cat#5741, 1:100; Twist, Abcam, Cat#ab50581, 1:100. After immunohistochemical staining, the tissue sections were scanned using Automated Quantitative Pathology Imaging System (Vectra, PerkinElmer, Hopkinton, MA, USA) and the total intensity score (TIS) was calculated each from six randomly chosen images at 40× magnification.

Wounding Healing Assay

MDA-MB-231 or MDA-MB-453 CSCs were seeded at a density of 2×10^5 cells/well in 6-well plates. When the cells have grown and fused to 80%, the tip head was used to scratch the central area of the plate well. Lovastatin (1 μM) was added to the cells cultured in DMEM medium supplemented with 3% fetal bovine serum. The cell migration distance in the scratch area was measured at 0 and 24 hours, respectively.

LC-MS/MS Analysis and Data Search

The CSCs were treated with lovastatin (1 μM) or vehicle in stem cell medium at 37°C for 48 h. The cells were then collected by centrifugation, washed with PBS, and snap-frozen in liquid nitrogen, followed by protein extraction and

trypsin digestion. The resulting peptides were labeled with tandem mass tag (TMT) isobaric reagents and fractionated by strong cation exchange chromatography. Succinylated peptides were immunoprecipitated with pan-Ksucc antibody-conjugated beads. Enriched peptides were analyzed by liquid chromatography coupled to an Orbitrap Q Exactive™ Plus. Non-enriched peptides (for proteomics) were fractionated by high pH reverse-phase HPLC using the Agilent 300 Extend C18 column followed by LC-MS/MS analysis. The resulting MS/MS data was processed using MaxQuant with integrated Andromeda search engine (v.1.5.2.8). Tandem mass spectra were searched against Swissprot human database concatenated with reverse decoy database. False discovery rate (FDR) thresholds for protein, peptide, and modification site were specified at 1%. Minimum peptide length was set at 7. For quantification method, TMT 6-plex was selected. The site localization probability was set at ≥ 0.75. The relative changes of Ksucc-modified proteins were normalized to the respective protein level revealed by global proteomic profiling.

Bioinformatics Analyses of Gene Expression Levels and Breast Cancer Patient Survival

To explore the expression levels of cytoskeleton-related genes in TNBC and non-TNBC, we analyzed the RNA-seq data of 115 TNBC and 982 non-TNBC clinical samples from the cBioPortal database (http://www.cbioportal.org/study/summary?id=brca_tcga). After obtaining the results, we plotted the value of the ordinate to the gene expression converted by log10. We then integrated four datasets, i.e., GSE42568, Nathan Kline Institute (NKI), and GSE3494-U133A, and GSE1456-GPL97 to analyze the overall survival (OS) of breast cancer patients of all molecular subtypes between high and low expression levels of cytoskeleton-related genes. Results were obtained with the PROGgeneV2 tool (http://genomics.jefferson.edu/proggene/). We next explored the relationship between these cytoskeleton-related genes and the TNBC patients' survival by Kaplan-Meier plotter database (http://kmplot.com/analysis/index.php?p=service&cancer=breast).

Transmission Electron Microscopy

To investigate the formation of pseudopodia, the CSCs were treated with lovastatin (1 μM) or vehicle for 48 h, fixed with 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide (OsO4) for 1 – 2 h at 4°C. The samples were then dehydrated in a graded series of acetone (50%, 70%, 90%, and 100%) and embedded in Epon-Araldite resin. Ultra-thin sections (50 – 100 nm) were cut using the ultramicrotome and stained with 3% uranyl acetate and lead nitrate. The cell morphology was observed, and the images acquired using an HT7700 Transmission Electron Microscope (Hitachi, Tokyo, Japan).

Statistical Analysis

All the quantitative data were presented as mean ± SEM. Statistical analyses (ANOVA, unpaired Student's *t* test) were carried out using SigmaPlot (version 12.5). $P < 0.05$ was considered as statistically significant. IC_{50} was calculated using the GraphPad Prism 5 software. Drug interaction between lovastatin and doxorubicin was assessed using the CompuSyn software to calculate the combination index (*CI*), with $CI < 1$, $CI = 1$, and $CI > 1$ indicating synergistic, additive, and antagonistic actions, respectively.

RESULTS

Lovastatin Dysregulates Lysine Succinylation of Cytoskeleton-Associated Proteins

Lysine acylations, novel forms of post-translational modifications, play a key role in drug-induced cytotoxicity (38). To investigate the pathways of targeted by lovastatin in TNBC CSCs, we compared lysine acylations of TNBC cells (MDA-MB-231 CSCs) with non-TNBC cells (MDA-MB-453 CSCs). We found that lysine succinylation (Ksucc) was a major lysine acylation type dysregulated by lovastatin in MDA-MB-231 CSCs compared with MDA-MB-453 CSCs (data not shown).

We next performed TMT labeling and immunoprecipitation using pan-Ksucc antibody followed by LC-MS/MS to uncover the changes of Ksucc modifications and the specific sites. Bioinformatics analyses were performed to annotate the proteins differentially modified by Ksucc in response to lovastatin treatment. Gene Ontology (GO) analysis showed that Ksucc-modified proteins were mainly involved in cytoskeleton organization (such as actin binding) (**Figure 1A**). Protein-protein interaction network analysis based on Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) showed the proteins (FLNA, TMSB10, STMN, TPM3, MSN, SPTAN1, DSTN, and EZR) of cytoskeleton organization as key mediators of lovastatin's action in MDA-MB-231 CSCs (**Figure 1B**). Subcellular localization analysis revealed that most of the succinylated proteins were distributed in the cytoplasm and the nucleus (**Figure 1C**), consistent with their localization and role in regulating the cytoskeleton organization.

Lovastatin Inhibits Epithelial-to-Mesenchymal Transition of TNBC CSCs

MDA-MB-231 and MDA-MB-453 CSCs were treated with different concentrations of lovastatin (0.3 – 3 μM) for 48 h. Western blot analysis revealed that the protein levels of Vimentin and Twist were decreased by lovastatin treatment in MDA-MB-231 but not MDA-MB-453 CSCs (**Figure 2A**). We noticed the increased protein level of Twist in MDA-MB-231 CSCs treated with 0.3 μM lovastatin. This may suggest a dose-dependent effect for lovastatin on some of its actions, which was consistent with other results obtained with this drug in our hands (unpublished observations). The expression of Vimentin and Twist was investigated by immunofluorescence and laser scanning confocal microscopy. The fluorescence intensity of Vimentin (green) and Twist (red) was decreased by lovastatin treatment in MDA-MB-231 but not MDA-MB-453 CSCs (**Figure 2B**).

The effect of lovastatin on EMT was further investigated by another cell model of induced EMT. Addition of TGF-β1 to

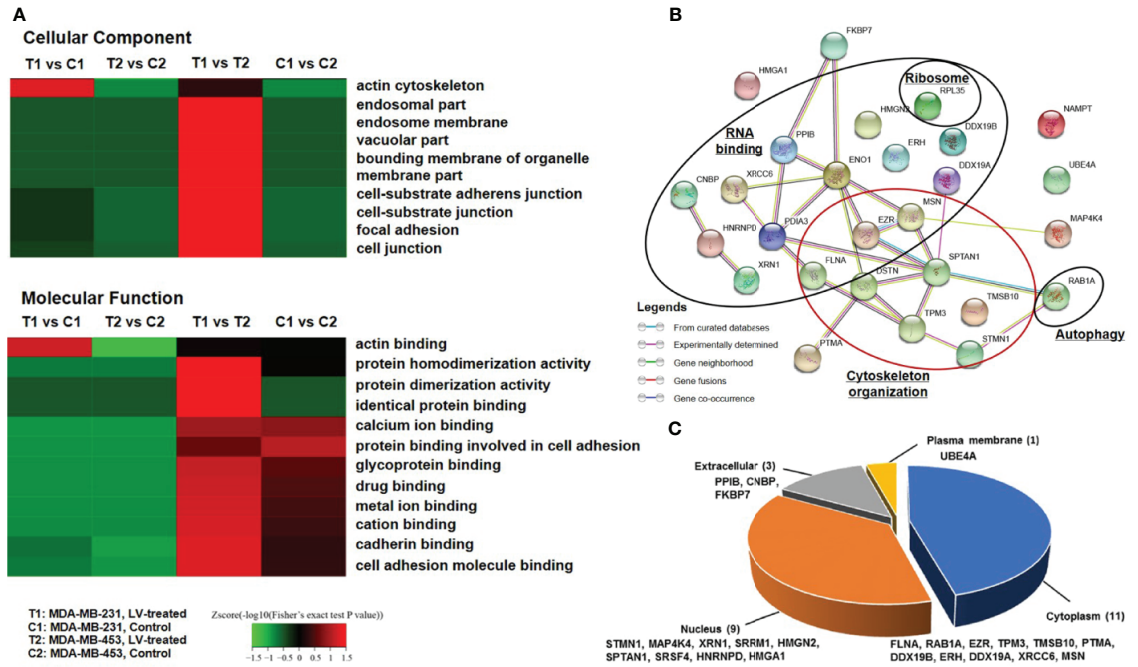

FIGURE 1 | Lovastatin induces lysine succinylation (Ksucc) of cytoskeleton-associated proteins. Gene Ontology (GO) analysis showing the enrichment of Ksucc-modified proteins involved in cytoskeleton organization in MDA-MB-231 CSCs treated with lovastatin (1 μM, 48 h) **(A)**. Protein-protein interaction network generated using the STRING database (https://string-db.org/) showing the functional groups of lovastatin-dysregulated Ksucc-modified proteins in MDA-MB-231 CSCs **(B)**. Pie-chart showing the distribution of Ksucc-modified proteins in different cellular components of MDA-MB-231 CSCs **(C)**. LV, lovastatin.

immortalized epithelial cells such as MCF10A is a well-recognized cell model of inducing EMT *in vitro* (39). When treated with TGF-β1 (160 pM), MCF10A cells could form the loose linked spindle morphology. Addition of lovastatin (0.3 – 1 μM) reversed the change of cell morphology induced by TGF-β1 (**Figure 2C**). These results demonstrate the inhibitory effect of lovastatin on the EMT of TNBC CSCs.

The orthotopic xenograft model of mammary fat pad injection was used to study the tumor growth and EMT phenotype (**Figure 3A**). Each of the tumors was spherical or irregular in shape and gray or gray-red in color. For the mice receiving MDA-MB-231 CSCs, the average tumor volume of the lovastatin-treated group was smaller than that of the control group ($P < 0.05$) (**Supplementary Figure 2A**). For the mice receiving MDA-MB-453 CSCs, the average tumor volume of the lovastatin-treated group was even larger compared with the control group ($P < 0.05$). Tumor weight analysis at the end of the experiment confirmed the results of tumor volume measurement (**Supplementary Figure 2B**). Immunohistochemical staining was performed to evaluate the EMT-related proteins on the orthotopic tumors. We found that in xenograft tumors derived from MDA-MB-231 CSCs, the lovastatin-treated group had a lower score of the mesenchymal markers Vimentin and Twist than the control group ($P < 0.05$) (**Figure 3B**). Again, in MDA-MB-453 CSCs tumors, there was no statistical difference in Vimentin and Twist between the lovastatin-treated group and the control group.

Lovastatin Promotes Chemosensitization and Inhibits Metastasis of TNBC CSCs

Since CSCs contribute to chemoresistance (40), we next investigated whether lovastatin synergizes with the standard chemotherapeutic drug to elicit greater inhibitory effect. We demonstrated that lovastatin sensitizes MDA-MB-231 CSCs to doxorubicin, a standard chemotherapeutic drug for breast cancer therapy. Confocal microscopy of autofluorescence revealed that lovastatin promoted intracellular accumulation of doxorubicin in MDA-MB-231 CSCs (**Figure 4A**). Furthermore, lovastatin synergized with doxorubicin to inhibit tumorsphere formation of MDA-MB-231 CSCs (**Figure 4B**).

Wounding healing assay was used to evaluate the effect of lovastatin on TNBC CSCs migration. CSCs were treated with lovastatin and photographed at 0 and 24 h respectively after cell scratching. We found that the migration area of the lovastatin-treated group was significantly larger than that of the vehicle-treated group in MDA-MB-231 CSCs. However, there was no obvious inhibitory effect on migration in MDA-MB-453 CSCs (**Figure 5A**).

Considering the formation of pseudopodia is supported by actin cytoskeleton, we evaluated whether lovastatin caused disruption of pseudopodia in TNBC cells. As expected, transmission electron microscopy (TEM) revealed that lovastatin reduced the number of pseudopodia in MDA-MB-231 but not MDA-MB-453 CSCs (**Figure 5B**). We then

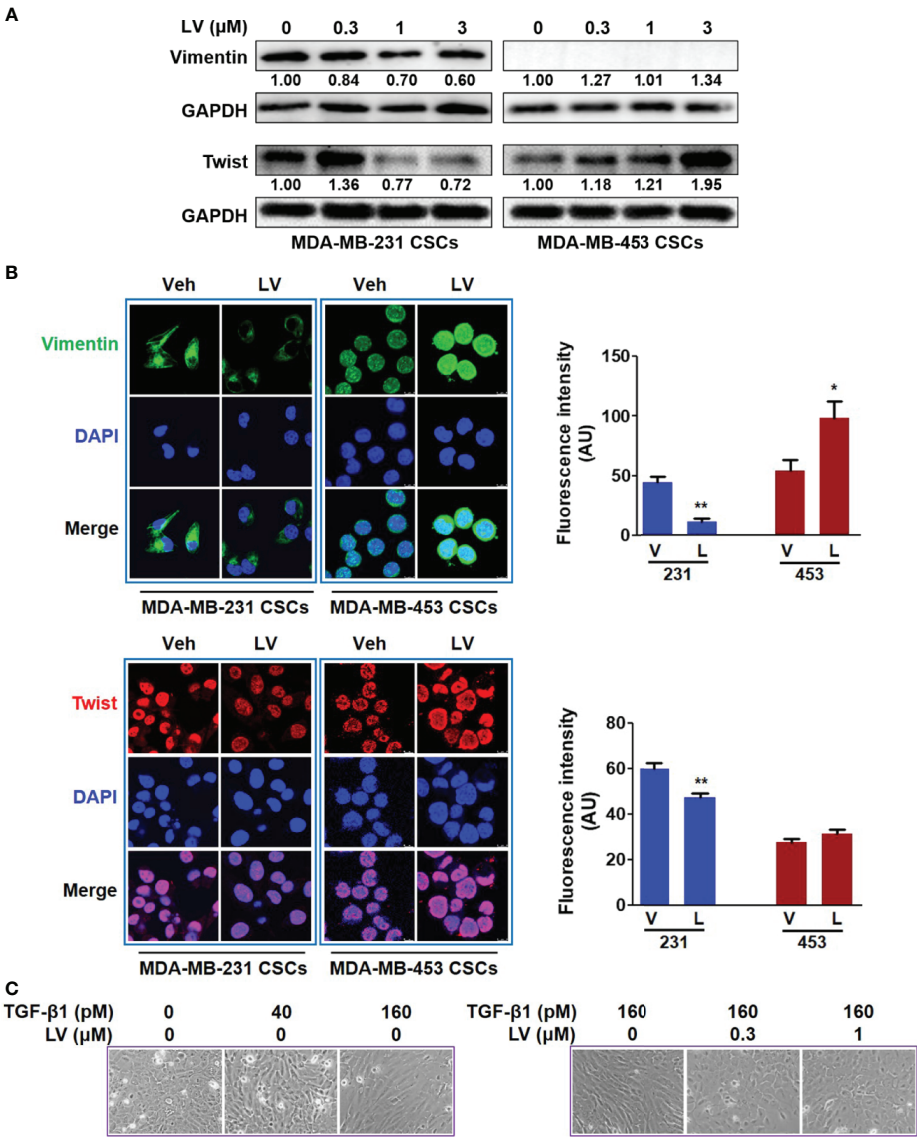

FIGURE 2 | Lovastatin inhibits EMT of TNBC CSCs *in vitro*. MDA-MB-231 CSCs and MDA-MB-453 CSCs were treated with lovastatin or vehicle for 48 h and the protein levels of Vimentin and Twist were analyzed by western blot analysis **(A)**. Representative confocal images immunofluorescence staining of Vimentin (green) and Twist (red) on MDA-MB-231 CSCs treated in a similar way as in **(A)**. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (blue). Original magnification: 63×. Right, quantification of immunofluorescence intensity **(B)**. MCF10A cells were cultured for 7 days with or without different concentrations of TGF-β1 and/or lovastatin, morphological changes were observed by microscopic examination. Original magnification: 200× **(C)**. *$P < 0.05$, **$P < 0.01$, compared with control; V or Veh, vehicle; L or LV, lovastatin; AU, arbitrary unit.

examined the effect of lovastatin on cytoskeleton by immunofluorescence-confocal microscopic examination of F-actin. Interestingly, we found F-actin seemed to be changed from diffuse distribution in the cytoplasm in untreated cells to nuclear or perinuclear localization in lovastatin-treated MDA-MB-231 CSCs (**Figure 5C**).

Another nude mouse model of tail vein injection (**Figure 6A**) was further used to evaluate the synergistic effect of combination treatment on metastasis of TNBC CSCs to distal organs. We found that doxorubicin alone had no inhibition and lovastatin alone showed 46.2 ± 21.7% inhibition on liver metastasis of MDA-MB-231 CSCs. However, combination of lovastatin with doxorubicin synergistically inhibited the majority of liver metastasis of MDA-MB-231 CSCs as demonstrated by a 81.5 ± 5.8% reduction of the macroscopic nodules (**Figure 6B**). Quantification of histopathological examination confirmed the synergistically inhibitory effect on cancer cell colonization in the liver of the combination treatment group (**Figure 6C**). Thus, these data suggest that lovastatin could cause disruption of the cytoskeleton and inhibit liver metastasis in TNBC CSCs.

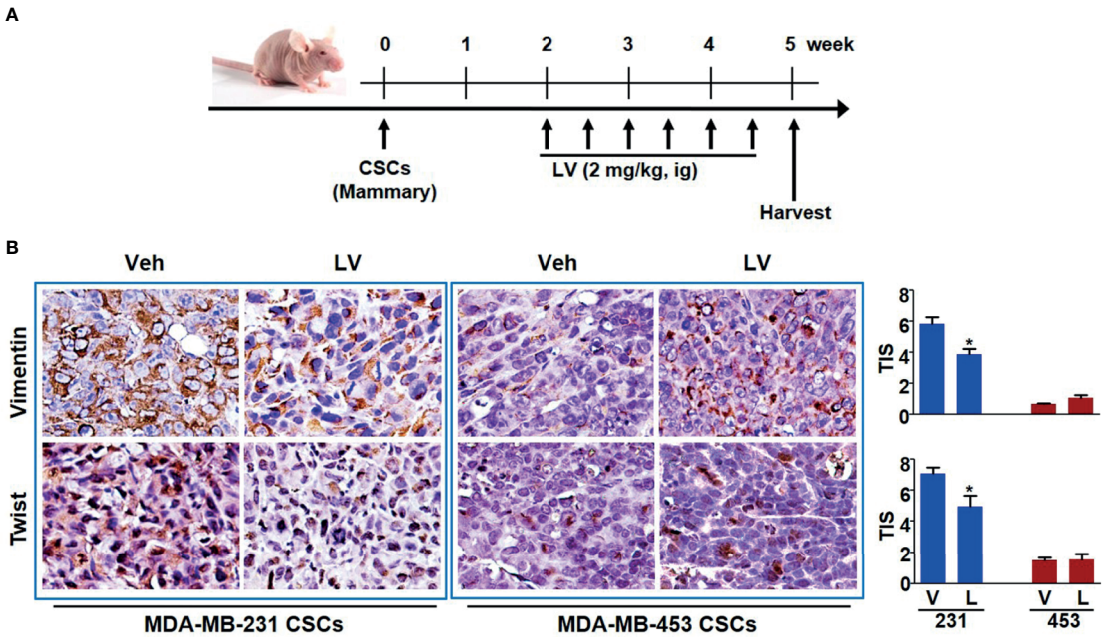

FIGURE 3 | Lovastatin inhibits EMT of TNBC CSCs *in vivo*. Schematic diagram showing the experimental procedure of mouse model of EMT phenotype **(A)**. Representative images of immunohistochemical staining for Vimentin and Twist in orthotopic tumors derived from MDA-MB-231 and MDA-MB-453 CSCs. The nucleus was counterstained by hematoxylin. Right, quantification of the total intensity score (TIS) **(B)**. *$P < 0.05$, compared with control; V or Veh, vehicle; L or LV, lovastatin; ig, intragastric administration.

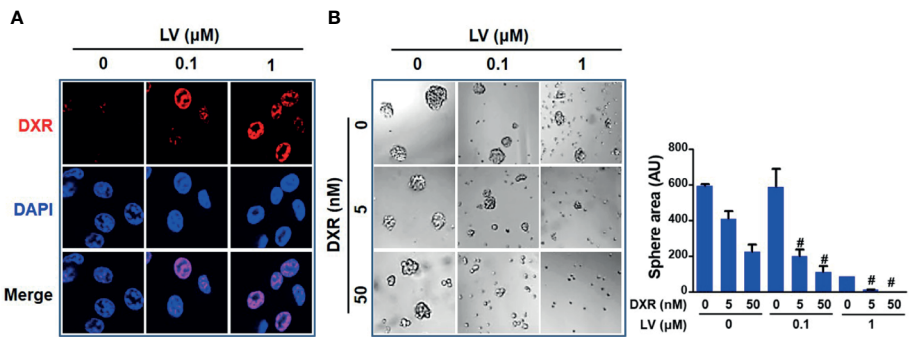

FIGURE 4 | Lovastatin increases the sensitivity of TNBC CSCs to doxorubicin. Intracellular accumulation of DXR promoted by lovastatin. MDA-MB-231 CSCs were treated for 24 h with DXR (5 μM), alone or in combination with lovastatin, and the intracellular fluorescence of DXR (red) was observed by laser scanning confocal microscopy. Original magnification: 63× **(A)**. Synergistic effect between lovastatin and DXR on inhibiting tumorsphere-forming activity. MDA-MB-231 CSCs were cultured in the presence or absence of lovastatin and/or DXR and the tumorspheres were observed and recorded 5 d after treatment. Right, quantifications of the areas of tumorspheres **(B)**. #*CI* < 1.0, showing synergism between the two drugs. LV, lovastatin; DXR, doxorubicin; AU, arbitrary unit.

Higher Expression Levels of Cytoskeleton-Associated Genes Are Characteristic of TNBC and Predict Survival Outcomes in Breast Cancer Patients

In order to explore how our results might be relevant to the clinic, we compared the expression levels of cytoskeleton-related genes between TNBC and non-TNBC and investigated their associations with breast cancer patient survival. We analyzed the RNA-seq data of TNBC and non-TNBC samples from The Cancer Genome Atlas (TCGA) database for the cytoskeleton-related proteins revealed in **Figure 1B**. We found that five out of the eight cytoskeleton-related genes, i.e., FLNA, TMSB10, STMN1, MSN, and TPM3, were expressed at significantly higher levels in TNBC compared with non-TNBC (**Figures 7A–E**).

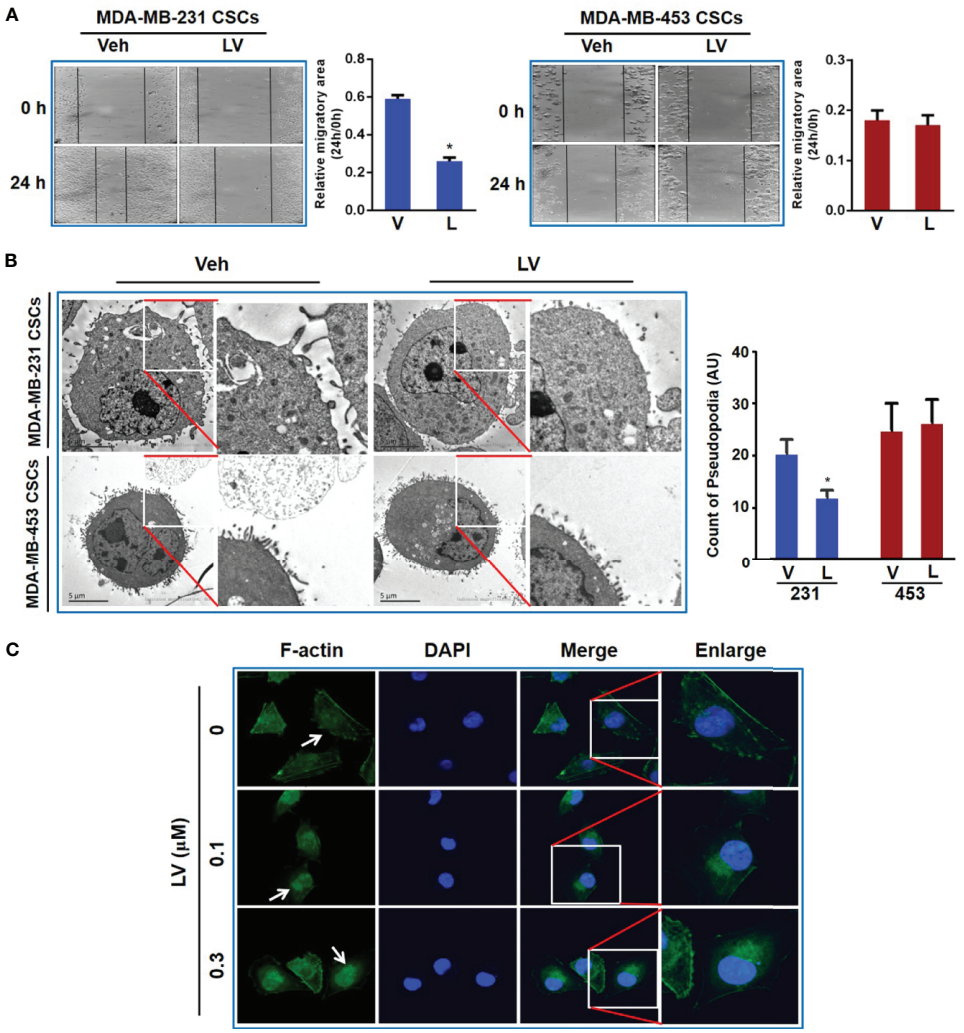

FIGURE 5 | Lovastatin inhibits migration of TNBC CSCs *in vitro*. Representative cell images of wound-healing assay in MDA-MB-231 and MDA-MB-453 CSCs treated with vehicle or lovastatin (1 μM, 24 h). Right, quantification of relative migratory area **(A)**. Representative TEM micrographs showing the pseudopodia in MDA-MB-231 and MDA-MB-453 CSCs after treatment with lovastatin (1 μM, 48 h). Scale bar = 5 μm **(B)**. Representative confocal images of immunofluorescence staining for F-actin in MDA-MB-231 after treatment with vehicle or lovastatin (0.1 or 0.3 μM, 48 h). Blue, DAPI staining of the nucleus. Original magnification: 63× **(C)**. *P < 0.05, compared with control; V or Veh, vehicle; L or LV, lovastatin; AU, arbitrary unit.

Consistent with the RNA-seq data, survival analysis using the PROGgeneV2 tool showed that breast cancer patients of all molecular subtypes who had higher levels of these cytoskeleton-related genes had poorer OS compared with those with lower levels (**Figures 7F–J**). We further explored the roles of these cytoskeleton-related genes in the survival of TNBC patients using the Kaplan-Meier plotter database. Our results revealed that the expression levels of these genes were associated with distant metastasis-free survival (DMFS) (**Figures 7K–O**) of TNBC patients. Except for MSN, the expression levels of four out of the five genes, i.e., FLNA, TMSB10, STMN1, and TPM3, were negatively associated with DMFS of TNBC patients. The clinical data suggest that these cytoskeleton-related genes might be potential targets for the treatment of TNBC.

DISCUSSION

It is well known that EMT and metastasis play an important role in the acquisition of the malignant phenotype of cancer cells (41). We set out to explore whether lovastatin could inhibit breast CSCs by reversal of the EMT program and inhibition of metastasis in TNBC. Our study demonstrated that both the expression of mesenchymal markers such as Vimentin and EMT-related transcription factors such as Twist could be down-regulated by treatment with lovastatin in MDA-MB-231 CSCs. Furthermore, EMT induced by TGF-β1 in immortalized mammary epithelial cells MCF10A could also be reversed by lovastatin. The present study also showed that lovastatin could inhibit liver metastasis as evidenced by reduced nodule

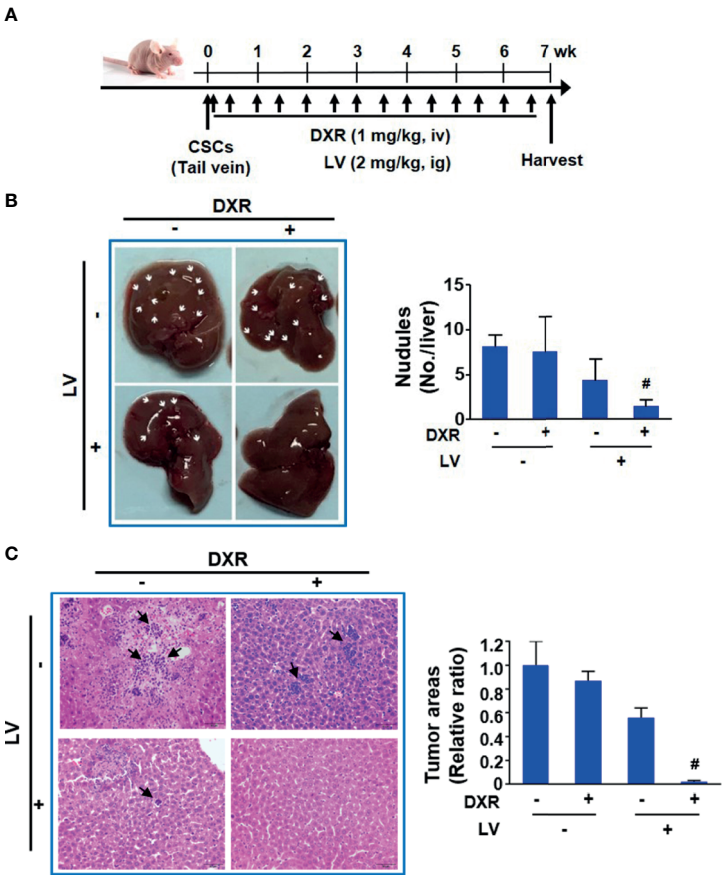

FIGURE 6 | Lovastatin inhibits metastasis of TNBC CSCs *in vivo*. Schematic of the mouse model of tumor metastasis **(A)**. Representative images of the livers from each group of mice. Arrows indicate the tumor nodules on the liver. Right, quantifications of the tumor nodules on liver surface **(B)**. Representative H&E-stained histopathological images showing reduced colonization of tumor cells in the liver by combination treatment with lovastatin and DXR. Arrows indicate the metastatic tumor cells in the liver. Right, quantifications of the metastatic tumor cells on liver surface. Original magnification: 40× **(C)**. $^{\#}CI < 1.0$, showing synergism between the two drugs. LV, lovastatin; DXR, doxorubicin; iv, intravenous injection; ig, intragastric administration.

formation and confirmed histopathologically by eliminated cancer cell colonization. In our animal model, we also examined the lungs for metastasis. Unexpectedly, we didn't observe metastatic cancer cells in the lungs as obviously did in the liver. One speculation is that the cells we used were CSCs, rather than bulk tumor cells, and the difference in signaling pathways between them may lead to the difference in metastatic spreading of tumor cells to different target organs. These results provided solid evidence that lovastatin could inhibit the EMT program and metastasis in TNBC CSCs *in vitro* and *in vivo*.

In the process of EMT, tumor cells gain migratory and metastatic properties that involve a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions required for invasive growth (41, 42). Emerging evidence suggests that cytoskeleton regulatory proteins are a convergent node of signaling pathways emanating from extracellular stimulus to cell movement. The coordinated activity of various cytoskeleton-binding proteins regulates a variety of cytoskeleton-based processes, including assembly of the microfilament and cell motility.

Actin cytoskeleton remodeling is an upstream regulator of EMT in metastatic breast cancer cells (43), and several studies clarified EMT was driven by actin cytoskeleton remodeling in hepatocellular and colorectal carcinoma (44, 45). F-actin cytoskeleton is regulated by various actin-binding proteins, one family of which are the filamins, with filamin A (FLNA), also called actin-binding protein 280 (ABP-280), being the most powerful actin-binding protein, together with actin microfilaments, direct the cell's elasticity and movement (46, 47). In our study, we demonstrated lovastatin induced rearrangement of the actin cytoskeleton favoring perinuclear and nuclear localization of F-actin filaments. Location of actin filaments underneath the plasma membrane is important for the formation of cellular protrusions such as lamellipodia and filopodia (48). Our results further showed that the number of pseudopodia of TNBC CSCs after lovastatin-treated were reduced, which confirmed cytoskeleton organization pathway play an important role in the lovastatin inhibition EMT and metastasis of TNBC CSCs. We have demonstrated that lovastatin inhibited the EMT and metastasis of TNBC CSCs. Therefore, it's

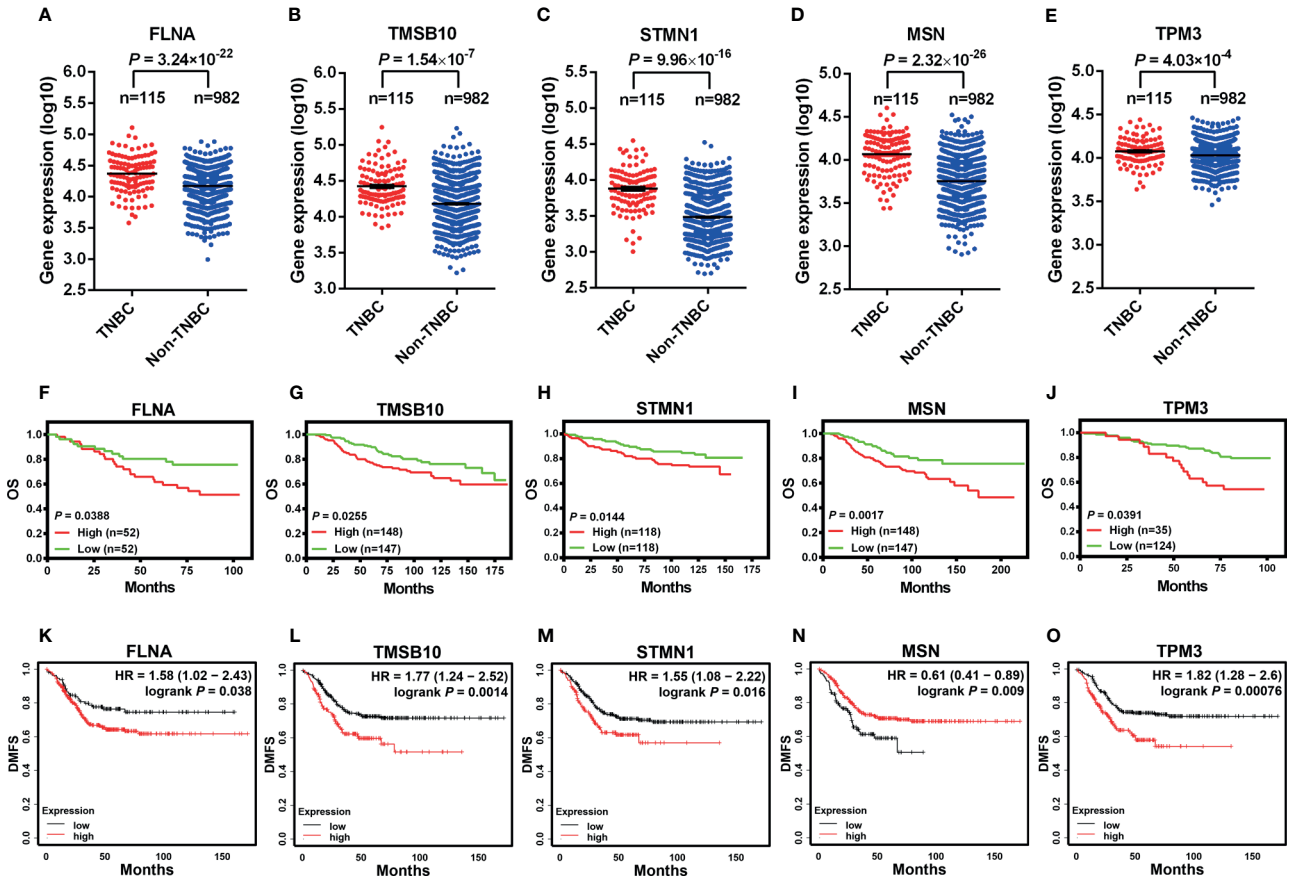

FIGURE 7 | Higher expression levels of cytoskeleton-associated genes are characteristic of TNBC and predict poorer survival outcomes in breast cancer patients. The expression of FLNA **(A)**, TMSB10 **(B)**, STMN1 **(C)**, MSN **(D)**, SPTAN1 **(E)** analyzed between TNBC and non-TNBC clinical samples from The Cancer Genome Atlas (TCGA), which was contained in the online cBioPortal for cancer genomics database **(A-E)**. OS in breast cancer patients retrieved from the online database (PROGgeneV2) between high and low expression of FLNA [**(F)**, dataset GSE42568], TMSB10 [**(G)**, dataset NKI], STMN1 [**(H)**, dataset GSE3494_U133A], MSN [**(I)**, dataset NKI], TPM3 [**(J)**, dataset GSE1456-GPL97] **(F–J)**. DMFS in TNBC patients between high and low expression of cytoskeleton-associated genes on the Kaplan-Meier plotter database **(K-O)**. OS, overall survival; DMFS, distant metastasis-free survival.

not surprising that lovastatin modulates these malignant behaviors of TNBC CSCs through dysregulation of cytoskeleton-associated proteins. This is supported by bioinformatics analysis showing that the cytoskeleton-associated genes are differentially expressed between TNBC and non-TNBC tissues samples and that higher expression levels of these genes are associated with survival outcomes in TNBC patients.

In summary, our present study has provided evidence, for the first time, that lovastatin, a natural HMG-CoA reductase inhibitor, inhibits TNBC CSCs *in vitro* and *in vivo* through inhibition of EMT phenotype and suppression of metastasis by dysregulation of cytoskeleton-associated proteins. This study lays the foundation for the understanding of the inhibitory effect of lovastatin on the EMT and metastasis of TNBC CSCs and has potential clinical implications for the future management of TNBC. Further studies are required to move forward our effort toward resolving the issues of how lovastatin causes disturbance of the cytoskeleton organization pathway and how protein Ksucc contributes to lovastatin-induced EMT and metastasis in TNBC CSCs.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://www.cbioportal.org/study/summary?id=brca_tcga.

ETHICS STATEMENT

The animal study was reviewed and approved by Hunan Normal University Institutional Animal Care and Ethics Committee.

AUTHOR CONTRIBUTIONS

CZ, SY, LL, HY, and GH performed the experiments. SC, YL, XP, and ZC collected and analyzed the data. CZ, SY, and LL drafted the manuscript. MW, QZ, GL, and SF reviewed the manuscript. XD conceived and designed the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Current Triple-Negative Breast Cancer Subtypes: Dissecting the Most Aggressive Form of Breast Cancer

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Triple-negative breast cancer (TNBC) is a highly heterogeneous disease defined by the absence of estrogen receptor (ER) and progesterone receptor (PR) expression, and human epidermal growth factor receptor 2 (HER2) overexpression that lacks targeted treatments, leading to dismal clinical outcomes. Thus, better stratification systems that reflect intrinsic and clinically useful differences between TNBC tumors will sharpen the treatment approaches and improve clinical outcomes. The lack of a rational classification system for TNBC also impacts current and emerging therapeutic alternatives. In the past years, several new methodologies to stratify TNBC have arisen thanks to the implementation of microarray technology, high-throughput sequencing, and bioinformatic methods, exponentially increasing the amount of genomic, epigenomic, transcriptomic, and proteomic information available. Thus, new TNBC subtypes are being characterized with the promise to advance the treatment of this challenging disease. However, the diverse nature of the molecular data, the poor integration between the various methods, and the lack of cost-effective methods for systematic classification have hampered the widespread implementation of these promising developments. However, the advent of artificial intelligence applied to translational oncology promises to bring light into definitive TNBC subtypes. This review provides a comprehensive summary of the available classification strategies. It includes evaluating the overlap between the molecular, immunohistochemical, and clinical characteristics between these approaches and a perspective about the increasing applications of artificial intelligence to identify definitive and clinically relevant TNBC subtypes.

Keywords: triple-negative breast cancer, TNBC, molecular subtype of breast cancer, epigenetics, clustering, artificial intelligence-AI, classification, precision medicine

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INTRODUCTION

Breast cancer (BC) is the most prevalent cancer in women, with a steadily increasing number of cases diagnosed every year (1). Traditionally, BC is classified and treated based on the status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. The presence of these markers has allowed the development of targeted and efficient therapies. Tumors without expression of ER, PR, and overexpression of HER2, collectively known as triple-negative breast cancer (TNBC), lack targeted therapies, leaving chemotherapy as the only systemic therapeutic alternative (2). TNBC exhibits a higher proliferation rate, higher incidence of metastases to the brain, liver, and lungs (3), and more often affects younger patients (4) than the other BC subtypes. This aggressiveness, added to the absence of targeted therapies, maintains TNBC as an unmet clinical challenge.

One of the main strategies employed to improve precision oncology involves a better understanding and rational classification of malignancies. During the last 20 years, researchers have characterized BC tumors and classified them into intrinsic molecular subtypes (5, 6). In these pivotal studies, the basal-like subtype was associated with the absence of ER and HER2 expression, and a higher expression of basal cytokeratin (CK 5/6). This classification, currently performed by the PAM50 test (7), is well accepted in the clinical setting. The TNBC and the PAM50 Basal-like subtype have frequently been considered synonymous; however, this is not always the case. Although nearly 85% of PAM50 basal-like tumors have a TNBC phenotype, the remaining 15% exhibit other express ER, PR, or HER2 markers (8–10).

Despite TNBC being grouped as a single disease, clinical, histological, and molecular profiling highlight its intrinsic heterogeneity. Nevertheless, from a clinical perspective and based purely on the absence of ER, PR, and HER2 positivity, patients with TNBC are uniformly considered for treatment with chemotherapy. However, response to treatment is markedly variable, and patients with TNBC still have higher rates of distant relapse than patients with any other BC subtype. Therefore, a greater understanding of the heterogeneity of these tumors and a more efficient classification system that highlights targetable differences is urgently needed to improve the treatment and outcome of patients with TNBC.

THE ORIGINS OF TNBC SUBTYPING

During the last decade, several groups invested their efforts into characterizing TNBC at different molecular levels. The first attempts to stratify TNBC were based on histology, immunohistochemistry (IHC), and transcriptomic profiling (**Table 1**). In 2013, TNBC was classified using 13 IHC markers by Elsawaf et al. This study identified four groups according to the expression patterns of cytokeratins (CK). After subsetting, patients with luminal (20%) or basoluminal (28%) displayed a significantly worse survival than patients with basal A (26%) or basal B (26%) TNBC tumors (11).

The Legacy of BC Subtyping Using Gene Expression Patterns

In 2011, Lehmann et al. identified six TNBC subtypes (TNBCtype-6 classification) based on gene expression profiling and ontology analyses (12). The novel subtypes included basal-like (BL) 1 and BL2, which were enriched in cell cycle genes and growth factor signaling, respectively; Immunomodulatory (IM), with high expression of immune-related pathways; mesenchymal (M), which presented genes of mesenchymal differentiation and proliferation; mesenchymal stem-like (MSL), which had mesenchymal features and low proliferation; and luminal androgen receptor (LAR), characterized by the activation of hormone-related pathways. Importantly, LAR and M subtypes had a significantly lower relapse-free survival than the rest of the subtypes. Five years later, the same group refined the classification since they observed an important presence of tumor-infiltrating lymphocytes (TILs) and stromal cells in the IM and MSL subtypes, respectively. Thus, the TNBC subtypes were refined as BL1, BL2, M, and LAR (TNBCtype-4 classification). BL1 displayed the best prognosis among the four subtypes (13). Similarly, Burstein et al. subdivided TNBC tumors using gene expression profiling and copy number variations (CNVs). They identified four stable groups with distinct prognoses and suggested putative subtype-specific targets. These subtypes were named LAR, mesenchymal (MES), basal-like immune-suppressed (BLIS), and basal-like immune-activated (BLIA). BLIS showed the worst survival, and BLIA the best survival compared to the rest (14). A recent study by Jézéquel et al. also employing transcriptomic profiling identified three different TNBC subtypes (C1, C2, and C3), taking advantage of the fuzzy clustering strategy. The C1 cluster included TNBC tumors with a molecular apocrine phenotype that showed a better prognosis, and C2 and C3 were enriched in basal-like properties. C2 displayed biological aggressiveness and an immune-suppressive phenotype, whereas C3 outlined the adaptive immune response and immune checkpoint upregulation (15).

Long-non-coding RNAs (lncRNAs) were considered to classify TNBC tumors by Liu et al., given their role as regulators of gene expression. They combined mRNA and lncRNA expression profiles (16) to construct the Fudan University classification (FUSCC) system. Similar to the findings by Lehman et al. and Burstein et al., four subtypes were identified: IM, enriched in immune cell signaling pathways; LAR, enriched in hormone-related pathways; MES, whose main features were low levels of cell proliferation-related genes and enriched pathways associated with epithelial-mesenchymal transition (EMT); and BLIS, showing upregulation of proliferative pathways and the downregulation of genes involved in the immune response. Again, patients with BLIS TNBC showed a worse overall prognosis. The authors compared these clusters with the TNBCtype-6 classification. They found that the IM groups were nearly identical in both studies, and LAR

TABLE 1 | Examples of TNBC stratification methods.

Classification Method	Subtypes	Freq (%)	Effect on prognosis	Characteristics
Histochemistry (11)	Luminal	20	Worse	EGFR<10%, Ki-67<50%, 2 or more luminal CK+
	Basoluminal	28	Worse	EGFR>10%
	Basal A	26	Better	EGFR<10%; high proliferation (Ki-67>50%)
	Basal B	26	Better	EGFR<10%, Ki-67<50%, 2 or luminal CK-
Gene expression from microarray (12)	BL1	18-26	Neutral	Cell cycle, DNA damage
	BL2	10-15	Neutral	Growth factor signaling
	IM	10-20	Neutral	Immune-related pathways
	M	12-20	Worse	Mesenchymal differentiation and proliferation
	MSL	8-16	Better	Mesenchymal features, low proliferation
	LAR	10-15	Worse	Hormone-related pathways, inflammation
Gene expression from microarray (13)	BL1	35	Better	Cell cycle, DNA damage
	BL2	22	Neutral	Growth factor signaling
	M	25	Neutral	Mesenchymal differentiation and proliferation
	LAR	16	Neutral	Hormone-related pathways, inflammation
Gene expression and CNV (14)	BLIA	49	Better	High proliferation, immune activation
	BLIS	23	Worse	High proliferation, immune suppression
	LAR	15	Neutral	Hormone-related pathways, inflammation
	MES	13	Neutral	Mesenchymal differentiation and proliferation
Gene expression (15)	C1	23	Better	Apocrine
	C2	41	Neutral	Basal-like, Immune suppression
	C3	36	Neutral	Basal-like, Immune checkpoint upregulation
mRNA and lncRNA expression (16)	MES	34	Neutral	EMT, lower levels of proliferation
	BLIS	32	Worse	Proliferative pathways, immunosuppression
	LAR	17	Neutral	Hormone-related pathways, inflammation
	IM	17	Neutral	Immune signaling
Alternative Polyadenylation (17)	LAR	22	Neutral	Hormone-related pathways
	MLIA	22	Neutral	Mesenchymal and Immune-related pathways
	BL	40	Neutral	DNA-damage response
	S	16	Worse	Cell growth, immune-related pathways
DNA methylation, 450K (18)	Epi-CL-A	25	Neutral	Mesenchymal differentiation and proliferation
	Epi-CL-B	33	Worse	DNA-damage response Cell division
	Epi-CL-C	22	Neutral	Hypoxia, protein degradation
	Epi-CL-D	20	Neutral	Immune-related pathways
DNA methylationMBDCap-Seq (19)	Cluster 1	58	Better	Largely hypomethylated
	Cluster 2	18	Neutral	High methylated
	Cluster 3	24	Worse	Medium methylated
Protein levels (20)	I/H-subtype	66	Neutral	Hormone-related pathways, inflammation
	DD-related	34	Neutral	DNA-damage response
Metabolic pathways (21)	MPS1	26	Neutral	Lipogenic
	MPS2	37	Worse	Glycolytic
	MPS3	37	Neutral	Mixed phenotype

and BLIS partially covered Lehmann's LAR and BL1 groups, respectively. The authors also designed an IHC-based approach to classify TNBC patients (22). Quist et al. employed a four-gene signature to cluster TNBC tumors into six subtypes. The MC6 subtype, which comprised nearly 50% of TNBC samples, was associated with a higher sensitivity to platinum-based chemotherapy. Importantly, this correlation was further validated in TNBC cell lines (23).

From DNA to Metabolites for TNBC Clustering

New ways of stratifying TNBC patients have arisen thanks to the advent of next-generation sequencing, computing systems, and the exponential increase of available data sources during the following years. Thereby, new data types have been used to classify TNBC into novel subtypes (**Table 1**). Different single nucleotide variant (SNV) patterns have been identified in TNBC tumors (24) and circulating DNA from TNBC patients (25). Jiang et al. explored these differences in the FUSCC cohort. They discovered that somatic mutations and CNVs events were not homogeneously distributed among TNBC subsets. For instance, FUSCC LAR tumors were enriched in PI3K pathway mutations. High genomic instability was associated with the FUSCC BLIS subtype. Given the mutational differences, this study defined four genetic subtypes: Homologous recombination deficiency (HRD), clock-like, APOBEC, and mixed (26). Interestingly, the HDR subtype showed a greater proportion of germline variants than other mutation subtypes. *BRCA1*, *RAD51D*, and *BRCA2* were the most frequently mutated genes (27).

The mRNA processing machinery has also been considered to establish TNBC subtypes with analysis of alternative polyadenylation events in a TNBC cohort using a Bayes-based strategy. The gene expression of these four subtypes was then compared with TNBCtype-6 subtypes. Subtype 1, named LAR,

was enriched in hormone-regulated pathways and displayed a significant overlap with the TNBCtype-6 LAR subtype. Taking this classification as a reference, gene expression patterns found in M, MSL, and IM groups were found in subtype 2, termed mesenchymal-like immune activated (MLIA). Subtype 3 was called basal-like (BL) due to increased proliferation and DNA damage-related genes, similar to the TNBCtype-6 BL subtype. Subtype 4, which exerted the worst prognosis, showed downregulation of cell growth and immune-related pathways and was identified as the Suppressed (S) subtype (17). Alternative splicing was also used to stratify TNBC tumors and identify a model to predict the overall survival. Alternative promoter signature significantly separated TNBC patients into high- and low-risk groups, suggesting that it might play a special role in the development and progression of TNBC (28).

DNA methylation (DNAm) patterns are also being implemented for tumor characterization (29, 30). Initially, three different TNBC DNAm clusters were identified using MBDCap-Seq. This study identified 865 TNBC differentially-methylated regions (DMR), most of them hypermethylated. Survival analysis showed that the TNBC subgroup, which included hypomethylated tumors, displayed a better prognosis (19). Most recently, we tried to explain the TNBC heterogeneity using DNAm profiles. Four TNBC epitypes were identified using machine learning (18). The Epi-CL-A was mainly composed of samples identified as mesenchymal according to the TNBCtype-6 classification. Epi-CL-B, which presented a worse prognosis, partially overlapped with the TNBCtype-6 LAR and the Burstein et al. BLIS subtypes. Consistent with the overlap mentioned above, Epi-CL-A showed activation of mesenchymal cell differentiation and proliferation pathways. In contrast, most of the differentially expressed genes in Epi-CL-B were involved in DNA damage response, maintaining chromatin structure, and cell division. Epi-CL-C was characterized by the activation of hypoxia-related pathways and the modulation of protein homeostasis. Finally, Epi-CL-D showed enrichment in immune-related pathways such as response to interferon-beta, positive regulation of T cell-mediated cytotoxicity, or antigen processing and presentation (18).

The tumor proteome analysis is another feature that is starting to see precision medicine applications as an approach for patient stratification. For example, two stable clusters of TNBC tumors were identified by reverse-phase protein array (RPPA). Analysis of the protein signatures revealed that one of the clusters was enriched in growth arrest and DNA damage (GADD45 genes) and p53 signaling pathways. This subtype was identified as the DNA damage (DD)-related subtype. Another cluster was designated as I/H-subtype due to its association with inflammation, hormonal receptor, and MAPK signaling pathways (20). Deeper subsetting defined the existence of 5 RPPA subtypes. Researchers found significant agreement between the RPPA classification system and the TNBCtype-4 system (13) and intrinsic subtypes by PAM50. Thus, the TNBCtype-4 subtypes BL1 and BL2 were enriched in the RPPA subclusters 2 and 1, respectively, classified as DD-related. The RPPA cluster 4, a subset of I/H-subtype, was enriched in PAM50 normal-like TNBC tumors (20). Additionally, integrative analysis of the proteome and genome identified potential protein markers of drug sensitivity and drug resistance. For example, enrichment with mitochondrial proteins was associated with sensitivity to drugs that might depend on mitochondrial protein expression, like belinostat (31). Similarly, a comprehensive quantitative proteome profile of BC cell lines identified two major subgroups within TNBC cell lines (basal A and B) with different functional signatures (32).

Most recently, metabolic pathways were also exploited as a differential feature to classify TNBC. Gene expression from the FUSCC cohort (26) was analyzed to identify transcriptional differences in genes involved in metabolic pathways (21). Thus, TNBC tumors were classified into three different molecular pathway subtypes (MPS) based on the enrichment scores of metabolic pathways. MPS1 was defined as the lipogenic subtype; MPS2 was characterized as a glycolytic subtype and showed the worst relapse-free survival among the three metabolic subtypes. Tumors that showed a mixed enrichment were identified as MPS3 subtype. Untargeted metabolomic analysis on frozen TNBC samples revealed that MPS1 presented higher amounts of fatty acids, whereas MPS2 showed higher levels of glycolysis mediators. Furthermore, they found that cell lines classified as MPS1 showed a higher fatty acid uptake and a higher sensitivity against C75, a *de novo* lipid synthesis inhibitor. In contraposition, glycolysis inhibitors displayed a more powerful growth inhibitory effect in those cells stratified as MPS2. Interestingly, based on this classification, the researchers were able to sensitize MPS2 against PD-1-targeted therapy through an LDH inhibitor, which decreased lactate levels, promoting immune recognition (21).

The Microenvironment, a Novel Source of Information and Noise for Subtype Discovery

TNBC tumors have also been stratified according to their immunogenomic profile. The analysis of 29 immune-associated gene sets defined three clusters in four BC datasets. The subsets were called immunity low, immunity medium, and immunity high. The latter group was characterized by greater immune cell infiltration and anti-tumor immune activities associated with a better prognosis (33). The tumor microenvironment is also known to impact TNBC outcome, defining response subtypes. TNBC presents the highest proportion of TILs in comparison with other BC subtypes (34). Its presence is associated with a better prognosis (35, 36), higher rates of complete pathological response (pCR) to neoadjuvant chemotherapy, and better response to immunotherapy (37, 38). In another study, a high number of TILs was associated with enhanced survival. In contrast, increased levels of the immunosuppressor markers, such as PD-L1, CD163, and FOXP3, or a glycolytic microenvironment, determined by MCT4 expression, predicted a worse outcome. Together, these parameters were used to subset 174 TNBC tumors into four clusters. Clusters 1 and 2 defined by high TILs and low PD-L1 and FOXP3 showed better survival

than clusters 3 and 4, both associated with increased PD-L1, FOXP3, and stromal MCT4 (39).

CURRENT AND FUTURE APPLICATIONS

Precision treatment of TNBC is not utopic, and it is only just knocking on the door. Characterization of driver alterations in potentially druggable genes is essential to assess TNBC heterogeneity and tailor the best treatment for each patient (40). Molecular stratification and differential treatment assignment have been used in patients with refractory metastatic TNBC (ClinicalTrials.gov identifier: NCT03805399). The study revealed clinical benefits in IM and BLIS+BRCA1/2 wild-type subsets. Patients whose malignancies were classified as IM received a combination of paclitaxel + anti-PD-1, which promoted an objective response rate (ORR) in 50% of patients (41). Previous trials using monotherapy in TNBC without stratification showed an ORR of around 5-10% (42, 43). These preliminary data suggest that the overly simplistic current IHC classification of TNBC alone betrays the diverse heterogeneity of this subgroup and risks leaving on the table potential treatment options that can be effective if directed toward specific intrinsic subtypes.

In early-stage TNBC, TILs evaluation has been standardized (44) and should be routinely performed due to its prognostic value (45). In advanced TNBC, germline BRCA1/2 mutations are associated with higher response to platinum compounds (46). These mutations are routinely assessed to identify candidates for PARP inhibitor therapy (47, 48). Ongoing studies evaluate the incorporation of PARP inhibitors in other germline mutations beyond BRCA1/2, like PALB2, or in somatic BRCA1/2 mutations. PD-L1 expression by IHC is assessed to select patients for immunotherapy associated with chemotherapy (49, 50). Patients with metastatic TNBC harboring PIK3CA/AKT1/PTEN alterations have longer progression-free survival when treated with AKT inhibitors and chemotherapy (51, 52). Identifying tumor-associated antigens overexpressed in a subpopulation of TNBC may prompt the generation of new therapeutic strategies (53).

For TNBC that are classified into the immune-activated subtype, new biomarkers are emerging to predict response to immunotherapy in addition to PD-L1, such as the presence of TILs (54), tumor mutation burden (55), expression of immune genes (56), or through the construction of personalized cancer immunograms that integrate multiple variables (57).

What Is Clear About the Still Fuzzy TNBC Subtyping?

Perhaps, the several strategies employed to construct systems that identify clinically useful TNBC subtypes reflect the lack of proper definition of this disease. From the quantitative variables such as gene and protein expression or metabolic and epigenomic profiling to the qualitative traits such as gene mutation, basic and translational researchers have explored a problem that still appears to remain unsolved. We see a common factor, independently of the approach, that clear overlaps exist between the different classification systems (**Figure 1A**). This is encouraging as it points towards the existence of stable entities identified in diverse patient populations. However, the fact that there is still a large variability, added to several subtle similitudes between some of the current subtyping systems (grey ribbons **Figure 1A**), suggests that there is still a long way to go.

In Pursuit of the Consensus TNBC Subtypes

In the upcoming years, integrating different data sources will be key in identifying definitive TNBC subtypes that will help guide clinicians toward specific treatment recommendations for their patients. Integrative analyses comparing TNBC and non-TNBC patients from the TCGA cohort have been performed combining gene expression, DNAm, and somatic mutations, revealing differential signatures between these two types of BC (58). Thus, a similar approach combining even more layers of information may identify consensus TNBC subtypes. There is already one attempt to stratify TNBC patients using multiple data types, specifically using transcriptome (RNA-seq), microRNA expression (miRNA-seq), and CNV (59). However, this study did not use metabolomics, proteomics, imaging, or histomolecular features, which have independently proven to be informative for subtyping TNBC.

Beyond the Human's Good Intentions, Is Artificial Intelligence the Key?

The efforts of several scientists led to the generation of a substantial amount of knowledge about TNBC heterogeneity, which is intended to improve precision treatments. Nevertheless, there is still a wealth of static and dynamic data due to clinical parameters and treatment perturbations that escape from the analytical skills employed to construct the subtyping systems. It seems clear that the key to constructing a definitive and clinically useful classification of TNBC subtypes will incorporate integration of all the datasets and subtyping systems created to date. We believe that recent advances in artificial intelligence (AI) will accelerate this process and provide the largely anticipated rational stratification system for TNBC patients (**Figure 1B**).

Most of the current subtyping systems have relied on information from a single data source. However, the complexities of TNBC biology are unlikely captured sufficiently by a single data type. Instead, the combined information across multiple data types can provide a more holistic view of the complexities of TNBC biology. Advancements in the AI subfields of machine learning and deep learning have produced powerful methods that can be leveraged to construct models using diverse molecular data types (60, 61). The power of these methods lies in the ability to capture more complex relationships within data than traditional statistical approaches. Thus, these methods provide the necessary tools to integrate the diverse molecular data of current TNBC subtype systems. Furthermore, deep learning methods effectively extract information from non-

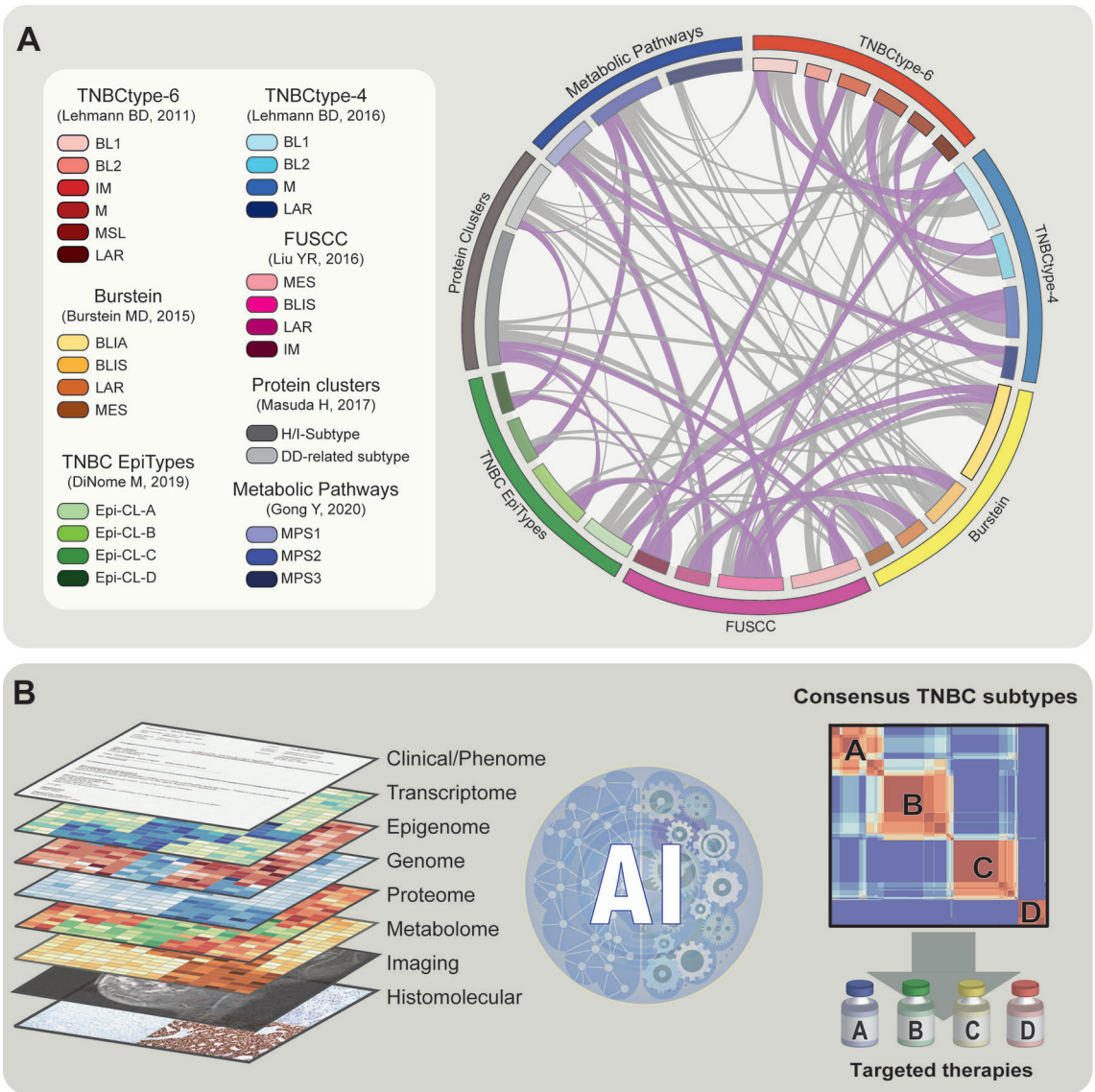

FIGURE 1 | Illustrative representation of current subtypes and the future of subsetting in TNBC. **(A)** Left panel: Summary of TNBC classification methods described and their subtypes. Right panel: Representation of the similitude between the different classification systems that reported comparisons with existing methods. Ribbons represent the partial overlap between different subtypes. Ribbons referring to strong overlaps are shown in purple. **(B)** Left panel: Schematic representation of the different layers of information to construct the definitive TNBC subtypes. This includes clinical, molecular, and histological data. Middle plot: Representation of application of artificial intelligence (AI) algorithm to integrate diverse datasets and construct TNBC subtypes. Right panel: Schematic correlation plot representing consensus integrative TNBC subtypes. TNBC stratification can be applied to improve subtype-specific therapies.

molecular data types such as clinical imaging and histopathology (62), significantly improving the current stratification methods. As the speed and scale at which biological data is collected increases and new advancements in computational technology emerge, AI-based methods will increasingly provide a powerful analytical framework for analyzing molecular and clinical data. Without question, these parallel advancements will constitute a breakthrough in TNBC precision diagnosis and treatment, addressing the most aggressive form of BC.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Ki67 Index Changes and Tumor-Infiltrating Lymphocyte Levels Impact the Prognosis of Triple-Negative Breast Cancer Patients With Residual Disease After Neoadjuvant Chemotherapy

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Purpose: The aim of this study was to assess the prognostic influence of Ki67 index changes in patients with primary triple-negative breast cancer (TNBC) treated with neoadjuvant chemotherapy (NAC), and to evaluate whether the combination of Ki67 index changes and residual disease (RD) tumor-infiltrating lymphocytes (TILs) provides additional prognostic information for this group.

Materials and Methods: Data from 109 patients with primary TNBC and RD after NAC were analyzed retrospectively. Ki67 changes and RD TIL levels were investigated for associations with recurrence-free survival (RFS) and overall survival (OS) using Kaplan–Meier and Cox analyses.

Results: Ki67 index decreased after NAC in 53 patients (48.6%) and high RD TIL levels (≥30%) were observed in 54 patients (49.5%). In multivariate Cox analyses, no Ki67 decrease status and low RD TIL levels were significantly associated with reduced RFS (hazard ratio (HR): 2.038, 95% confidence interval (CI): 1.135–3.658, P = 0.017; HR: 2.493, 95% CI: 1.335–4.653, P = 0.004), and OS (HR: 2.187, 95% CI: 1.173–4.077, P = 0.014; HR: 2.499, 95% CI: 1.285–4.858, P = 0.007), respectively. Notably, low RD TIL levels were significantly associated with reduced RFS (HR: 3.567, 95% CI: 1.475–8.624, P = 0.005) and reduced OS (HR: 3.873, 95% CI: 1.512–9.918, P = 0.005) in only the no Ki67 decrease group. The differences in 3-year RFS and OS between patients with no Ki67 decrease and low or high RD TIL levels were 24.4% *vs* 79.1% (P = 0.0001) and 33.1% *vs* 87.5% (P = 0.0001), respectively.

Conclusion: Ki67 index changes and RD TIL levels were associated with the prognosis of patients with primary TNBC with RD after NAC. RD TIL levels had greater prognostic significance in the no Ki67 decrease group.

Keywords: triple-negative breast cancer, Ki67, tumor-infiltrating lymphocytes, neoadjuvant chemotherapy, residual disease, prognostic factor

INTRODUCTION

Triple-negative breast cancer (TNBC) is a specific subtype with an aggressive clinical manifestation that accounts for approximately 15–20% of breast cancers. TNBCs tend be a higher clinical stage and are more prone to recurrence and metastasis than other breast cancer subtypes (1). Neoadjuvant chemotherapy (NAC) has become an integral part of the systematic treatment of TNBC. A major advantage of this strategy is the ability to observe the tumor response to chemotherapy regimens before surgery (2). Patients with TNBC who achieve pathological complete response (pCR) after NAC have better prognosis than those who do not reach pCR (3, 4); however, numerous patients with TNBC have residual disease (RD) after NAC, which is associated with a higher risk of relapse and distant metastasis (5, 6). Novel prognostic biomarkers that can stratify these patients will be valuable for making individualized treatment decisions and maximizing therapeutic efficacy in specific patient groups.

Tumor-infiltrating lymphocytes (TILs) are key tumor immune-related factors, which can communicate with the tumor microenvironment and mediate immune responses against the tumor (7, 8). There is currently significant research interest in the prognostic impact of TIL levels in patients with breast cancer. Growing evidence shows that higher pre-treatment TIL levels are associated with better prognosis in patients with breast cancer in both neoadjuvant and adjuvant settings (9–11). Moreover, several studies have evaluated residual lesions in patients with TNBC and RD after NAC and found that high RD TIL levels are associated with better relapse-free survival (RFS) and overall survival (OS) (12, 13). Real-world data from patients with TNBC in our region may provide new information regarding the prognostic significance of RD TIL levels.

Ki67 index is an indicator of malignant proliferation activity, which has been extensively investigated as a prognostic indicator in breast cancer (14). It is established that the Ki67 index in breast cancer changes dynamically after NAC, indicating that tumor proliferation ability may alter following NAC (15, 16). The Ki67 index is closely related to local recurrence and distant metastasis of breast cancer and there is some evidence that a decreased Ki67 index after NAC is associated with favorable clinical outcomes (16–18); however, there have been limited studies on the impact of this biomarker on the prognosis of patients with TNBC and RD.

The primary objective of the present study was to assess the independent prognostic influence of changes in Ki67 index in patients with primary TNBC following NAC. The secondary objective was to evaluate whether the combination of changes in Ki67 index and RD TIL levels provides additional prognostic information for this group.

MATERIALS AND METHODS

Patients and Treatments

In this retrospective study, 180 consecutive female patients with non-metastatic TNBC treated with NAC at the First Affiliated Hospital of Chongqing Medical University between November 2012 and August 2018 were assessed. The exclusion criteria were as follows: (1) patients with previous cancer, concomitant cancer, or bilateral breast cancer; (2) patients who received <three cycles of NAC or did not undergo surgery; (3) patients with incomplete clinical data; (4) patients who achieved pCR after NAC; and (5) patients with unevaluable RD TIL levels or Ki67 index. Finally, 109 patients were included in this study (**Figure 1**). All 109 included patients underwent NAC every 21 days [mean number of cycles: 4 (range, 3–8)]. The majority (90.8%) received an anthracycline plus taxane regimen. Four patients were treated with a taxane-based regimen, and six with an anthracycline-based regimen. Medical records were reviewed to collect clinicopathological data, including age, menopausal status, tumor size, lymph node involvement, histological subtype, histological grade, and surgical procedure.

This research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (No. 2020-59), who deemed that written informed consent was not necessary due to the retrospective nature of this research.

Histological Evaluation and Immunohistochemistry

All pathological results were re-evaluated independently by two pathologists with no knowledge of patient outcomes. RD molecular subtype was confirmed as TNBC in all participants before inclusion. TNBC was defined as estrogen-receptor (ER), progesterone-receptor (PR), and human epidermal growth factor receptor 2 (HER2) -negative (19). ER and PR status were considered negative if <1% of tumor cells were stained, and HER2 status was considered negative if a score of 0 or 1+ was confirmed by immunohistochemistry, or no HER2/*neu* gene amplification was detected by fluorescence *in situ* hybridization. pCR was defined as the absence of residual invasive tumor lesions in any breast tissue or lymph node (ypT0ypN0 or ypT0/is ypN0) (20).

Regarding the Ki67 index, between 500 and 1,000 cells were counted to calculate the percentage of positive tumor cells in the invasive front of the tumor with nuclear staining, as advised by the International Ki67 in Breast Cancer Working Group (using the Global Scoring method) (21). To evaluate changes in Ki67 after NAC, the Ki67 indices were assessed in biopsy specimens before NAC and surgical specimens after NAC from the same

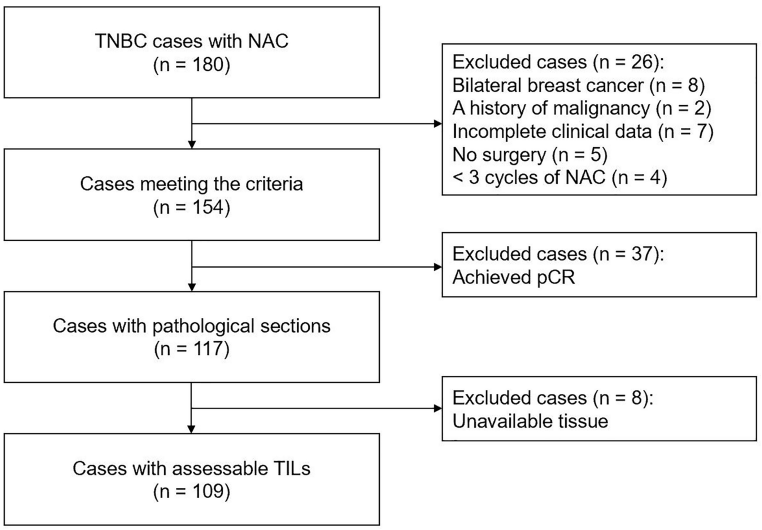

FIGURE 1 | Flowchart of patient selection in present study. TNBC, triple-negative breast cancer; NAC, neoadjuvant chemotherapy; pCR, pathological complete response; TILs, tumor-infiltrating lymphocytes.

patient. According to the report of Matsubara et al. (22), Ki67 decrease was defined as a decrease in the baseline Ki67 index of >1% after NAC. Histopathological evaluation of the percentage of TILs was conducted using hematoxylin and eosin (H&E)-stained sections from surgical specimens, according to the recommendations of the International TILs Working Group 2014 (23). Briefly, quantification of TILs in the tumor stroma was recorded as the percentage of occupied stromal areas (13). Based on the study of Liu et al. (24), the cut-off value applied for the percentage of TILs was 30% (**Figure 2**).

Follow-Up

Follow-up investigations, including a clinical examination and a radiological assessment, were performed at regular intervals (3-month intervals in years 1–3, 6-month intervals in years 4–5, and 12-month intervals in years 6–10 after surgery). Detailed information on patients with recurrence, metastasis, or death was accurately recorded. OS and RFS were defined as per the STEEP classification (25). OS was defined as the length of time from the date of tumor diagnosis to the date of death from any cause, or to the date of the last visit. RFS was calculated from the date of surgery to local, regional, or distant recurrence, or to the date of death from any cause. The deadline for follow-up was February 1, 2021.

Statistical Analysis

All data were analyzed using SPSS statistics software, version 25.0 (SPSS Inc., Chicago, IL, USA). Categorical variables are presented as numbers and percentages and were compared *via* Chi-square and Fisher's exact tests. The Kaplan–Meier method was used to estimate the distributions of OS and RFS, and the log-rank test was used to compare survival distributions among groups. Univariate Cox proportional hazards models were fit to assess the association between baseline variables and clinical outcomes. Multivariate Cox proportional hazards models were fit to assess the association of each baseline covariate with clinical outcomes, while adjusting for patient and disease characteristics. Results are expressed as hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical significance was defined as a two-sided P value <0.05.

RESULTS

Patient Characteristics

A total of 109 TNBC cases with evaluable RD TIL levels were eligible for analysis. Patient baseline characteristics are shown in **Table 1**. Mean age was 47.8 years (range: 20–76 years), 41 patients (37.6%) were postmenopausal and 68 (62.4%) were premenopausal or perimenopausal. Invasive ductal carcinoma constituted the most frequent histopathological subtype (90.8%). Excluding 15 unavailable cases, the most common histological grade was II (53.2%), followed by III (32.1%), and I (0.9%). The majority of patients (90.8%) received combination anthracycline and taxane chemotherapy, with mastectomy (98.1%) the most frequent operation. Before NAC, mean tumor size was 4.7 ± 2.9 cm, the most common tumor size was 2–5 cm (66.1%), followed by >5 cm (27.5%), and ≤2 cm (6.4%). Baseline nodal status before NAC was positive and negative in 69.7 and 30.3% of patients, respectively. After NAC, mean residual tumor size was 2.9 ± 2.1 cm, and the most common tumor size was ≤2 cm (47.7%), followed by 2–5 cm (40.4%), and >5 cm (11.9%). Nodal status after NAC was positive and negative in 59.6 and 40.4% of patients, respectively. Before NAC, mean Ki67 index was 36.8% ± 22.5%, while after NAC the corresponding value was 30.6% ± 20.9%. Relative to baseline status, 53 patients (48.6%) had a decreased Ki67 index after NAC and high RD TIL levels (≥30%) were observed in 54 patients (49.5%).

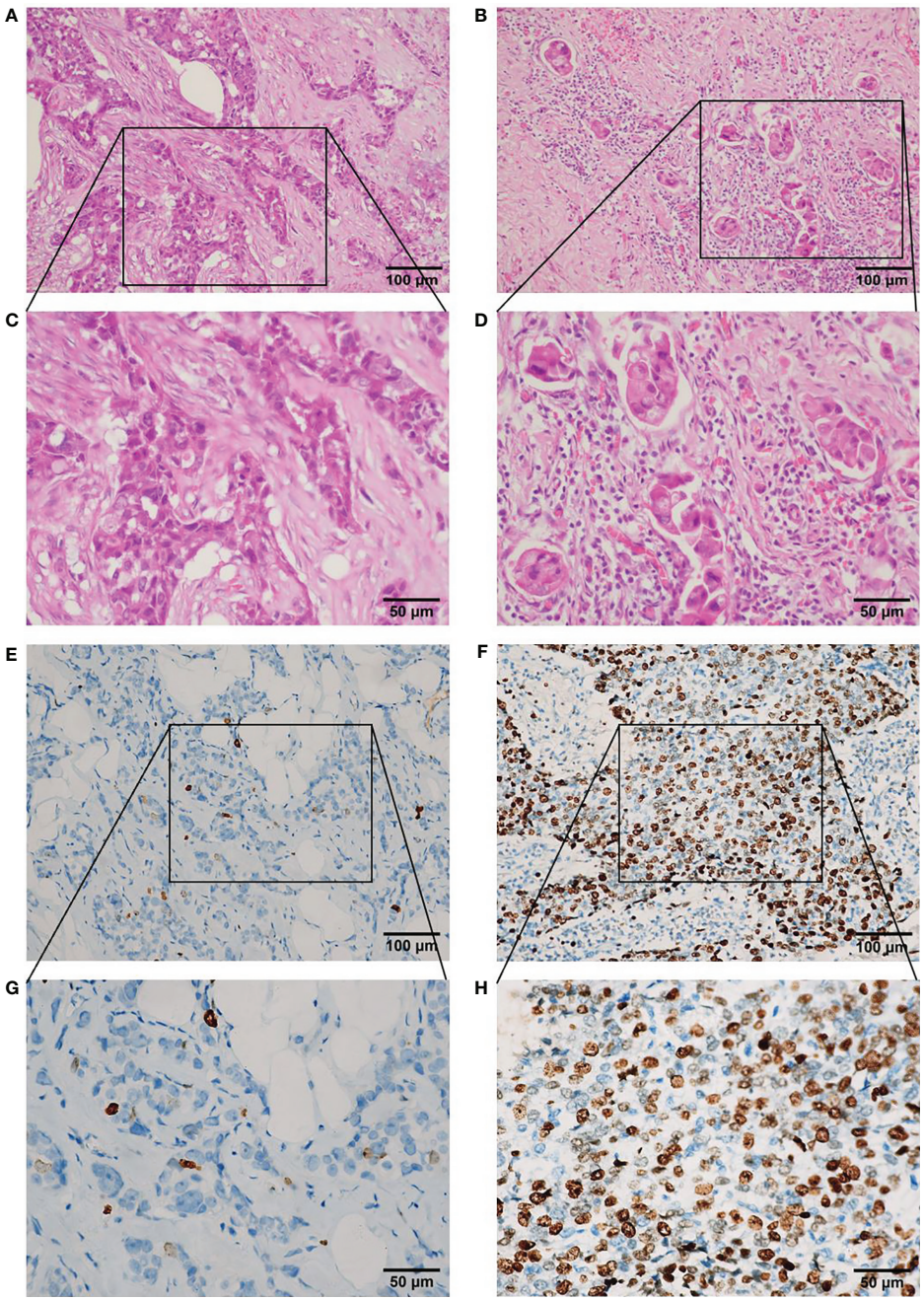

FIGURE 2 | Representative photomicrographs of TILs in hematoxylin and eosin sections and Ki67 index in immunohistochemical sections in residual disease in triple-negative breast cancer after neoadjuvant chemotherapy. **(A)** Low level (<30%) of TILs (×200 magnification). **(B)** High level ≥30%) of TILs (×200 magnification). **(C)** Low level (<30%) of TILs (×400 magnification). **(D)** High level (≥30%) of TILs (**x** 400 magnification). **(E)** Low level (<14%) of Ki67 index (×200 magnification). **(F)** High level (≥14%) of Ki67 index (×200 magnification). **(G)** Low level (<14%) of Ki67 index (×400 magnification). **(H)** High level (≥14%) of Ki67 index (×400 magnification). TILs, tumor-infiltrating lymphocytes.

During a median follow-up period of 51 months (range, 1 to 97 months) for RFS and 54 months (range, 4 to 101 months) for OS, there were 48 RFS events and 43 deaths. The 3-year RFS and OS rates were 69.7 and 72.0%, respectively. Bias due to loss during follow-up represented 8.26% (nine patients).

Associations of Changes in Ki67 Index and RD TIL Levels With Clinicopathological Characteristics

Relative to baseline status, 53 patients (48.6%) had a decreased Ki67 index after NAC. We compared the clinicopathological

TABLE 1 | Clinicopathological characteristics of patients (n = 109).

Characteristics	Mean ± SD	N (%)
Patients		109 (100)
Age at diagnosis (year)	47.8 ± 10.5	
≤50		73 (67.0)
>50		36 (33.0)
Menopausal status		
Pre/peri		68 (62.4)
Post		41 (37.6)
Histologic subtype		
Ductal		99 (90.8)
Lobular		3 (2.8)
Others*		7 (6.4)
Grade		
I		1 (0.9)
II		58 (53.2)
III		35 (32.1)
Unknown		15 (13.8)
Neoadjuvant therapy		
Anthracycline plus taxane		99 (90.8)
Taxane-based		4 (3.7)
Anthracycline-based		6 (5.5)
Surgery		
Mastectomy		107 (98.1)
Conservative surgery		2 (1.9)
Tumor size before NAC (cm)	4.7 ± 2.9	
≤2		7 (6.4)
2–5		72 (66.1)
>5		30 (27.5)
Nodal status before NAC		
Positive		76 (69.7)
Negative		33 (30.3)
Ki67 before NAC (%)	36.8 ± 22.5	
<14		17 (15.6)
14–30		44 (40.4)
>30		48 (44.0)
Residual tumor size (cm)	2.9 ± 2.1	
≤2		52 (47.7)
2–5		44 (40.4)
>5		13 (11.9)
Nodal status after NAC		
Positive		65 (59.6)
Negative		44 (40.4)
Ki67 after NAC (%)	30.6 ± 20.9	
<14		35 (32.1)
14–30		29 (26.6)
>30		45 (41.3)
Ki67 status		
Decrease		53 (48.6)
No decrease		56 (51.4)
RD TILs level		
Low		55 (50.5)
High		54 (49.5)

**Other histological types and distribution were as follows: two medullary carcinoma; two metaplastic carcinoma; one invasive carcinoma with apocrine differentiated carcinoma; one sarcomatous carcinoma; one pleotypic carcinoma. SD, standard deviation; NAC, neoadjuvant chemotherapy; RD, residual disease; TILs, tumor-infiltrating lymphocytes.*

features of patient groups with Ki67 decrease and no Ki67 decrease using the chi-square and Fisher's exact tests. No differences were identified in age, menopausal status, histological subtype, histological grade, residual tumor size, nodal status after NAC, or RD TIL levels (all P >0.05; **Table 2**).

RD TIL levels were evaluated based on examination of H&E-stained specimens (**Figure 2**). High RD TIL levels (≥30%) were detected in 49.5% of cases. Relationships between RD TIL level (low or high) and clinicopathological characteristics were assessed using chi-square and Fisher's exact tests (**Table 3**).

TABLE 2 | The relationship between Ki67 status and other factors.

Characteristics	Ki67 status		P
	Decrease (n = 53)	No decrease (n = 56)	
Age at diagnosis (year)			0.840
≤50	35 (66.0)	38 (67.9)	
>50	18 (34.0)	18 (32.1)	
Menopausal status			0.711
Pre/peri	34 (64.2)	34 (60.7)	
Post	19 (35.8)	22 (39.3)	
Histologic subtype			0.927
IDC	48 (90.6)	51 (91.1)	
No IDC	5 (9.4)	5 (8.9)	
Grade			0.380
I–II	30 (56.6)	29 (51.8)	
III	14 (26.4)	21 (37.5)	
Unknown	9 (17.0)	6 (10.7)	
Residual tumor size (cm)			0.571
≤2	28 (52.8)	24 (42.9)	
2–5	19 (35.8)	25 (44.6)	
>5	6 (11.3)	7 (12.5)	
Nodal status after NAC			0.531
Positive	16 (29.1)	28 (51.9)	
Negative	39 (70.9)	26 (48.1)	
RD TILs level			0.504
Low	25 (47.2)	30 (53.6)	
High	28 (52.8)	26 (46.4)	

IDC, invasive ductal carcinoma; NAC, neoadjuvant chemotherapy; RD, residual disease; TILs, tumor-infiltrating lymphocytes.

TABLE 3 | The relationship between TILs and other factors.

Characteristics	RD TILs level		P
	Low (n = 55)	High (n = 54)	
Age at diagnosis (year)			0.734
≤50	36 (65.5)	37 (68.5)	
>50	19 (34.5)	17 (31.5)	
Menopausal status			0.786
Pre/peri	35 (63.6)	33 (61.1)	
Post	20 (36.4)	21 (38.9)	
Histologic subtype			0.742
IDC	49 (89.1)	50 (92.6)	
No IDC	6 (10.9)	4 (7.4)	
Grade			0.792
I–II	28 (50.9)	31 (57.4)	
III	19 (34.5)	16 (29.6)	
Unknown	8 (14.5)	7 (13.0)	
Residual tumor size (cm)			0.049
≤2	21 (38.2)	31 (57.4)	
2–5	24 (43.6)	20 (37.0)	
>5	10 (18.2)	3 (5.6)	
Nodal status after NAC			0.015
Positive	16 (29.1)	28 (51.9)	
Negative	39 (70.9)	26 (48.1)	
Ki67 status			0.504
Decrease	25 (45.5)	28 (51.9)	
No decrease	30 (54.5)	26 (48.1)	

TILs, tumor-infiltrating lymphocytes; RD, residual disease; IDC, invasive ductal carcinoma; NAC, neoadjuvant chemotherapy.

High RD TIL levels were significantly associated with residual tumor size ≤2 cm (P = 0.049) and negative nodal status after NAC (P = 0.015). No associations were detected with age, menopausal status, histologic subtype, histological grade, or change in Ki67 index (all P >0.05).

Association of Changes in Ki67 Index and RD TIL Levels With Prognosis

Univariate analyses indicated that there were no significant associations of age, menopausal status, histological subtype, histological grade, or residual tumor size with RFS or OS (all P >0.05; **Table 4**). Taking into consideration clinical practice and statistical power, residual tumor size, nodal status after NAC, RD TIL levels, and Ki67 status were included in multivariate Cox proportional hazard regression models for RFS and OS. On multivariate analyses, no Ki67 decrease status, low RD TIL levels, and positive nodal status after NAC were significantly associated with reduced RFS, with estimated HR values of 2.038 (95% CI: 1.135–3.658, P = 0.017), 2.493 (95% CI: 1.335–4.653, P = 0.004), and 3.207 (95% CI: 1.574–6.535, P = 0.001), respectively (**Table 4**). Moreover, no Ki67 decrease status, low RD TIL levels, and positive nodal status after NAC were also significantly associated with reduced OS, with estimated HR values of 2.187 (95% CI: 1.173–4.077, P = 0.014), 2.499 (95% CI: 1.285–4.858, P = 0.007), and 3.842 (95% CI: 1.756–8.408, P = 0.001), respectively (**Table 4**).

Patients with decreased Ki67 status had higher 3-year RFS and OS rates compared with patients with no Ki67 decrease (RFS: 62.8% vs 47.7%, log-rank P = 0.0250; OS: 78.9% vs 58.8%, log-rank P = 0.0147) (**Figures 3A, B**). In addition, patients with low RD TIL levels exhibited reduced 3-year RFS and OS relative to those with high RD TIL levels (RFS: 41.1% vs 68.8%, log-rank, P = 0.0002; OS: 53.1% vs 84.6%, log-rank, P = 0.0004) (**Figures 3C, D**).

Prognostic Value of RD TIL Levels According to Ki67 Index Status

In univariate analyses there were no significant associations of age, menopausal status, histological subtype, histological grade, or residual tumor size with RFS or OS in either the Ki67 decrease or no decrease groups (all P >0.05; **Tables 5**, **6**). Taking into consideration clinical practice and statistical power, residual tumor size, nodal status after NAC, and RD TIL levels were included in multivariate Cox proportional hazard regression models. In the no Ki67 decrease group, low RD TIL levels were significantly associated with reduced RFS and OS, with estimated HR values of 3.567 (95% CI: 1.475–8.624, P = 0.005) and 3.873 (95% CI: 1.512–9.918, P = 0.005), respectively. Moreover, positive nodal status after NAC was significantly associated with reduced RFS and OS, with estimated HR values of 2.955 (95% CI: 1.167–7.481, P = 0.022) and 3.335 (95% CI: 1.227–9.068, P = 0.018), respectively (**Table 5**); however, in the Ki67

TABLE 4 | Univariate and multivariate analyses for RFS and OS in all TNBC patients.

Factor	RFS						OS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age at diagnosis (year)			0.831						0.780			
≤50	1						1					
>50	0.937	0.514–1.707					1.094	0.583–2.052				
Menopausal status			0.843						0.783			
Pre/peri	1						1					
Post	0.942	0.525–1.691					1.090	0.591–2.009				
Histologic subtype			0.796						0.509			
IDC	1						1					
No IDC	1.130	0.447–2.854					1.370	0.538–3.484				
Grade												
I–II	1						1					
III	1.475	0.791–2.748	0.221				1.544	0.799–2.982	0.196			
Unknown	1.150	0.495–2.670	0.745				1.147	0.465–2.834	0.766			
Residual tumor size (cm)			0.237			0.739			0.121			0.331
≤2	1						1					
>2	1.414	0.796–2.511					1.625	0.880–3.001				
Nodal status after NAC			<0.001			0.001			<0.001			0.001
Negative	1			1			1			1		
Positive	3.739	1.868–7.527		3.207	1.574–6.535		4.503	2.079–9.754		3.842	1.756–8.408	
Ki67 status			0.029			0.017			0.018			0.014
Decrease	1			1			1			1		
No decrease	1.910	1.069–3.413		2.038	1.135–3.658		2.114	1.138–3.928		2.187	1.173–4.077	
RD TILs level			<0.001			0.004			0.001			0.007
High	1			1			1			1		
Low	2.974	1.612–5.487		2.493	1.335–4.653		3.060	1.592–5.882		2.499	1.285–4.858	

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; IDC, invasive ductal carcinoma; NAC, neoadjuvant chemotherapy; RD, residual disease; TILs, tumor-infiltrating lymphocytes.

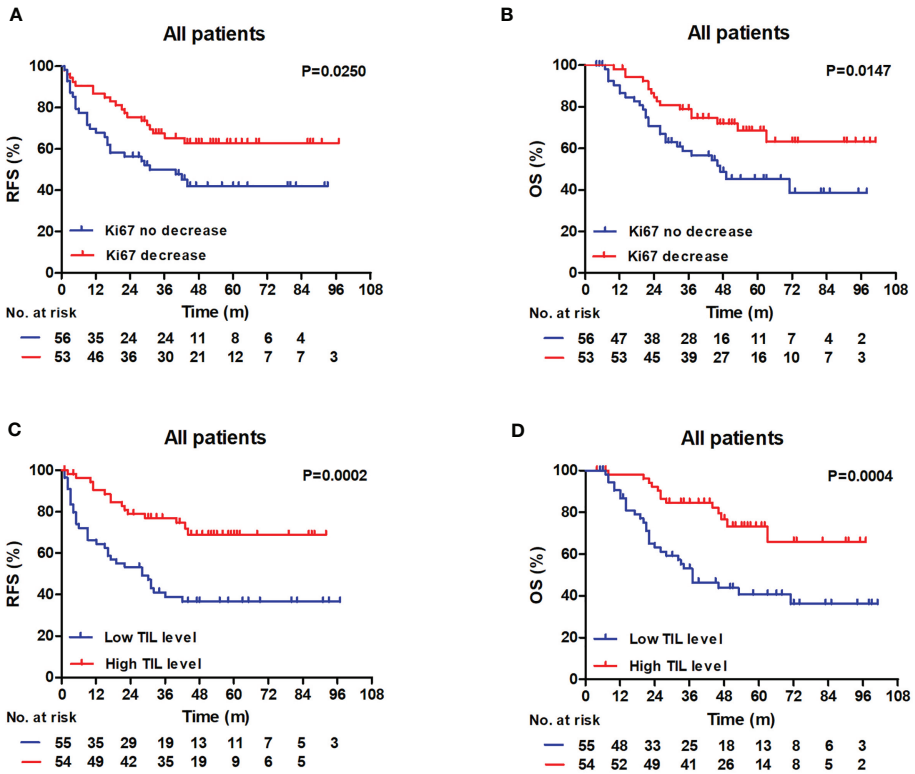

FIGURE 3 | RFS and OS outcomes in the whole patient cohort according to the TIL level and the Ki67 status. **(A, B)** Patients with decreased Ki67 status exhibited raised 3-year RFS and 3-year OS compared with patients without (RFS: 62.8% vs 47.7%, log-rank, P = 0.0250; OS: 78.9% vs 58.8%, log-rank, P = 0.0147). **(C, D)** Patients with low RD TIL levels exhibited reduced 3-year RFS and 3-year OS compared with patients with high RD TIL levels (RFS: 41.1% vs 68.8%, log-rank, P = 0.0002; OS: 53.1% vs 84.6%, log-rank, P = 0.0004). RFS, recurrence-free survival; OS, overall survival; TIL, tumor-infiltrating lymphocyte.

decreased group, only positive nodal status after NAC was associated with reduced RFS and OS, with estimated HR values of 3.517 (95% CI: 1.165–10.614, P = 0.026) and 4.391 (95% CI: 1.241–15.534, P = 0.022), respectively (**Table 6**).

In Kaplan–Meier analyses, patients with high RD TIL levels had significantly better RFS and OS rates than those with low RD TIL levels in the no Ki67 decrease group (RFS: log-rank P = 0.0001; OS: log-rank P = 0.0001) (**Figures 4A, B**). The differences in 3-year RFS and OS between patients with low or high RD TIL levels were 24.4% vs 79.1% and 33.1% vs 87.5%, respectively; however, in the Ki67 decrease group, no significant differences in RFS or OS were detected (RFS: log-rank P = 0.2318; OS: log-rank P = 0.3436) (**Figures 4C, D**).

DISCUSSION

In this study, we examined 109 patients with TNBC who did not achieve pCR after NAC, to investigate the prognostic significance of changes in Ki67 index and RD TIL levels. We found that no Ki67 decrease status and low RD TIL levels after NAC were significantly associated with worse RFS and OS in patients with TNBC and RD. Moreover, the magnitude of the prognostic value of RD TIL levels differed according to Ki67 status, with the greatest absolute differences observed in patients with no decrease in Ki67 index.

TIL levels are associated with TNBC patient prognosis, with high TIL levels linked to better treatment response and clinical outcomes in both neoadjuvant (9, 26, 27) and adjuvant (28–30) settings. In a retrospective study involving 375 TNBC RD cases, Luen et al. (13) reported that RD TIL levels provided independent and additional prognostic information beyond pre-treatment TIL levels in patients with primary TNBC treated with NAC, for both RFS (χ^2 9.88, P = 0.002) and OS (χ^2 8.02, P = 0.005). These findings were supported by other studies (12, 31). In this study we focused on the prognostic impact of RD TIL levels in patients with TNBC treated with NAC. Our results show that low (<30%) RD TIL levels are an independent prognostic factor associated with reduced RFS and OS in patients with primary TNBC, with estimated HR values of 2.493 (95% CI: 1.335–4.653, P = 0.004) and 2.499 (95% CI: 1.285–4.858, P = 0.007), respectively, consistent with published studies (12, 13). Moreover, we found that low RD TIL levels were associated with larger residual tumor size and positive nodal status. Overall, evidence indicates that low RD TIL levels are associated with more aggressive tumors, possibly because RD TIL levels are directly related to the magnitude of host anti-tumor adaptive immune responses following NAC (13).

TABLE 5 | Univariate and multivariate analyses for RFS and OS in Ki67 no decrease group.

Factor	RFS						OS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age at diagnosis (year)			0.716						0.786			
≤50	1						1					
>50	0.864	0.393–1.899					1.118	0.500–2.500				
Menopausal status			0.690						0.942			
Pre/peri	1						1					
Post	0.858	0.405–1.819					1.029	0.476–2.223				
Histologic subtype			0.801						0.957			
IDC	1						1					
No IDC	0.857	0.259–2.841					0.967	0.290–3.221				
Grade												
I–II	1						1					
III	1.177	0.540–2.565	0.682				1.225	0.544–2.760	0.624			
Unknown	0.722	0.209–2.503	0.608				0.854	0.245–2.981	0.805			
Residual tumor size (cm)			0.664			0.441			0.816			0.950
≤2	1						1					
>2	0.850	0.409–1.769					1.096	0.508–2.366				
Nodal status after NAC			0.003			0.022			0.002			0.018
Negative	1			1			1			1		
Positive	4.020	1.623–9.957		2.955	1.167–7.481		4.641	1.746–12.339		3.335	1.227–9.068	
RD TILs level			0.001			0.005			0.001			0.005
High	1			1			1			1		
Low	4.577	1.932–10.841		3.567	1.475–8.624		5.093	2.033–12.755		3.873	1.512–9.918	

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; IDC, invasive ductal carcinoma; NAC, neoadjuvant chemotherapy; RD, residual disease; TILs, tumor-infiltrating lymphocytes.

TABLE 6 | Univariate and multivariate analyses for RFS and OS in Ki67 decrease group.

Factor	RFS						OS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age at diagnosis (year)			0.873						0.817			
≤50	1						1					
>50	1.079	0.425–2.742					1.127	0.409–3.104				
Menopausal status			0.995						0.900			
Pre/peri	1						1					
Post	1.003	0.395–2.549					1.067	0.387–2.943				
Histologic subtype			0.558						0.314			
IDC	1						1					
No IDC	1.550	0.357–6.721					2.151	0.484–9.555				
Grade												
I–II	1						1					
III	1.739	0.618–4.895	0.295				1.889	0.609–5.862	0.271			
Unknown	1.935	0.595–6.290	0.273				1.755	0.462–6.669	0.409			
Residual tumor size (cm)			0.064			0.055			0.093			0.079
≤2	1						1					
>2	2.420	0.950–6.161					2.391	0.865–6.611				
Nodal status after NAC			0.026			0.026			0.022			0.022
Negative	1			1			1			1		
Positive	3.517	1.165–10.614		3.517	1.165–10.614		4.391	1.241–15.534		4.391	1.241–15.534	
RD TILs level			0.248			0.397			0.350			0.457
High	1						1					
Low	1.711	0.688–4.257					1.602	0.596–4.309				

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; IDC, invasive ductal carcinoma; NAC, neoadjuvant chemotherapy; RD, residual disease; TILs, tumor-infiltrating lymphocytes.

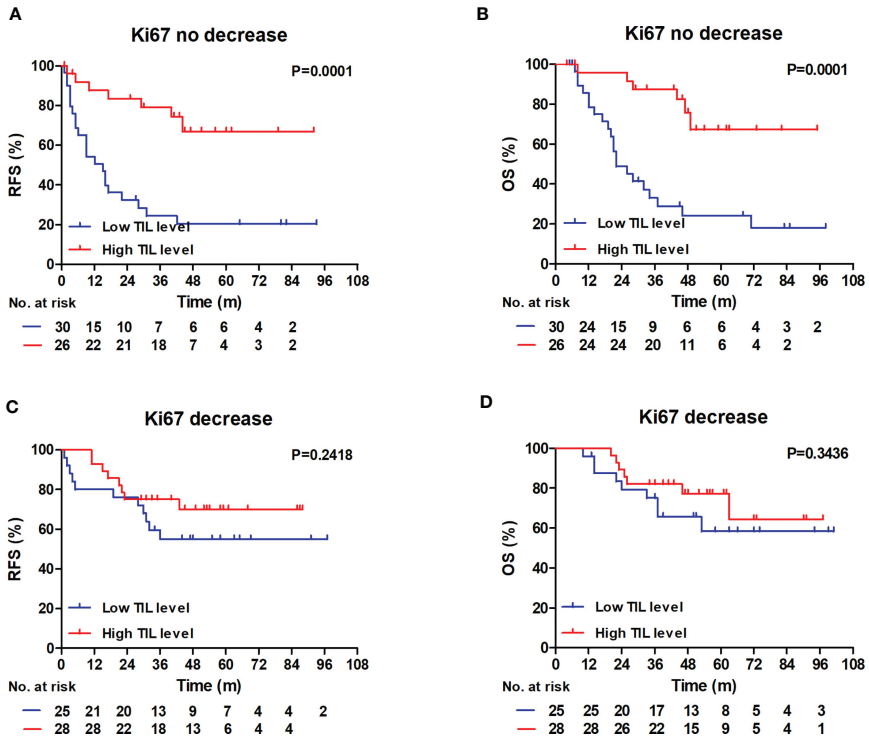

FIGURE 4 | RFS and OS outcomes in the Ki67 no decrease and decrease groups according to the TIL level. **(A, B)** In Ki67 no decrease group, Patients with low RD TIL levels exhibited reduced 3-year RFS and 3-year OS compared with patients with high RD TIL levels (RFS: 24.4% *vs* 79.1%, log-rank, P = 0.0001; OS: 33.1% *vs* 87.5%, log-rank, P = 0.0001). **(C, D)** In Ki67 decrease group, no significant differences were showed in RFS and OS graphs (RFS: log-rank, P = 0.2418; OS: log-rank, P = 0.3436). RFS, recurrence-free survival; OS, overall survival; TIL, tumor-infiltrating lymphocyte.

Many previous investigations have shown that breast cancer Ki67 index status changes after NAC (15, 16). In this study, we also detected differences in Ki67 index before and after NAC; 48.6% of residual tumors exhibited a decrease in Ki67 index after NAC. Furthermore, we explored the correlation between pre-NAC Ki67 index and Ki67 changes after NAC and found that decreased Ki67 status was related to high Ki67 index before NAC (**Table S1**; P = 0.014). Decreased Ki67 index after NAC has been reported as significantly associated with better prognosis in patients with breast cancer (16, 18, 32); however, prognostic information regarding Ki67 changes is limited in patients with TNBC and RD, and other studies (17, 18) showed that decreased Ki67 expression after NAC had clear prognostic significance in patients with TNBC and RD although, unfortunately, they did not provide the results of multivariate analysis of the TNBC group. Hence, our study may provide some new information. During median follow-up periods of 51 and 54 months for RFS and OS, respectively, we found that no Ki67 decrease status after NAC was significantly associated with worse RFS and OS in patients with TNBC and RD. In multivariate Cox analyses, no Ki67 decrease status was significantly associated with reduced RFS (HR: 2.038, 95% CI: 1.135–3.658, P = 0.017) and OS (HR: 2.187, 95% CI: 1.173–4.077, P = 0.014). In Kaplan–Meier analyses, patients with decreased Ki67 status had higher 3-year RFS and OS rates than patients without (RFS: 62.8% vs 47.7%, log-rank P = 0.0250; OS: 78.9% vs 58.8%, log-rank P = 0.0147). In contrast, a retrospective study of 435 patients with breast cancer who did not achieve pCR after standard NAC with anthracycline and paclitaxel reported no prognostic significance of Ki67 changes in the TNBC group (32); the difference between these findings and our data may be due to differences in sample source and the definition of Ki67 decrease.

Interestingly, in this investigation we observed that the prognostic significance of RD TIL levels differed markedly according to Ki67 status in patients with TNBC who received NAC. In the no Ki67 decrease group, low RD TIL levels were significantly associated with reduced RFS and OS, with estimated HR values of 2.733 (95% CI: 1.122–6.658, P = 0.027) and 4.114 (95% CI: 1.335–12.673, P = 0.014), respectively; however, this is in contrast with the lack of significant prognostic influence of RD TIL levels in the Ki67 decrease group (P >0.05). The relationship between Ki67 changes and RD TIL levels remains somewhat unclear. We explored the correlation between RD TIL level and Ki67 status after NAC, and found that there was no significant statistical correlation between the two factors (**Table 3**; P = 0.504). A larger patient sample may be required to further explore this correlation. In addition, we found that RD TIL level had stronger prognostic significance in the no Ki67 decrease group. We suspect that this finding may be related to changes in tumor proliferation and the tumor microenvironment that occur after NAC. TIL levels

reflect the tumor immune microenvironment, and high TIL levels in RD may indicate a strong anti-tumor immune response after NAC. Ki67 index reflects the ability of tumor cells to proliferate. No decrease in Ki67 status after NAC may reflect a limited effect of NAC on tumor proliferative capacity and activity, and the observed prognostic correlation with high RD TIL levels is logical in this context. Increased understanding of the interactions between cancer cell proliferation regulation and tumor immune responses may advance treatment of TNBC in the future.

This study has some limitations. First, the sample size was small and patients were recruited from a single center; therefore, selection bias was unavoidable. Second, due to the amount of work and the retrospective nature of this study, we evaluated RD based on tumor size and nodal status, which is less robust than modern methods, such as residual cancer burden index. Third, in our study cohort, four patients had a potential follow-up period of <36 months (range, 30–35 months). Therefore, a large-scale, multi-center, prospective validation study, with longer follow-up period is needed to further clarify the results of this study.

CONCLUSION

In summary, in this study we found that decreased Ki67 index and high RD TIL levels were associated with superior RFS and OS in patients with primary TNBC and RD following NAC. Larger positive effects of TILs on RFS and OS were observed in patients with no Ki67 decrease status. Hence, assessment of Ki67 index changes and RD TIL levels after NAC could provide valuable prognostic information for patients with TNBC.

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 Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YiW was responsible for original conception and design, analysis of data, search of the literature, correction, and editorship of the manuscript. BZ was responsible for original conception, acquisition of clinical data, correction, and English editing. YY was responsible for acquisition and re-evaluation of pathological sections, analysis of pathological data, and search of the literature. YuW, RC, ZT, and MH were responsible for acquisition of clinical data, correction, and English editing. SL was responsible for design, English editing, correction, and approval of the final version. 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.2021.668610/full#supplementary-material

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Protein Tyrosine Kinase 7 Regulates EGFR/Akt Signaling Pathway and Correlates With Malignant Progression in Triple-Negative Breast Cancer

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Purpose: Triple-negative breast cancer (TNBC), the most aggressive subtype of breast cancer, is associated with high invasiveness, high metastatic occurrence and poor prognosis. Protein tyrosine kinase 7 (PTK7) plays an important role in multiple cancers. However, the role of PTK7 in TNBC has not been well addressed. This study was performed to evaluate the role of PTK7 in the progression of TNBC.

Methods: Correlation of PTK7 expression with clinicopathological parameters was assessed using tissue microarray immunohistochemistry (IHC) staining in 280 patients with breast cancer. PTK7 expression in TNBC (MDA-MB-468, MDA-MB-436 and MDA-MB-231) and non-TNBC (MCF7 and SK-BR-3) breast cancer cell lines were examined using immunoblotting assay. PTK7 correlated genes in invasive breast carcinoma were analyzed using cBioPortal breast cancer datasets including 1,904 patients. PTK7 overexpressed or knockdown TNBC cell lines (MDA-MB-468 and MDA-MB-436) were used to analyze the potential roles of PTK7 in TNBC metastasis and tumor progression. A TNBC tumor bearing mouse model was established to further analyze the role of PTK7 in TNBC tumorigenicity *in vivo*.

Results: PTK7 is highly expressed in breast cancer and correlates with worse prognosis and associates with tumor metastasis and progression in TNBC. Co-expression analysis and gain- or loss-of-function of PTK7 in TNBC cell lines revealed that PTK7 participates in EGFR/Akt signaling regulation and associated with extracellular matrix organization and migration genes in breast cancer, including COL1A1, FN1, WNT5B, MMP11, MMP14 and SDC1. Gain- or loss-of-function experiments of PTK7 suggested that PTK7 promotes

proliferation and migration in TNBC cell lines. PTK7 knockdown MDA-MB-468 cell bearing mouse model further demonstrated that PTK7-deficiency inhibits TNBC tumor progression *in vivo*.

Conclusion: This study identified PTK7 as a potential marker of worse prognosis in TNBC and revealed PTK7 promotes TNBC metastasis and progression *via* EGFR/Akt signaling pathway.

Keywords: PTK7, triple-negative breast cancer (TNBC), migration, progression, EGFR

INTRODUCTION

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer characterized by high invasiveness, metastasis and heterogeneous clinical behavior (1–3). Due to lacking expression of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2), TNBC patients are not sensitive to endocrine therapy or HER2-targeted therapy (4, 5). Resistance to conventional systemic radiotherapy and chemotherapy and high occurrence of post-chemotherapy metastasis make it urgent to develop new TNBC treatment strategies (6–8). Therefore, the importance of understanding the molecular biology of TNBC has gained considerable attention.

Protein tyrosine kinase 7 (PTK7), a member of the receptor tyrosine kinase (RTK) superfamily, is a catalytically inactive RTK that plays a role in multiple cellular processes including polarity and adhesion (9–12). PTK7 interacts with Wnt3a and Wnt8 and acts as an important regulator of both non-canonical and canonical Wnt signaling in multiple developmental contexts (13, 14). PTK7 activates AP-1 and NF-κB signaling and upregulates matrix metalloproteinase-9 (MMP9) which results in increasing invasive properties of esophageal squamous cell carcinoma cells (15). PTK7 binds and activates FGFR1 and increases tumorigenicity (16). Furthermore, PTK7 regulates the activity of kinase insert domain receptor (KDR) and thereby participates in VEGF induced tumor angiogenesis (17).

The expression and function of PTK7 have been investigated in several human cancers, although controversial results have been obtained (18–24). PTK7 is highly expressed and plays an oncogenic role in lung adenocarcinoma (18). PTK7 is overexpressed and contributes to thyroid (19) and cervical (22) cancer progression. A bioinformatics analysis reported that PTK7 is highly expressed in stage I-IV hepatocellular carcinoma (HCC) and considered as an independent prognostic marker for reduced overall survival (21). Another investigation of PTK7 expression in 79 consecutive invasive breast cancer tissues by immunohistochemistry found that PTK7 expression level negatively associates with tumor grade and lymph node metastasis (23). However, Gartner and colleagues found elevated PTK7 mRNA expression level in TNBC cell lines and PTK7 overexpression in metastatic lymph node predicts shorter disease-free survival (DFS) in breast cancer patients (24). The controversy of PTK7 function in breast cancer may be due to its multiple molecular subtypes and heterogeneity.

Although some lines of evidence revealed the important role of PTK7 in tumor progression, the molecular functions of PTK7 in metastasis and motility in TNBC remains elusive. Here we demonstrate that PTK7 were predominantly upregulated in breast cancer tissues. Expression levels of PTK7 predict a poor outcome and an increased risk for cancer metastasis in TNBC patients. Moreover, PTK7 regulates tumor metastasis and collagen fibril organization *via* EGFR-Akt pathway.

Abbreviations: BP, Biological Process; ER, estrogen receptor; GO, Gene Ontology; HER2, human epidermal growth factor receptor 2; IHC, Immunohistochemistry; KDR, kinase insert domain receptor; KEGG, Kyoto Encyclopedia of Genes and Genomes; MMP9, matrix metalloproteinase-9; PR, progesterone receptor; PTK7, protein tyrosine kinase 7; RFS, recurrence-free survival; RPTK, receptor protein tyrosine kinase; TNBC, triple-negative breast cancer.

MATERIAL AND METHODS

Plasmid Constructs and Reagents

Antibodies for PTK7 (25618, 1:1,000) and phosphor-Akt (S473) (4060, 1:1,000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody for β-actin (AC026, 1:20,000) was from Abclonal (Wuhan, Hubei, China). Antibody for Tubulin (10068-1-AP, 1:1,000) was from Proteintech (Chicago, IL, USA). Antibody for EGFR (1114-1, 1:1,000) was from Epitomics (Burlingame, CA, USA). Antibody for phosphor-EGFR (Y1173) (ET1610-4, 1:1,000) was from HuaBio (Hangzhou, Zhejiang, China). Antibody for Akt (B-1, 1:1,000) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human PTK7 expression plasmid was from Addgene (Watertown, MA, USA). LV3 lentiviral vectors encoding shRNAs silencing PTK7 or a nonsilencing control shRNA (shNC) were purchased from GenePharma (Suzhou, Jiangsu, China). The sequences of PTK7 shRNAs: shPTK7#1: 5'-GGATGATGTCACTGGAGAAGA-3'; shPTK7#2: 5'-GGAGGGAGTTGGAGATGTTTG-3'. For gene silencing, 293T cells were transfected with lentiviral vectors together with packaging plasmids and packaged lentiviral particles were prepared and used to infect indicated cells followed by puromycin selection.

Patients and Tissue Microarray

Two tissue microarrays containing 280 cases of breast cancer tissues collected from 2006 to 2016 with overall survival time (3- to 11-year follow-up, mean follow-up time was 106 months) were purchased from BioChip (Shanghai, China). The breast cancers were divided

into the four intrinsic subtypes, Luminal A, Luminal B, HER2(+) and TNBC, based on immunohistochemistry (IHC) results for ER, PR, HER2 and Ki67 provided by BioChip. ER, PR and HER2 positivity was defined using 2018 ASCO/CAP guidelines. ER and PR positivity was defined as ER ≥ 1%, PR ≥ 1%, respectively. For HER2, IHC 3+ or IHC 2+/ISH+ was defined as HER2 positive. ER positive, PR ≥ 20% and Ki67 < 15% was defined as Luminal A. ER positive, PR < 20% and Ki67 > 30% was defined as Luminal B. All the patients provided informed consent. The study was approved by Institutional Review Board of Hebei University Affiliated Hospital. The patient information and histological features were displayed in **Tables 1** and **2**. The analysis of clinicopathological features were based on 280 breast cancer cases or 49 TNBC cases where indicated, excluding a few cases because of missing data.

TABLE 1 | Patient characteristics.

Variable	No. of Patients (%)
No. of BC patients	280 (100)
Age: Median [range]	59 [29-88]
Molecular typing	
Luminal A	96 (37.5)
Luminal B	37 (14.5)
HER2(+)	74 (28.9)
TNBC	49 (19.1)
TNM stage	
I	57 (20.4)
II	138 (49.3)
III	81 (28.9)
Lymphatic metastasis	
Negative	143 (51.3)
Positive	136 (48.7)
Distant metastasis	
Negative	280 (100)
Positive	0 (0)
Prognosis	
Survival	208 (74.3)
Death	72 (25.7)

IHC Staining

Tissue microarrays were treated with heat-induced antigen-retrieval procedures and IHC staining was performed using the avidin–biotin complex method. The tissue sections were blocked with 10% goat serum and incubated with anti-PTK7 antibody (25618; 1:1,000 dilution; Cell Signaling Technology) at 4°C overnight. Then, the slides were washed three times using PBS followed by biotinylated-secondary antibody incubation for 2 hours at room temperature. The slides were washed three times and incubated with streptavidin/HRP. DAB peroxidase substrate was utilized for visualization. The IHC staining was assessed by two pathologists who were blinded to clinical information. PTK7 IHC score was assessed according to the staining intensity (no staining = 0; weak staining = 1, moderate staining = 2 and strong staining = 3) and the percentage of stained cells (0–4% = 0, 5%–25% = 1, 26%–50% = 2, 51%–75% = 3 and 76%–100% = 4). IHC score = stained cell percentage score × staining intensity score. PTK7 protein expression was divided into low expression (IHC score 0~4), medium expression (IHC score 4~8) and high expression (IHC score 8~12) according to the IHC score.

PTK7 Gene Expression Profiling

GEPIA: Gene Expression Profiling Interactive Analysis system (http://gepia.cancer-pku.cn/), a newly developed interactive web server for analyzing the RNA sequencing expression data was used to analyze PTK7 expression in breast invasive carcinoma (n = 1,085) and matched normal breast tissues (TCGA normal and GTEx dataset, n = 291). PTK7 expression according to triple-negative status using Breast Cancer Gene-Expression Miner v4.3 system (http://bcgenex.centregauducheau.fr/BC-GEM/). TNBC (n = 572) and non-TNBC breast cancer (n = 6,739) DNA microarray data were selected. For PTK7 genetic alteration analysis in invasive breast carcinoma, cBioPortal for Cancer Genomics (http://www.cbioportal.org/) breast cancer datasets were used which includes 1,904 patients with Agilent

TABLE 2 | Molecular subtyping and clinical characteristics.

Variable	Molecular subtyping			
	Luminal A	Luminal B	HER2(+)	TNBC
No. of subtyping patients: n (%)	96 (100)	37 (100)	74 (100)	49 (100)
Age: Median [range]	61 [37-88]	66 [41-88]	57 [33-87]	57 [32-84]
TNM stage: n (%)				
I	21 (22.1)	7 (18.9)	16 (22.2)	12 (25)
II	46 (48.4)	24 (64.9)	33 (45.8)	18 (37.5)
III	28 (29.5)	6 (16.2)	23 (32.0)	18 (37.5)
Lymphatic metastasis: n (%)				
Negative	47 (51.6)	20 (58.8)	36 (50.7)	26 (53.1)
Positive	44 (48.4)	14 (41.2)	35 (49.3)	23 (46.9)
Prognosis: n (%)				
Survival	79 (82.3)	34 (91.9)	52 (70.3)	30 (61.2)
Death	17 (17.7)	3 (8.1)	22 (29.7)	19 (38.8)
PTK7 expression				
IHC score: Mean ± s.d.	5.06 ± 2.42	5.12 ± 2.39	6.20 ± 2.41	7.46 ± 2.68
Low PTK7 level: n (%)	36 (37.5)	13 (35.1)	20 (27.0)	6 (12.2)
Medium PTK7 level: n (%)	41 (42.7)	14 (37.8)	25 (33.8)	12 (24.5)
High PTK7 level: n (%)	19 (19.8)	10 (27.0)	29 (39.2)	31 (63.3)

microarray data (METABRIC, Nature 2012 & Nat Commun 2016).

Recurrence-Free Survival (RFS) Assay by Kaplan-Meier Plotter

The prognostic value of PTK7 mRNA expression was evaluated using an online database, Kaplan-Meier Plotter (http://www.kmplot.com/). To analyze RFS of patients with Luminal A, Luminal B, HER2(+) and TNBC subtypes of breast cancer, patients were divided into two groups (high versus low expression) and assessed by a Kaplan-Meier survival plot, with the hazard ratio (HR) with 95% confidence intervals (CIs) and log rank P-value.

KEGG, GO and PTK7 Correlated-Gene Analysis

PTK7 correlated genes were investigated using breast cancer datasets including 1,904 patients with Agilent microarray data (http://www.cbioportal.org/). Positively- (Spearman's correlation > 0.3, $P < 0.01$) and negatively- (Spearman's correlation < -0.3, $P < 0.01$) correlated genes were selected as candidate PTK7 correlated genes. PTK7 correlated genes were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) by DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp) and Gene Ontology (GO) was performed using DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp). Pair-wise gene correlation of PTK7 with EGFR, COL1A1, FN1, WNT5B, MMP11, MMP14 and SDC1 in breast invasive carcinoma were analyzed using GEPIA Correlation Analysis tools (http://gepia.cancer-pku.cn/detail.php?clicktag=correlation).

Cell Culture

Human TNBC cell lines MDA-MB-436, MDA-MB-468, MDA-MB-231, MCF7 and SK-BR-3 were obtained from Cell Resource Center (IBMS, CAMS/PUMC, Beijing, China). Human embryo kidney 293T cell line was obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Science, China. MDA-MB-436, MDA-MB-468 and MDA-MB-231 were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. HEK293T, MCF7 and SK-BR-3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. All cell lines were cultured in a humidified atmosphere of 5% CO_2, 95% air at 37°C.

Gene Silencing

For gene silencing, HEK293T cells were transfected with LV3 lentiviral vectors encoding specific shRNAs targeting PTK7 (shPTK7#1 and shPTK7#2) or control shRNAs (shNC) along with packaging plasmids psPAX2 and pMD2.G. The supernatant was collected at 48 hours after transfection and filtered through a 0.45 μm polysulfone filter for lentiviral particles preparation. MDA-MB-436 and MDA-MB-468 cells were than transduced with the packaged virus and selected by puromycin to establish stable cell lines. Immunoblotting assays were performed to examine the silencing efficiency.

Immunoblot Assay

Total cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, protease inhibitor cocktail). Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, blocked with 5% non-fat milk and incubated with a specific primary antibody. The membranes were then washed and incubated with HRP-conjugated secondary antibody and visualized by chemiluminescent detection (ECL, Roche Diagnostics, Penzberg, Germany) and exposure to X-ray film (Thermo Fisher Scientific, Waltham, MA, USA). The experiment was repeated independently 3 times.

Actin Cytoskeleton Staining

Cells were fixed in 4% paraformaldehyde at room temperature for 10 min followed by permeabilization with 0.1% Triton X-100. Cells were incubated with TRITC-tagged phalloidin in the dark at room temperature for 30 min and stained with 4',6-diamidino-2-phenylindole (DAPI) for 3 min to visualize nuclear. Confocal microscopy was performed with the Confocal Laser Scanning Microscope Systems (FV3000, Olympus, Shinjuku, Japan). The experiment was repeated independently 3 times.

Cell Proliferation Assays

For MTT assay, 1×10^4 cells were seeded into 96-well plates and cultured for 0, 24, 48 or 72 hours. Before detection, each well was added with 20 μL MTT reagent (0.5 mg/mL in PBS) followed by an additional 2 hours incubation. The medium was removed and purple-blue MTT formazan precipitate was dissolved in 100 μL DMSO for 10 min at room temperature. The absorbance was measured at 490 nm using a BioTek Epoch Spectrophomometer (BioTek, Winooski, VT, USA). For colony formation, a single-cell suspension was prepared and cells were seeded into a 6-well plate in a concentration at 750 cells/mL and incubated for 2 weeks. Cells were stained with crystal violet and colony formation was photographed under a phase-contrast microscope and colony numbers and diameters were measured. All the experiments were repeated independently 3 times.

Cell Invasion Assay

Cell invasion assay were performed using a modified Boyden transwell system. The transwell permeable supports chambers (Corning Incorporated, Corning, NY, USA) with 8-μm pore size were pre-coated with 10 mg/L Matrigel overnight at 4°C and 1×10^5 cells were seeded to the upper chamber of the transwell system and incubated at 37°C for 24 hours. Cells remaining on the upper chamber were mechanically erased with a cotton swab and the cells migrated to the lower surface of the filter were stained with crystal violet and counted under the microscope. The experiment was repeated independently 3 times.

Tumor Xenograft

Male BALB/c-nu mice at 4-5 weeks old were used to establish TNBC mouse model *in vivo*. All the mice were purchased from

the Beijing HFK Bioscience Co., Ltd (Beijing, China) and housed in a specific pathogen-free environment at Hebei University Laboratory Animal Research Center. All experiments were approved by the Animal Research Ethics Committee of the authors' institution. Briefly, MDA-MB-468 cells (5×10^5) were injected s.c. into the right mammary fat pad of nude mice. Each group consisted of six mice. The challenged mice were monitored every 2 days for tumor growth. The tumor volume was estimated according to the formula: Volume = $0.5 \times a \times b^2$, where a and b represent the largest and smallest diameters, respectively. All the mice were sacrificed 61 days after injection and the tumors were weighted, measured and photographed. The experiment was repeated independently 2 times.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software 8.0 (GraphPad Software, San Diego, CA, USA). Two-tailed Student's t tests or one-way ANOVA according to the number of groups compared. P-values < 0.05 were considered significant and the level of significance expressed as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

RESULTS

PTK7 Is Highly Expressed and Correlates With Worse Prognosis in TNBC

To explore the potential role of PTK7 in breast cancer, we analyzed PTK7 expression in breast cancer using an RNA-Seq datasets GEPIA: Gene Expression Profiling Interactive Analysis system (http://gepia.cancer-pku.cn/) and found that PTK7 transcription levels are significantly higher in breast invasive carcinoma (BRCA) tissues (n = 1,085) than that in matched non-tumor tissues (n = 291), suggesting a potential role of PTK7 in breast cancer (**Figure 1A**).

To further investigate the clinical relevance of PTK7, we evaluated breast cancer tissue samples from 280 human subjects (**Table 1**) and performed IHC staining against PTK7 (**Figure 1B**). IHC staining showed that PTK7 was expressed both in the cytosol and the nucleus of breast cancer cells (**Figure 1C**). The samples were divided into four subtypes, Luminal A, Luminal B, HER2(+) and TNBC, based on ER, PR, HER2 and Ki67 expression. Interestingly, PTK7 expression was distinctively higher in TNBC subtype than that in Luminal A, Luminal B and HER2(+) molecular subtypes (**Figures 1C, D**). Next, three TNBC cell lines (MDA-MB-468, MDA-MB-436 and MDA-MB-231),

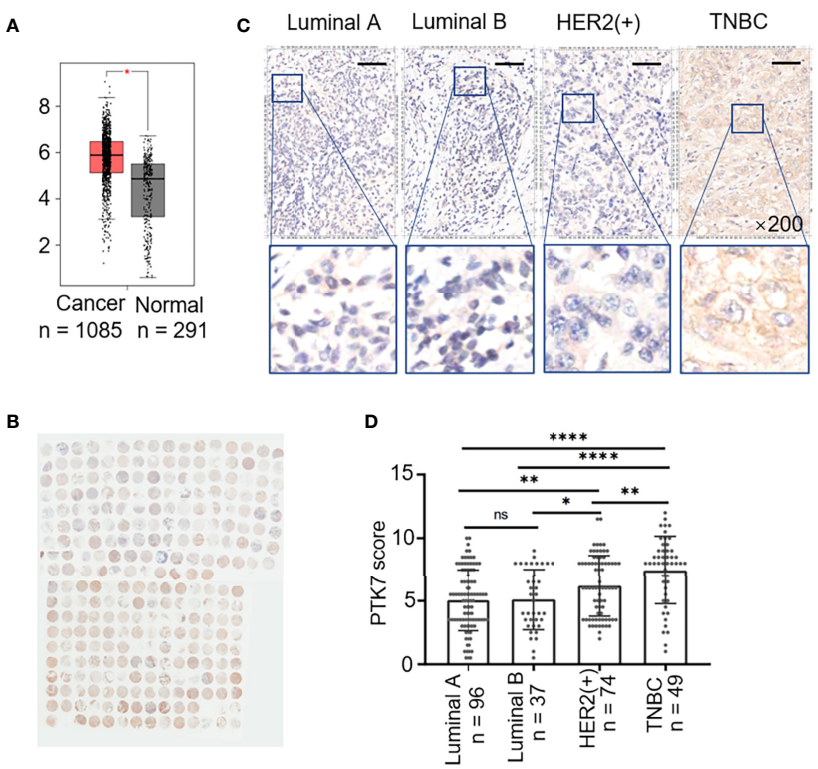

FIGURE 1 | PTK7 expression is upregulated in breast cancer. **(A)** Box plots of PTK7 expression in breast invasive carcinoma (BRCA) using GEPIA: Gene Expression Profiling Interactive Analysis system (http://gepia.cancer-pku.cn/). BRCA tumor (T) and non-tumor (N) TCGA normal and GTEx dataset included 1,085 tumor cases (T) and 291 non-tumor cases (N) was selected to observe the expression of PTK7. **(B)** IHC staining of PTK7 expression in breast cancer tissue microarray. **(C)** Representative images from PTK7 IHC staining in Luminal A, Luminal B, HER2(+) and TNBC subtypes of breast cancer tissues. Magnification, ×200; scale bars, 100 μm. **(D)** Scatter dot plots of PTK7 expression in tumors with different molecular subtypes. Data were analyzed using one-way ANOVA and Tukey's multiple comparisons test and are shown as mean ± s.d. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. ns, no significance.

ER(+) breast cancer cell line (MCF7) and HER2(+) breast cancer cell line (SK-BR-3) were used to analyze PTK7 expression and the result showed significantly higher PTK7 levels in TNBC cells than that in MCF7 and SK-BR-3 cells (data not shown).

PTK7 genetic alteration and expression levels were further analyzed using online database in different molecular subtypes of breast cancer. TNBC (n = 572) and non-TNBC breast cancer (n = 6,739) DNA microarray data were selected from Breast Cancer Gene-Expression Miner v4.3 system (http://bcgenex.centregauducheau.fr/BC-GEM/) and PTK7 expressions were higher in TNBC than that in non-TNBC (**Supplementary Figure S1A**). PTK7 genetic alterations in invasive breast carcinoma were analyzed using cBioPortal for Cancer Genomics (http://www.cbioportal.org/) breast cancer datasets and the results revealed that PTK7 genetic amplification exists in 1.6% cases (n = 30) of invasive breast carcinoma patients (n = 1,904), most of which are ER(-), PR(-) and HER2(-) (TNBC, n = 22) (**Supplementary Figure S1B**).

PTK7 expression was qualified as low, medium and high according to IHC score and a follow-up analysis of patient overall survival showed that higher expression of PTK7 in TNBC breast cancer tissue correlated with a worse outcome (**Figure 2D**). However, there was no statistical difference in Luminal A, Luminal B and HER2(+) subtypes (**Figures 2A–C**). Next, we performed RFS analysis using online database Kaplan-Meier Plotter (http://www.kmplot.com/) to assess the effect of PTK7 on breast cancer prognosis. Breast cancer samples were divided into two groups based on PTK7 expression and no significant difference was found Luminal A, Luminal B and HER2 subtypes of breast cancer (**Supplementary Figure S1C**). Interestingly, a significantly worse RFS was found in PTK7 high expressed TNBC (**Supplementary Figure S1C**). These data indicated that PTK7 plays an important role in TNBC and correlated with breast cancer prognosis.

Elevated PTK7 Is Associated With Tumor Growth and Metastasis in TNBC

Next, we selected all the TNBC tissue samples (n = 49) from 280 subjects of breast cancer tissue microarray (**Table 1**) and divided them into groups based on TNM stages and lymph node metastasis. PTK7 expression was significantly higher in TNM II and TNM III groups than that in TNM I group (**Figures 3A, B**). Moreover, elevated PTK7 was observed in TNBC with lymph node metastasis (**Figures 3D, E**). When dividing TNBC tumor samples into groups based on PTK7 IHC staining score, the percentage of high PTK7 expression samples was significantly higher in TNBC with TNM stage III and lymph node metastasis groups (**Figures 3C, F**). These data therefore collectively suggested that PTK7 participates in tumor metastasis in TNBC.

PTK7 Upregulates EGFR/Akt Signaling Activation

We next analyzed PTK7 co-expression genes using breast cancer datasets including 1,904 patients with Agilent microarray data

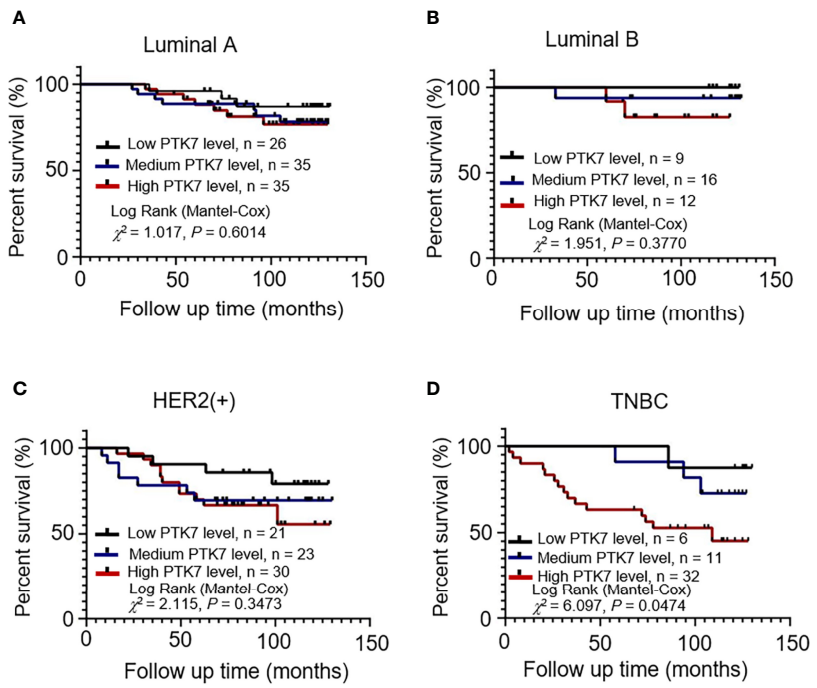

FIGURE 2 | PTK7 upregulation is associated with poor patient survival in TNBC. Breast cancer samples were divided into groups based on PTK7 expression [low expression (IHC score 0~4), medium expression (IHC score 4~8) and high expression (IHC score 8~12)]. Kaplan-Meier overall survival curve analysis and two-sided log-rank tests were performed in Luminal A **(A)**, Luminal B **(B)**, HER2(+) **(C)** and TNBC **(D)** breast cancer molecular subtypes, respectively. Marks on graph lines represent censored samples.

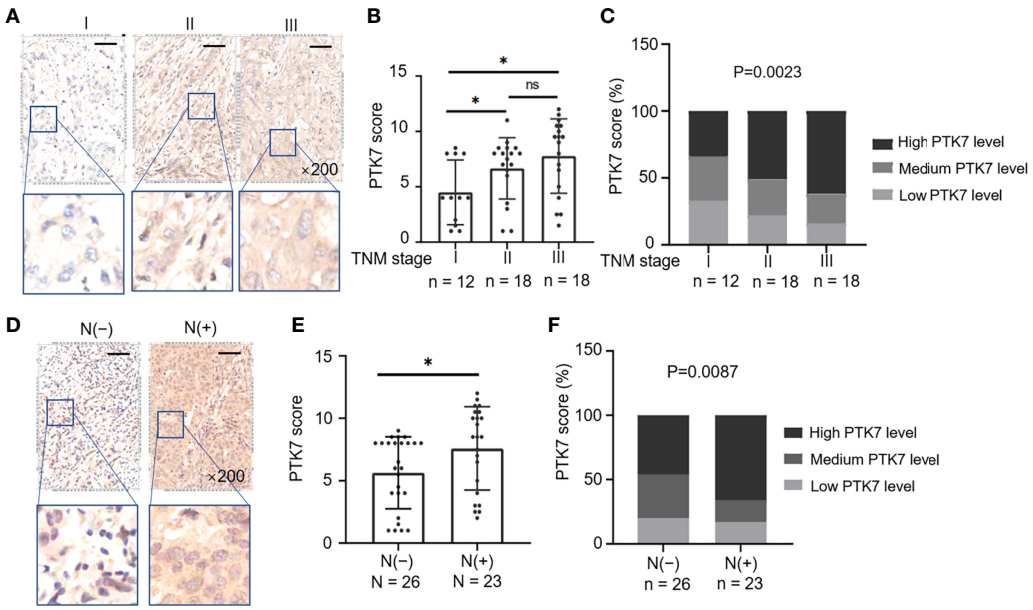

FIGURE 3 | Upregulation of PTK7 is related to metastasis and TNM stage in TNBC. **(A)** TNBC samples from 280 subjects of breast cancer tissue microarray were selected and divided into three groups based on TNM stages (AJCC staging). Representative images of IHC staining of PTK7 expression in the three groups (stage I, II and III) are shown. Magnification, ×200; scale bars, 100 μm. **(B)** Scatter dot plots of PTK7 scores in the three groups described in **(A)**. Data were analyzed using one-way ANOVA and are shown as mean ± s.d. *$P < 0.05$. **(C)** The percentage of cases in the groups described in **(A)**. Data were analyzed using Pearson's χ^2 test. Light grey, low PTK7 level (IHC score 0~4); dark grey, medium PTK7 level (IHC score 5~8); black, high PTK7 level (IHC score 8~12). **(D)** TNBC samples were divided into two groups based on lymph node metastasis. Representative images of PTK7 staining in TNBC with or without lymph node metastasis are shown. Magnification, ×200; scale bars, 100 μm. **(E)** Scatter dot plots of PTK7 scores in the two groups described in **(D)**. Data were analyzed using one-way ANOVA and are shown as mean ± s.d. *$P < 0.05$. **(F)** The percentage of cases in the groups described in **(D)**. Data were analyzed using Pearson's χ^2 test. Light grey, low PTK7 level (IHC score 0~4); dark grey, medium PTK7 level (IHC score 5~8); black, high PTK7 level (IHC score 8~12). ns, no significance.

(http://www.cbioportal.org/). As shown in **Figure 4A**, **Supplementary Figure S2** and **Supplementary Table S1**, 374 PTK7 positively-correlated genes (Spearman's correlation > 0.3, $P < 0.01$) and 289 PTK7-negatively-correlated genes (Spearman's correlation < -0.3, $P < 0.01$) was found. The functions of PTK7 positively-correlated genes were predicted by the analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) by DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp) and 9 pathways related to the functions of PTK7 alterations in invasive breast cancer were found (**Figure 4B**, right panel). PI3K/Akt signaling pathway (hsa04151) and actin cytoskeleton regulation (hsa04810) were significantly enriched in PTK7 positively-correlated genes and the associated genes are listed (**Figure 4B**, left panel). To further investigate function of PTK7 in EGFR-PI3K-Akt signaling pathway in breast cancer, we performed PTK7 and EGFR pair-wise gene correlation analysis using GEPIA (**Figure 4C**) and further confirmed EGFR expression positively correlated with PTK7 (R = 0.42, P = 2e-48). Then, wild type or PTK7-knockdown TNBC cells (MDA-MB-468 and MDA-MB-231) were stimulated with EGF (500 ng/ml) and phosphor-EGFR and phosphor-Akt levels were significantly lower in PTK7-knockdown cells than that in control cells (**Figures 4D, E**), which suggested that PTK7 regulates EGFR/Akt signaling pathway.

PTK7 Is Associated With Extracellular Matrix Organization and Cytoskeleton Remodeling in Breast Cancer Cells

We further investigated Gene Ontology (GO) using DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp). Biological Process (BP) of PTK7 positively- and negatively-correlated genes showed that 16 biological processes, including extracellular matrix organization (GO:0030198), cell adhesion (GO:0007155), actin filament organization (GO:0007015) and positive regulation of cell migration (GO:0030335) were related to PTK7 positively-correlated genes (**Figure 5A**). To further exam the molecular mechanism, pair-wise gene correlation analysis of PTK7 and key migration associated genes in breast cancer were analyzed using GEPIA correlation analysis tool. As shown in **Figure 5B**, PTK7 expression in breast cancer was significantly positively correlated with COL1A1, FN1, WNT5B, MMP11, MMP14 and SDC1 in breast cancer.

To further identify the potential role of PTK7 in TNBC cytoskeleton remodeling, MDA-MB-231 and MDA-MB-468 cells were transduced with shNC, shPTK7#1 or shPTK7#2. F-actin filaments were stained with phalloidin and the result showed that the actin filaments were recruited into thick and long actin bundles aligned along the long axis in shNC MDA-

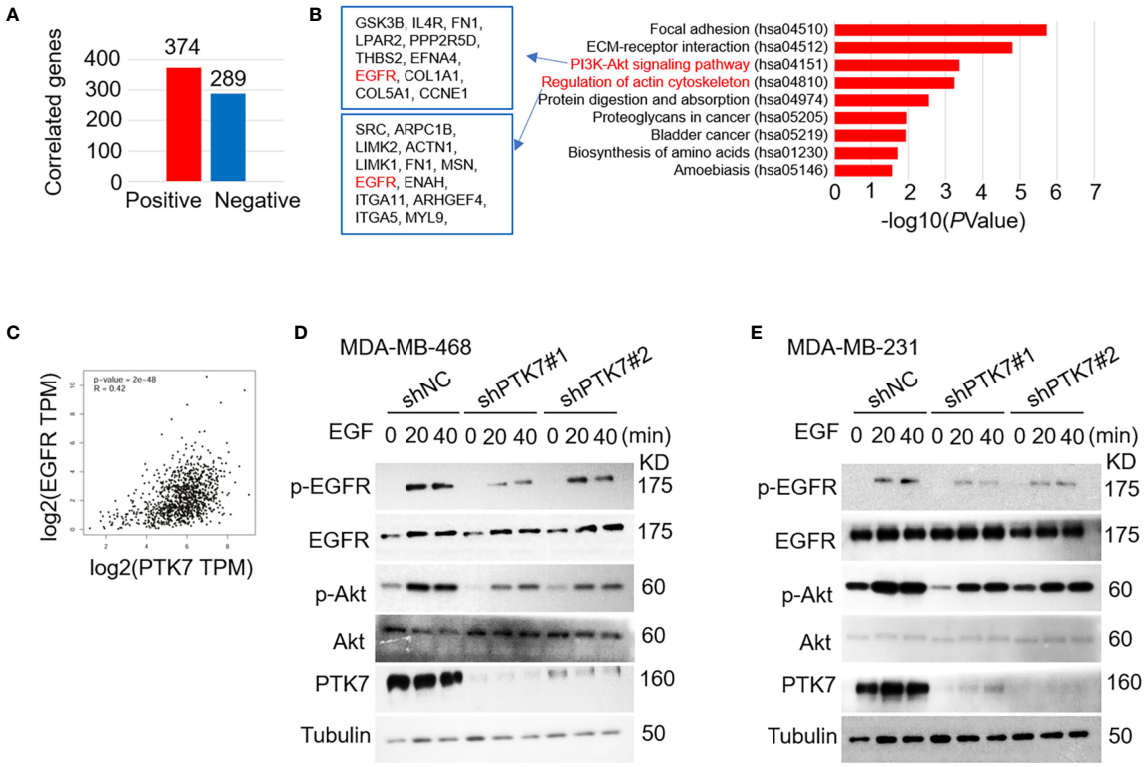

FIGURE 4 | PTK7 regulates EGFR-PI3K-Akt pathway in breast cancer. **(A)** PTK7 co-expression analysis in invasive breast carcinoma using breast cancer datasets including 1,904 patients with Agilent microarray data (http://www.cbioportal.org/) and 374 PTK7 positively- (Spearman's correlation > 0.3, $P < 0.01$) and 289 PTK7 negative- (Spearman's correlation < -0.3, $P < 0.01$) correlated genes were selected and used as candidate genes in the following analysis. **(B)** PTK7 positively-correlated genes were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) by DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp). PTK7 positively correlated genes enriched in PI3K-Akt signaling (hsa04151) and Regulation of actin cytoskeleton (hsa04810) pathways are listed in the frames, respectively. **(C)** PTK7 and EGFR pair-wise gene correlation in breast invasive carcinoma were analyzed using GEPIA Correlation Analysis tools (http://gepia.cancer-pku.cn/detail.php?clicktag=correlation). **(D, E)** MDA-MB-468 **(D)** and MDA-MB-231 **(E)** cells were transduced with a non-targeting control shRNA (shNC) or two different PTK7-specific shRNAs (shPTK71 and shPTK7#2). Cells were stimulated with EGF (500 ng/ml) for 0, 20 or 40 minutes and phospho-EGFR and phosphor-Akt levels were evaluated using immunoblotting assay.

MB-231 and MDA-MB-468 cells; PTK7-knockdown markedly reduced thick stress fibers (**Figure 5C**).

PTK7 Promotes Proliferation and Migration in TNBC Cell Lines

To identify the consequences of PTK7 in TNBC progression, human PTK7 overexpression or knockdown TNBC cell lines MDA-MB-436 and MDA-MB-468 were used and MTT cell proliferation assay were performed. As expected, overexpression of PTK7 in MDA-MB-436 and MDA-MB-468 cells significantly promotes proliferation activity (**Figures 6A, B**) and knockdown of PTK7 resulted in a decrease of cell growth (**Figures 6C, D**). Colony formation assay showed that both the colony numbers and colony diameters significantly decreased in PTK7-knockdown cells (**Figure 6E**). Matrigel pre-coated Boyden chamber was then used to analyze the roles of PTK7 in TNBC cell migration and invasion. Knockdown of PTK7 in MDA-MB-436 and MDA-MB-468 cells exhibited decreased migration ability (**Figures 6F, G**), and overexpression of PTK7 promoted transwell migration in TNBCs (**Supplementary Figure S3**).

PTK7-Deficiency Inhibits TNBC Tumor Growth *In Vivo*

To further analyze the role of PTK7 in TNBC tumorigenicity *in vivo*, shNC, shPTK7#1 and shPTK7#2 stable transduced MDA-MB-468 cells were used to establish TNBC tumor bearing mouse model. The challenged mice were monitored every two days and sacrificed at day 61 after injection (**Figure 7A**). PTK7 knockdown dramatically inhibited tumor growth (**Figures 7B, C**). These results suggested that PTK7 is required for TNBC progression *in vivo*.

DISCUSSION

RTKs, a protein kinase family transducing extracellular signals across the cell membrane, were known to be grouped into 20 subfamilies and play pivotal roles in diverse cellular activities including growth, differentiation, motility, and death (25–28). Many RTKs are involved in oncogenesis (29, 30). PTK7 is a particular member of the RTK family that lacks detectable catalytic tyrosine kinase activity. Although PTK7 plays a role in multiple

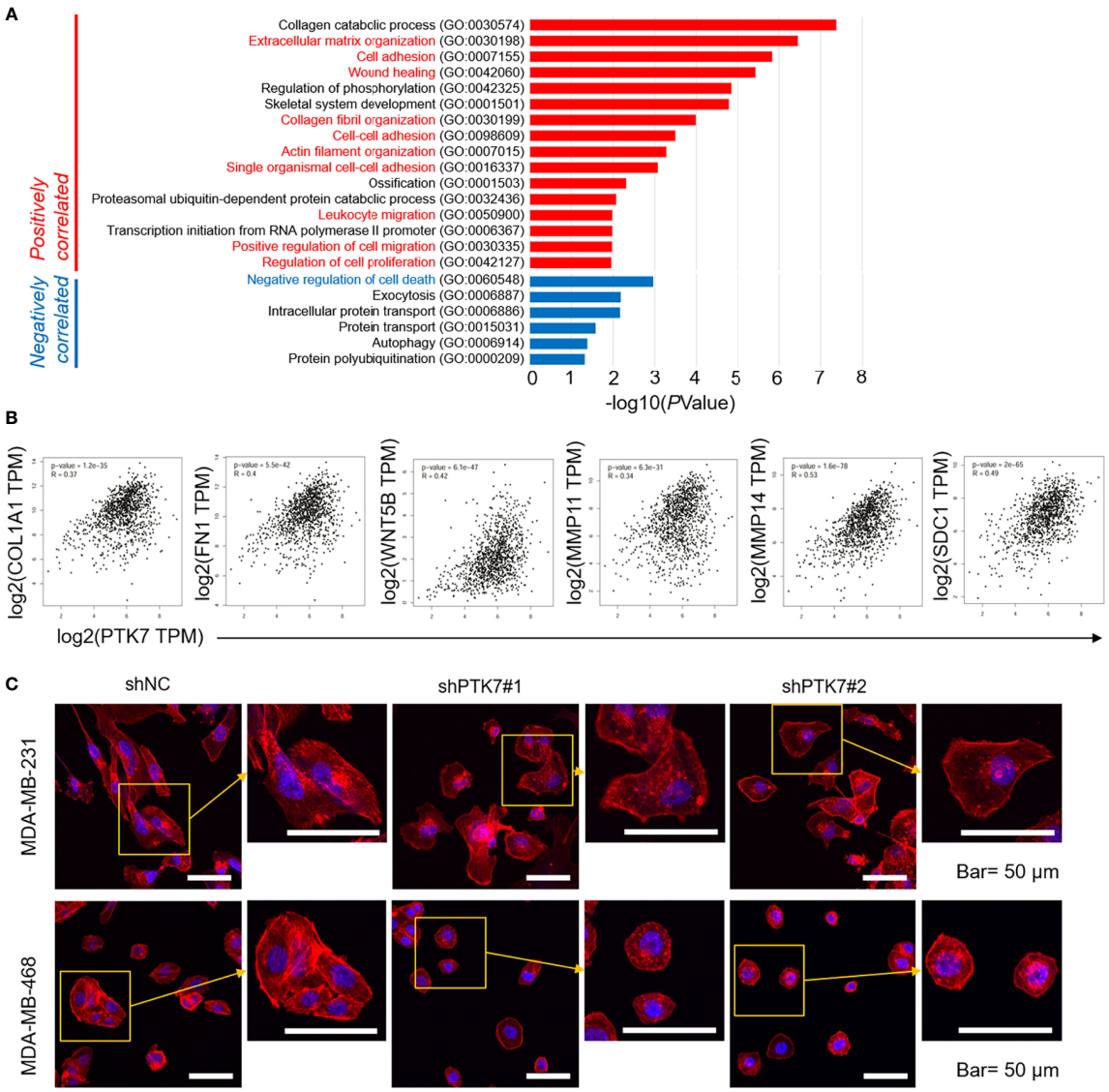

FIGURE 5 | PTK7 associates with extracellular matrix organization and migration in breast cancer cells. **(A)** Gene Ontology (GO) was performed using DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp) and Biological Process (BP) of PTK7 positively- and negatively-correlated genes were shown. **(B)** Pair-wise gene correlation of PTK7 with COL1A1, FN1, WNT5B, MMP11, MMP14 or SDC1 in breast invasive carcinoma were analyzed using GEPIA Correlation Analysis tools (http://gepia.cancer-pku.cn/detail.php?clicktag=correlation). **(C)** MDA-MB-231 and MDA-MB-468 cells were transduced with shNC, shPTK71 or shPTK72. Cells were stained for F-actin with TRITC-phalloidin. Pictures show the TRITC-tagged Phalloidin (red) and DAPI (purple). Presentative images are shown. Magnification, 400×; scale bars, 50 μm.

cellular processes during tumor progression, the definite role of PTK7 in breast cancer progression remains unclear.

A recent meta-analysis of the prognostic value of PTK7 expression in human malignancies revealed that higher expression of PTK7 significantly indicates worse prognosis in human malignancies in 11 studies published with a total sample size of 2431 participants (31). The expression and function of PTK7 in breast cancer have been well investigated, however, controversial results were obtained. Several studies suggested that PTK7 is highly expressed in TNBC cell lines and associates with resistance to anthracycline-based chemotherapy in TNBC (32). PTK7 expression in breast cancer predicts poor prognosis (24). Recent evidence including 79 consecutive invasive breast cancer tissues demonstrated that PTK7 expression is negatively associated with tumor grade and lymph node metastasis and may serve as a tumor suppressor in breast cancer (23).

To reveal the clinical relevance of PTK7 in breast cancer, in the present study, we evaluated breast cancer tissue samples from 280 human subjects and performed tissue microarray IHC staining against PTK7. There was no significant associate of PTK7 expression with TNM stages from totally 280 breast cancer tissues. Interestingly, either correlations of PTK7 expression with

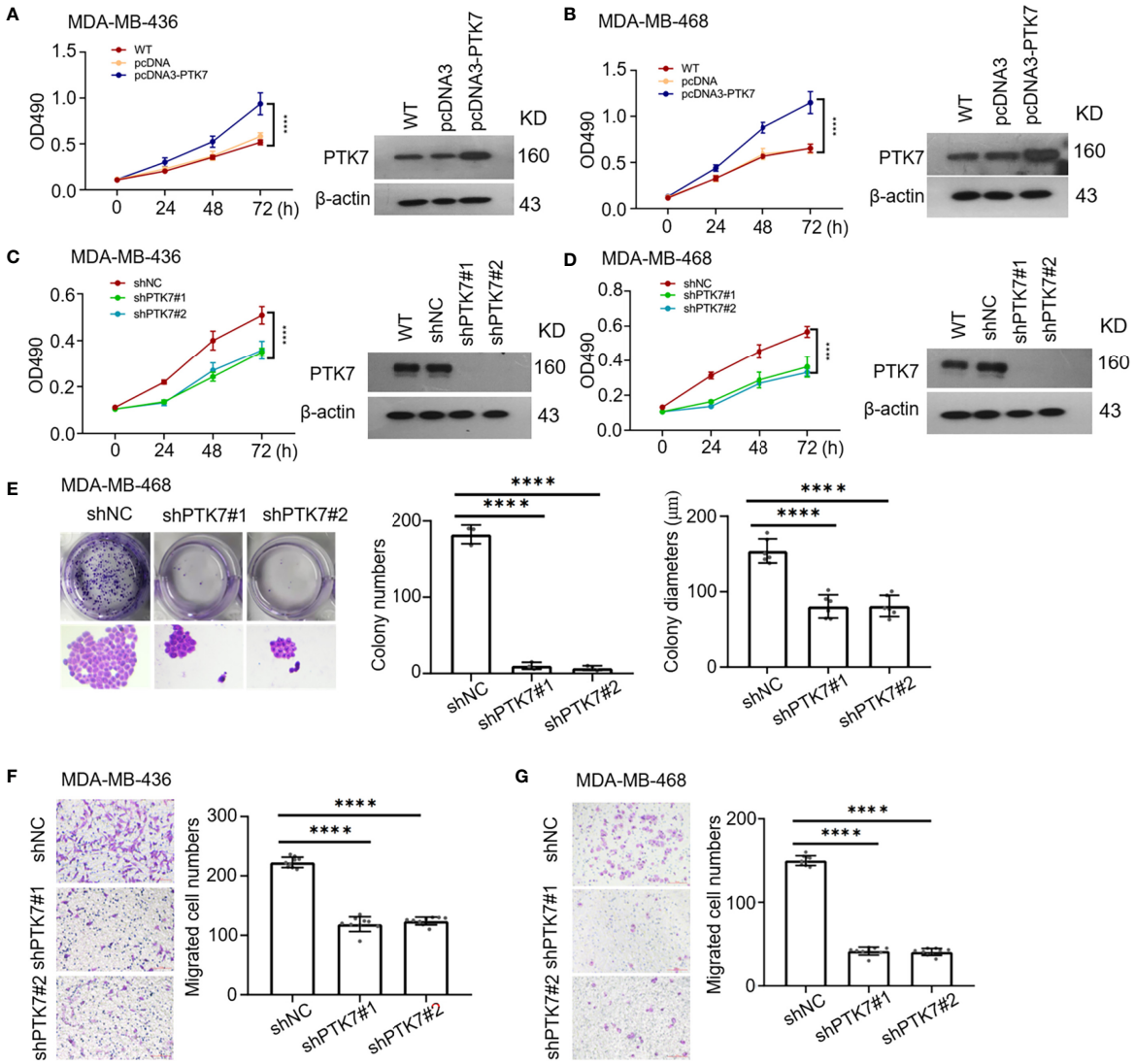

FIGURE 6 | PTK7 participates in cell proliferation and migration in TNBC cell lines. **(A, B)** TNBC cell lines MDA-MB-436 **(A)** and MDA-MB-468 **(B)** were transfected with control vector pcDNA3 or PTK7 expression vector pcDNA3-PTK7 for 48 h and proliferation was evaluated using MTT assay. Data are shown as mean ± s.d. ****$P < 0.0001$. **(C, D)** MDA-MB-436 **(C)** and MDA-MB-468 **(D)** were transduced with a non-targeting control shRNA (shNC) or two different PTK7-specific shRNAs (shPTK71 and shPTK7#2). Cell proliferation was evaluated using MTT assay. Data are shown as mean ± s.d. ****$P < 0.0001$. **(E)** Colony formation assay was performed to determine proliferation in shNC-, shPTK7#1- or shPTK7#2-transduced MDA-MB-468 cells. Presentative images are shown (left) and colony numbers and colony diameters were shown as mean ± s.d. Magnification, ×100; ****$P < 0.0001$. **(F, G)** Transwell migration assay using Boyden chamber in shNC-, shPTK7#1- or shPTK7#2-transduced MDA-MB-436 **(F)** and MDA-MB-468 **(G)** cells was performed and photographed under a light microscope. Presentative images are shown (left) and migrated cells were counted and shown as mean ± s.d. Magnification, ×100; ****$P < 0.0001$.

clinicopathological parameters by tissue microarray IHC staining or online RFS analysis by Kaplan-Meier Plotter (http://www.kmplot.com/) demonstrated that PTK7 expression extraordinarily correlates with worse prognosis in ER/PR/HER2-negative (TNBC) breast cancer, which suggested a special relationship of PTK7 expression with worse prognosis in TNBC. The function of PTK7 in breast cancer exhibits heterogeneity in multiple molecular subtypes may due to different cell context and intracellular signaling mechanisms.

Compared with Luminal A, Luminal B and HER2(+) breast cancer subtypes, patients with TNBC were always recognized to have the worst overall survival data due to its rapid progression, high probabilities of early recurrence, and distant metastasis resistant to standard treatment (33). According to the present data, TNBC with high PTK7 expression level predicts worse outcome. KEGG analysis and PTK7 gain- or loss-of-function TNBC cell lines revealed that PTK7 regulates EGFR/Akt signaling pathway. GO assay further demonstrated PTK7

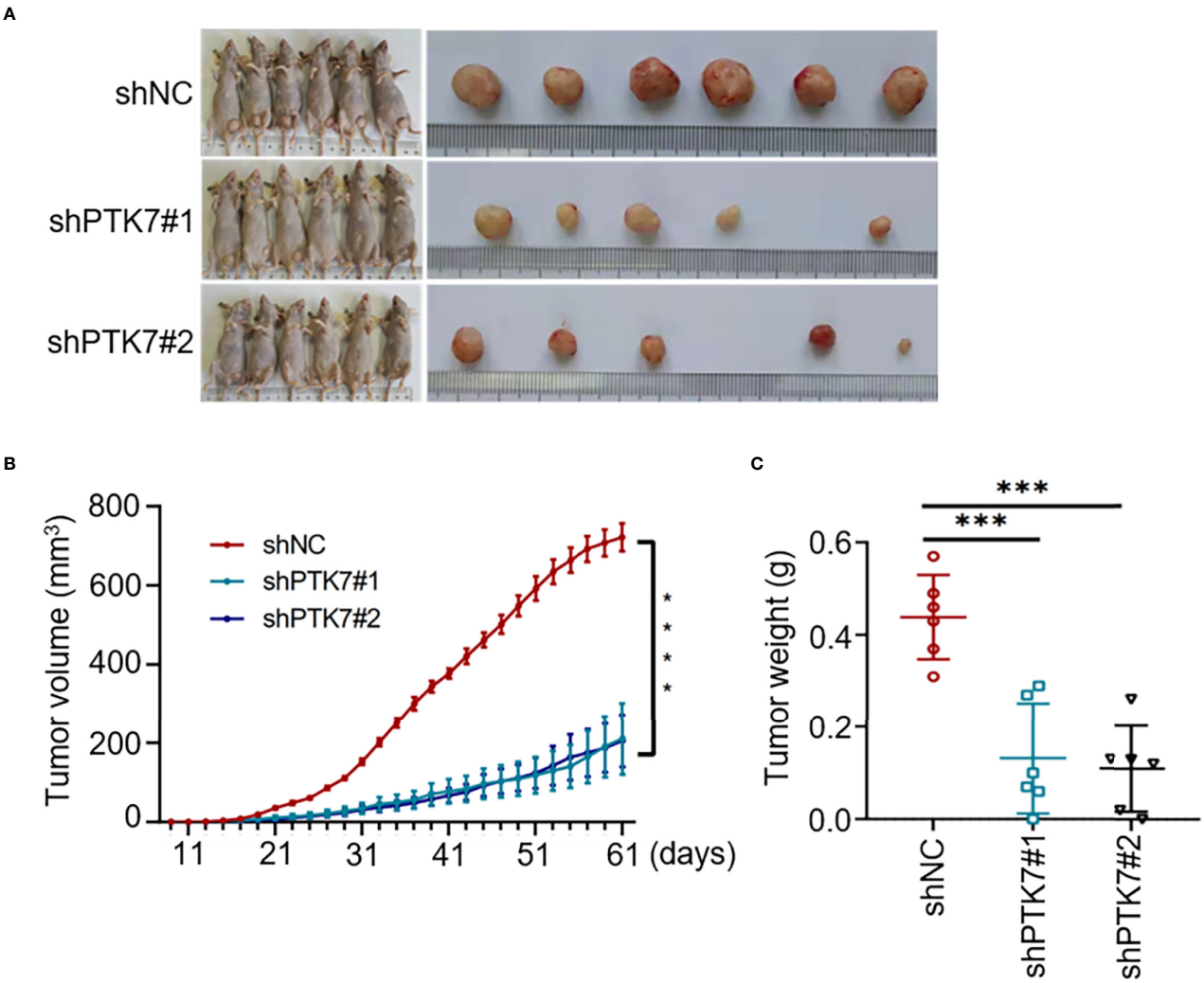

FIGURE 7 | PTK7 promotes in *vivo* TNBC tumorigenicity. **(A)** Nude mice were injected into the mammary fat pad with shNC-, shPTK7#1- or shPTK7#2-transduced MDA-MB-468 cells at 5×10^5 cells per site. Mice were sacrificed and tumors were photographed 61 days after subcutaneous injection. **(B)** Tumor volume was monitored every 2 days after injection and tumor growth curve is shown. Tumor volume was calculated by the formula: V = 1/2×*a* (length)×b^2 (width). **(C)** The harvest tumor weight was shown as mean ± s.d. ***$P < 0.001$; ****$P < 0.0001$.

participates in extracellular matrix organization and migration in TNBC cells. A recent study revealed that PTK7 expression is associated with EGFR mutations and plays an oncogenic role in lung adenocarcinomas (18). The role of PTK7-targeted antibody-drug conjugate has been investigated in several solid tumors, including TNBC, and exhibits potential therapeutic activity (34–36). In addition, our present data demonstrated that loss of PTK7 expression in TNBC cells results in a downregulated EGFR/Akt signaling and reduced tumor growth in MBA-MD-468 TNBC cancer xenografts. These findings may have significant implicants for the treatment of TNBC *via* targeting PTK7.

Taken together, this study identified PTK7 as a potential marker of worse prognosis in TNBC. PTK7 promotes extracellular matrix organization and migration *via* EGFR/PI3K/Akt signaling pathway in TNBC. Strategies targeting PTK7 may inform the development of novel therapies to fight against TNBC. To further define the independent predictive role and targeted therapy strategy of PTK7 in TNBC, a larger sample of patients with TNBC treatment information should be investigated.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GEPIA: Gene Expression Profiling Interactive Analysis system (http://gepia.cancer-pku.cn/) cBioPortal for Cancer Genomics (http://www.cbioportal.org/) Kaplan-Meier Plotter (http://www.kmplot.com/) DAVID: Functional Annotation Tools (https://david.ncifcrf.gov/tools.jsp).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Affiliated Hospital of Hebei University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Welfare and Ethical Committee of Hebei University.

AUTHOR CONTRIBUTIONS

N-PC, SQ, SJ, and J-HS designed and carried out the experiments. SQ and SJ analyzed the data. N-PC, SQ, YQ, Y-NW, and L-SZ performed immunohistochemical staining, tumor xenograft. SJ, J-LH, T-TW, and W-WL performed cell culture, gene silencing, immunoblotting, actin cytoskeleton staining, cell proliferation assays, cell proliferation and invasion assays. J-HS, N-PC, SQ, SJ, and Y-NW analyzed and interpreted the data. J-HS, J-CZ, Y-PM, and B-PC provided supervision and guidance. J-HS, Y-PM, and B-PC 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.2021.699889/full#supplementary-material

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A Novel Seven Gene Signature-Based Prognostic Model to Predict Distant Metastasis of Lymph Node-Negative Triple-Negative Breast Cancer

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Background: The prognosis of lymph node-negative triple-negative breast cancer (TNBC) is still worse than that of other subtypes despite adjuvant chemotherapy. Reliable prognostic biomarkers are required to identify lymph node-negative TNBC patients at a high risk of distant metastasis and optimize individual treatment.

Methods: We analyzed the RNA sequencing data of primary tumor tissue and the clinicopathological data of 202 lymph node-negative TNBC patients. The cohort was randomly divided into training and validation sets. Least absolute shrinkage and selection operator Cox regression and multivariate Cox regression were used to construct the prognostic model.

Results: A clinical prognostic model, seven-gene signature, and combined model were constructed using the training set and validated using the validation set. The seven-gene signature was established based on the genomic variables associated with distant metastasis after shrinkage correction. The difference in the risk of distant metastasis between the low- and high-risk groups was statistically significant using the seven-gene signature (training set: $P < 0.001$; validation set: $P = 0.039$). The combined model showed significance in the training set ($P < 0.001$) and trended toward significance in the validation set ($P = 0.071$). The seven-gene signature showed improved prognostic accuracy relative to the clinical signature in the training data (AUC value of 4-year ROC, 0.879 *vs.* 0.699, $P = 0.046$). Moreover, the composite clinical and gene signature also showed improved prognostic accuracy relative to the clinical signature (AUC value of 4-year ROC: 0.888 *vs.* 0.699, $P = 0.029$; AUC value of 5-year ROC: 0.882 *vs.* 0.693, $P = 0.038$). A nomogram model was constructed with the seven-gene signature, patient age, and tumor size.

Conclusions: The proposed signature may improve the risk stratification of lymph node-negative TNBC patients. High-risk lymph node-negative TNBC patients may benefit from treatment escalation.

Keywords: triple-negative breast cancer, distant metastasis, prognostic biomarker, modeling, transcriptomics

INTRODUCTION

Breast cancer is estimated to be the most common cancer diagnosed in women and the second leading cause of cancer-related death in the United States in 2021 (1). Triple-negative breast cancer (TNBC) is characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), representing 10%-20% of all breast cancers (2, 3). TNBC is more likely to show lymph node involvement at diagnosis and exhibit invasive and metastatic tendencies (2, 4). Nonetheless, the incidence of lymph node-negative TNBC has markedly increased owing to early detection and initiated screening programs (5–8).

To date, lymph node-negative TNBC is generally considered at moderate risk of disease recurrence and is often recommended for adjuvant chemotherapy (9). Small lymph node-negative tumors tend to have an excellent prognosis without chemotherapy (10). However, the risk of metastasis and death of partial lymph node-negative TNBC patients is still high despite the high proportion of adjuvant chemotherapy (2, 11, 12). A more quantitative approach is required to inform the risk of distant metastasis and individualized treatment in lymph node-negative TNBC.

Several multigene assays have been developed to facilitate prognosis prediction and treatment planning in early-stage breast cancer, but most of the enrolled patients are hormone receptor-positive (13–15). Although many publications have attempted to identify gene signatures that predict the prognosis of TNBC patients, several limitations need to be considered due to the limited sample size and incomplete follow-up information (16–20). Above all, most previous studies include all TNBC patients as a cohort. Because lymph node status is a well-known prognostic value, there is an urgent need to identify a robust risk stratification tool for lymph node-negative TNBC patients (21, 22). Based on detailed clinicopathological information, well-documented follow-up, and complete RNA-sequencing data, we constructed a gene expression-based prognostic signature combined with clinicopathological factors to provide quantitative predictions of short- and long-term disease outcomes for Chinese lymph node-negative TNBC patients.

MATERIALS AND METHODS

Patient Samples and Study Design

We included 202 eligible patients from our previously published cohort of 465 primary TNBC patients treated at Fudan University Shanghai Cancer Center (FUSCCTNBC) (23). Patients were included based on the following criteria: histologic diagnosis of lymph node-negative TNBC with RNA-sequencing data and follow-up information for recurrence and metastasis. The RNA-sequencing data are available in the Sequence Read Archive (RNA-seq: SRP157974). Patients with contralateral breast cancer, lymph node recurrence, and unknown sites of recurrence were excluded. Lymph node status was independently confirmed by two experienced pathologists. The date of diagnosis of metastasis was defined when metastasis was either confirmed by biopsy or clinically diagnosed. The follow-up of this cohort was completed on June 11, 2019. Distant metastasis-free survival (DMFS) was defined as the interval between diagnosis and the first distant metastasis (viscera/bone/brain). Patients without events were censored from the time point of the last follow-up.

Ethics Statement

The present study was reviewed and approved by the Ethics Committee of Fudan University Shanghai Cancer Center (Ethics number: 050432-4-1212B). The patients provided written informed consent to participate in this study.

Gene Selection and Risk-Score Algorithm

To identify mRNAs of prognostic value, analysis for differentially expressed mRNAs between two groups was performed using the *limma* package (version 3.48.0) in R software. We also performed Gene Set Enrichment Analysis (GSEA) of differentially expressed genes between the two groups with or without distant metastasis using the RNA-sequencing data and GSEA software (GSEA_4.1.0) (24, 25).

The cohort was randomly divided into the training set (n=142) and validation set (n=60) at a ratio of 7 to 3 by the *caret* package (version 6.0-88) in R software. Pearson chi-square test or Fisher's exact test was used to ensure that there was no significant difference and that no bias was introduced in clinicopathological characteristics between the two sets. Least absolute shrinkage and selection operator (LASSO) Cox regression analysis was performed to further filter the differentially expressed mRNAs. A multivariate Cox regression model was used to determine the coefficient of each factor. The risk score of each model was used to estimate the probability of distant metastasis. The genomic risk score was calculated from individual gene expression measurements as follows: Genomic risk score = ($\beta_{B3GALT5\text{-}AS1} \times$ *B3GALT5-AS1*) + ($\beta_{DNER} \times$ *DNER*) + ($\beta_{CSN1S1} \times$ *CSN1S1*) + ($\beta_{KIF5A} \times$ *KIF5A*) + ($\beta_{SIX3} \times$ *SIX3*) + ($\beta_{NOTUM} \times$ *NOTUM*) + ($\beta_{CPS1} \times$ *CPS1*). The clinical risk score was calculated as follows: Clinical risk score = $\beta_{\text{Age}} \times$ Age (years)+ $\beta_{\text{Tumor size}} \times$ Tumor size (cm). The combined risk score was calculated as follows: Combined risk score = $\beta_{\text{Gene score}} \times$ Genomic risk score + $\beta_{\text{Clinical score}} \times$ Clinical risk score.

Validation of Different Prognostic Models

Patients were stratified into high- and low-risk groups based on optimum cutoff risk scores determined by the "surv_cutpoint" function in the *survminer* package (version 0.4.9) in R software. Kaplan-Meier analyses and log-rank tests were performed to assess the differences in DMFS between the high- and low-risk groups. The time-dependent receiver operating characteristic (ROC) curve was used to measure the prognostic performance by comparing the area under the ROC curve (AUC) values.

Construction and Validation of a Nomogram Model

Based on data availability and clinical evidence (9, 26, 27), a nomogram was constructed integrating the seven-gene risk score, age of the patients at surgery, and pathological tumor size. We measured the predictive accuracy of the nomogram *via* Harrell's concordance index (C-index) in the training and validation sets. In addition, the predictive capacity of the nomogram was also evaluated using calibration curve and decision curve analysis (DCA).

Statistical Analysis

Pearson's chi-square test or Fisher's exact test was used to compare the clinical and pathological characteristics between the training set and validation set. All statistical analyses were performed using the SPSS 22.0 (SPSS Inc.) or R software (version 4.1.0, www.r-project.com). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

The clinical and pathological characteristics of 202 patients and their primary tumors are summarized in **Table 1**. Of 202 lymph node-negative TNBC patients, the median follow-up was 68.2 months (interquartile range, 57.6-80.6 months). Overall, 12 (5.9%) cases with distant metastasis were observed. Of the 12 patients, 4 (33.3%) patients had multisite metastasis, and 7 (58.3%) patients died due to breast cancer during follow-up. The median tumor size and age of the patients at surgery in this study cohort were 2.5 centimeters (range 0.8-12.0) and 53 years (range 25-82), respectively.

TABLE 1 | Clinicopathological characteristics of patients and their tumors.

Characteristics	Number of patients (%)			P^a
	Whole set	Training set	Validation set	
Age, years				0.865
≤50	86 (42.6%)	61 (43.0%)	25 (41.7%)	
>50	116 (57.4%)	81 (57.0%)	35 (58.3%)	
Menopausal status				0.468
Premenopausal	75 (37.1%)	55 (38.7%)	20 (33.3%)	
Postmenopausal	127 (62.9%)	87 (61.3%)	40 (66.7%)	
Histological grade				0.183
I	35 (17.3%)	27 (19.0%)	8 (13.3%)	
II	13 (6.4%)	9 (6.3%)	4 (6.7%)	
III	134 (66.3%)	96 (67.6%)	38 (63.3%)	
Unknown	20 (9.9%)	10 (7.0%)	10 (16.7%)	
Tumor size				0.239
≤2cm	85 (42.1%)	64 (45.1%)	21 (35.0%)	
>2-5cm	111 (55.0%)	75 (52.8%)	36 (60.0%)	
>5cm	6 (3.0%)	3 (2.1%)	3 (5%)	
Ki-67				0.820
≤20%	28 (13.9%)	20 (14.1%)	8 (13.3%)	
>20%	169 (83.7%)	119 (83.8%)	50 (83.3%)	
Unknown	5 (2.5%)	3 (2.1%)	2 (3.3%)	
Chemotherapy				0.644
No	6 (3.0%)	4 (2.8%)	2 (3.3%)	
Yes	188 (93.1%)	131 (92.3%)	57 (95.0%)	
Unknown	8 (4.0%)	7 (4.9%)	1 (1.7%)	
Radiotherapy				0.861
No	180 (89.1%)	127 (89.4%)	53 (88.3%)	
Yes	21 (10.4%)	14 (9.9%)	7 (11.7%)	
Unknown	1 (0.5%)	1 (0.7%)	0 (0.0%)	
Metastasis				0.345
No	190 (94.1%)	135 (95.1%)	55 (91.7%)	
Yes	12 (5.9%)	7 (4.9%)	5 (8.3%)	

[a]P values were calculated using Pearson's chi-square test or Fisher's exact test to compare the clinical and pathological characteristics between the training set and validation set.

Construction and Validation of the Novel Seven-Gene Signature

An overview of the study design is shown in **Figure 1**. Using $\log_2$ (fold change) > 1 or < -1 and $P < 0.05$, we identified 71 differentially expressed mRNAs between the two groups with or without distant metastasis. We also performed Gene Set Enrichment Analysis of differentially expressed genes between the two groups with or without distant metastasis using the RNA-sequencing data. In patients with distant metastasis, 25 gene sets were significantly enriched at nominal P value < 0.05. The top ten gene sets enriched in 12 lymph node-negative TNBC patients with distant metastasis compared to 190 patients without distant metastasis were illustrated in **Figure S1**. In patients with distant metastasis, 56 mRNAs were upregulated, whereas 15 mRNAs were downregulated (**Figure 2**). We constructed a matrix integrating RNA-sequencing data of 71 differentially expressed mRNAs and clinicopathological data of all 202 patients. Next, patients were randomly classified into the training set (n = 142) and validation set (n = 60). There was no difference in all characteristics between the training and internal validation sets (**Table 1**). Seven genes, including *B3GALT5-AS1*, *DNER*, *CSN1S1*, *KIF5A*, *SIX3*, *NOTUM*, and *CPS1*, were selected using the LASSO Cox regression model in the training set. The summary of $\log_2$(fold change), multivariable Cox regression coefficient, hazard ratio, 95% confidence interval, and P value for selected genes are presented in **Table 2**. Time-dependent ROCs and Kaplan–Meier curves were used to evaluate the prognostic potential of the seven-gene signature for DMFS (**Figures 3A, B**). The AUC values for 3-, 4-, and 5-year DMFS were 0.823, 0.879, and 0.870 in the training set and 0.727, 0.705, and 0.689 in the validation set, respectively (**Figure 3A**). The formula of genomic risk score is as follows: genomic risk score = 0.18801037 × *DNER* + 0.28358112 × *CSN1S1* + 0.36011127 × *KIF5A* + 0.57677377 × *SIX3* + 0.70105693 × *NOTUM* + 0.74508978 × *CPS1* - 0.06761698 × *B3GALT5-AS1*. Patients were stratified into high- (n = 15) and low-risk groups (n = 127) by selecting the optimal cutoff value (1.78) in the training set (**Figures 3B, C**). Using the same cutoff value (1.78), the patients

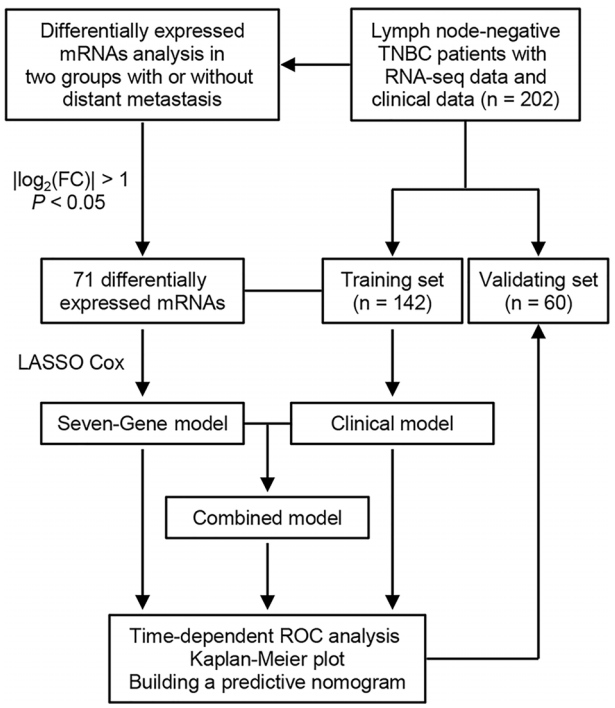

FIGURE 1 | Flowchart of study design. TNBC, triple-negative breast cancer; FC, fold change; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic.

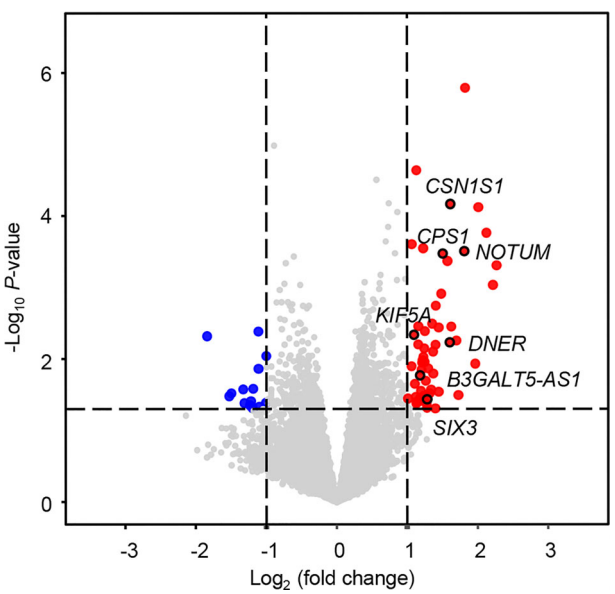

FIGURE 2 | Volcano plot for differentially expressed mRNAs between patients with and without distant metastasis. In total, 71 differentially expressed mRNAs were screened out with $\log_2$(fold change) > 1 or < -1 and $P < 0.05$. Significantly upregulated and downregulated mRNAs are shown as red and blue dots, respectively.

TABLE 2 | Genes included in the seven-gene prognostic signature.

Gene symbol	Log_2 FC[a]	Coefficient[b]	HR (95% CI)[b]	P[b]
B3GALT5-AS1	1.18	-0.06761697	0.93 (0.41-2.15)	0.87
DNER	1.60	0.18801037	1.21 (0.39-3.73)	0.74
CSN1S1	1.61	0.28358112	1.33 (1.03-4.30)	0.11
KIF5A	1.10	0.36011127	1.43 (0.79-2.61)	0.24
SIX3	1.28	0.57677377	1.78 (0.92-3.44)	0.09
NOTUM	1.81	0.70105693	2.02 (1.22-3.33)	0.01
CPS1	1.51	0.74508978	2.11 (1.03-4.30)	0.04

FC, fold change; HR, hazard ratio; CI, confidence interval.
[a]The difference in the expression of seven genes between the group with and without distant metastasis was calculated using the limma package in R software.
[b]The coefficients, hazard ratios, 95% confidence intervals, and P values of seven genes were calculated using a multivariate Cox proportional hazards regression model.

were also divided into high-risk (n = 8) and low-risk (n = 52) groups in the validation set (**Figures 3B, C**). The Kaplan-Meier analyses for DMFS as a function of the seven-gene signature showed highly significant differences between the high- and low-risk groups (**Figure 3B**, $P < 0.001$ in the training set; $P = 0.039$ in the validation set).

Construction and Validation of the Combined Gene and Clinical Model

We also created a clinical prognostic model using the following clinically significant predictors: age and tumor size. The summary of multivariable Cox regression coefficient, hazard ratio, 95% confidence interval, and P value for age and tumor size are presented in **Table S1**. The formula of clinical risk score is as follows: clinical risk score = 0.21532 × Tumor size (cm) - 0.04466 × Age (years). The AUC values of the clinical model for 3-, 4-, and 5-year DMFS were 0.755, 0.699, and 0.693 in the training set and 0.574, 0.651, and 0.631 in the validation set, respectively (**Figure 4A**). The genomic risk score remained an independent prognostic factor in the multivariate Cox analysis after adjusting for patient age and tumor size in both the training set (hazard ratio = 2.64, 95% CI: 1.76-3.96, $P < 0.001$) and validation set (hazard ratio = 1.63, 95% CI: 1.07-2.49, $P = 0.02$). The combined risk score was derived from the genomic and clinical risk score as follows: combined risk score = 0.9702 × Genomic risk score + 1.0854 × Clinical risk score. After integrating the clinical model with the genomic risk score, the AUC values for 3-, 4-, and 5-year DMFS were 0.836, 0.888, and 0.882 in the training set, respectively (**Figure 4B**). The AUC values of the combined model remained high in the validation set with values of 0.801, 0.793, and 0.768 for 3-, 4-, and 5-year DMFS, respectively (**Figure 4B**). Patients were stratified into high- (n = 15 or 9) and low-risk groups (n = 127 or 51) in the training set or validation set (**Figure 4C**). The Kaplan-Meier analyses for DMFS as a function of the combined model showed a significant difference between the high- and low-risk groups in the training set (**Figure 4C**, $P < 0.001$). Likewise, the trend was also observed in the validation set (**Figure 4C**, $P = 0.071$).

Construction and Validation of a Predictive Nomogram

We integrated the seven-gene signature with age and tumor size to construct a prognostic nomogram in the training set (**Figure 5A**). The C-index value for the combined models was

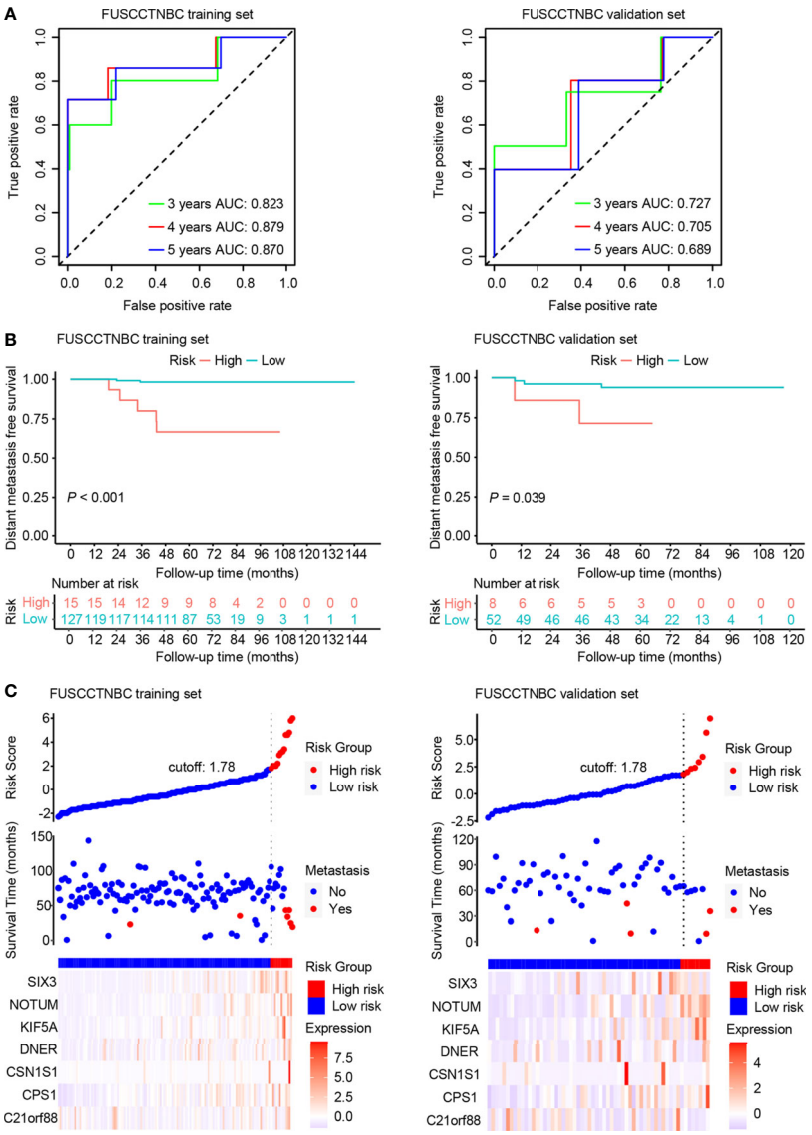

FIGURE 3 | Time-dependent receiver operating characteristic (ROC), Kaplan–Meier survival analysis, and risk score analysis for the seven-gene signature in the training set and validation set of the lymph node-negative triple-negative breast cancer (TNBC) cohort. AUC, area under the curve. **(A)** Time-dependent ROC curves of the seven-gene signature for 3-, 4-, and 5-year distant metastasis-free survival (DMFS). **(B)** Kaplan–Meier plots of the seven-gene signature illustrating that the patients in the high-risk group showed poorer DMFS than those in the low-risk group. **(C)** Distribution of genomic risk score, DMFS status of patients, and heat map of seven differentially expressed mRNA expression profiles.

0.874 in the training set and 0.805 in the validation set. The 4- and 5-year time-dependent ROC curves for the seven-gene, clinical, and combined models are illustrated in **Figure 5B**. Both the seven-gene model and combined model showed better prognostic performance than the clinical model for predicting 4-year DMFS (P = 0.046 for the gene model; P = 0.029 for the combined model). The combined model showed significantly better prognostic performance than the clinical model for predicting 5-year DMFS (P = 0.038), and the seven-gene model also trended toward significance (P = 0.065). The calibration analysis of the 4-year DMFS prediction is shown in **Figure 5C**. The solid blue line has a closer fit to the dotted gray line, indicating great predictive accuracy of the nomogram. Decision curve analysis (DCA) revealed that compared to the clinical model, the seven-gene model and combined model were superior in predicting 4-year DMFS (**Figure 5D**).

DISCUSSION

We constructed a novel seven-gene signature (*B3GALT5-AS1*, *DNER*, *CSN1S1*, *KIF5A*, *SIX3*, *NOTUM*, and *CPS1*) and a combined prognostic model integrating a seven-gene signature with patient age and tumor size to quantify the likelihood of

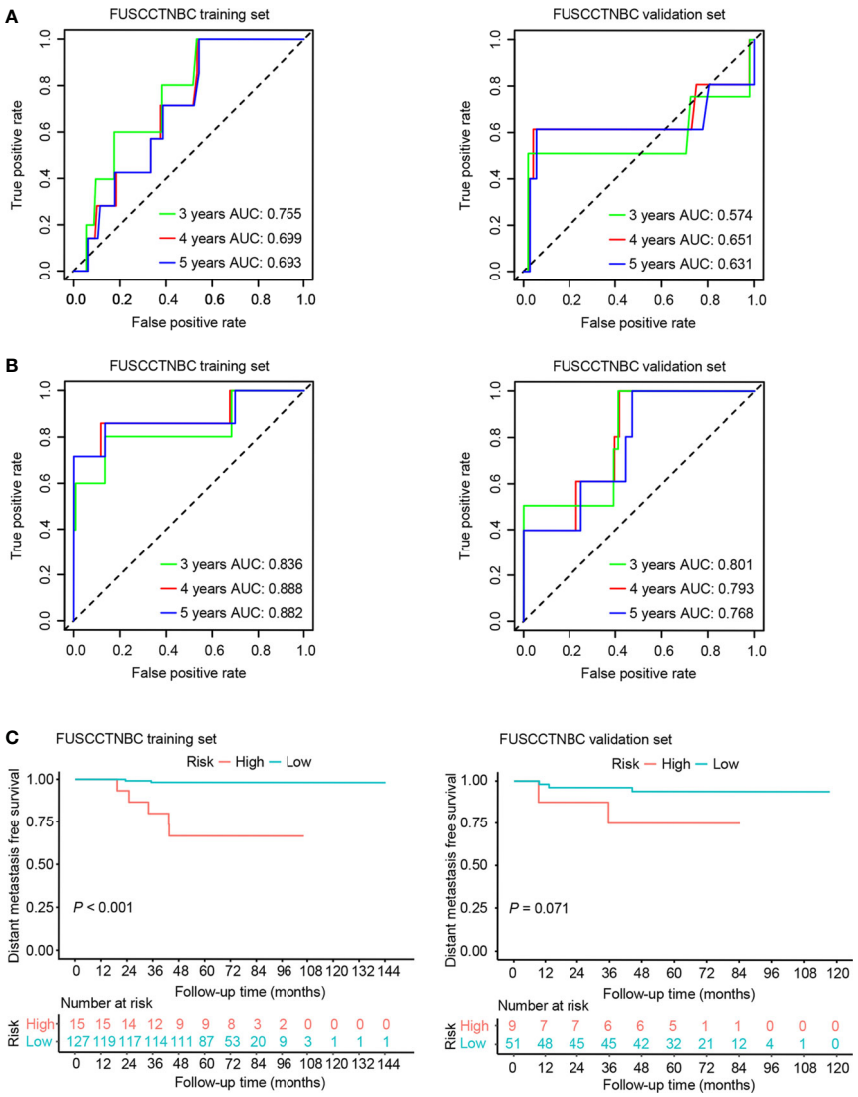

FIGURE 4 | Time-dependent receiver operating characteristic (ROC) and Kaplan–Meier survival analysis for the clinical model and combined model in the training set and validation set of the lymph node-negative triple-negative breast cancer (TNBC) cohort. AUC, area under the curve. **(A)** Time-dependent ROC curves of the clinical model for 3-, 4-, and 5-year distant metastasis-free survival (DMFS). **(B)** Time-dependent ROC curves of the combined model for 3-, 4-, and 5-year DMFS. **(C)** Kaplan–Meier plots of the combined model illustrating that the patients in the high-risk group showed poorer DMFS than those in the low-risk group.

distant metastasis in lymph node-negative TNBC. Both the seven-gene signature and the combined prognostic model had higher AUC values for 4- and 5-year survival than the clinical model. Patients were divided into low- and high-risk groups based on optimal cutoff values. Compared to the low-risk group, patients in the high-risk group had significantly poorer DMFS in both the training set and validation set. Finally, we constructed a prognostic nomogram and validated it in an internal validation set.

Several multigene assays have been employed in breast cancer, including the 76-gene signature, MammaPrint® (70-gene profile), Breast Cancer Index (BCI) test, Oncotype® DX Breast Recurrence Score (RS), EndoPredict® (EP), and Prosigna® (Risk Of Recurrence, ROR) (13, 28–32). None of the above is specifically designed and validated for TNBC patients. Most previous prognostic evaluation studies have focused on all TNBC patients (20, 33–37). One publication has reported the first validated proteomic signature of lymph node-negative TNBC patients (38), but all patients involved in this study were adjuvant treatment-naive, differing from actual clinical practice. The present study focused only on lymph node-negative TNBC patients with more than 90% of patients receiving adjuvant treatment. Apart from the study cohort, the flowchart to construct the gene signature in our study differed from previous studies. The seven differentially expressed mRNAs between the two groups with or without distant metastasis were utilized in our study, while we constructed our previous integrated mRNA-lncRNA signature after comparing the

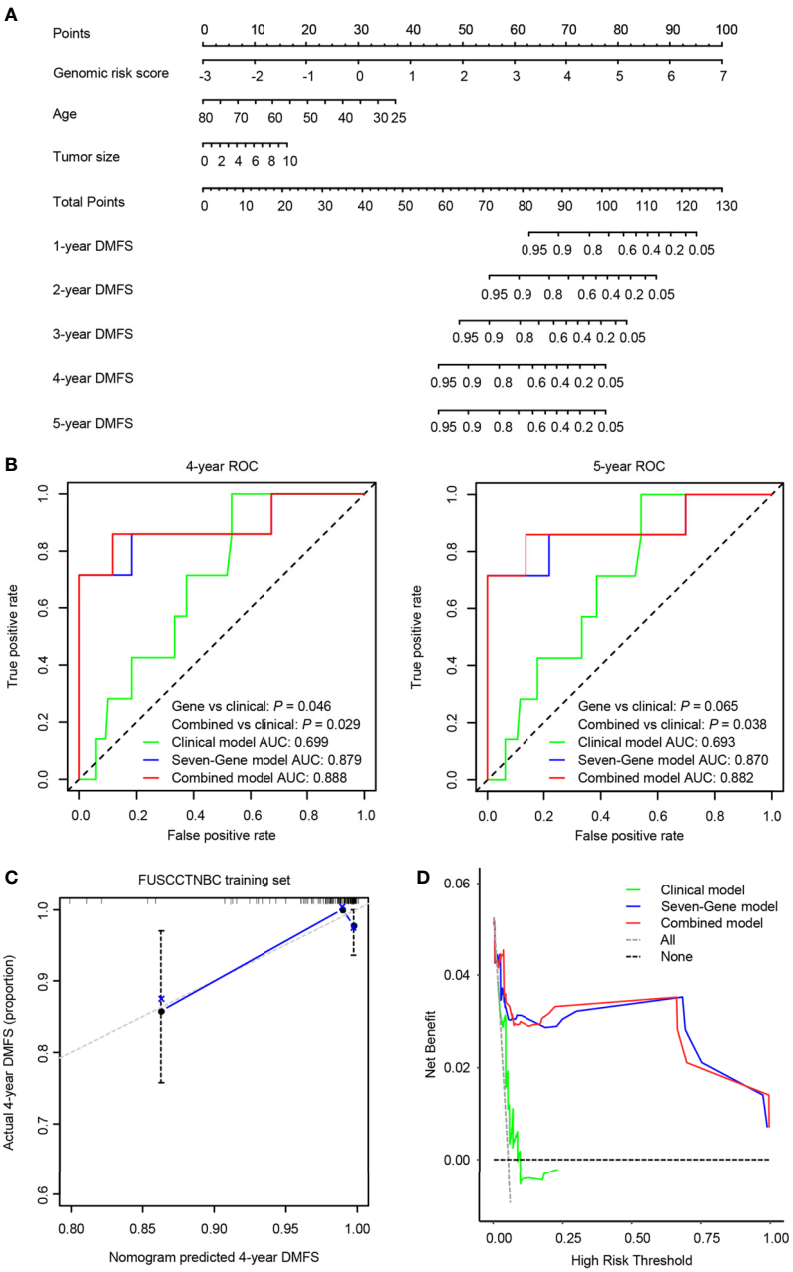

FIGURE 5 | A predictive nomogram was established in the training set. AUC, area under the curve. **(A)** The nomogram was built by the seven-gene risk score and clinical characteristics, including age and tumor size. **(B)** The time-dependent receiver operating characteristic (ROC) curves of the seven-gene model, clinical model, and combined model for 4- and 5-year distant metastasis-free survival (DMFS). The combined model was better than the clinical model for predicting 4-year (P = 0.029) and 5-year (P = 0.038) DMFS. **(C)** Calibration plots of the nomogram for 4-year DMFS. **(D)** Decision curve analysis (DCA) of the seven-gene model, clinical model, and combined model for 4-year DMFS.

tumor tissues with the paired normal tissues as in most previous studies (39, 40). Therefore, genes selected for model development in the present study correlated more closely to prognosis based on well-documented follow-up information. Although more than 90% of patients received adjuvant chemotherapy in our cohort, the high-risk groups classified by the seven-gene signature and combined model presented poor DMFS within four years after surgery. Chemotherapy escalation may be required for these patients.

Among the seven genes, *B3GALT5-AS1* was the only RNA gene. A previous study has revealed the suppressive roles of the B3GALT5-AS1/miR-203/epithelial-mesenchymal transition (EMT) regulation axis in colon cancer liver metastasis (41). Similarly, *B3GALT5-AS1* was the only gene with a negative

correlation coefficient in the present study. Delta/Notch-like EGF repeat containing (*DNER*) is a transmembrane protein that regulates EMT to enhance the proliferation and metastasis of breast cancer cells *via* the Wnt/β-catenin pathway (42). The other three genes, *SIX3*, *NOTUM*, and *CPS1*, have also been reported in other types of tumors. A systematic meta-analysis of non-small cell lung cancer has indicated that higher expression of SIX homeobox 3 (*SIX3*) is associated with a greater probability of tumorigenesis and a higher TNM stage (43). *NOTUM* acts as a key negative regulator of the Wnt signaling pathway, and knockdown of *NOTUM* genes inhibits the proliferation and migration of colorectal cancer cells (44). Previous studies have demonstrated that *CPS1* expression is upregulated in glioblastoma multiforme and that overexpression of *CPS1* is associated with poor therapeutic response and adverse outcomes among rectal cancer patients receiving concurrent chemoradiotherapy (45, 46). Inconsistent with our study, Mou et al. found a positive correlation between the lower expression of *CSN1S1* and patients surviving with breast cancer (47). Kinesin family member 5A (*KIF5A*) encodes a member of the kinesin family of proteins. Previous research has confirmed that kinesin overexpression correlates with specific taxane resistance in basal-like breast cancer cell lines and tissues (48). Investigational kinesin protein inhibitors, such as GSK-923295, may be promising drugs in the future.

Our study had several limitations. First, external validation is required to ensure generalization. Second, our study did not explore the expression and prognostic effects of the seven genes at the protein level due to the incomplete protein expression information of partial genes. Finally, the reliability of our prognostic model needs further clinical validation.

In conclusion, we identified and validated a novel seven-gene signature model and constructed a nomogram combined with the patient age and tumor size for predicting DMFS in lymph node-negative TNBC patients. A higher risk score may indicate an increased likelihood of distant metastasis and vice versa. After taking the potential benefits and increased risks of distant metastasis into account, treatment escalation may be considered as an alternative strategy for lymph node-negative TNBC patients with a high-risk score. In contrast, de-escalation chemotherapy might be taken into consideration in patients with a low-risk score.

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 studies involving human participants were reviewed and approved by Fudan University Shanghai Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WP, CL, SJ, XJ, GD, and ZS: study concept and design. WP, CL, SJ, and GS: data analysis and interpretation. WP: wrote the first draft of the manuscript. WP, CL, and GS: visualization. XJ and ZS: funding acquisition. XJ, GD, and ZS: final approval. 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.2021.746763/full#supplementary-material

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Harnessing DNA Repair Defects to Augment Immune-Based Therapies in Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) has poor prognosis with limited treatment options, with little therapeutic progress made during the past several decades. DNA damage response (DDR) associated therapies, including radiation and inhibitors of DDR, demonstrate potential efficacy against TNBC, especially under the guidance of genomic subtype-directed treatment. The tumor immune microenvironment also contributes greatly to TNBC malignancy and response to conventional and targeted therapies. Immunotherapy represents a developing trend in targeted therapies directed against TNBC and strategies combining immunotherapy and modulators of the DDR pathways are being pursued. There is increasing understanding of the potential interplay between DDR pathways and immune-associated signaling. As such, the question of how we treat TNBC regarding novel immuno-molecular strategies is continually evolving. In this review, we explore the current and upcoming treatment options of TNBC in the context of DNA repair mechanisms and immune-based therapies, with a focus on implications of recent genomic analyses and clinical trial findings.

Keywords: TNBC, DNA repair, immunotherapy, PARP inhibition (PARPi), PD-1 - PD-L1 axis, DDR (DNA damage response), breast cancer

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INTRODUCTION

Triple-negative breast cancer (TNBC) is defined by the absence of estrogen and progesterone receptors (ER and PR) and human epidermal growth factor receptor 2 (HER2). This aggressive variant, which accounts for 15-20% of all breast cancers (BC), exhibits a high propensity for early recurrence and metastasis (1, 2). Despite relatively better initial response rates to taxane- and anthracycline-based chemotherapy, durable responses are limited as a result of poorly differentiated tumors with higher rates of acquired resistance to systemic chemotherapy and radiotherapy as compared to other BC subtypes, with median overall survival in metastatic TNBC ranging from 12-18 months (1, 3).

Understanding of specific heterogeneity in TNBC has served as the basis for certain targeted therapies based on particular molecular subtypes previously identified through genomic and transcriptomic profiling (1, 4): The 1) basal-like (BL) subtype exhibits higher rates of BRCA1/2 mutations and expression of DNA damage response (DDR) genes; 2) mesenchymal-like (MES)

subtype exhibits stem-like properties, and increased epithelial-mesenchymal transition (EMT), phosphoinositide 3-kinase (PI3K), and Janus kinase (JAK) pathway activation; 3) immunomodulatory (IM) subtype is associated with increased immune checkpoint expression and tumor-infiltrating lymphocytes (TILs); and 4) luminal androgen receptor (LAR) subtype is associated with increased androgen receptor (AR) signaling. For instance, the BL subtype may be potentially more sensitive to alkylating agents, platinums, or poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi's) as the result of higher rates of BRCA1/2 mutations and DDR deficiency, whereas the MES subtype may be sensitized to protein tyrosine kinase (PTK) and PI3K inhibition given increased activation of these pathways. Likewise, the IM subtype may have increased response to immunotherapy given increased TILs and expression of immune checkpoints, whereas the LAR subtype is potentially more sensitive to AR inhibitors given increased androgen-dependent metabolic activity in this molecular variant of TNBC. However, targeted therapies in TNBC have failed to achieve the remarkable efficacy as observed in other cancers (1, 5).

Pervasive therapeutic resistance in TNBC is another significant challenge, contributing to higher recurrence rates and decreased survival as compared to other BC subtypes (5). Therapeutic resistance in TNBC subtypes occurs through a variety of mechanisms. These include greater antioxidant and autophagy capacity resulting in resistance to radiation- or drug-induced oxidative stress, chemoresistance through upregulation of O-6-methylguanine-DNA methyltransferase (MGMT)-associated activity and mismatch repair (MMR)-deficiency allowing for base mismatched DNA replication (6–8), increased Mcl-1 and Bcl-2-related antiapoptotic activity, and high degree of immunosuppression in part through recruitment of regulatory T cells (Tregs) (9), anti-inflammatory M2 macrophages (10), and increased immune checkpoint (e.g. PD-L1) expression (6, 11–13).

Nevertheless, based on the frequency of DDR deficiency in TNBC, investigation of novel strategies targeting DNA repair defects have generated hope for improved outcomes. PARPi's aimed at DDR-deficiency in TNBC have been approved for patients with metastatic HER2-negative BC with an inherited BRCA1 or BRCA2 mutation previously treated with chemotherapy (NCT02000622 using Olaparib), and those with deleterious or suspected deleterious germline BRCA-mutated HER2-negative, locally advanced, or metastatic BC (NCT01945775 using Talazoparib). However, these have restricted application and demonstrate modest albeit intriguing clinical benefit at present (14–16). A recent report also suggested benefit of PARPi in patients with metastatic breast cancer beyond germline BRCA1/2 mutations (NCT02032823 using Olaparib) (17).

Another promising therapy for TNBC exploits the immune system. Given the immunogenic characteristics of TNBC, immunotherapy represents a promising treatment strategy for this aggressive breast cancer with few efficacious systemic options at present. The most successful immunotherapeutic agents to date consist of immune checkpoint inhibitors (ICIs), which block immune co-inhibitory receptors, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), or associated ligands such as programmed cell death ligand 1 (PD-L1), to dis-inhibit TILs and permit tumor-specific cytotoxicity. However, highly immunosuppressive tumor microenvironment (TME) competes with ICI-enhanced anti-tumor immunity and significantly contribute to inconsistent clinical responses. Immunotherapies, particularly combination strategies, represent a refined approach to treating cancers with immune modulating DDR defects, high tumor mutational burden (TMB), and intact anti-tumor immunity, which are all characteristics frequently observed in TNBC. Tumors with intact interferon-gamma (IFN-γ) pathway signaling, robust TILs, increased immune co-inhibitory receptor expression, and high TMB/neoantigen expression have been shown to respond better to immune checkpoint inhibition than weakly immunogenic tumors with inadequately established anti-tumor immunity (18). As such, TNBC typically exhibits properties favorable to immunotherapy response, including increased TILs (19), which correlates with improved outcomes in early-stage TNBC (20), higher PD-L1 expression as compared to hormone receptor positive BC (12, 13), and increased TMB giving rise to tumor neoantigen-specific T cells (2, 18, 21, 22). The PD-L1 mAb, Atezolizumab, is an FDA-approved ICI for patients with PD-L1 positive, unresectable, locally advanced, or metastatic TNBC (NCT02425891). However, ICI monotherapy efficacy is limited in TNBC, with response rates in the 5-25% range (23), suggesting coexisting immunosuppressive or tumorigenic factors at play that overwhelm or subvert ICI-enhanced anti-tumor immunity. Thus, improved strategies that augment the immunotherapeutic potential of ICIs are needed.

Given the immunosuppressive phenotype associated with TNBC (6, 10, 11, 13), it is feasible that innate and acquired immune resistance mechanisms have in part curbed robust outcomes using various approved inhibitors in TNBC patients. Furthermore, DDR-targeting therapies have been shown to augment anti-tumor immunity as well as immune checkpoint signaling (24–27), potentially opening the door to combination immunotherapy in TNBC patients with DDR-deficiency and inadequate or exhausted TILs.

This review summarizes the promising role of DNA repair deficiency as a surrogate biomarker to guide the use of ICI therapy in TNBC, discusses underlying mechanisms that link DDR signaling to anti-tumor immunity, and outlines the emerging evidence describing the relationship and potential cooperative therapeutic potential between DDR-pathway targeting agents and immunotherapy.

1 DNA DAMAGE REPAIR AND ASSOCIATED DEFECTS IN TNBC

Cells routinely undergo DNA damage as the result of cytotoxic stress. In normal physiology, mechanisms of DNA damage detection and repair are critical to preserve genomic integrity and thwart malignancy when DNA damage exceeds the cellular repair threshold. DDR accomplishes this by arresting proliferation and facilitating removal of damaged cells through activation of senescence or apoptosis. As such, defects in DDR genes permit mutations and chromosome rearrangements

advantageous for tumor initiation and progression. In TNBC, with alkylating chemotherapies and radiation as major components of therapy, aberrant DDR signaling represents a dominant mechanism of tumorigenesis and treatment resistance, while also yielding potential therapeutic synergies with platinum chemotherapies or targeted therapies. An overview of the DNA damage response and repair pathways is detailed below and shown in **Figure 1**.

1.1 DNA Damage Response and Repair Pathways

Depending on the mechanism of DNA damage and lesion formation, DDR is achieved by various pathways (28, 29). DNA single-strand break (SSB) damage is remedied by three main pathways: base excision repair (BER), nucleotide excision repair (NER), and mismatch-repair (MMR). More severe DNA double-strand breaks (DSBs) are restored by two additional pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (28, 29). Ataxia telangiectasia mutated (ATM), ATM- and RAD3-related (ATR), and DNA-dependent protein kinase (DNA-PK), in cooperation with many other mediators, act as core sensors that regulate DDR and coordinate DSB signaling. ATM and ATR protein kinases, operating together *via* downstream targets Checkpoint Kinase 1 (CHK1) and Checkpoint Kinase 2 (CHK2), respectively, play a vital role in DDR signaling by maintaining replication fork stability and the regulation of cell cycle control checkpoints (30). Additionally, DNA-PK activity is required for NHEJ, and a WEE1 nuclear kinase regulates mitotic entry and nucleotide reservoirs during DNA damage response (30, 31). Loss of function mutations in crucial genes involved in DDR, such as BRCA1/2, BRD4, PTEN or TP53, are associated with cancer-prone cellular behavior and malignant phenotypes. Consequently, failure in DDR results in impaired removal of genome mutations, accumulation of DNA damage and increases the risk of oncogenesis (32). In reflexive response to DDR deficiency, tumor cells activate alternate DDR pathways, thereby counteracting sensitivity to genomic insult by

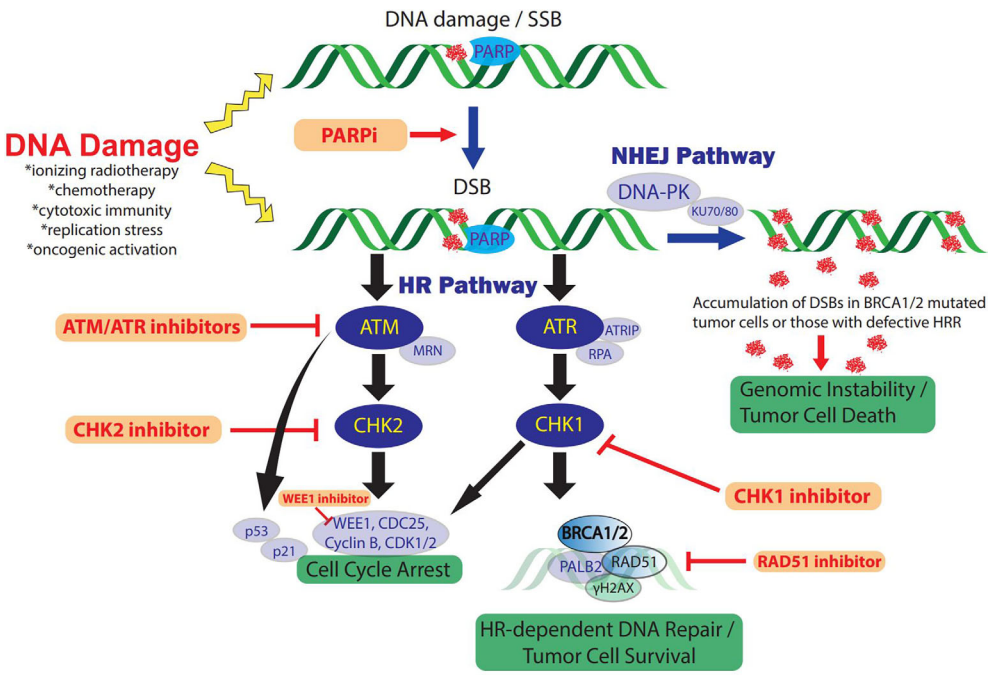

FIGURE 1 | **The DDR and therapeutic strategies in TNBC**. DNA-damaging therapies or endogenous replication dysfunction result in SSBs and DSBs which activate the DDR and repair signaling pathways. Distinct DSB DDR signaling pathway initiation depends on the type of DNA damage and is mediated by three central DDR kinases: DNA-PK, ATM, and ATR. In addition, PARP enzymes play a key role in DDR and facilitate SSB repair efficiency and functions in DSB repair *via* HRR and NHEJ pathways. The ATM and ATR pathways cross-talk extensively and only key intersections are highlighted here for pragmatic purposes. ATM/CHK2 signaling induces cell cycle arrest, preventing cell cycle progression in tumor cells with DNA damage. In addition, ATR/CHK1/WEE1 signaling initiates DNA DSB repair by inducing checkpoints and activating key components of HRR, including BRCA1/2 activity. Alternatively, DSB repair occurs through NHEJ *via* DNA-PKcs recruitment. Inhibition of PARP to treat TNBC with defects in HRR such as BRCA1/2 mutations, induces DSBs from unrepaired SSBs *via* PARP trapping and collapsed replication forks. Accumulated PARPi-induced DNA damage cannot be effectively repaired due to the HRR deficiency, resulting in genomic instability and cell cycle arrest. In addition, loss of function or inhibitors (red) against other key mediators of HRR also constrain NHEJ dependence which can be overwhelmed in the setting of concomitant PARPi *via* accumulation of DSB and genomic instability. RAD51 inhibition also suppresses HRR and sensitizes TNBC to PARPi. ATM/ATR/CHK1/WEE1 inhibition increases DSBs and impairs cell cycle arrest checkpoints and DNA damage repair, ultimately resulting in tumor cell death. DDR, DNA damage response; SSB, single-strand breaks; DSB, double-stranded breaks; DNA-PK, DNA-dependent protein kinase; ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia and Rad3-related protein; NHEJ, non-homologous end-joining; HR(R), homologous recombination repair; PARP, poly (ADP-ribose) polymerase; PARPi, PARP inhibitor/inhibition; MRN complex, Mre11, Rad50, Nbs1; ATRIP, ATR-interacting protein; RPA, replication protein A.

preventing lethal cytotoxic stress and perpetuating oncogenesis, which is altogether a problematic mechanism of resistance to DNA-damaging cancer treatments. As a result of tumor cells often harboring oncogenic defects in DDR pathways and therefore increased dependence on alternate DDR mechanisms to survive, there is increased susceptibility to DDR inhibition and subsequent accumulation of lethal levels of DNA damage as compared to normal cells (5). These DDR defects will cause accumulation of significant DNA alterations that not only can facilitate oncogenesis, but it is becoming ever more evident that these changes can modify the TME and inflammatory cascade (33).

Therapeutic targeting of DDR pathways in TNBC is therefore a promising strategy given the propensity for therapeutic resistance and DDR deficiency. Furthermore, increasing evidence demonstrates a link between DDR deficiency and activation of anti-tumor immunity, and we will discuss the potential for combined approaches targeting genomic and immunologic aspects of TNBC tumorigenesis later in this review.

1.2 The Role of PARP in DNA Damage Repair

DNA base damage, such as base loss or SSBs, results in BER. Poly (ADP-ribose) polymerases (PARP1/2) are important DNA-damage sensors and regulators of BER-mediated SSB repair as well as other DDR pathways (25). These enzymes bind *via* zinc finger domains to SSBs *via* co-factor nicotinamide (β-NAD+) and catalyze the synthesis of PARP chains (auto-poly (ADP-ribosylation), resulting in activation of intracellular signaling pathways that enable chromatin remodeling and recruitment of DDR-related protein machinery, thereby preventing accumulation of SSBs (34–36). In the setting of a HR deficiency, PARP inhibition disrupts efficient DNA damage repair resulting in increased genomic instability, stalled replication fork extension and lethal DSBs.

1.3 Synthetic Lethality and Clinical Utility of PARP Inhibitors in TNBC

Clinical use of PARP inhibitors (PARPi's) is an important example of DDR-specific targeting of HR defective cancers (14, 37). PARP1 inhibition can cause the accumulation of SSBs and subsequent DSBs. HR is required for DSB repair, and HR-deficiency is a typical pathological feature of the BRCA1/2-mutated tumor and enables enhanced response to PARP1 inhibition due to synthetic lethality. PARPi's in cells deficient in HR are unable to effectively undergo DDR, whereas PARPi is well-tolerated by normal cells. As such, this effect of PARPi is more likely observed in tumor cells with a BRCA-deficient background or tumors with underlying deficiencies in HR (38). Tumors cells with intact HR signaling can overcome PARP inhibition preferentially by HR rather than NHEJ (34), whereas cells with HR deficiency (HRD), including those with mutations in BRCA1/2, BRD4, and PTEN, demonstrate sensitivity to PARP inhibition resulting in cell death (35, 36, 38). PARPi therefore represents a synthetic lethal therapeutic approach for the treatment of cancers with compromised ability to repair double-strand DNA breaks by HR, including those with defects in BRCA1/2 (17, 34, 38). Numerous PARPi's have been developed, including Olaparib, Rucaparib, Niraparib, Talazoparib, and Veliparib, which are primarily applied in cancer patients with BRCA1/2 mutations (14, 16, 17, 39). Altogether these studies demonstrate that sensitivity of HRD-TNBC tumor cells to DNA-damaging agents may be the direct result of associated defective DDR mechanisms.

Although the greatest efficacy of PARPi has been observed in tumors with BRCA1/2 mutations, consensus is that synthetic lethality insufficiently explains PARPi-related anti-tumor activity. For example, the degree of PARP catalytic inhibition is poorly correlated to PARPi-induced cell-killing in HRD cells (40). In addition, PARPi induces cytotoxicity to a greater extent than PARP depletion, suggesting associated mechanisms contribute to anti-tumor activity (40, 41). In addition, loss of other tumor suppressor DDR proteins, many of which are involved in HR, such as RAD51, ATR, ATM, CHK1, CHK2, and partner and localizer of BRCA2 (PALB2), also have been shown to permit sensitization to PARPi (35, 40). HRD has also been shown to regulate sensitivity to alkylating chemotherapy in some TNBC patients (42), whereas the ATR-CHK1 cascade may conversely regulate resistance to chemotherapy by preventing replication stress. Further emphasizing the role of these accessory molecules in preventing susceptibility to DNA repair targeting, it was reported that ATR inhibition was effective in sensitizing both HR-proficient and deficient TNBC cells to ionizing radiation therapy (43). These results suggested that PARPi might be a useful therapeutic strategy not only for the treatment of BRCA-mutated tumors but also for the treatment of a wider range of non-BRCA-mutated tumors that are inherently HRD or 'BRCAness/HRDness' (15, 34).

In the context of TNBC, there is a higher degree of 'BRCAness' as compared to other breast cancer subtypes (1, 5), As such, PARPi's have demonstrated the potential for increased therapeutic efficacy in TNBC patients with HRD/BRCAness, due to increased accumulation of DSBs and incidence of synthetic lethality (35, 36). Olaparib, an orally active PARPi, was the first to be shown to induce synthetic lethality in BRCA-deficient cells and exhibit potential clinical benefit in patients with TNBC having BRCA deficiency. At present, Olaparib and Talazoparib are FDA-approved as single-agent regimens for previously chemotherapy-treated, HER2 negative, metastatic breast cancers with germline BRCA mutations, which primarily constitutes TNBC. In addition to exploiting BRCAness in TNBC, PARPi's have been shown to radiosensitize breast cancer cells through DDR inhibition, and clinical trials in breast cancer patients explore their potential to enhance the response of cancers to ionizing radiation (44).

Use of PARPi's in TNBC is supported by findings from the phase III OlympiAD trial of metastatic breast cancer (16), which demonstrated an approximately two-fold increase in response rate (59.5%), increased median progression-free survival (PFS; 7.0 months), and less toxicity as compared to conventional chemotherapy in patients with metastatic HER2-negative breast cancer with germline BRCA1/2 mutations treated with Olaparib (NCT02000622). A phase I study of Talazoparib demonstrated promising efficacy and safety profiles in

advanced cancers with deleterious BRCA1/2 mutations including breast cancer (NCT01945775). A phase III trial EMBRACA comparing Talazoparib versus physician's choice standard of care in metastatic TNBC revealed significant benefit of Talazoparib with better PFS and objective response rates (ORRs) (14, 39). Other PARPi's, including Veliparib, and Rucaparib, have been investigated in metastatic breast cancer. Trials of veliparib in combination with alkylating agents are currently underway for advanced or metastatic TNBC (45–47). In early TNBC, the phase III OlympiAD trial (NCT02032823) is currently ongoing to evaluate adjuvant Olaparib monotherapy after standard neoadjuvant therapy in high-risk TNBC with germline BRCA1/2 mutations. Another phase I trial of neoadjuvant monotherapy with the novel PARPi Niraparib is underway (NCT03329937). The phase II/III PARTNER trial of neoadjuvant Olaparib in combination with carboplatin followed by the standard chemotherapy is under investigation in patients with TNBC and/or germline BRCA mutations (NCT03150576). The I-SPY 2 trial, which evaluated neoadjuvant Veliparib and carboplatin in addition to the standard chemotherapy in patients with high-risk breast cancer and TNBC, demonstrated significant benefit from this combination therapy (pathologic complete response (pCR) rates: 52% *vs* 24%) (48). In a recent biomarker analysis of the I-SPY2, a BRCA1ness gene signature was identified as a significant predictive biomarker of response to neoadjuvant combination Veliparib and carboplatin (49). Conversely, a phase II neoadjuvant trial in high-risk, residual TNBC after standard neoadjuvant chemotherapy failed to show a significant therapeutic benefit from the combination of low-dose Rucaparib and cisplatin compared with cisplatin alone; although the lack of benefit may be due to a therapeutically insufficient rucaparib dose (NCT01074970). Altogether, additional studies are required to elucidate the clinical benefit of PARPi addition to platinum-based chemotherapy in TNBC as platinum alone demonstrates efficacy either as monotherapy or in combination (50, 51). This also further emphasizes the need to identify additional therapies that sidestep resistance to therapeutic targeting of DDR deficiency.

Other strategies to exploit HR include inducing a synthetic lethality by generating a BRCAness phenotype. These promising preclinical studies include combinations with inhibitors of EGFR, PI3K, BET, and others (52–54). We recently reported promising results of a clinical trial with lapatinib and veliparib in non-BRCA1/2 mutated TNBC based on an induced DNA repair deficiency with EGFR inhibition (NCT02158507) (55).

1.4 Role of MMR and NHEJ in TNBC

In TNBC, defective MMR allows DNA replication with mismatched bases and facilitates resistance to anti-metabolites and alkylating agents. Whole-genome sequencing studies have shown that approximately 5-7% of TNBC patients are MMR-deficient (6, 8), as compared to approximately 2% in other breast cancers. Furthermore, MMR status corresponds to PD-L1 expression and $CD8^+$ T cells in the TNBC TME versus poor correlation in other subsets of breast cancers. Altogether, these findings indicate the immunotherapeutic efficacy potential in TNBC with MMR deficiency (6). In the context of immunotherapy, MMR deficiency not only has the potential to elicit more tumor antigens and improved immune checkpoint inhibitor response (56). The TMB/neoantigen/IFN-γ pathway is a well understood cancer pathway that results in PD-L1 upregulation, supported by the finding that even partial loss of MMR significantly correlates with increased PD-L1 expression suggesting a therapeutic vulnerability in HRD TNBC (6). Mounting evidence indicates that DDR defects are also important in driving sensitivity and response to ICI. Given that MMR deficient (dMMR) tumors harbor a large number of mutations, which are associated with high neoantigen load and T-cell infiltration, it is not surprising that dMMR tumors can respond well to immune checkpoint blockade. Indeed in many cancers, MMR deficiency predicts efficacy of anti-PD-L1 (Pembrolizumab), and microsatellite instability (MSI)/dMMR is a validated DDR defect biomarker for predicting response to ICI therapy (56). Furthermore, Pembrolizumab is FDA-approved for solid tumors based solely on the presence of MSI-status as a biomarker, irrespective of cancer type (56). Although MSI or dMMR rarely appears in breast cancer (57), as we will discuss further, the therapeutic potential in combining with immune-stimulating DNA repair inhibitors remains intriguing.

The NHEJ signaling pathway is an important mediator of DSB repair. The Ku70-Ku80 heterodimer and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) initiate NHEJ, and these complexes have been shown to be regulated by EGFR amplification and/or p53 mutation-induced overexpression of long non-coding RNA in the NHEJ pathway 1 (LINP1), resulting in NHEJ-mediated chemo- and radiation resistance (58, 59). Doxycycline, an FDA-approved agent that can inhibit DNA-PK, has been shown to reduce DNA-PKcs expression and sensitize breast cancer cells to radiation (60). Although more investigation is necessary, these findings suggest that targeting of NHEJ-related mediators may be useful in TNBC, particularly those with EGFR, p53 and/or DDR-associated mutations resistant to DNA-damaging agents.

1.5 Role of Radiation Therapy in DNA Damage Signaling and Immune Strategies

Most breast cancer patients receive ionizing radiotherapy (RT) as part of their treatment to improve locoregional control by inducing tumor cell death predominately through the generation of DSBs, which in turn can elicit either protective anti-tumor immune responses or immunosuppression (61). Unfortunately, positive immune effects of radiation are often insufficient to shift the balance of the immunosuppressive TME to achieve tumor rejection, especially in the absence of targeted immunotherapy. Combining immune checkpoint blockade with radiotherapy has thus emerged as an exciting dual modality treatment approach for a myriad of cancer types, although clinical outcomes are highly variable.

1.5.1 Impact of Radiation Therapy on Anti-Tumor Immunity and Immunosuppression

RT-enhanced tumor immunogenicity can occur through multiple mechanisms, including increased antigen availability, inflammatory cell infiltration into tumors, and increased priming

and exposure of phagocytic and cytotoxic cells to tumor-associated antigens (62). Specifically, RT can up-regulate FAS (death receptor) and MHC class I on tumor cell surfaces, alter the repertoire of peptides presented by MHC, cause translocation of calreticulin to tumor cell surfaces resulting in enhanced antigen uptake by antigen presenting cells, and induce release of HMGB1 from dying tumor cells. These actions induced by RT can result in dendritic cell maturation and chemokine and cytokine secretion that promotes TIL trafficking (62, 63). Furthermore, RT-induced DSBs and subsequent ATM activation has been shown to regulate pattern recognition receptors that activate interferon and innate immune system signaling (64, 65). Local and systemic immune effects include RT-induced alteration of chemokine signaling, cell trafficking, and secondary immune system activation *via* dendritic cell cross-presentation of tumor-derived antigens to T cells (63, 66).

The link between radiation and both local and systemic anti-tumor immune effects has been investigated in many preclinical and clinical studies (61, 63, 65). It has been reported that immune-related therapeutic effects of locally ablative RT require intact immunity, type I interferon production and infiltration of $CD8^+$ T cells (67), highlighting the importance of functional anti-tumor immunity in the current era of radio-immunotherapy. However, RT and the resultant tumor cell death can also potentiate immunosuppressive TMEs, as studies have shown that radiation can induce lymphopenia, immune dysfunction through release of immunosuppressive cytokines (TGF-β, IL-10) and chemokines, and induction of immunosuppressive immune cells including myeloid-derived suppressor cells (MDSCs), M2 tumor-associated macrophages (TAMs), T regulatory cells (Tregs), which can all result in immune escape and tumor progression (62, 66).

Importantly, radiation can further induce immunosuppression and adaptive immune resistance *via* upregulation of checkpoint pathways, including PD-L1 expression on the tumor cell surface (65, 68). Although the neoantigen-T cell activation-IFN-γ-STAT1/3-IRF1 pathway of PD-L1 induction has historically been viewed as the chief mediator of this adaptive immune resistance, recent work has implicated DNA damage and repair signaling in the regulation of tumor PD-L1, including through radiation-mediated DSBs and cytosolic DNA sensing. DNA damage dependent PD-L1 expression is upregulated by ATM/ATR/CHK1 kinase activities and the cyclic-GMP-AMP ((cGAMP) synthase (cGAS))/stimulator of interferon genes (STING)-dependent pathway. Altogether, tumor cell PD-L1 expression is controlled by the STAT-IRF pathway which is regulated by distinct DNA damage mechanisms: 1) DSB-induced ATM/ATR/CHK1 kinase activities, 2) DDR deficiency/high MSI/increased TMB resulting in neoantigen-induced T cell activation and IFN-γ production, and 3) cytosolic DNA fragments that induce the cGAS/STING pathway resulting in type I interferon activity (68).

RT induced PD-L1 expression *via* activation of the cytosolic DNA sensing cGAS/STING pathway represents a novel mechanism of adaptive immune resistance. The cGAS/STING, with subsequent type I interferon production, is a fundamental immunostimulatory pathway in antimicrobial innate immunity (64), and has been found to mediate the TME and immune milieu, including immune surveillance, dendritic cell function and $CD8^+$ T cell function (69). Interestingly, STING-activity is also upregulated in the setting of DDR deficiencies including BRCA1/2 and ATM mutant tumor cells (69). This STING-dependent interferon signaling can initially facilitate immune activation; however chronic STING pathway activation and/or IFN-γ signaling can ultimately lead to T cell exhaustion *via* PD-L1-dependent resistance to anti-tumor immunity (70), potentiating cancer immune escape.

RT-induced DSBs and subsequent ATM/ATR/CHK1 kinase activities have also been implicated in upregulation of tumor PD-L1 expression through direct STAT1/3-IRF1 activation (26, 66, 68), independent of neoantigen production. Consistent with this, Ku or BRCA2 defects were found to augment RT-induced PD-L1 expression (26, 68), and ATR inhibition reduced upregulation of PD-L1 following RT. Interestingly, ATR inhibition potentiated $CD8^+$ T cell activity and reduced RT-induced T cell exhaustion (71). Furthermore, RT-induced interferon signaling has been shown to be dependent on cGAS/STING pathway activation (65). This evidence suggests a novel PD-L1-dependent, immunosuppressive consequence of DNA damaging therapies (e.g., chemotherapy, RT, DDR inhibitors). In relation to immune-activating properties of RT, the disadvantageous PD-L1 induction following RT represents a therapeutic opportunity with combination ICI therapy that would result in more durable clinical responses.

1.5.2 Clinical Application of Radio-Immunotherapy Combinations in TNBC

Observations in patients receiving ICI and RT have demonstrated the potential for improved clinical responses in various primary and metastatic malignancies, and numerous clinical trials are underway investigating potential synergy. Clinical trials evaluating patients with metastatic cancer have established that RT combined with ICI is safe and well-tolerated, and can potentially halt tumor growth by stimulating anti-tumor immunity (61, 66). In TNBC, a phase II trial evaluated PD-L1 inhibition (Pembrolizumab) plus RT in patients with metastatic TNBC patients who were unselected for PD-L1 expression. In this study, the ORR for the entire cohort was 17.6% (3 of 17 patients; 95% CI: 4.7%-44.2%), with 3 complete responses of tumors outside of the irradiated portal (72). The context dependence of the robust synergistic effects of RT and ICI are potentially consistent with fluctuating immune-tolerance and suppression mechanisms, particularly in the locally advanced or metastatic setting. This altogether highlights the need for larger clinical trials assessing predictive biomarkers and investigation of additional targeted strategies. For instance, the phase I RADIOPARP trial is investigating PARP1 inhibitors (Olaparib) in combination with RT in the setting of advanced or metastatic TNBCs (73). Neoadjuvant Veliparib combined with RT is under exploration in a phase I study for node-positive, residual BC following neoadjuvant chemotherapy (NCT01618357). These and additional studies are needed to optimize radiotherapy modulation of DDR-dependent immune augmentation and anti-tumor immunity in the context of ICIs.

2 CANCER IMMUNOLOGY IN TNBC

Immune evasion is a hallmark of cancer that is the result of a complex TME consisting of stroma, myeloid and lymphoid immune cells, dysregulated lymphovascular networks. The interaction of these components often plays roles in tumorigenesis, tumor heterogeneity, and adaptive and therapeutic resistance. Central to immune-mediated tumor rejection are TILs, a heterogeneous population that contributes to competing innate and adaptive anti-tumor and immunosuppressive effects. TILs, including $CD8^+$ T and NK cells that are central to anti-tumor immunity in breast cancer, have prognostic significance even in systemically untreated early TNBC, suggesting that the presence of TILs may delineate candidates most likely to benefit from adjuvant chemotherapy or immunotherapy (74).

The TME of TNBC is often abundant in TILs because of inherent genomic instability and high mutational burden. As the result of these genetic and epigenetic aberrations, anti-tumor TILs engage in immune-mediated tumor cell killing and tumor cell immunoediting, often times resulting in subset(s) of immune resistant tumor cells (2, 75, 76). In metastatic TNBC, response rate and overall survival after Atezolizumab significantly correlated with TIL levels (77). However, in early TNBC, retrospective studies demonstrated significantly worse survival outcomes in patients harboring high PD-L1 expression and a low number of TILs or a high ratio of PD-L1/CD8 expression (12, 78), suggesting that TIL alone is not indicative of the immune activity or suppression status. Consistent with this, immunologic signatures associated with higher mutational burden positively correlated with higher TILs and a more favorable prognosis (12), suggesting antigen-specific anti-tumor TILs likely play a significant role in coordinating the functional state of anti-tumor immunity and response to immunotherapy (12). Furthermore, TILs are shown to be a robust predictive biomarker of long-term survival in TNBC patients treated with neoadjuvant therapies and to facilitate improved response to cytotoxic agents (79–81). However, effective anti-tumor TIL activity is frequently hindered by immunosuppressive immune cells types such as regulatory Tregs and MDSCs, which are also typically found in higher concentrations in TNBCs. Nevertheless, compared with other BC subtypes, TNBC exhibits a higher degree of lymphocytic infiltration (19), and studies to date indicate that TILs are useful biomarkers and potential therapeutic targets in TNBC.

2.1 Immune Co-Inhibitory Pathways in TNBC

Upon activation, T cells begin to express co-inhibitory cell surface receptors that control T cell function, such as CTLA-4 and PD-1. The balance between co-stimulatory and co-inhibitory signals is crucial for cytotoxic T cell activation and immunologic tolerance. Tumors can exploit this balance to escape T cell-mediated, tumor antigen-specific immunity. Importantly, therapeutically targeting these co-inhibitory pathways with immune checkpoint inhibitors (ICIs) is capable of unleashing anti-tumor activity (78, 82). In TNBC, immune co-inhibitory signaling is often upregulated and is associated with immunosuppression, MMR-status and mutational burden, chemoresistance and overall poor prognosis (6, 11, 12).

CTLA-4, an immune checkpoint constitutively expressed on Tregs and transiently upregulated on activated T cells, inhibits early T cell priming by antigen-presenting cells (APCs) in the lymph nodes (83). The expression of CTLA-4 on Treg cells competitively blocks the binding of CD28 to the CD80/86 proteins on APCs, thereby turning off T cell activation (82). CTLA-4 blockade has demonstrated efficacy in anti-tumor immune activity in some cancers by allowing tumor antigen-specific T cell stimulation. CTLA-4 ICI has demonstrated durable response in a small subset of patients with metastatic TNBC (84, 85), and CTLA-4 mAbs, including Ipilimumab and Tremelimumab, are being investigated with the PD-1-axis immunotherapies Durvalumab and Nivolumab, respectively, for TNBC.

PD-1, another immune checkpoint, is widely expressed on activated anti-tumor immune cells, including T and natural killer (NK) cells, and APCs, and yields inhibitory signals through binding of its two ligands, namely PD-L1 and PD-L2 (86). PD-L1 is highly inducible and expressed on many cancers in response to anti-tumor immune activity and inhibits $PD\text{-}1^+$ tumor antigen-specific $CD8^+$ T cells (87), representing a key mechanism underlying cancer adaptive immune resistance. Correlation between TILs and PD-1/PD-L1 expression is well studied, as tumor-associated inflammation promotes adaptive upregulation of immunosuppressive PD-L1 expression in response to anti-tumor immune cell production of IFN-γ and tumor cell STING pathway activation (22, 88). Blockade of PD-1/PD-L1 interaction is capable of restoring T cell function and tumor elimination. However, in breast and other cancer cell types, meaningful response is inconsistent as a result of reduced or heterogeneous PD-L1 expression, immunosuppressive mechanisms, impaired immune cell function and trafficking of TILs (87, 89), resulting in paradoxical $PD\text{-}L1^+$ "non-responders" and $PD\text{-}L1^{low/null}$ "responders".

There is compelling evidence that resistance to DNA-damaging agents may play a meaningful role in immunotherapy outcomes. For example, defects in BRCA1/2 correlates to higher levels of PD-L1 expression (90, 91). In addition to inactivation of $PD\text{-}1^+$ anti-tumor immune cells, tumor PD-L1 also mediates diverse cell-intrinsic functions that increase cancer virulence, including mTORC1 promotion and autophagy suppression (92–94), that can not only alter immune infiltrates and enable immune escape (94–98), but may also play a role in response to DNA-damaging therapies. Indeed, it has been shown that tumor-intrinsic PD-L1 can regulate IFN-γ-induced apoptosis, DDR, RT and chemotherapy resistance, and effects on Ras/Mek/ERK, PI3K/AKT, JAK/STAT (94, 99–101); which, altogether may create treatment-exploitable immune signaling effects.

The interaction of these pathways to modulate the immune system is depicted in **Figure 2**.

2.2 Role of Immune Checkpoint Inhibition in TNBC

ICIs, including monoclonal antibodies against PD-1 (Pembrolizumab, Nivolumab), PD-L1 (Atezolizumab, Durvalumab, Avelumab), and CTLA-4 (Ipilimumab), have

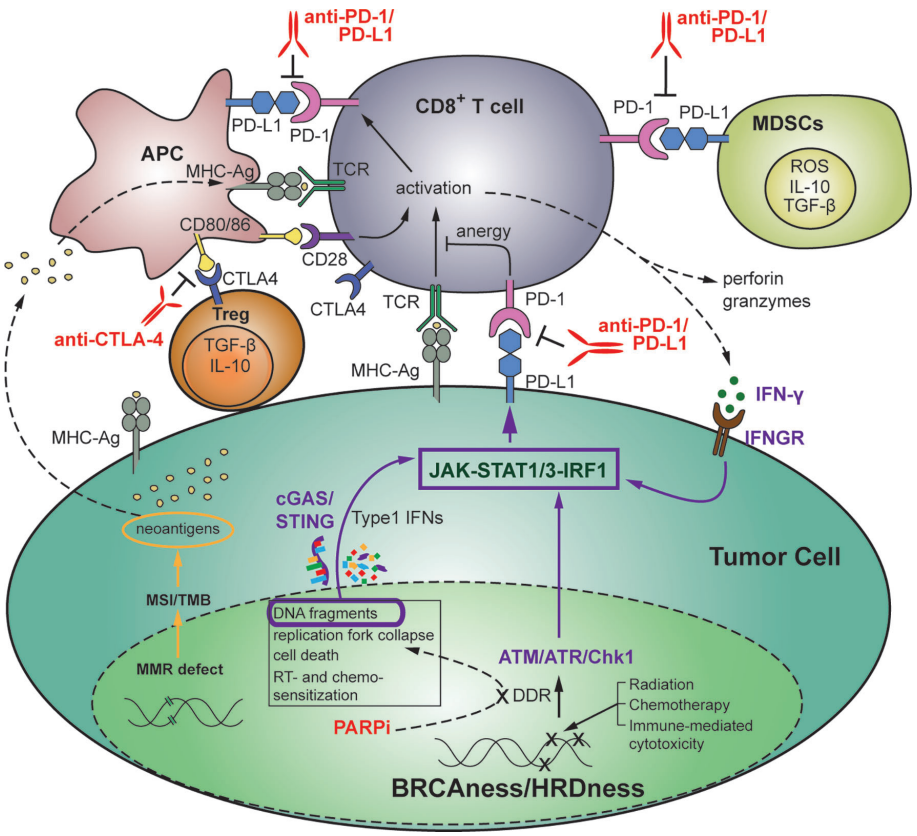

FIGURE 2 | **Therapeutic strategies targeting the interplay between DDR and anti-tumor immunity in the setting of HR-deficient triple-negative breast cancer.** DNA damage affects the balance between tumor progression and immune surveillance. Genomic stress induced by DNA-damaging treatments or by defects in DDR or MMR results in accumulation of chromosomal abnormalities, higher TMB, oncogene activation and tumorigenesis, as well as immune recognition, activation of immunostimulatory genes, and increased TILs including anti-tumor immune cells ($CD8^+$ T cells, APCs, $CD4^+$ T cells, and NK cells). Immunosuppressive immune cells such as $CD4^+$ Tregs, MDSCs and M2 macrophages can also be increased. Targeting of SSB and DSB repair with inhibitors of DDR, including PARPi's, in the setting of TNBC with BRCA or HR-related mutations (BRCAness/HRDness) can, in addition to inducing synthetic lethality, increase generation of cytosolic DNA fragments. This results in activation of the immunomodulatory cGAS/STING pathway that promotes anti-tumor immunity through activation of T and NK cells, neoantigen recognition, and increased PD-L1 expression *via* the JAK-STAT1/3-IRF1 pathway. Anti-tumor immunity can further contribute to tumor PD-L1 expression *via* IFN-γ-dependent activation of IRF1. Tumor and immune cell expressed PD-L1 subsequently suppresses $PD\text{-}1^+$ cytotoxic anti-tumor immune cells *via* inhibitory binding. Thus, DNA-damage induced anti-tumor immune response is often overwhelmed by coexisting immunosuppressive factors, and the balance in favor of anti-tumor immune rejection can be mediated by ICIs such as anti-PD-1/PD-L1 and anti-CTLA-4 mAbs. APC, antigen presenting cells; ICI, immune checkpoint inhibitor; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cells; MMR, mismatch repair; NK, natural killer; TIL, tumor-infiltrating lymphocyte; TMB, tumor mutational burden; Treg, T regulatory cell.

generated durable responses across many tumor types (102). Clinical studies using PD-1/PD-L1 mAb therapies have demonstrated promise in patients with PD-L1 positive TNBC, and Atezolizumab is FDA-approved for patients with $PD\text{-}L1^+$, unresectable, locally advanced, or metastatic TNBC (NCT02425891). Studies of Atezolizumab in advanced solid cancers, including heavily pretreated TNBC, demonstrate limited but impressive outcomes, as only 10% of patients experienced clinically meaningful response, with 100% survival rate at 2 years in these responders, and median PFS of $PD\text{-}L1^+$ TNBC patients treated with Atezolizumab plus nab-PTX was significantly increased by 50% (7.5 months *vs.* 5 months) (103). KEYNOTE-012 and KEYNOTE-086 studies demonstrated durable response with Pembrolizumab in approximately 20% of patients with metastatic TNBC (104, 105). Patients with positive PD-L1 expression treated with first-line Pembrolizumab showed a higher response rate than patients with any level of PD-L1 expression. Using Avelumab in patients with heavily pre-treated metastatic TNBC, the phase I JAVELIN trial demonstrated promising efficacy outcomes with a 31% control rate, and PD-L1 expression correlated with response (106). Currently, a phase II trial of Pembrolizumab as monotherapy for BRCA-mutated breast cancer is underway (NCT03025035). These studies using PD-1-axis inhibitors demonstrate therapeutic benefit in some patients, but future studies are required to address inconsistent responses, better define the therapeutic ceiling of ICIs in upfront treatment of early-stage TNBC, elucidate the role of targeted therapies in increasing therapeutic index of ICIs, and identify reliable biomarkers to guide the imperfect prognostic value of PD-L1 expression.

Based on the remarkably durable responses in a small subset of TNBC responders in ICI monotherapy studies, many studies using combination ICI with conventional therapies are currently ongoing and have shown early signs of benefit. Interim data from Impassion130 trial, using nab-paclitaxel in combination with Atezolizumab showed a 40% ORR in metastatic TNBC, and early data suggests a clinically meaningful overall survival benefit in patients with PD-L1 immune cell-positive disease (NCT02425891). Trials investigating the combination of Eribulin and Pembrolizumab in heavily pretreated metastatic TNBC are ongoing, with interim analysis demonstrating a 41.2% ORR to first-line treatment and a 27.3% ORR to later-line treatment (107). However, PD-L1 status failed to predict treatment response to either combination. These trials investigating ICI efficacy in heavy-treated TNBC patients altogether have highlighted the need for earlier intervention with ICI therapy in advanced or metastatic TNBC. KEYNOTE-355, a phase III trial evaluating the combination of Pembrolizumab plus conventional chemotherapy compared with chemotherapy alone as first-line treatment in metastatic TNBC is ongoing (NCT02819518). The combination of Durvalumab and nab-paclitaxel followed by dose-dense conventional chemotherapy as well as the combination of Avelumab and an antibody to the immune modulator, 41BB, is under investigation in advanced solid tumors, including TNBC (NCT02489448).

In early TNBC, preliminary results from the neoadjuvant I-SPY 2 trial demonstrated that pCR rates increased from 22.3% to 62.4% by adding neoadjuvant Pembrolizumab to paclitaxel followed by anthracycline-based chemotherapy, which represents an approximately 40% improvement in pCR compared with standard chemotherapy alone (108). The KEYNOTE-173 trial also showed a remarkably increased pCR rate from 60% to 90% in high-risk patients by combining Pembrolizumab with paclitaxel or conventional chemotherapy (109). In the adjuvant setting, the SWOG1418 phase III trial is evaluating adjuvant monotherapy with Pembrolizumab after neoadjuvant chemotherapy followed by curative surgery. Another phase III trial for high-risk patients with early TNBC is investigating the addition of Avelumab after standard curative treatment including adjuvant chemotherapy (NCT02926196).

Clinical success using immune checkpoint inhibitors has led to the identification of additional checkpoints that mediate tumor immunosuppression, such as the lymphocyte-activation gene 3 (LAG3), T cell immunoglobulin and mucin-domain 3 containing-3 (TIM3), Siglec-15, indoleamine 2, 3-dioxygenase 1 (IDO1), and glucocorticoid-induced tumor necrosis factor receptor (GITR). Targeted therapies for these are undergoing clinical trials in TNBC patients. For example, Siglec-15 is an immune checkpoint that inhibits antigen-specific T cell responses, and is expressed, independent of PD-L1 status, on both tumor and tumor-infiltrating myeloid cells (110), and a mAb for Siglec-15 is currently being evaluated in a phase I/II study for advanced or metastatic solid tumors (NCT03665285).

Although clinical trials for immunotherapy in breast cancer have not shown that same high efficacy as in other carcinomas, TNBC is likely to have increased benefit as compared to other types of breast cancer given high mutational load, DDR-deficiency and increased PD-L1 expression. This may be especially true in early-stage TNBC with potentially more favorable tumor immune microenvironments, as studies thus far have mostly evaluated immune checkpoint inhibitors in advanced staged TNBC. However, innate, and adaptive resistance to immunotherapy remains a challenge, and targeted therapies that synergize with the immune-activating potential of immune checkpoint inhibitors is a promising strategy to maximize immunotherapeutic potential in TNBC patients.

3 DDR DEFICIENCY-ASSOCIATED ANTI-TUMOR IMMUNITY IN TNBC

Recent work has highlighted the important interaction between genomic instability and the immunogenicity and activation of anti-tumor immunity (111). Highly mutated tumors often exhibit one or several mutations in key components of DDR or replicative pathways, including MSH2 for MMR/MSI, BRCA1/2 for HR and DNA polymerase epsilon (POLE) for DNA replication. Targeting of DSB repair proteins with DDR inhibitors has also been shown to increase the TMB (111). Likewise, DDR defects result in accumulation of chromosomal abnormalities, leading to higher TMB, oncogene activation and tumorigenesis (112, 113). However, this DDR-defect-dependent genomic instability and increased TMB can also result in immune recognition, activation of immunostimulatory genes, increased TIL, and anti-tumor immune production of IFN-γ with resultant immunosuppressive tumor PD-L1 upregulation (18, 90, 114, 115). Similar effects can be observed as a result of genomic stress induced by DDR defects or DNA-damaging treatments, including RT, PARPi or platinum-based chemotherapies. This is due to generation of chromosomal fragments that stimulate the cytosolic sensing cGAS/STING pathway that promotes anti-tumor immunity through activation of T and NK cells, neoantigen recognition, and increased PD-L1 expression, and this immune system stimulation is enhanced in the background of BRCAness/HRDness (24, 27, 90, 116–118). It is also evident that in response to DNA damage, ATM/ATR/CHK1 kinase activity regulates the transition from DDR to immunostimulatory signaling directly through STAT1/3-IRF1-mediated transcription of PD-L1 (26). Thus, DDR signaling and DNA-damaging treatments result in robust immune modulation and significantly affect the balance between tumor progression and immune surveillance.

In support of the notion that tumor cells with extensive genomic instability orchestrate a high octane anti-tumor immune response that is smothered by coexisting immunosuppression, DNA damage and DDR-defects associated with increased TMB and neoantigen production correlate with STING-induced PD-L1 expression and improved ICI response (25). In some studies including in invasive breast carcinoma, defects in BER or BRCA1/2 were associated with increased neoantigen load, increased TILs, and elevated PD-L1 expression (26, 90, 91, 119), and a genome wide genetic screen identified BRCA2 inactivation as a mediator of cGAS/STING-induced IFN response and pro-inflammatory cytokine production

(116). Consistent with these findings, DDR deficient breast tumors exhibited increased immune infiltration. However, elevated PD-L1 expression was driven predominantly by cGAS/STING pathway activation as opposed to the canonical neoantigen/activated T cell/IFN-γ pathway of PD-L1 induction (69), which is significant given that STING activation mediated by DNA-damaging agents is implicated in response to ICI therapy. Although some studies report elevated TILs in BRCA1/2 mutant breast cancer (21, 24), a pooled analysis of five phase II studies showed that TIL density was not associated with HR defect or BRCA1/2 mutation in early stage patients with TNBC (20). It is therefore likely that neoantigen-independent mechanisms of immune augmentation are involved in TIL density and PD-L1 expression in DDR-deficient TNBC, which is compatible with numerous studies that have shown tumors with low TMB can also be sensitive to ICIs (22). Importantly, patients with BRCA1/2 and other HR-related gene deficiencies demonstrate higher response rates to ICI as compared to MMR deficient tumors despite relative lower TMB, corroborating the possibility of additional immunologic mechanisms related to DDR-deficiency (22, 120, 121). Furthermore, the observation that HR intact tumors may also respond to the PARPi and ICI combination could perhaps be explained by the activation of the cGAS/STING and subsequent neoantigen-independent immune activation (122, 123).

Despite the significant clinical activity of PARPi in breast cancers harboring germline loss-of-function BRCA mutations (14, 16, 39), the majority of patients treated with PARPi's alone do not significantly benefit (115). Unrepaired chromosomal damage following PARPi further promotes immune activation and adaptive upregulation PD-L1 expression *via* cGAS/STING pathway activation or ATM/ATR/CHK1 kinase activity (26, 119), which may result in immune escape and explain variable results. Altogether these studies support the hypothesis that use of PARPi together with ICI will retain immune activating consequences of DDR defect targeting while also preventing T cell inactivation.

In the setting of DDR deficiency, a consequence of tumor cell DNA damage and sustained inflammatory activity is recruitment and activation of immunosuppressive immune phenotypes as the result of chronic, low level, DNA damage, potentially resulting in cancer progression and immunotherapy resistance (117). It is proposed that PARPi may potentially shift to more substantial DDR-mediated cytotoxic anti-tumor immune milieu more favorable for ICI efficacy (124). In support, it is reported that PARPi efficacy is enhanced by $CD8^+$ T cell activity *via* cross-talk with STING pathway activation in BRCA-deficient models of TNBC (123). Collectively, numerous studies indicate PARPi-dependent immunologic effects may prime a vigorous albeit imbalanced anti-tumor immune response and set the stage for improved ICI efficacy.

The combination of enhanced immune activation resulting from deficient DDR pathway signaling and the immunosuppressive consequences, including PD-L1 upregulation of unrepaired DNA damage *via* HR-deficiency and/or the use of DDR inhibitors such as PARPi, suggests potentially targetable immunological susceptibilities in TNBC patients (**Figure 2**). Tumor immune evasion mechanisms in response to genomic instability subvert immune-mediated elimination of DDR hindered cancers, serving as rationale for targeting the immunosuppressive arm of DDR signaling in response to DNA damaging therapies *via* ICI combinations. This approach may be highly lethal to immunogenic tumor cells with DDR defects and impinge upon these immunosuppressive mechanisms of therapeutic resistance (24, 68, 71, 125, 126). TNBC often harnesses DDR defects, TMB load, and PD-L1 expression, and these characteristics have been found to be amongst the strongest predictors of response to ICI (18, 22, 113).

3.1 DDR Inhibitors and Immunotherapy in TNBC

Therapeutic targeting of genomic instability through the use of DDR-inhibitors, including PARPi, have been shown to not only induce synthetic lethality in DDR-deficient tumor cells, but also to augment the tumor immune microenvironment through increased TMB and activation of immunostimulatory genes (21, 25, 114). Accruing evidence supports the potential association between DDR defects and ICI efficacy. Interestingly, preclinical TNBC studies demonstrated PARPi-mediated PD-L1 upregulation with expected attenuation of anti-tumor immunity, that PD-L1 blockade re-sensitized PARPi-treated cancer cells to T-cell killing, and the combination of PARPi and anti-PD-L1 therapy demonstrated greater antitumor activity and tumor control compared with each agent alone (127), further indicating a potential synergistic effect of combination DNA damage response inhibitors (DDRi's) and ICI. Combination PARPi with PD-1/PD-L1 targeted therapies demonstrated increased TILs and enhanced antitumor immunity in both BRCA-proficient and BRCA-deficient mouse models of TNBC (123, 127), indicating additional PARPi-mediated immunologic factors associated with ICI outcomes. Interestingly, whole exome sequencing of cancer patients previously treated with PD-1 inhibitors revealed that ICI responders are enriched for BRCA mutations (8). Altogether, this dual effect of DDRi-induced immune activation and PD-L1-dependent immunosuppression suggests immunologic vulnerability that may be exploited through the use of ICI, and serves as the rationale for studies investigating the clinical efficacy of combination therapy with PARPi's and anti-PD-1/PD-L1 in multiple cancers, including TNBC (121, 128).

A summary of ongoing clinical trials combining DDR targeting agents with immunotherapy is listed in **Table 1**.

3.2 Exploiting BRCA1/2 Deficiency and Immunotherapy in TNBC

Given the potential of tumor cell HR defects, including BRCAness, to increase susceptibility to ICI through enhanced immune activation and expression of PD-1 or PD-L1 (103), ICI response is being studied in cancers, including breast cancers, with germline mutations in BRCA1 or BRCA2 (NCT01772004, NCT03025035). In previously treated, platinum-resistant recurrent ovarian cancer, Durvalumab and Olaparib demonstrated clinical activity, irrespective of BRCA mutation status (NCT02484404) (129). Interestingly, analysis of core biopsy and blood samples revealed

TABLE 1 | Ongoing clinical trials of combination DNA targeting and/or immunotherapy agents in TNBC or BC with DDR mutations.

Phase	Trial ID	BC subtype	Biomarkers	Regimen	Targets	Clinical endpoint
I	NCT03544125	mTNBC	Pre- and post-tumor biopsy (CLIA) analytics	Olaparib + Durvalumab	PARP PD-L1	Safety, ORR, DOR, PFS, OS
I	NCT03101280	Advanced or mTNBC	–	Rucaparib + Atezolizumab	PARP PD-L1	DLTs, PK, ORR, CR, PFS
I/II	NCT03964532 TALAVE	Advanced BC	Germline BRCA1/2 Deleterious mutation OR BRCA1/2 wild status TNBC; Serial biopsies for PD-L1	Talazoparib + Avelumab	PARP PD-L1	Safety, ORR, PFS, OS
II	NCT04584255	BRCAm Stage I-III BC	BRCA mutations, pre- and post-TILs, STING activation, serum immune	Niraparib + Dostarlimab	PARP PD-1	pCR, RCB
II	NCT02849496	HER- mBC	BRCA 1/2 mutation, HRD, PD-L1, TILs, ctDNA	Olaparib + Atezolizumab	PARP PD-L1	PFS, TTF, ORR, DOR, irBOR
II	NCT03801369	mTNBC	Tumor characteristics, predictive biomarkers	Olaparib + Durvalumab	PARP PD-L1	ORR, OS
II	NCT03025035	Advanced BRCAm BC	germline mutations in BRCA1 or BRCA2	Olaparib + Pembrolizumab	PARP PD-1	ORR, PFS, OS, irRECIST
II	NCT03167619 DORA	Advanced or mTNBC	Molecular biomarkers, TILs, PD-L1 status, cTC, plasma DNA	Olaparib + Durvalumab	PARP PD-L1	PFS, CR, PR, SD, OS
II/III	NCT04191135 KEYLYNK-009	Advanced TNBC	-	Olaparib + Pembrolizumab	PARP PD-1	PFS, OS
I/II	NCT03594396 MEDIOLA	Stage II/III TNBC	Serial tumor and serum biopsy study	Olaparib + Durvalumab	PARP PD-L1	pCR, ORR
I/II	NCT02484404	Advanced or mTNBC	gBRCAm status	Olaparib + Durvalumab	PARP PD-L1	Safety, ORR, PFS
I/II	NCT02657889 TOPACIO	Advanced or mTNBC	–	Niraparib + Pembrolizumab	PARP PD-1	DLTs, ORR, DOR, PFS, OS, PK
II	NCT04169841 GUIDE2REPAIR	HR-mutated advanced or metastatic BC	HR repair gene mutations	Olaparib + Durvalumab + Tremelimumab	PARP PD-L1 CTLA-4	Safety, PFS
II	NCT03330847	mTNBC	BRCA1/2 mutations or HRRm	Olaparib + Ceralasertib or Adavosertib	PARP ATR WEE1	PFS, ORR, OS, DOR, PK
I	NCT03945604	Advanced or mTNBC	-	Apatinib + Fluzoparib + Camrelizumab	VEGF PARP PD-1	DLT, ORR, PFS, OS
II	NCT04837209 NADiR	mTNBC	TILs, ctDNA	Niraparib + Dostarlimab + RT	PARP PD-1 DNAx	ORR, irRECIST, OS, PFS
I/II	NCT02264678	Her2- BC with BRCAm or TNBC	BRCA mutations HRRm ATR inhibition, ctDNA, CTCs	Ceralasertib + Durvalumab	ATR PD-L1 DNAx	Safety, PK, ORR, PFS, OS
I	NCT01618357	Stage II-IV BC, residual after NAC	Apoptosis/proliferation biomarkers	Pre-operative Veliparib + RT	PARP DNAx	Safety, MTD
I	NCT03945721 UNITY	Non-mTNBC	HRD status	Niraparib + post-op RT	PARP DNAx	MTD, LRR, DFS, cosmesis
I	NCT02227082	Advanced or mTNBC	–	Olaparib + RT	PARP DNAx	Toxicity
I	NCT03542175	Post-op TNBC	-	Rucaparib + RT	PARP DNAx	MTD
I	NCT04052555	Non-mTNBC	DDR mutations	Berzosertib + RT	ATR DNAx	MTD, DFS, OS
I	NCT02977468 Pembro/IORT	Treatment naïve TNBC	TILs	Pembrolizumab + intra-op RT	PD-L1 DNAx	-
II	NCT03464942 AZTEC	Advanced TNBC	–	Atezolizumab + stereotactic RT	PD-L1 DNAx	PFS, ORR, DOR, OS
I	NCT02826434	Stage II/III TNBC, HLA-A2+	Immune response rate, vaccine-specific CTLs	Peptide vaccine + Durvalumab	XBP1, CD138 PD-L1	Safety, tolerability

mTNBC, Metastatic triple-negative breast cancer; BC, breast cancer; (CLIA) analytics, Proportion of completion of Clinical Laboratory Improvement Act; ORR, objective response rate; DOR, duration of response; PFS, progression-free survival; OS, overall survival; DLTs, Dose-Limiting Toxicities; MTD, maximum tolerated dose; PK, Pharmacokinetics; pCR, pathologic complete response; TILs, tumor infiltrating lymphocytes; STING, stimulator of interferon genes; RCB, pathway, residual cancer burden; HRD, homologous recombination deficiencies; HRRm, HRR-related gene mutation; ctDNA, circulating tumor DNA; TTF, time to treatment failure; irBOR, immune-related best overall response; irRECIST, immune-related; cTC, circulating tumor cells; SD, stable disease; gBRCAm, germline BRCA1 and BRCA2 mutation; DDR, DNA-damage response; DCR, pathway, disease control rate; LRR, locoregional relapse; DFS, distant relapse; CTLs, Cytotoxic T Lymphocytes; DNAx, therapeutic targeting of DNA DSBs; (HLA)-A2+, Human Leukocyte Antigen.

this combination created a stronger immunostimulatory phenotype with enhanced IFN-γ and CXCL9/CXCL10 expression, systemic IFN-γ/TNF-α production and TILs (128, 129). Combination treatment with Durvalumab with the PARPi Olaparib is currently under exploration in a phase I/II trial of women's cancers, including patients with TNBC, with biomarker evaluation ongoing (128). The phase II MEDIOLA basket trial assessed the efficacy and safety of combination Olaparib and Durvalumab in patients with solid tumors, including ovarian cancer, breast cancer and gastric cancer (NCT02734004). In germline BRCA mutant, platinum-sensitive relapsed ovarian cancer, this combination demonstrated an overall response rate (ORR) of 63% and a 12-week disease control rate (DCR) of 81% (15). In gBRCAm HER2 negative metastatic breast cancer, the DCR was 80% at 12 weeks and 50% at 28 weeks, with ORR of 63%. Median PFS (mPFS) was 9.2 months and median overall survival (mOS) was 21.5 months. Moreover, patients with no prior line of chemotherapy had higher ORR and longer OS than those with two prior lines (respectively 78% *vs.* 50% for ORR and 21.3 *vs.* 16.9 months for OS) (15). Although there is no observed association between PD-L1 positivity and TILs at this point in the trial, there was a trend of higher PD-L1 and increased TILs observed in archival samples in patients who had SD/PR/CR, which was not observed in patients with progressive disease. Furthermore, high PD-L1 was observed in patients with DCR at 12 weeks (15, 33). In the phase II TOPACIO trial (NCT02657889), Niraparib and Pembrolizumab combination therapy has demonstrated clinical benefit in platinum-resistant TNBC, with numerically higher response rates in those with BRCA-mutated TNBC tumors (ORR of BRCAm *vs.* BRCA wild-type, 47% *vs.* 11%) (130). A phase II multicenter study of Durvalumab and Olaparib is underway for patients with advanced TNBC that is inoperable, locally advanced, or metastatic, and is not amenable to resection with curative intent, and who have received at least 4 cycles of platinum-based chemotherapy with demonstrated clinical benefit (NCT03167619). Other trials combining PARPi's, including Olaparib, Rucaparib, and Fluzoparib, with ICIs, such as Pembrolizumab (NCT03101280), Atezolizumab (NCT04191135), and Camrelizumab (NCT03945604), respectively, for locally advanced or metastatic TNBC are also underway. A phase II study will evaluate safety and efficacy of combination of PARPi (niraparib), PD-1 mAb (Dostarlimab), and RT in metastatic TNBC (NCT04837209). Though the relationship between endogenous or PARPi-induced BRCAness and immunotherapy response is still being investigated, these ongoing clinical trials will help establish the effect HR-deficiency and DDR targeting therapies on ICI outcomes in TNBC.

3.3 Other DDR Targets and Immunotherapy in TNBC

Evidence that unrepaired DNA damage induced by PARPi expands the anti-tumor activity of the ICI has prompted investigation of other key mediators implied in DNA replication and repair, such as ATM, ATR, CHK1, CHK2, DNA-PK, and WEE1 (31, 120, 121). Given the immunomodulatory effects seen with PARPi, these additional DDR mediators are exciting targets for combined immunotherapy. In preclinical breast cancer studies, the combination of a selective ATR inhibitor with Avelumab and platinum-based chemotherapy resulted in antitumor effect in syngeneic tumor models, leading to overall survival benefit compared to any dual-combination group, and also provided protective antitumor immunity with immunological memory in cured mice (131). In a preclinical model of lung cancer, CHK1 inhibition potentiated the anti-tumor effect of PD-L1 blockade and augmented cytotoxic T cell infiltration (27). In other studies, inhibition of DNA-PK upregulated PD-L1 in a cGAS-STING-dependent manner in irradiated p53-mutant cancer cells, suggesting selective blockade of NHEJ repair of DSB exhibits immunomodulatory effects similar to those seen in HR-inhibition. Preclinical studies of combined DNA-PK inhibition, radiation and PD-L1 blockade demonstrated increased anti-tumor activity in a p53-mutant cancer, suggesting that inhibition of DNA-PK inhibits repair of radiation-induced DSBs resulting in potentiation of anti-tumor immunity, adaptive PD-L1 expression through DDR-dependent mechanisms, and subsequent responsiveness to immune checkpoint blockade (132).

These promising preclinical studies have led to several early phase clinical trials. A clinical study in patients with advanced or metastatic cancers, use of Ceralasertib, a potent and selective ATR inhibitor in combination with Durvalumab is being evaluated (NCT02264678). A selective ATR kinase inhibitor, AZD6738, is undergoing a phase II study with Olaparib for metastatic TNBC patients with BRCA1/2 mutations or HRD (NCT03330847). The phase Ib BISCAY study, Durvalumab and Olaparib or the WEE1 inhibitor Adavosertib in patients with metastatic cancer with any detected HR-deficiency (NCT02546661). A phase I study combining the CHK1 inhibitor, Prexasertib, with a PD-L1 mAb demonstrated the potential for enhanced therapeutic activity and increased cytotoxic T cell activation (125).

Further highlighting the indication that DDR-inhibitors and DNA-damaging agents may enhance immunotherapeutic response, a phase II clinical trial is evaluating the efficacy of Atezolizumab with stereotactic RT for advanced TNBC (NCT03464942), and a phase I study for the feasibility of adjuvant Durvalumab with a peptide vaccine is underway for patients with stage II and III TNBC after completion of standard adjuvant therapy (NCT02826434). Altogether, early studies indicate a potential therapeutic benefit of DDR-pathway targeting/inhibition in combination with immunotherapy, and ongoing trials will provide new insights into and establish clinical efficacy of the immune potentiating efficacy of DDR-inhibitors.

4 SUMMARY

TNBC represents a highly diverse set of breast cancers with complicated molecular and immunologic landscapes, and thus remains a challenging oncologic entity to tackle effectively. However, advances in genomic profiling and our understanding of the interplay between DNA damage response and cancer immunity has resulted in exciting immuno-molecular

therapeutic opportunities. Of these, DDR-deficiencies including BRCAness have been shown to promote immunologic vulnerability through DNA damage-induced high TMB, immune-stimulatory and suppressive features, as well as adaptive immune resistance *via* PD-L1 upregulation. DDR deficiencies represent a frequent aberration in TNBC, and exploitation of immunologic consequences offers potential therapeutic leverage that combines favorable immune effects of DNA/DDR-targeted therapies with restoration of cytotoxic anti-tumor immune cells. The role for endogenous as well as therapy-induced DNA damage signaling in PD-L1 induced expression, and the possibility of circumventing DNA targeted therapy-induced immune suppression with concomitant immunotherapy provide rationale for combining agents targeting the DDR and the immune system. Immunotherapy, chiefly ICI, represents an opportunity to flip the switch back to immune activation, particularly in the context of concomitant DDR pathway targeting therapies, such as PARP inhibitors and others.

PARP inhibitor monotherapies, as well as therapeutic combinations, have demonstrated promising clinical benefit, and their effects on enhancing lethal DNA damage vulnerabilities have been shown. Nonetheless, the underlying mechanisms of PARPi-mediated sensitization of tumors to immunotherapy and/or radiotherapy remain to be fully elucidated. Furthermore, rapid translation of these potential breakthroughs in TNBC treatment will require thoughtful incorporation and thorough dissection of clinical trial outcomes and their implications into everyday clinical practice. Despite preclinical and clinical studies that have demonstrated PARPi-mediated immunosuppression *via* PD-L1 induction and complementary restoration of PARPi sensitivity *via* PD-L1 inhibition, with the added possibility of enhanced anti-tumor immunity, many unanswered questions remain regarding the potential benefit of combined targeted therapies and ICIs in TNBC. In addition to PARPi, other repair pathway mediators such as ATR, and CHK1, are being investigated in combination with immune-based strategies, and thus careful consideration of promising therapeutics as well as other immunotherapeutic strategies in the pipeline should not be overlooked. Optimization of treatment schemas for combined immunotherapeutic strategies remains a challenge, as does validation of biomarkers that will identify which patients will most benefit from either PARP inhibitors in combination with immunotherapy, radiotherapy, or other targeted therapies.

Lastly, identifying additional key mediators of DNA damage-associated immune modulation that regulate disease progression, therapeutic response and resistance will require further preclinical investigation and careful analysis of clinical samples to assess DDR deficiencies in certain tumor subsets, with the ultimate goal of personalizing DNA targeting and immune-based therapies in combination with conventional DNA- and immune-augmenting therapies, such as chemotherapy and radiation, to maximize the combined benefits of each approach and effectively target immunosuppressive pathways that contribute to immune escape and tumor progression. It will also be important to identify mediators of poor response to ICIs and improved prognostic markers for existing therapies to select patients that may benefit from alternative therapeutic strategies and explore options for TNBC refractory to ICI or PD-L1 negative TNBC. Furthermore, the role of less studied DDR mechanisms related to ICI is still unclear, and future work is needed to better predict which DNA damage response and repair pathways are most suitable for therapeutic targeting in specific subsets of patients.

AUTHOR CONTRIBUTIONS

Both authors initiated the concept and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Targeted Therapeutic Strategies for Triple-Negative Breast Cancer

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Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, which is characterized by the absence of estrogen receptor (ER) and progesterone receptor (PR) expression and the absence of human epidermal growth factor receptor 2 (HER2) expression/amplification. Conventional chemotherapy is the mainstay of systemic treatment for TNBC. However, lack of molecular targeted therapies and poor prognosis of TNBC patients have prompted a great effort to discover effective targets for improving the clinical outcomes. For now, poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi's) and immune checkpoint inhibitors have been approved for the treatment of TNBC. Moreover, agents that target signal transduction, angiogenesis, epigenetic modifications, and cell cycle are under active preclinical or clinical investigations. In this review, we highlight the current major developments in targeted therapies of TNBC, with some descriptions about their (dis)advantages and future perspectives.

Keywords: targeted therapy, triple-negative breast cancer, poly (ADP-ribose) polymerase, immune checkpoint, epigenetic modification

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INTRODUCTION

Breast cancer is the type of cancer with the best-characterized molecular classification or subtyping. Clinical therapeutic efficacies vary enormously among the different subtypes, with luminal A/B subtypes and triple-negative breast cancer (TNBC) showing the best and worst outcomes, respectively (1). For TNBC, although initially responsive to chemotherapy, which is the mainstay of systemic treatment in TNBC, resistance occurs eventually in a significant portion of patients, leading to relapse of these patients. Due to the aggressive nature and lack of defined molecular targets, the poor overall survival (OS) of metastatic TNBC has remained essentially unchanged over the past two or three decades. Generally speaking, metastatic TNBC has a median OS of approximately 13 months (2), rendering improvement of the clinical outcomes an urgent task in the management of TNBC. Fortunately, we are now seeing encouraging clinical results from molecularly targeted approaches in TNBC, which include poly (ADP-ribose) polymerase inhibition and, most recently, immune checkpoint inhibition. Other potential promising targeted therapeutic strategies that are being actively investigated for TNBC include inhibition of signaling kinases (serine/threonine- or tyrosine-type), angiogenesis, epigenetic modifications, and cell cycle. The targeted therapeutic strategies of TNBC examined in clinical and preclinical studies are summarized in **Table 1**.

TABLE 1 | Overview of Potential Targeted Therapeutic Strategies for TNBC.

Targets	Agents	Phase	Main Results	Advantages	Disadvantages	References
PARP	Olaparib	I/II/III	•**The OlympiA trial:** 3-year IDFS, 3-year DDFS, and OS were significantly higher in the olaparib group. •**The TBCRC 048 trial:** The ORR was 33% in germline mutations of non-BRCA1/2 HR-related genes and 31% in somatic mutations of BRCA1/2 or other HR-related genes; the median PFS for gPALB2 and sBRCA1/2 mutation carriers were 13.3 and 6.3 months; among the gPALB2 and sBRCA1/2 mutation carriers, responses occurred in 67% of TNBC patients. •**The olaparib combination with carboplatin trial:** Hematologic toxicity was the most common AE, with 36% of patients having Grade 3 and 4 neutropenia.	•Increase efficacy of BRCA-mutated breast cancer •Improve ORR, PFS and OS when combined with chemotherapy drugs	•Not suitable for patients with defective BRCA genes •High incidence of adverse events	(3–9)
	Veliparib	II	•**The I-SPY 2 trial:** Veliparib combined with carboplatin had higher rate of pCR than standard therapy alone.			
	Iniparib	II	•**The PrECOG 0105 trial:** The mean HRD-LOH scores were higher in responders compared with non-responders in iniparib clinical trails.			
Immune checkpoints	PD1: pembrolizumab	FDA-approved	•**The KEYNOTE-522 trial:** Higher percentage of patients having grade 3 or more serious AEs in the pembrolizumab plus chemotherapy group; patients in the pembrolizumab plus chemotherapy group had a higher pCR rate, which also occurred in PDL1-positive and PDL1-negative population. •**The KEYNOTE-355 trial:** Among patients with CPS of 10 or more, median PFS was significantly prolonged in the pembrolizumab plus chemotherapy group.	•Improve the OS and PFS rate of patients •Benefit from companion diagnostic	•High incidence of adverse events •Not all patients express PDL1 •No increasing pCR in combination with chemotherapy drugs	(10–15)
	PDL1: atezolizumab, durvalumab	II/III	•**The IMpassion050 trial:** Atezolizumab combination with chemotherapy didn't increase pCR either in the intention-to-treat population or in the PDL1-positive population; in the neoadjuvant phase, patients with Grade 3/4 or more serious AEs were increased in the atezolizumab group. •**The IMpassion130 trial:** Atezolizumab plus nab-paclitaxel prolonged PFS in both intention-to-treat population and PDL1-positive population. •**The GeparNuevo study:** Increased pCR rate was observed in both durvalumab and placebo group with higher stromal TILs or positive PDL1 expression; the pCR rate of patients with high TMB and high immune GEP or TILs was notably higher compared with patients with low TMB and low immune GEP or TILs.			
Antibody-drug conjugates	Trop2: sacituzumab govitecan	FDA-approved	•**The ASCENT trial:** In the sacituzumab govitecan group, PFS and OS were significantly prolonged and pCR rate was increased. According to the therapeutic effect, sacituzumab govitecan is recently approved for metastatic TNBC patients.	•Improve the prognosis of patients •Well tolerated	•A complex engineered	(16–18)
	LIV1: ladiratuzumab vedotin	II/III	•**A phase Ib/II trial:** Ladiratuzumab vedotin was well tolerated and the combination with pembrolizumab produced a synergistic effect through immunogenic cell death that might enhance anti-PD1 activity.			
Signaling pathways	EGFR: cetuximab	II	•**A phase II study:** The ORR was 20% with cisplatin plus cetuximab and 10% with cisplatin alone; patients treated with cisplatin plus cetuximab had longer PFS than those treated with cisplatin alone.	•Inhibite tumor metastasis and induce a change from mesenchymal to epithelial phenotype •Improve PFS	•Need to combine with medication for a better effect	(19–24)
	EGFR: erlotinib	Preclinical	•**Preclinical study:** Erlotinib inhibited tumor growth and metastasis and reversed a change from mesenchymal to epithelial phenotyp.			
	PI3K: BKM120	Preclinical	•**Preclinical study:** BKM120 led to significant tumor growth inhibition in PDX models (TNBC).			
	Akt: ipatasertib	II	•**A randomized, double-blind, phase II trial:** The median PFS in the ipatasertib group was 6.2 months, compared with 4.9 months in the placebo group.			
Angiogenesis	VEGF: bevacizumab	II/III	•**The RIBBON-2 trial:** Bevacizumab-containing therapy improved median PFS from 2.7 months to 6.0 months, median OS from 12.6 months to 17.9 months, and ORR from 18% to 41% and showed a 49% response rate, median TTP of 7.2 months, and median OS of 18.3 months. •**The GeparQuinto trial:** Bevacizumab to neoadjuvant anthracycline-taxane-containing chemotherapy significantly increased the pCR rate from 27.9% to 39.3% in TNBC patients. •**The BEATRICE study:** There are no differences in 3-year IDFS and OS, in which TNBC patients received chemotherapy with or without bevacizumab. •**The CALGB 40603 trail:** Patients treated with carboplatin had higher pCR breast and pCR breast/axilla rates, while patients received bevacizumab only had higher pCR breast rate.	•Show efficacy in the patient's tumor response and/or disease control	•Detrimental side-effects, along with acquired drug-resistance	(25–31)
	VEGFR: apatinib	II	•**A multicenter phase II study:** The ORR and clinical benefit rate were 10.7% and 25.0% and median PFS and OS were 3.3 months and 10.6 months in the apatinib trial.			
Epigenetic modifications	DNMT: 5-azacytidine/AZA, decitabine/DAC	Preclinical	•**Preclinical study:** PARPi's plus AZA/DAC increased PARPi efficacy and resulted in additional tumor inhibition in TNBC cells harboring wild-type BRCA1 compared with each drug alone.	•Increase PARPi efficacy •Enhance the effect of TNBC immunotherapy.	•Lack of adequate clinical trials	(32–37)

(Continued)

TABLE 1 | Continued

Targets	Agents	Phase	Main Results	Advantages	Disadvantages	References
	HDAC: suberoylanilide hydroxamic acid (SAHA),entinostat (ENT)	Preclinical	•**Preclinical study:** ENT increased the expression of ERα and aromatase and restored the sensitization of breast cancer cells to the aromatase inhibitor letrozole. SAHA could enhance the anti-tumor effects of the PARPi olaparib in TNBC cells by regulating the expression of homologous recombination repair (HRR)-related genes and hampering DNA repair.			
Cell cycle	CDK4/6: palbociclib	I/II	•**Phase I/II clinical trials** of the safety and efficacy of CDK4/6 inhibition with or without other agents in TNBC are ongoing. •**Preclinical study:** Dual blockade of PI3K and CDK4/6 had synergistic effect and could generate immunogenic cell death in TNBC cells; palbociclib could improve the sensitivity of Rb-positive TNBC cells to paclitaxel.	•Be benefited from high expression levels of mitotic checkpoint molecules in TNBC •Sufficient evidence from preclinical trials	•Not significant for patients lacking functional Rb protein •Lack of adequate clinical trials	(38–48)
	TTK: BOS172722	Preclinical	•**Preclinical study:** TTK inhibitors have anti-proliferative effects; combination of BOS172722 and paclitaxel results in significant tumor regression compared with either drug alone.			
	PLK4: CFI-400945	Preclinical	•**Preclinical study:** CFI-400945, in combination with radiation, exhibited a synergistic anti-cancer effect in TNBC cell lines and patient-derived organoids and led to a significant increase in survival to tumor endpoint in xenograft models.			
	ATR: VX-970	Preclinical	•**Preclinical study:** ATR or CHK1 inhibitor could delay the radiation-induced DNA repair and inhibit cell survival in TNBC cells.			
	CHK1: MK-8776	Preclinical				
	WEE1: MK-1775	Preclinical	•**Preclinical study:** WEE1 inhibition could overcome cisplatin resistance in TNBC cells.			
	TRAIL receptor agonist: drozitumab	Preclinical	•**Preclinical study:** TRAIL receptor agonist could induce apoptosis in TNBC cells that expressed vimentin and Axl.			

INHIBITION OF POLY (ADP-RIBOSE) POLYMERASES IN TNBC

Poly (ADP-ribose) polymerases (PARPs) are a family of proteins involved in DNA damage repair and multiple other cellular processes. So far, 17 PARP members have been identified in human (49). Among them, PARP1 is the best-characterized family member and is responsible for 85-90% of the total PARP activity. It is activated by single-strand breaks (SSBs), thus catalyzing the synthesis of poly (ADP-ribose) chains that serve as a signal and platform to recruit other DNA repair proteins. Failure to repair SSBs because of the PARP deficiency or inhibition leads to the formation of double-strand breaks (DSBs).

In the cells that are functional for breast cancer susceptibility gene products (BRCA1 and BRCA2), DSBs can be repaired by a process called homologous recombination (HR). Therefore, BRCA-mutated tumors are more sensitive to inhibition of PARPs due to combined loss of PARP and HR repair, an effect called "synthetic lethality" (50, 51). In the presence of PARP inhibitors (PARPi's), the cells with BRCA defects cannot repair the DNA damage and die, whereas the cells with functional BRCAs could perform effective DNA damage repair and survive (**Figure 1**). Up to 80% of ER/PR-negative breast cancers have reduced or undetectable BRCA1 expression (52). Although germline mutations in BRCA1/2 are generally low, these mutations can confer a lifetime risk of up to 85% of developing breast cancer, with the majority (around 90%) of these tumors being triple-negative (53). Therefore, TNBC can theoretically be treated by a strategy of synthetic lethality that is based on PARP inhibition in BRCA-mutated tumors.

PARP Inhibitors and Clinical Trials in TNBC

Various PARPi's, which hamper DNA repair by blocking PARP-mediated PARylation, have been developed to induce synthetic lethality. Up to now, four PARPi's, i.e., olaparib, rucaparib, niraparib, and talazoparib, have been approved by the US Food and Drug Administration (FDA) for cancer treatment. Two of them, olaparib and talazoparib, have been approved for BRCA-mutated metastatic breast cancer (54). While PARPi's have been evaluated in clinical trials for TNBC as monotherapies, combination of PARPi's with DNA-damaging chemotherapy appears to be a more promising approach due to increased efficacy of PARPi's in BRCA-mutated breast cancer patients.

Olaparib is effective for patients with germline/somatic BRCA mutation or other HR-related gene mutations. The phase III OlympiA trial (NCT02032823) accessed olaparib treatment in HER2-negative breast cancer patients with germline BRCA1/2 mutations who had received neoadjuvant or adjuvant chemotherapy. The 3-year invasive disease-free survival (IDFS), 3-year distant disease-free survival (DDFS), and OS were significantly higher in the olaparib group than in the placebo group (3).

The phase II TBCRC 048 trial (NCT03344965) assessed olaparib response in metastatic breast cancer patients with

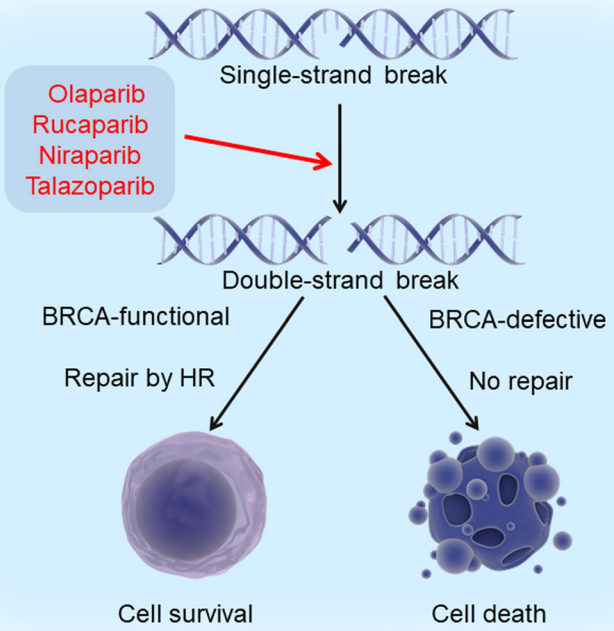

FIGURE 1 | Synthetic lethality induced by PARPi's and BRCA deficiency. Exposure of cells to PARPi's (olaparib, rucaparib, niraparib, and talazoparib) leads to the formation of double strand breaks (DSBs) from single strand breaks (SSBs). Cells with intact BRCA function could survive since these breaks can be repaired by homologous recombination, while those with defective BRCA die because DSBs cannot be repaired. This phenomenon is known as "synthetic lethality".

germline mutations of non-BRCA1/2 HR-related genes (cohort 1) and somatic mutations of BRCA1/2 or other HR-related genes (cohort 2). The objective response rate was 33% in cohort 1 and 31% in cohort 2. Confirmed responses were only seen in patients with gPALB2 or sBRCA1/2 mutations. The median progression-free survival (PFS) for gPALB2 and sBRCA1/2 mutation carriers were 13.3 and 6.3 months, respectively. Among the gPALB2 and sBRCA1/2 mutation carriers, responses occurred in 67% of TNBC patients. No responses were observed with ATM or CHK2 mutations alone (4). This study revealed that patients with mutations of other HR-related genes might benefit from PARP inhibition.

With no BRCA mutation, patients would benefit more from combination treatment with chemotherapy and olaparib. A phase I study (NCT01445418) investigated olaparib combined with carboplatin in metastatic or recurrent TNBC patients with no germline BRCA mutation or with BRCAPro scores < 10% and negative family history. The objective response rate was 22%, with 1 patient having complete response. Hematologic toxicity was the most common adverse event (AE), with 36% of patients having Grade 3 and 4 neutropenia (5).

Veliparib, a novel PARPi that has favorable toxicity profile but is not FDA-approved yet, has been extensively studied in combination with various chemotherapeutic drugs. In a phase I clinical trial, the combination of veliparib with cisplatin and vinorelbine (a microtubule-destabilizing agent) gave rise to an overall response rate (ORR) of 73% in TNBC patients with mutated BRCA1/2 (55). In a phase III trial (NCT02032277), veliparib has been combined with paclitaxel plus carboplatin for the treatment of TNBC in standard neoadjuvant chemotherapy (56).

Homologous Recombination Deficiency (HRD) as the Predictive Biomarker for PARP Inhibitors

BRCA1/2 and other HR-related gene mutations could cause a defect in DSB repair called homologous recombination deficiency (HRD), leading to genomic instability and thus enhanced sensitivity to PARPi's. Therefore, HRD status (including but not limited to BRCA1/2 mutations) could be evaluated to predict the response of PARPi's (5, 57, 58).

I-SPY 2 trial (NCT01042379) showed that the PARPi veliparib combined with carboplatin had higher rate of pathological complete response (pCR) than standard therapy alone, specifically in TNBC (6). Further study revealed that BRCA1/2 mutation carriers were more likely to achieve a pCR compared to wild-type patients in the veliparib/carboplatin arm (7). In the PrECOG 0105 (NCT00813956) trial, patients with TNBC were treated with iniparib and chemotherapy, and the

mean homologous recombination deficiency loss of heterozygosity (HRD-LOH) scores were higher in responders compared with non-responders (8). Jiang et al. reported that TNBC patients with higher HRD scores might have better prognosis and benefit from DNA repair inhibitors (9).

The above studies suggest that PARPi's have shown great promise in TNBC patients and may be used as an effective therapeutic strategy for the treatment of BRCA-mutated or even BRCA-intact TNBC. Further more excited clinical findings are expected with the optimization of the therapeutic regimen.

INHIBITION OF IMMUNE CHECKPOINTS IN TNBC

Recently, there is enormous interest in cancer immunotherapy, particularly immune checkpoint-based immunotherapy. This is demonstrated by the awarding of the Nobel Prize in Physiology or Medicine in 2018 to James P. Allison at the University of Texas MD Anderson Cancer Center and Tasuku Honjo at Kyoto University, for their seminal work in identification of immune checkpoint molecules, i.e., programmed cell death-1 (PD1), programmed death-ligand 1 (PDL1), and cytotoxic T lymphocyte-associated protein 4 (CTLA4).

Immune Checkpoint Inhibitors and Clinical Trials in TNBC

The discovery of these molecules led to the development of several FDA-approved humanized antibodies, so called immune checkpoint inhibitors, such as nivolumab, atezolizumab, and ipilimumab. These antibodies have demonstrated very well documented benefit for a variety of cancers (59) (**Figure 2**). Breast cancer, in general, is not an immunologically highly active cancer. However, the TNBC subtype shows higher presence of tumor-infiltrating lymphocytes (TILs) and is likely to respond to immunotherapy (60).

KEYNOTE-522 trial (NCT03036488) evaluated the safety and efficacy of pembrolizumab plus chemotherapy as neoadjuvant therapy, followed by definitive surgery and pembrolizumab as adjuvant therapy in patients who had early TNBC. Most treatment-related AEs occurred during the neoadjuvant phase, with higher percentage of patients having grade 3 or more serious AEs in the pembrolizumab plus chemotherapy group than in the placebo plus chemotherapy group. Consistent results were observed in the adjuvant phase. At the first and second interim analysis, patients in the pembrolizumab plus chemotherapy group had a higher pCR rate, which also occurred in PDL1-positive and PDL1-negative population, indicating that PDL1 expression was not a suitable predictor of response in early TNBC (10).

IMpassion050 trial (NCT03726879) evaluated the efficacy and safety of atezolizumab compared with placebo when it was combined with chemotherapy in high risk, HER2-positive early breast cancer. Results showed that this combination didn't increase pCR either in the intention-to-treat population or in the PDL1-positive population. In the neoadjuvant phase, patients with Grade 3/4 or more serious AEs were increased in the atezolizumab group. There were 4 patients with Grade 5 AEs, including alveolitis, septic shock, sepsis, and COVID-19, in the neoadjuvant phase and 1 patient in the adjuvant phase (11).

PDL1 Expression, Tumor Mutation Burden (TMB), and Immune Infiltration as Predictive Biomarkers of Immune Checkpoint Inhibitors

Clinical trials have shown a correlation between high expression of PDL1 and efficacy of immune checkpoint inhibitors in metastatic TNBC. Thus, PDL1 could be a potential predictive biomarker of response to immunotherapy. Two antibody-based companion diagnostics for PDL1 expression are available. The PDL1 IHC 22C3 pharmDx (Agilent Technologies) is approved for selecting patients for treatment with pembrolizumab, using a cutoff of combined positive score (CPS) of 10. The Ventana PDL1 (SP142) assay (Roche Diagnostics) is approved for treatment with atezolizumab in metastatic TNBC, using a cutoff of immune cell (IC) score of 1% (61, 62).

In the phase III KEYNOTE-355 trial (NCT02819518), metastatic TNBC patients were randomly assigned 2:1 to receive pembrolizumab plus chemotherapy or placebo plus chemotherapy. PDL1 expression of formalin-fixed tumor samples was assessed by the PDL1 IHC 22C3 pharmDx assay and characterised by CPS. Among patients with CPS of 10 or more, median PFS was significantly prolonged in the pembrolizumab plus chemotherapy group (12).

In the IMpassion130 trial (NCT02425891), patients with untreated metastatic TNBC were randomly assigned in a 1:1 ratio to receive PDL1 antibody atezolizumab plus nab-paclitaxel or placebo plus nab-paclitaxel. The PDL1 expression on tumor-infiltrating immune cells was evaluated by PDL1 (SP142) immunohistochemical assay (IC score ≥ 1%, PDL1-positive). Kaplan-Meier analysis showed that atezolizumab plus nab-paclitaxel prolonged PFS in both intention-to-treat population and PDL1-positive population (13).

Besides PDL1 expression, tumor mutation burden (TMB) and immune infiltration could also be predictors for immune checkpoint inhibitor response. In the phase II GeparNuevo study (NCT02685059), patients with early TNBC were randomly assigned to receive durvalumab or placebo in addition to chemotherapy. Increased pCR rate was observed in both durvalumab and placebo group with higher stromal TILs or positive PDL1 expression (14). Whole exome sequencing and RNA sequencing of these samples showed that median TMB was significantly higher in patients with a pCR. The pCR rate of patients with high TMB and high immune gene expression profile (GEP) or TILs was notably higher compared with patients with low TMB and low immune GEP or TILs, which indicated both TMB and immune GEP or TILs were pCR predictors (15).

These findings are expected to lead to new effective treatment options for patients with TNBC. The immune checkpoint-based strategy for the therapy of TNBC is the topic of our recently published review. For sake of saving time and space, immune checkpoint inhibition in TNBC will not be described redundantly here. Interested readers please refer to our review (63) and another review published last year by Keenan et al. (64).

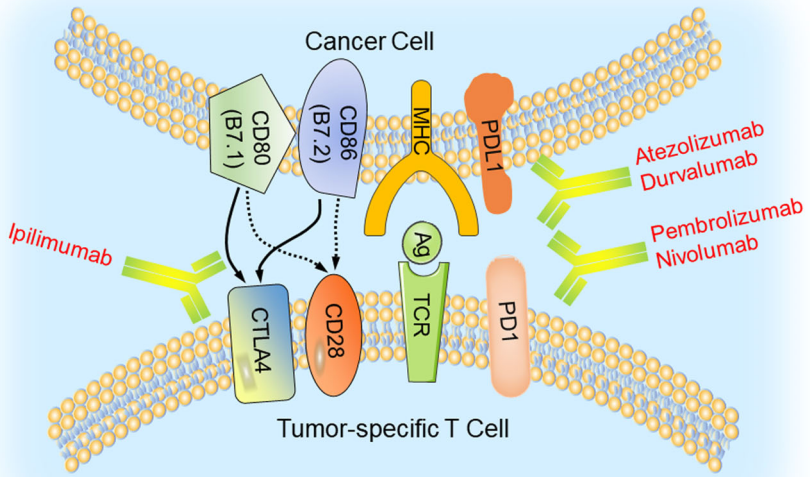

FIGURE 2 | Immune checkpoint blockade in TNBC. Major histocompatibility complexes (MHCs) are antigens on the surface of the cancer cell for recognition by the cytotoxic T lymphocyte (CTL) *via* the TCR. The binding of PD1 on the surface of the CTL with its ligand PDL1 functions to suppress the activation of the CTL, leading to its cell death. CTLA4 is another inhibitory immune checkpoint molecule expressed on CTL. Antibodies (anti-CTLA4/ipilimumab, anti-PD1/pembrolizumab and nivolumab, anti-PDL1/atezolizumab and durvalumab) inhibit these immune checkpoint proteins to restore the activity of CTLs and kill cancer cells.

It should be noted that the benefit of immune checkpoint inhibition in TNBC is dependent on the protein level of the immune checkpoint molecules. For example, patients with PDL1-positive immune cells had prolonged PFS treated with atezolizumab (13). Furthermore, the status of post-translational modifications such as glycosylation of the PDL1 protein also significantly impacts the therapeutic efficacy of immune checkpoint inhibition in TNBC (65). For patients with unresectable locally advanced or metastatic TNBC whose tumors have PDL1 expression ≥ 1%, atezolizumab plus nab-paclitaxel is an effective therapeutic option (66). Therefore, it will be pivotal to screen predictors of response to immune checkpoint inhibitors for better option. In addition, combination with chemotherapy would benefit more than immune checkpoint inhibition alone.

APPLICATION OF ANTIBODY-DRUG CONJUGATES IN TNBC

Antibody-drug conjugates (ADCs) are complex engineered therapeutics composed of monoclonal antibodies that specifically recognize tumor-associated antigens and cytotoxic agents that bind to the antibody *via* a linker. ADCs could precisely target the cells and are internalized through endocytosis. Then they are decomposed to release cytotoxic agents, which induce cell death eventually. This targeted therapeutic delivery approach could reduce off-target toxicity by limiting normal tissues exposed to the cytotoxic agents (67).

Sacituzumab govitecan comprises an antibody targeting trophoblast cell-surface antigen 2 (Trop2), which couples to SN-38, a topoisomerase I inhibitor, through cleavable CL2A linker. A phase III ASCENT trial (NCT02574455) evaluated the efficacy of sacituzumab govitecan comparing with single-agent chemotherapy in patients with relapsed or refractory metastatic TNBC. In the sacituzumab govitecan group, PFS and OS were significantly prolonged and pCR rate was increased (16). According to the therapeutic effect, sacituzumab govitecan is recently approved for metastatic TNBC patients who have received two prior lines of therapy.

Ladiratuzumab vedotin (or SGN-LIV1A) is an investigational anti-LIV1 antibody-drug conjugate. The antibody binds to monomethyl auristatin E *via* a protease-cleavable linker. A phase Ib/II trial (NCT03310957) studied the combination of ladiratuzumab vedotin with pembrolizumab in patients with metastatic TNBC. Preliminary results showed ladiratuzumab vedotin was well tolerated and the combination with pembrolizumab produced a synergistic effect through immunogenic cell death that might enhance anti-PD1 activity (17, 18).

INHIBITION OF SIGNALING PATHWAYS IN TNBC

In cancer cells, some signaling pathways are highly activated, such as EGFR and its downstream PI3K/Akt/mTOR pathway (**Figure 3**), which could accelerate tumor initiation and

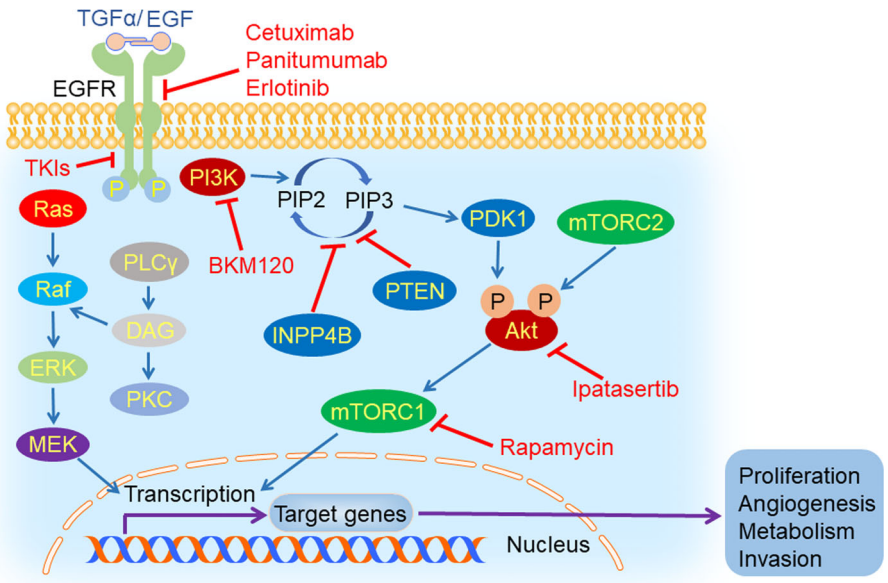

FIGURE 3 | EGFR and its downstream signaling pathways inhibition in TNBC. Epidermal growth factor receptor (EGFR) could be activated by its ligand EGF or transforming growth factor α (TGFα). After its activation, it can dimerize with all members of HER family and create homo- or hetero-dimers, triggering a myriad of downstream signaling pathways, such as PI3K/Akt/mTOR, Ras/Raf/MEK/ERK and PLCγ/PKC. EGFR inhibitors (cetuximab, TKIs, panitumumab, and erlotinib), PI3K inhibitor (BKM120), mTORC1 inhibitor (rapamycin) and Akt inhibitor (ipatasertib) could hamper tumorigenesis and tumor progression by suppressing the process of signal transduction.

progression. Thus, inhibiting these signaling pathways might be a potential therapeutic strategy for TNBC patients.

EGFR Inhibition

Epidermal growth factor receptor (EGFR) is a glycoprotein located on the surface of the cell membrane, which belongs to the HER family of transmembrane receptors. EGFR is activated by binding to its ligand including epidermal growth factor (EGF) and transforming growth factor α (TGFα). Following ligand binding, it can dimerize with all members of the HER family and generate homo- or hetero-dimers which could be autophosphorylated (68). The autophosphorylation triggers a myriad of downstream signaling pathways, such as PI3K/Akt, Ras/Raf/MEK/ERK and PLCγ/PKC, that play an important role in cell survival, proliferation, differentiation, motility, apoptosis, migration, adhesion, and angiogenesis (69). In TNBC, EGFR was overexpressed and was closely related with carcinogenesis and tumor progression (70). The expression of EGFR was negatively correlated with prognosis of TNBC patients (71).

EGFR could be targeted by monoclonal antibodies (cetuximab, panitumumab) and tyrosine kinase inhibitors (TKIs). Monoclonal antibodies and TKIs are approved for the treatment of advanced cancers, such as colorectal cancers and non-small cell lung cancers (72). However, two randomized phase II trials targeting EGFR in TNBC have not demonstrated significant beneficial effects. In the TBCRC 001 study, metastatic TNBC patients were treated with cetuximab alone and then plus carboplatin in progression compared to the combination therapy from the beginning. In another phase II study (NCT00463788), patients with metastatic TNBC received cisplatin plus cetuximab or cisplatin alone. The ORR was 20% with cisplatin plus cetuximab and 10% with cisplatin alone. Patients treated with cisplatin plus cetuximab had longer PFS than those treated with cisplatin alone (19).

Despite the unsatisfactory clinical data, the results should not be ignored when considering the potential of anti-EGFR agents in TNBC. A preclinical study from MD Anderson Cancer Center demonstrated that the EGFR tyrosine kinase inhibitor erlotinib inhibited tumor growth and metastasis and reversed a change from mesenchymal to epithelial phenotype by increasing the expression of E-cadherin and decreasing the expression of vimentin in TNBC cells (20). Another preclinical research showed that erlotinib inhibited tumor growth and metastasis in a SUM149 xenograft mouse model, which might be non-specific effect of EGFR inhibition since erlotinib could inhibit other kinases (21). The above results suggest that EMT modulation by targeting EGFR may reduce metastasis of TNBC, and inhibiting EGFR may be a potential therapeutic approach to patients with TNBC.

PI3K/Akt/mTOR Inhibition

Phosphoinositide 3-kinase (PI3K) is a lipid kinase which is activated by receptor tyrosine kinases (RTKs) and catalyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) subsequently. Phosphoinositide-dependent kinase 1 (PDK1) and Akt are both recruited by IP_3 and located

near the plasma membrane. Then, Akt is phosphorylated at Thr308 by PDK1, leading to its partial activation. Full activation of Akt occurs upon the phosphorylation at Ser473 by mTORC2 (73). The PI3K/Akt/mTOR signaling pathway plays a vital role in cell growth, proliferation, angiogenesis, and metabolism (74), which is negatively regulated by PTEN and INPP4B (75).

The PI3K/Akt/mTOR pathway is an important oncogenic driver in TNBC. The activation mutations of PIK3CA, the gene encoding the catalytic subunit of PI3K (76), are 23.7% in TNBC. The inhibition of the PI3K/Akt/mTOR signaling pathway has exhibited a promising prospect in treating TNBC. In patient-derived xenograft (PDX) models originating from TNBC, the PI3K inhibitor BKM120 was used to evaluate their response by measuring tumor growth. It has been shown that BKM120 therapy led to significant tumor growth inhibition in all models, with the percentage of tumor growth inhibition (%TGI) ranging from 35% in the least sensitive model WHIM12 (PTEN-deficient) and 84% in the most sensitive model WHIM4 (PTEN-normal) (22). Lin et al. proposed another strategy for using an mTORC1 inhibitor, rapamycin, to combat metastatic TNBC with upregulated Gαh, also known as tissue transglutaminase (tTG) or transglutaminase 2 (TG2) (23). Patients from a randomized, double-blind, phase II trial (NCT02162719) received intravenous paclitaxel with or without Akt inhibitor ipatasertib until disease progression or unacceptable toxicity. Results showed that median PFS in the ipatasertib group was 6.2 months, compared with 4.9 months in the placebo group. These are the first results supporting Akt-targeted therapy for TNBC (24). The development of drugs targeting the PI3K/Akt/mTOR pathway for the treatment of TNBC is an emerging field, and we look forward to more promising clinical trials.

INHIBITION OF ANGIOGENESIS IN TNBC

Solid tumors couldn't grow beyond a certain size or metastasize to another organ without blood vessels (77). Thus, blocking tumor angiogenesis could cut off intertumoral oxygen and nutritional supply and arrest tumor growth (**Figure 4**). Vascular endothelial growth factor (VEGF) and its receptor VEGFR have been demonstrated to be major contributors to angiogenesis (78). The VEGF signaling stimulates cellular pathways that promote the formation of intertumoral blood vessels, leading to rapid tumor growth and metastatic potential (79).

VEGF is highly expressed in TNBC and a higher VEGF content is significantly correlated with shorter relapse-free survival (RFS) as well as OS (80). Bevacizumab is a humanized antibody binding to VEGF-A, the prototype VEGF family member, which prevents VEGF from interacting with its receptor, VEGFR. A randomized phase III RIBBON-2 trial revealed that second-line bevacizumab-containing therapy for TNBC patients improved median PFS from 2.7 months to 6.0 months, median OS from 12.6 months to 17.9 months, and ORR from 18% to 41%, respectively (25). A first-line bevacizumab-containing therapy showed a 49% response rate,

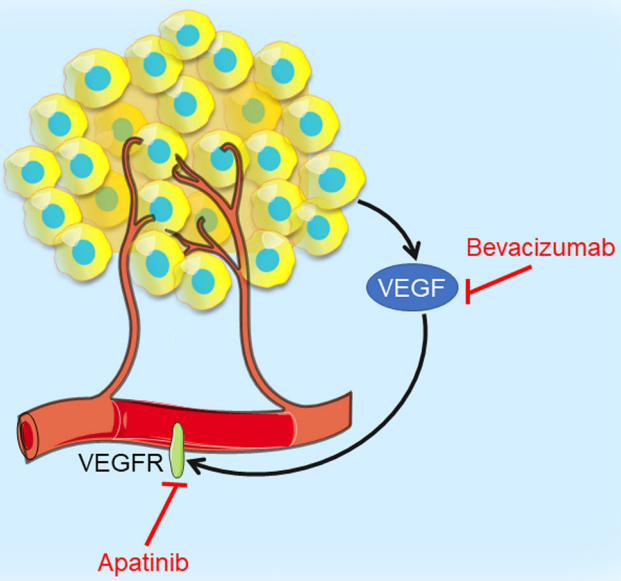

FIGURE 4 | Angiogenesis inhibition in TNBC. Tumor cells produce VEGF which interacts with VEGFR contributing to angiogenesis. VEGF inhibitor bevacizumab and VEGFR inhibitor apatinib could prevent VEGF interacting with VEGFR, thus blocking tumor angiogenesis.

median time to progression (TTP) of 7.2 months, and median OS of 18.3 months, respectively, for metastatic TNBC (26). In the GeparQuinto trial indicated the addition of bevacizumab to neoadjuvant anthracycline-taxane-containing chemotherapy significantly increased the pCR rate from 27.9% to 39.3% in TNBC patients (27). Results from a phase II neoadjuvant trial showed bevacizumab combined with docetaxel and carboplatin as neoadjuvant chemotherapy resulted in an encouraging pCR rate (42%) in TNBC (28). However, no differences in 3-year invasive disease-free survival (IDFS) and OS were noted in a phase III BEATRICE study (NCT00528567), in which TNBC patients received chemotherapy with or without bevacizumab (29). Moreover, in CALGB 40603 trail (NCT00861705), the efficacy of carboplatin or bevacizumab combined neoadjuvant chemotherapy were evaluated in stage II to III TNBC. Patients treated with carboplatin had higher pCR breast and pCR breast/axilla rates, while patients received bevacizumab only had higher pCR breast rate. Those received both agents had the highest pCR rate, with no significant interaction between their effects (30). A multicenter phase II study (NCT01176669) of VEGFR inhibitor apatinib treating metastatic TNBC patients revealed that the ORR and clinical benefit rate were 10.7% and 25.0%, respectively. Median PFS and OS were 3.3 months and 10.6 months, respectively (31). These angiogenesis inhibitors have shown objective efficacy in clinical trials of TNBC and had controllable toxicity, but testing in breast cancer that is highly angiogenesis-dependent might provide more convincing evidence for novel strategy of TNBC treatments.

INHIBITION OF EPIGENETIC MODIFICATIONS IN TNBC

Epigenetic modifications often specify stably heritable changes in phenotype resulting from changes in a chromosome without alterations in the DNA sequence (81). With decades of research, epigenetic modifications have emerged as fundamental players in cancer development and progression, which mainly include DNA modifications (such as DNA methylation) and histone modifications (such as histone deacetylation) (**Figure 5**) (82). DNA methylation recruits proteins involved in gene repression or inhibits the binding of transcription factors to DNA to regulate gene expression (83). Histone modifications could influence chromatin compaction and accessibility through many ways, including acetylation, phosphorylation, ubiquitinylation, and sumoylation (84). Additionally, epigenetic modifications are being developed as clinical biomarkers for diagnostic, prognostic, and therapeutic applications in tumors (85, 86). Therefore, inhibiting DNA methylation and histone deacetylation may be a probable targeted therapeutic strategy.

DNMT Inhibition

DNA methylation refers to the process that a methyl group is added to the 5′ position of the cytosine ring in CpG dinucleotides. Tumor suppressor genes, such as BRCA1, could be inhibited in tumors by promoter hypermethylation, which may be an important mechanism of primary breast cancer progression (87, 88). A research based on the analysis of a large number of breast cancer

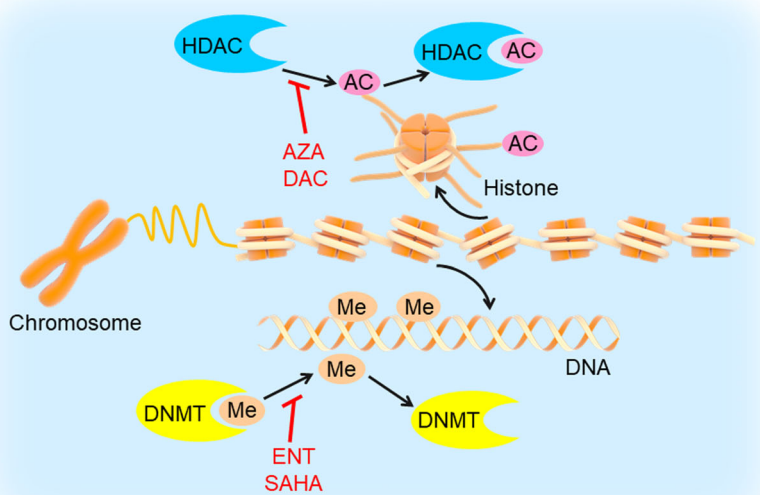

FIGURE 5 | DNA methylation and histone deacetylation inhibition in TNBC. DNA is methylated by DNMT and histone is deacetylated by HDAC, which could be inhibited by DNMTi's (entinostat/ENT, suberoylanilide hydroxamic acid/SAHA) and HDACi's (5-azacytidine/AZA, decitabine/DAC), respectively. This would induce tumor cell apoptosis and inhibit angiogenesis, cell migration and invasion.

cases confirmed that BRCA1 is abnormally methylated in sporadic tumors and methylation of BRCA1 played a key role in breast tumorigenesis. Moreover, methylation of BRCA1 is negatively correlated with ER and PR expression (89).

DNA methylation is initiated by DNA methyltransferases (DNMTs). The DNMT family enzymes consist of DNMT1, DNMT2, DNMT3A, and DNMT3B, among which DNMT1 is the crucial maintenance methyltransferase in humans (90). DNMT1 was highly expressed in TNBC compared to other subtypes. The expression of DNMT1 was negatively associated with OS in breast cancer (91). A preclinical study showed that PARPi's plus DNMT inhibitors (DNMTi's, 5-azacytidine/AZA, decitabine/DAC) increased PARPi efficacy and resulted in additional tumor inhibition in TNBC cells harboring wild-type BRCA1 compared with each drug alone (32). Although it was only a preclinical study in TNBC, DNMTi's had been approved by the US FDA for treating other cancers, such as myeloid malignancies and could be promising agents for TNBC treatment (33).

HDAC Inhibition

Histone deacetylase (HDAC) is an enzyme that deacetylates histone proteins. The deacetylation of histones leads to chromatin condensation, which ultimately represses the transcription of gene expression. The negative regulation of tumor suppressor gene is associated with tumor cell invasion, migration, proliferation, and angiogenesis. In contrast, HDAC inhibitors (HDACi's) could reverse the gene expression suppression through histone hyperacetylation and chromatin relaxation. More specifically, HDACi's could induce tumor cell apoptosis and inhibit angiogenesis, cell migration, and invasion (92, 93).

In a preclinical study, researchers found the HDACi entinostat (ENT) increased the expression of estrogen receptor-α (ERα) and aromatase in breast cancer cells and restored the sensitization of breast cancer cells to the aromatase inhibitor letrozole both *in vitro* and *in vivo*. These results suggested that combination of histone deacetylase and aromatase inhibitors could be used to treat ER-negative and endocrine therapy-resistant breast cancer (34). Sulaiman et al. have revealed that the expression of mTORC1 and HDAC were higher in TNBC than in luminal breast cancer. Co-inhibition of mTORC1 and HDAC with rapamycin plus valproic acid reproducibly promoted estrogen receptor 1 (ESR1) gene expression in TNBC cells (35). HDACi's increase PDL1 and HLA-DR expression in TNBC and reduce the proportion of $CD4Foxp3^+$ T cells. PD1 and CTLA4 blockade promoted TIL infiltration, cell apoptosis, and tumor regression. Thus, HDAC inhibition by HDACi's could potentiate the tumor-suppressive effects of immunotherapy in TNBC (36). Another study has demonstrated that the HDACi suberoylanilide hydroxamic acid (SAHA) could enhance the anti-tumor effects of the PARPi olaparib in TNBC cells by regulating the expression of homologous recombination repair (HRR)-related genes and hampering DNA repair (37).

INHIBITION OF CELL CYCLE IN TNBC

The cell cycle involves four ordered phases denoted G_1 (resting stage), S (DNA synthesis), G_2 (protein synthesis), and M (mitosis) (**Figure 6**). To ensure the fidelity of the cell cycle, several checkpoints arrest cell cycle to allow cells to properly repair defects during DNA synthesis and chromosome segregation (94). Cyclin-dependent kinases (CDKs) are activated and promote cell cycle progression with binding to cyclins that are synthesized and cleared during the cell cycle (95). Tumors with dysregulated CDKs often induce unscheduled proliferation (94).

It is well-known that the CDK4/6 inhibitors, blocking the cell cycle at the G_1 to S transition by triggering the dephosphorylation of retinoblastoma tumor suppressor protein (Rb) (96), play a vital role in preventing the proliferation of cancer cells. For now, three CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) received FDA approval for the treatment of HR-positive or HER2-negative breast cancer (97–100). However, the therapeutic effect of CDK4/6 inhibitors in TNBC is poor since loss of Rb often occurs. Extensive studies have revealed that combination with other molecules inhibition or therapy, such as PI3K inhibition, AR inhibition, immune checkpoint blockage, and chemotherapy, might help to overcome drug resistance in TNBC (38). In a preclinical study, dual blockade of PI3K and CDK4/6 had synergistic effect and could generate immunogenic cell death in TNBC cells (39). Pretreatment with palbociclib could improve the sensitivity of Rb-positive TNBC cells to paclitaxel (40). Phase I/II clinical trials of the safety and efficacy of CDK4/6 inhibition with or without other agents (anti-androgen medication, anti-PDL1 antibody, and chemotherapeutic drugs) in TNBC are ongoing (38).

Another class of agents targeting the cell cycle is TTK protein kinase inhibitors. TTK, namely monopolar spindle 1 (MPS1), controls the spindle assembly checkpoint (SAC) that ensures the integrity and stability of the genome in mitosis (101). TNBC has high expression levels of mitotic checkpoint molecules, and consequently, TTK inhibitors might prevent TNBC growth and proliferation (41). A preclinical trial demonstrated MPS1/TTK inhibitors have anti-proliferative effects in basal BC cell lines, with the half-maximal inhibitory concentration (IC_{50}) values ranging from 0.05 to 1.0 μM (42). Anderhub et al. showed that in multiple xenograft models of human TNBC, the combination of MPS1 inhibitor BOS172722 and paclitaxel results in significant *in vivo* efficacy, showing significant tumor regression compared with either drug alone (43).

Polo-like kinase 4 (PLK4), a regulator of the centriole duplication, is crucial to the maintenance of centriole and centrosome numerical integrity. PLK4 inhibitors would potentiate aneuploidy and genomic instability and lead to cancer cell death (102). An *in vitro* experimental study showed that a novel inhibitor of PLK4, CFI-400945, in combination with radiation, exhibited a synergistic anti-cancer effect in TNBC cell lines and patient-derived organoids and led to a significant increase in survival to tumor endpoint in xenograft models *in vivo*, compared to control or single-agent treatment (44). However, overactivation of PLK4 is always correlated with centrosome amplification (CA) promoting a high risk of breast cancer (103). Further preclinical studies are warranted to characterize molecular mechanisms of action of this combination and its potential clinical applications, and lay a theoretical foundation for PLK4 to be used as a promising target in TNBC.

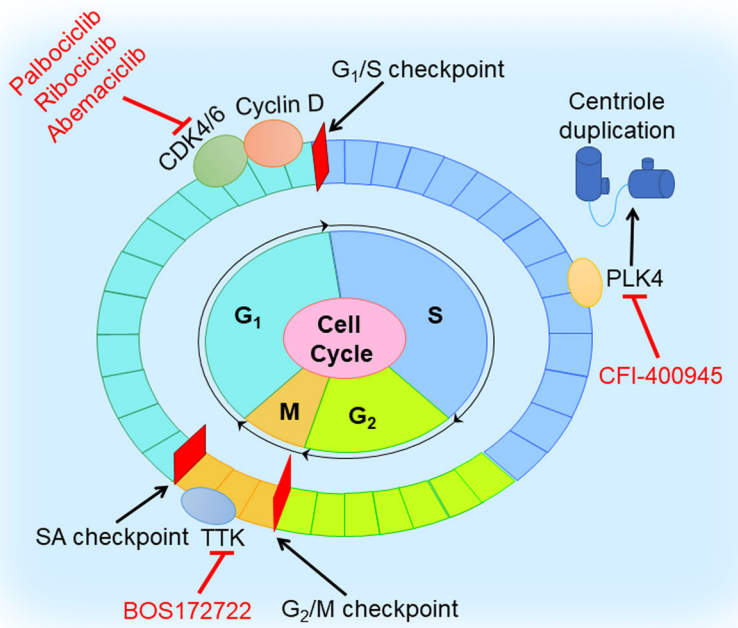

FIGURE 6 | Cell cycle inhibition in TNBC. Cell cycle involves G_1, S, G_2, and M phases. CDK4/6 inhibitor (palbociclib, ribociclib, and abemaciclib) blocks the cell cycle at G_1 to S transition by triggering dephosphorylation of retinoblastoma tumor suppressor protein (Rb). TTK inhibitor (BOS172722) binds to TTK that controls the spindle assembly checkpoint. PLK4 inhibitor (CFI-400945) reduces centriole duplication to prevent tumor growth.

Beyond this, ATR, CHK1, WEE1, and TRAIL might also be targets in TNBC. Preclinical studies showed that ATR or CHK1 inhibitor could delay the radiation-induced DNA repair and inhibit cell survival in TNBC cells (45, 46), while WEE1 inhibition could overcome cisplatin resistance in TNBC cells (47), and TRAIL receptor agonist could induce apoptosis in TNBC cells that expressed vimentin and Axl (48).

CONCLUDING REMARKS

TNBC is a complex disease with poor prognosis and rare effective targeted therapy. It is urgent to explore novel targeted therapeutic strategies. For now, PARP inhibition has shown great promise in BRCA1/2-mutated TNBC patients. It is of great hope to combine PARPi's with DNA-damaging chemotherapy for TNBC patients harboring wild-type BRCA1/2. Meanwhile, results of clinical and preclinical studies have revealed that immunotherapy with checkpoint blockage gives rise to a good outcome in PD1/PDL1-positive TNBC patients. Targeting VEGF/VEGFR alone provides potential efficacy by inhibiting angiogenesis. However, many patients develop drug resistance while interconnected or compensatory pathways could overcome VEGF/VEGFR-targeted inhibition (78). As the "genomic medicines", epigenetic drugs (DNMTi's, HDACi's, etc) have shown great application prospects in treating TNBC patients. Targeting epigenetic modifications have exhibited great efficacy when used jointly with other therapies such as chemotherapy or immunotherapy (104). CDK4/6 is the main target of cell cycle in breast cancer. When combined with other targeted therapeutic agents, CDK4/6 inhibitors could benefit more TNBC patients.

In summary, each targeted therapy in TNBC has its advantages and disadvantages when applied alone. Thus, combination of various targeted therapies would be a better strategy to enhance the therapeutic effectiveness and benefit more TNBC patients. Additionally, it is also warranted to conduct more and in-depth studies to identify novel effective therapeutic targets in TNBC. Hopefully, TNBC patients will have more individualized treatment options and better outcomes in the near future.

AUTHOR CONTRIBUTIONS

YL, ZZ, and XY: Reviewing the literature and writing the original manuscript. SF: Writing, revising, and editing the manuscript. XD: Revising and reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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Mapping Intellectual Structures and Research Hotspots of Triple Negative Breast Cancer: A Bibliometric Analysis

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Background: Triple negative breast cancer (TNBC) is a highly heterogeneous breast cancer subtype with a poor prognosis due to its extremely aggressive nature and lack of effective treatment options. This study aims to summarize the current hotspots of TNBC research and evaluate the TNBC research trends, both qualitatively and quantitatively.

Methods: Scientific publications of TNBC-related studies from January 1, 2010 to October 17, 2020 were obtained from the Web of Science database. The BICOMB software was used to obtain the high-frequency keywords layout. The gCLUTO was used to produce a biclustering analysis on the binary matrix of word-paper. The co-occurrence and collaboration analysis between authors, countries, institutions, and keywords were performed by VOSviewer software. Keyword burst detection was performed by CiteSpace.

Results: A total of 12,429 articles related to TNBC were identified. During 2010-2020, the most productive country/region and institution in TNBC field was the USA and The University of Texas MD Anderson Cancer Center, respectively. Cancer Research, Journal of Clinical Oncology, and Annals of Oncology were the first three periodicals with maximum publications in TNBC research. Eight research hotspots of TNBC were identified by co-word analysis. In the core hotspots, research on neoadjuvant chemotherapy, paclitaxel therapy, and molecular typing of TNBC is relatively mature. Research on immunotherapy and PARP inhibitor for TNBC is not yet mature but is the current focus of this field. Burst detection of keywords showed that studies on TNBC proteins and receptors, immunotherapy, target, and tumor cell migration showed bursts in recent three years.

Conclusion: The current study revealed that TNBC studies are growing. Attention should be paid to the latest hotspots, such as immunotherapy, PARP inhibitors, target, and TNBC proteins and receptors.

Keywords: triple negative breast cancer, bibliometric analysis, co-word analysis, co-citation analysis, research hotspots

INTRODUCTION

Breast cancer is one of the most common malignancies in women worldwide, and its mortality rate ranks second in cancer-related deaths (1). Triple negative breast cancer (TNBC) is a subtype of breast cancer where there is reduced expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) receptor. TNBC accounts for 20% of all newly-diagnosed breast cancers (2). Among highly heterogeneous diseases, TNBC has highly invasive biological characteristics and earlier age of onset, and early recurrence and distant metastasis are common (3, 4). Most TNBC treatments are limited as therapeutic targets have not been elucidated (5). Adjuvant chemotherapy is currently the standard treatment for TNBC, but the optimal chemotherapy regimen is still controversial due to drug resistance and tolerance issues (3, 6). Therefore, it is urgent to find specific therapeutic targets to improve the clinical outcomes, which has become a hotspot of TNBC research (7).

Bibliometrics is a quantitative analysis method that uses co-word and co-citation analyses of existing research to help scholars quickly identify popular themes and emerging trends in a particular field of study (8, 9). Among them, CiteSpace, VOSviewer, Bibliographic Items Co-occurrence Matrix Builder (BICOMB), and BibExcel are commonly used tools for bibliometric analysis and visualization (10). In recent years, many scholars have conducted bibliometric analysis on diseases, such as coronavirus disease, Alzheimer's disease, pancreatic cancer, and obesity (11–15). However, there is no bibliometric study on TNBC. Therefore, we collated the last ten years' scientific publications on TNBC from the Web of Science (WoS) database and systematically summarized the studies using Citespace, VOSviewer, BICOMB, and BibExcel software. We present the field structure and the development of knowledge and highlight the research hotspots and future directions in this field to provide a reference for further clinical research on TNBC.

METHODS

Data Sources and Retrieval Strategies

The WoS core collection was used as the data source. The retrieval strategy was as follows: subject words = triple-negative breast cancer or subject words = triple negative breast cancer, literature type = article or review, language = English, year = 2010–2020. A total of 12,429 studies were retrieved. All records and references were downloaded in a TXT format. To avoid deviation caused by the frequent update of the database, all literature retrievals and data extractions were finalized on October 17, 2020 and introduced into the Bibliometrics analysis software for further analysis.

Co-Citation Analysis

CiteSpace software was developed by Dr. Chaomei Chen using Java. It is mainly applied to visualization analysis of scientific literature, which is usually applicable to "co-citation analysis" of large volumes of literature data in a particular field of study. The settings were as follows: from 2010 to 2020, years per slice = 1, and the top 50 of the most cited papers in a year per individual network. Based on our research goals, each node represented a citation, with the larger size of the node denoting a greater frequency (16, 17). The author co-citation and literature co-citation networks were constructed, and keyword burst detection was performed. Additionally, the java program VOSviewer (Leiden University, Leiden, Netherlands) was used to visualize the cooperative networks and keyword co-occurrence between countries/regions and institutions (18).

Co-Word Analysis

The TXT files were imported to BICOMB (14, 19) for fetching high-frequency keywords. Based on this, binary matrices of word-paper and co-word matrices of high-frequency words were generated. gCLUTO was used to produce a biclustering analysis on the binary matrix of word-paper to determine the research hotspots of TNBC (20, 21). To improve the display of the clustering results, visualized mountain maps and heat maps were generated as per the results of the biclustering analysis.

Strategic Diagram Analysis

In 1998, John Law proposed a series of strategic diagrams to reveal the current development situation on each research topic in specific fields and predict their future development trends (22). Using Excel, we imported the cluster information from gCLUTO into the co-word matrix and calculated the intra-class and inter-class link averages for each hotspot category. The centrality and density were then calculated. Subsequently, a two-dimensional strategic diagram was established. The X-axis and Y-axis represent the centrality and the density, respectively. Among them, the centrality was a criterion of interaction among various clusters, and with greater centrality, the cluster had a greater central tendency in a research field. The density represents the strength of the internal connections of a cluster, which is used to measure the ability to maintain the internal integration within the cluster.

RESULTS

Annual Analysis of Publications

Between 2010 and October 17, 2020, 12,429 TNBC articles were published and listed on WoS (**Figure 1**). The cumulative number of posts related to TNBC has maintained a rapid growth every year since 2010. The annual growth trend is in line with the fitting curve y=313.63e0.1951X (R2 = 0.9545). This indicates that TNBC is arousing increasing attention and has clinical significance and development potential.

Distribution Characteristics of Countries/Regions and Institutions

Since 2010, 101 countries/regions have participated in TNBC studies. The maps created by CiteSpace and Google Earth have shown the distributions and numbers of countries/regions of

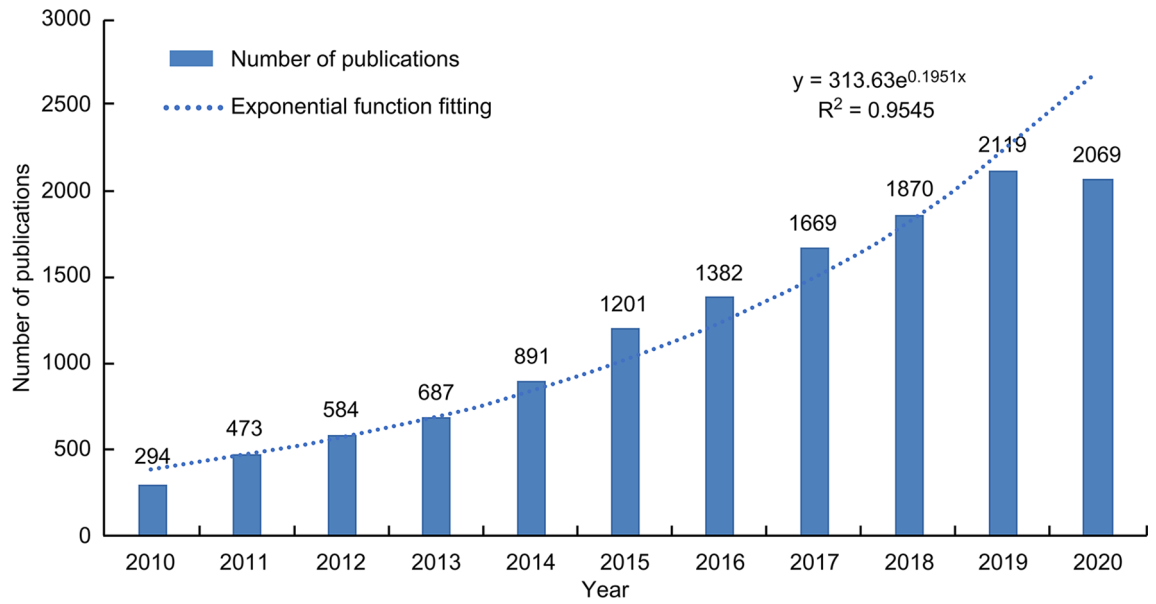

FIGURE 1 | The trend of TNBC research from 2010 to 2020.

publications (**Figure 2A**). The top 10 countries published a total of 12,327 articles over the past decade. The United States had the most publications (n=5420), followed by China (n=2555), Italy (n=643), and South Korea (n=634). Centrality was used to evaluate the importance of nodes in a network. **Table 1** revealed that the United States also had the highest centrality (0.27). This suggests that the United States is the most prolific and influential country in TNBC research. According to the collaborative visualization network of publishing countries/regions (**Figure 2B**), the United States and China are the two largest network nodes located at the central connection point of the collaborative relationship map, i.e., they are most closely connected with other major publishing countries. In the collaborative network map, the lines between China and the United States are the widest, indicating the large partnership community between the two countries for TNBC research. In contrast, the cooperative ties among other countries could be strengthened.

A total of 9,187 institutions participated in the TNBC study. The University of Texas MD Anderson Cancer Center published the maximum number of papers (n=478), followed by Dana-Farber Cancer Institute (n=239), Fudan University (n=230), and Memorial Sloan Kettering Cancer Center (n=204). The H index is primarily used to evaluate the comprehensive influential power of a specific institution. The results from **Table 1** show that although Chinese institutions were high on the ranking list of total publications, their H indices and total and average numbers of citations were significantly lower. Thus, although China has been relatively active in TNBC research in recent years and produced numerous papers, its global attention and international influence are still low. Further, we used VOSviewer software to analyze the collaborative visualization networks among these institutions (**Figure 2C**). The results showed a scattered distribution and insufficient cooperation among the international institutions.

Journal Analysis

Since 2010, 1,056 journals have published articles on TNBC. We identified the top 10 most popular journals with 4,359 published articles over the past decade, accounting for 35.01% of all articles (**Table 2**). Thus, emphasizing posts from these key journals helped us keep abreast of the latest trends. Cancer Research, Journal of Clinical Oncology, and Annals of Oncology were not only among the first three periodicals with maximum publications but also among the first three journals in the Impact Factor list. They were classified as Q1 by the Journal Citation Reports standard and were important sources of knowledge for TNBC. The analysis of the core author and intellectual basis of the research field is shown in **Supplementary Material** (10, 16, 17, 23, 24). (**Supplementary Figure 1** and **Supplementary Table 1**).

Research Hotspots: Co-Word Analysis and Clustering Analysis of Keywords

As a general overview of the literature theme, keywords are highly refined and generalized to a specific topic and can fully interpret the literature. Using high-frequency keywords to elucidate the research hotspots in a discipline can effectively determine the research hotspots and other important issues.

The literature search identified 12,429 TNBC-related publications and extracted 11,535 keywords with BICOMB. The frequency of the 50th word was equal to its ordinal number, so terms ranked above 50 could be defined as high-frequency keywords (**Table 3**). The top 10 most frequent keywords after excluding the keywords without actual

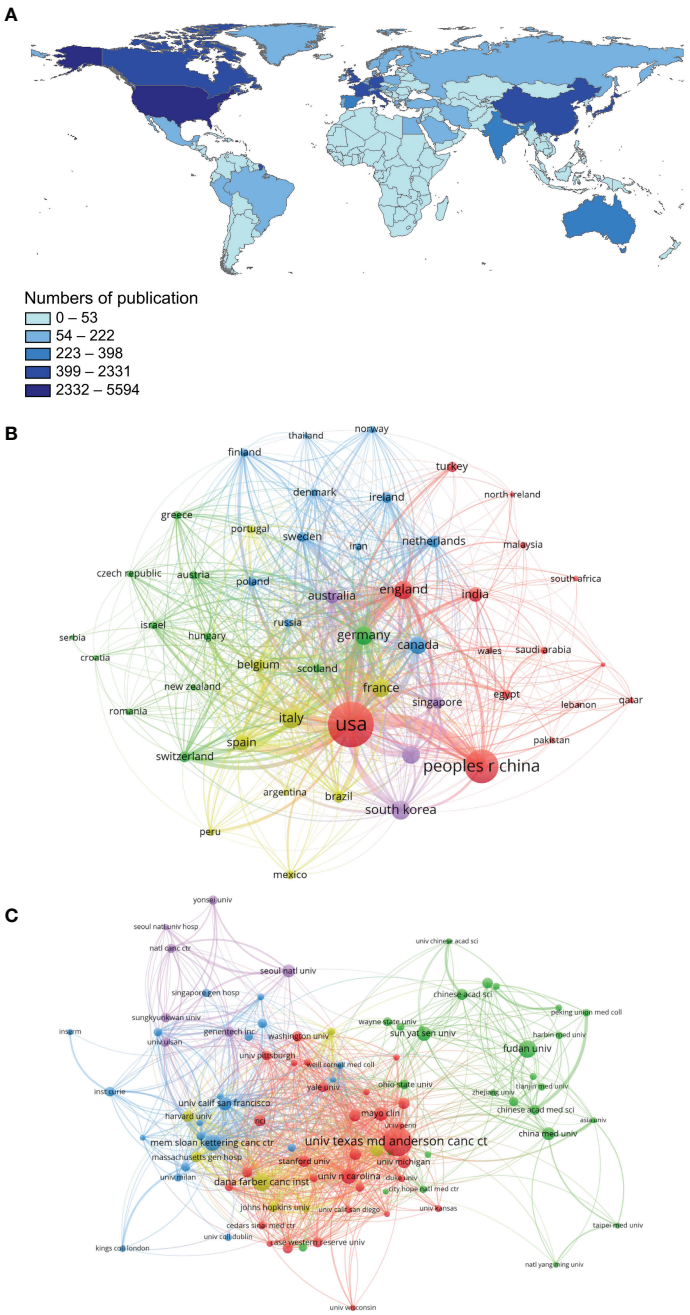

FIGURE 2 | Main countries/regions and institutions of TNBC research and their interrelationships. **(A)** Countries/Regions distribution of TNBC-related research results; **(B)** A visualization network of collaboration between countries/regions in TNBC research; **(C)** A visualization network of collaboration among institutions in TNBC research.

referential meanings are prognosis, apoptosis, metastasis, epithelial-mesenchymal transition, chemotherapy, biomarker, immunotherapy, neoadjuvant chemotherapy, *Brca1*, and survival. We constructed the binary matrix (**Supplementary Table 2**) and co-word matrix (**Supplementary Table 3**) based on high-frequency keywords. Subsequently, gCLUTO was used for biclustering analysis, and the mountain and heat maps were drawn based on this. Additionally, VOSviewer was used for visualization analysis of the keywords that co-occurred at least 15 times or more.

Keyword co-occurrence analysis refers to counting the frequency of appearance of keywords in the same literature and analyzing the intrinsic relationships and degree of intimacy among keywords. Based on this, closely related keywords are grouped into different clusters through clustering analysis. These clusters reflect the key

TABLE 1 | The main countries, regions, and institutions contributing to publications in TNBC research.

Rank	Country/Region	Article Counts	Proportion	Centrality	Institutions	Article Counts	Proportion	H Index	Total Number of Citations	Average Number of Citations
1	USA	5420	44.0%	0.27	Univ Texas Md Anderson Canc Ctr	478	5.45%	52	10830	22.7
2	People's Republic of China	2555	20.7%	0.01	Dana Farber Canc Inst	239	2.72%	53	9382	39.3
3	Italy	643	5.22%	0.1	Fudan Univ	230	2.62%	27	2449	10.7
4	South Korea	634	5.14%	0.01	Mem Sloan Kettering Canc Ctr	204	2.33%	33	4849	23.8
5	Germany	583	4.73%	0.18	Univ N Carolina	190	2.17%	39	8027	42.2
6	England	560	4.54%	0.11	Sun Yat Sen Univ	176	2.01%	30	2606	14.8
7	Japan	546	4.43%	0.06	Mayo Clin	168	1.92%	32	3863	23.0
8	France	522	4.23%	0.17	Univ Calif San Francisco	158	1.80%	40	6178	39.1
9	Canada	482	3.91%	0.12	Univ Michigan	157	1.79%	26	2348	15.0
10	Spain	382	3.10%	0.06	China Med Univ	153	1.74%	23	2086	13.6

research contents and core research fields that the keywords refer to (21). To ensure a visual effect and analysis emphasis, any keywords without actual referential meanings were excluded from this article, and the keywords that appeared at least 15 times were selected for visualization. A total of 186 keywords appeared at least 15 times, and the co-occurrence map of keywords was drawn (**Figure 3**). The nodes in the map indicated the corresponding keywords, and the size of node indicated how many publications in TNBC field included the corresponding keywords. The bigger the node size, the greater the popularity of the keyword. The link line between 2 keyword nodes indicated the relationships between the keywords. The keywords, such as prognosis, metastasis, apoptosis, had many link lines with other nodes, indicating that these keywords have a close relationship with other keywords in this field (**Figure 3**).

In the visualized mountain map (**Figure 4**), the marked number corresponds to the cluster number. The volume of the mountain is directly proportional to the number of keywords within the cluster, and the height is also directly proportional to the intra-class similarity of the cluster. A sharp peak signifies high intra-class similarity. The peaks are shown in five colors: red, yellow, green, light blue, and dark blue. The standard deviation of the intra-class similarities represented by these colors increased in turn. The distance between the peaks was used to evaluate the similarity between the two clusters. The eight peaks were relatively independent and clearly distributed, indicating a satisfactory clustering effect.

In the visualized heat map (**Figure 5**), the rows represent high-frequency words and columns represent published literature. The colors represent values in the original data matrix. The values in the original data matrix are represented by color depth. The white area in the figure represents a value close to zero. The gradually deepened red area represents larger values. The clustering tree represents article clusters containing high-frequency words. By identifying the semantic connections among high-frequency words and their source articles, we confirmed eight research hotspots in the TNBC research field:

Cluster 0: Immunotherapy for TNBC.

Cluster 1: The role of EMT in TNBC tumor cell metastasis.

Cluster 2: The effect of neoadjuvant chemotherapy in TNBC treatments.

Cluster 3: The Application of Paclitaxel in TNBC Treatments.

Cluster 4: PARP inhibitors in TNBC treatments.

Cluster 5: Tumor stem cell studies of TNBC.

Cluster 6: Tumor microenvironment of TNBC.

Cluster 7: Molecular subtypes of TNBC.

Strategic Diagram Analysis

Based on the gCLUTO blustering analysis, we calculated each cluster's centrality and density (**Supplementary Table 4**) (22).

TABLE 2 | The top 10 highly-productive journals in TNBC research.

Rank	Journal	Number of publications	Proportion	IF [2019]	Quartile in category [2019]
1	Cancer Research	1886	15.2%	9.72	Q1
2	Journal of Clinical Oncology	616	5.00%	33.0	Q1
3	Annals of Oncology	369	2.98%	18.3	Q1
4	Breast Cancer Research and Treatment	340	2.74%	3.83	Q2
5	PLOS One	276	2.23%	2.74	Q2
6	European Journal of Cancer	191	1.54%	7.28	Q1
7	Modern Pathology	176	1.42%	5.99	Q1
8	Scientific Reports	165	1.33%	3.99	Q3
9	Breast	163	1.31%	3.75	Q2
10	Breast Cancer Research	159	1.28%	4.99	Q1

TABLE 3 | High-frequency Keywords in the TNBC Study.

Rank	Keywords	Frequency, n	Percentage, %	Cumulative Percentage, %
1	Triple negative breast cancer	3044	10.0	10.0
2	Breast cancer	1842	6.08	16.1
3	Prognosis	421	1.39	17.5
4	Triple negative	351	1.16	18.7
5	Apoptosis	339	1.12	19.8
6	Metastasis	315	1.04	20.8
7	Epithelial-mesenchymal transition	195	0.64	21.5
8	Chemotherapy	189	0.62	22.1
9	Biomarker	150	0.49	22.6
10	Immunotherapy	149	0.49	23.1
11	Neoadjuvant chemotherapy	144	0.48	23.6
12	Brca1	127	0.42	24.0
13	Survival	124	0.41	24.4
14	Immunohistochemistry	121	0.40	24.8
15	Invasion	120	0.40	25.2
16	Targeted therapy	120	0.40	25.6
17	Egfr	117	0.39	26.0
18	Proliferation	101	0.33	26.3
19	Migration	95	0.31	26.6
20	Cancer	92	0.30	26.9
21	Estrogen receptor	90	0.30	27.2
22	HER2	89	0.29	27.5
23	Autophagy	89	0.29	27.8
24	Molecular subtype	86	0.28	28.1
25	Androgen receptor	80	0.26	28.3
26	Cancer stem cells	79	0.26	28.6
27	PD-L1	76	0.25	28.9
28	Angiogenesis	76	0.25	29.1
29	MDA-MB-231	70	0.23	29.3
30	Breast neoplasms	70	0.23	29.6
31	MicroRNA	69	0.23	29.8
32	Doxorubicin	69	0.23	30.0
33	Paclitaxel	69	0.23	30.2
34	PARP inhibitor	66	0.22	30.5
35	Metastatic breast cancer	65	0.21	30.7
36	Pathological complete response	65	0.21	30.9
37	Cisplatin	65	0.21	31.1
38	Cell cycle	63	0.21	31.3
39	Akt	62	0.20	31.5
40	Tumor-infiltrating lymphocyte	62	0.20	31.7
41	Overall survival	58	0.19	31.9
42	Brain metastases	58	0.19	32.1
43	P53	57	0.19	32.3
44	Stat3	56	0.18	32.5
45	Breast	55	0.18	32.7
46	Tumor microenvironment	53	0.17	32.8
47	Drug resistance	52	0.17	33.0
48	Basal-like breast cancer	51	0.17	33.2
49	Cancer stem cells	51	0.17	33.3
50	Triple negative breast neoplasms	50	0.17	33.5

The coordinates of each cluster were obtained accordingly. With "centrality" as the abscissa and "density" as the ordinate, we considered the mean value of centrality and density from all clusters as the origin of the coordinate (7.62, 3.05) and drew the strategic diagram. Each point in **Supplementary Figure 2** represents a cluster. The coordinate value of each cluster is the difference between centrality/density and their mean values. As shown in **Supplementary Figure 2**, the above eight clusters were distributed in four different quadrants. We then analyzed the research status of various hot topics on TNBC. The clusters in the first quadrant (Cluster 2, neoadjuvant chemotherapy, Cluster 3, Paclitaxel and Cluster 7 molecular subtypes) had high centrality and density and close internal and extensive connections with the rest of the clusters, indicating the maturity of these three types of research and their core positions in the research field. Though in a marginal position, a fairly completed research system has taken shape in the cluster of the second quadrant (Cluster 1, EMT). The centrality and density of clusters in the third quadrant (Cluster 5, tumor stem cell, and Cluster 6, special tumor microenvironment) were low. The internal connections of the research topic are not closely related to each other, and connections with other

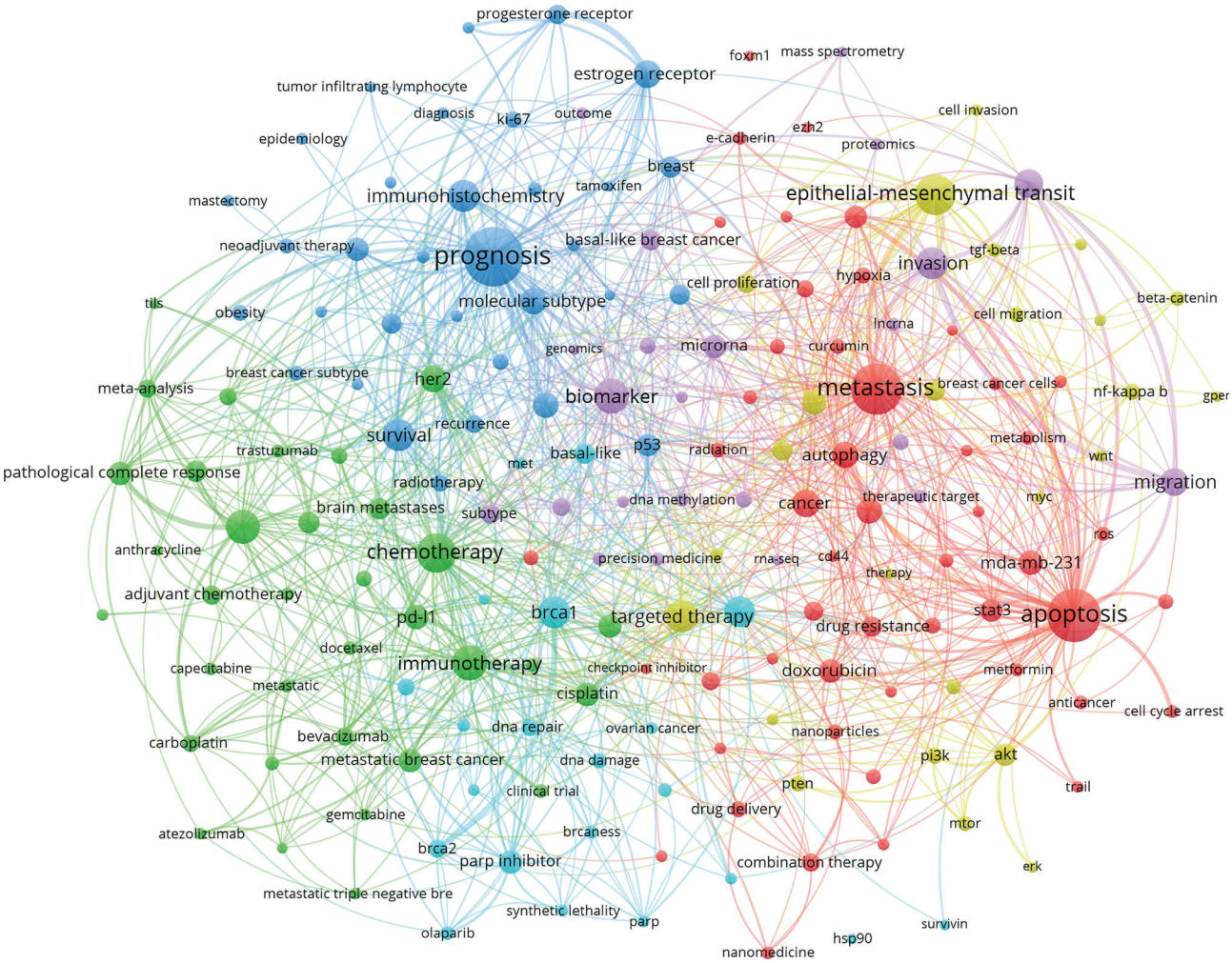

FIGURE 3 | Keyword Co-occurrence Map Based on VOSviewer in TNBC research field.

groups are relatively loose, which puts them on the edge of the whole research field. The clusters in the fourth quadrant (Cluster 0, immunotherapy and Cluster 4, PARP inhibitor) were lower in density but higher in centrality, indicating that the studies of immunotherapy and PARP inhibitors for TNBC are not yet mature but are the current focus of this field. These emerging topics will be the future trend of TNBC research. We usually focus on hot topics in the fourth quadrant. It should be noted that the locations of various clusters in strategic diagrams are not fixed. The maturity of certain research themes or the emergence of new knowledge points may lead to a transfer of the first quadrant to the second quadrant. Similarly, with the development of themes in the second and third quadrants, they also have the potential to move to the first quadrant.

Research Fronts: Keyword Burst Analysis

Research fronts are the newest and most potential research topics or fields in scientific research. The burst of keywords reflects the sudden increase in a citation in a certain period, displays the time distribution and dynamic variability of keywords, and accurately reveals the evolution trend of hotspots in the research field (17, 25). Additionally, burst detection of keywords is also helpful in finding keywords that have not reached the frequency threshold but may have academic contributions to analyze the hotspots and fronts of TNBC research more comprehensively. The stronger the burst intensity, the greater the attention the research topic arises and the more it reflects research published during a given period. We detected keywords of 12,429 TNBC articles from 2010 to 2020 using the burst detection algorithm of CiteSpace. In the diagram generated by CiteSpace, the timeline was shown as a blue line, and the interval during which a keyword showed a burst was depicted as a red segment in a specific location of the blue timeline. The specific burst words, burst strengths, and starting and ending years are indicated. The top 30 keywords with the highest burst strength are shown in **Figure 6**.

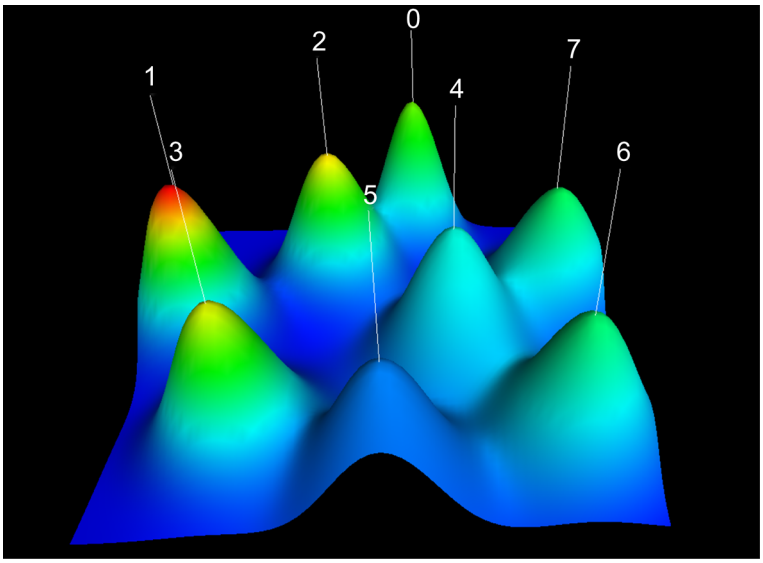

FIGURE 4 | Visualized Mountain Map based on the Biclustering analysis of TNBC Binary Matrix of Word-paper. Cluster 0: Immunotherapy for TNBC. Cluster 1: The role of EMT in TNBC tumor cell metastasis; Cluster 2: The effect of neoadjuvant chemotherapy in TNBC treatments; Cluster 3: The Application of Paclitaxel in TNBC Treatments; Cluster 4: PARP inhibitors in TNBC treatments. Cluster 5: Tumor stem cell studies of TNBC; Cluster 6: Tumor microenvironment of TNBC; Cluster 7: Molecular subtypes of TNBC.

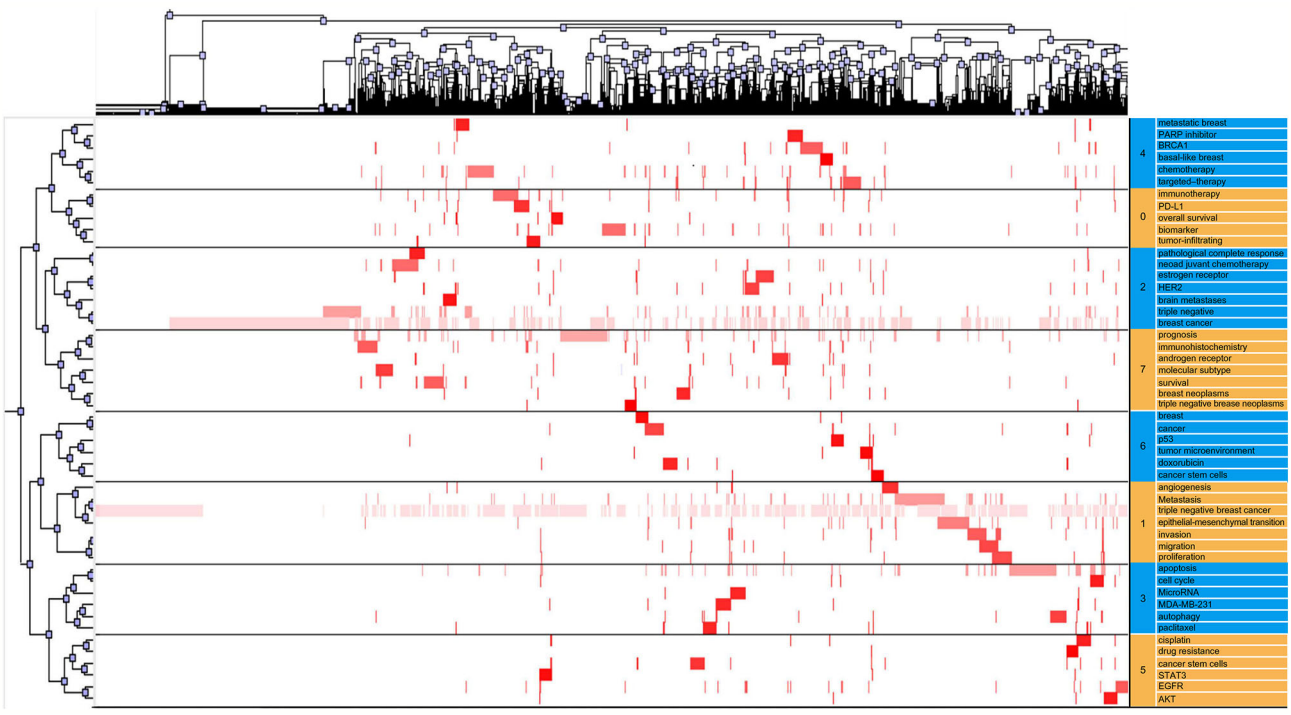

FIGURE 5 | Visualized matrix based on the biclustering analysis of TNBC binary matrix of Word-paper.

The TNBC research field presents a diversified characteristic, and different burst words appear in different periods. The keywords with the strongest intensity were "Pattern" (62.2415), followed by "Estrogen Receptor" (52.1277) and "Phenotype" (50.7964). The bursts of these 3 keywords all began in 2010 and last for 3-5 years. In the recent three years, the keywords with the strongest intensity were "Target" (48.7595) and "Immunotherapy" (48.5009). The keywords with the longest

burst time were "Estrogen Receptor" (2010 - 2015) and "Neoadjuvant Chemotherapy" (2013 - 2018). It is noteworthy that the five keywords bursting in the past two years were receptor (2019 - 2020), target (2019 - 2020), immunotherapy (2019 - 2020), protein (2018 - 2020), and migration (2017 - 2020). This indicates that these research themes are relatively active in recent TNBC studies and may become research fronts in the future.

Top 30 Keywords with the Strongest Citation Bursts

Keywords	Strength	Begin	End	2010 - 2020
pattern	62.2415	2010	2014	
her2	15.9598	2010	2011	
progesterone receptor	21.7171	2010	2011	
molecular subtype	8.0713	2010	2010	
brca1	35.6067	2010	2012	
phenotype	50.7964	2010	2013	
paclitaxel	5.3795	2010	2010	
estrogen receptor	52.1277	2010	2015	
basal like subtype	28.1215	2010	2011	
trastruzumab	6.0523	2010	2010	
phase ii	9.4176	2010	2010	
Poly (adp ribose) polymerase	17.1778	2010	2012	
marker	15.9598	2010	2011	
basal like	7.3982	2010	2010	
growth factor receptor	12.9561	2012	2012	
recurrence	13.6047	2012	2012	
mutation	12.3076	2012	2012	
gene expression	19.2456	2012	2015	
subtype	7.7523	2013	2013	
prognostic factor	12.7992	2013	2013	
neoadjuvant chemotherapy	21.5901	2013	2018	
risk	22.3046	2015	2015	
tumor infiltrating lymphocyte	30.3125	2017	2018	
stem cell	35.9622	2017	2018	
migration	28.5177	2017	2020	
Progression	18.7521	2018	2018	
Protein	23.22	2018	2020	
immunotherapy	48.5009	2019	2020	
target	48.7595	2019	2020	
receptor	11.3268	2019	2020	

FIGURE 6 | The evolution trend of burst words on TNBC from 2010 to 2020.

DISCUSSION

In the present study, we analyzed the publications of triple negative breast cancer between 2010 and 2020 using information visualization methods. A total of 12,429 articles related to TNBC were identified. During 2010-2020, the most productive country/region in TNBC field was the USA, followed by China and Italy. The University of Texas MD Anderson Cancer Center had the maximum number of publications in TNBC field, followed by Dana-Farber Cancer Institute, Fudan University, and Memorial Sloan Kettering Cancer Center. Cancer Research, Journal of Clinical Oncology, and Annals of Oncology were the first three periodicals with maximum publications in TNBC research. Co-word analysis and clustering analysis of keywords identified eight research hotspots in TNBC field, that is, Cluster 0: Immunotherapy for TNBC; Cluster 1: The role of EMT in TNBC tumor cell metastasis; Cluster 2: The effect of neoadjuvant chemotherapy in TNBC treatments; Cluster 3: The application of Paclitaxel in TNBC treatments; Cluster 4: PARP inhibitors in TNBC treatments. Cluster 5: Tumor stem cell studies of TNBC; Cluster 6: Tumor microenvironment of TNBC; and Cluster 7: Molecular subtypes of TNBC.

Cluster 0: The Immunotherapy for TNBC

Breast cancer has always been considered a "cold tumor" with low immunogenicity. However, more studies have found that due to a high genomic instability and mutational burden of TNBC (26), the expression levels of the programmed cell death ligand 1 (PD-L1) protein are high, and the tumor-infiltrating lymphocytes (TIL) are rich in the microenvironment. Thus, it is assumed that breast cancer might be a "hot tumor" with a positive immune response (27).

PD-L1 inhibitors are currently the most thoroughly studied and widely used immune checkpoint inhibitors (28). Although PD-L1 expression is also observed in primary breast cancer, it is more prevalent in TNBC (20% to 30%). The immune escape mechanism of TNBC makes it more suitable for immune checkpoint blockade therapy (29, 30). Although TNBC had these characteristics that enhance anticancer immune responses, the single-agent efficacy of immune checkpoint inhibitors in TNBC is low (31). Combination regimens of PD-1/L1 inhibitors plus chemotherapy have demonstrated more success in metastatic TNBC than single-agent PD-1/L1 inhibitors. In March 2019, following the results of IMpassion130, a phase III clinical trial, the combined application of the PD-L1 antibody atezolizumab and the albumin-binding paclitaxel was approved by the FDA as the first-line therapy for metastatic or unresectable locally advanced TNBC (32, 33). This was the first immunotherapy approved for breast cancer. This therapy achieved clinically significant overall survival benefits in PD-L1-positive TNBC patients, and most adverse events (AEs) were lowered (34). This was followed by the KEYNOTE-355 study which showed that pembrolizumab in combination with chemotherapy had a significant and clinically meaningful improvement in PFS versus placebo-chemotherapy among patients with metastatic TNBC with CPS of 10 or more (35). Based on results of KEYNOTE-355, pembrolizumab + chemotherapy was approved by FDA in November 2020 to treat patients with locally recurrent unresectable or metastatic TNBC expressing PDL1. It is noteworthy that introducing immune checkpoint inhibitors in the early stages of TNBC may be a potential therapy because the primary tumor seems more immunogenic than the metastatic tumor (36). These results indicate that TNBC treatment has entered the era of immunotherapy.

Additionally, many studies have shown significant infiltration of TIL in TNBC, and high levels of TIL are significantly associated with a reduced distant recurrence rate of primary TNBC (37). Moreover, TILs in TNBC are strong independent indicators of prognosis, and the extended disease-free survival and overall survival periods can be touted (38, 39).

Immunotherapy has brought a new hope and option for TNBC patients, which is expected to alter the existing clinical treatment standard for advanced TNBC. In coming years, more biomarkers should be explored to accurately screen the population benefiting from single-agent immunotherapy and improve the prognosis (29, 33, 38, 40). Besides, novel therapeutic strategies to overcome a lack in anticancer immunity in TNBC are urgently needed and likely to be a research focus in future.

Cluster 1: The Role of EMT in TNBC Tumor Cell Metastasis

Unlike primary tumors, metastatic diseases are not curable because of their systemic nature and inoperable features. Therefore, the spread of breast cancer tumor cells and eventual distant metastasis (mainly lung, bone, and brain) are clinical problems to be resolved. Despite standardized adjuvant chemotherapy, the 5-year survival rate of patients with metastatic TNBC was still less than 30% (41, 42). Metastasis occurs through a series of complex cellular biological events, among which the spread of tumor cells to distant organs is one of the most critical steps.

Currently, it is well accepted that the epithelial-mesenchymal transition (EMT) is a critical mechanism for the initiation of tumor cell metastasis in TNBC. EMT is a process wherein cells lose their epithelial features and gain mesenchymal features. The loss of connections and apical-basal polarity of epithelial cells, as well as cytoskeletal reorganization, occurs in this process, which increases the activity and aggressiveness of the cells (43). However, EMT is believed to limit cell migration and promote colonization and growth of metastasized tumor cells. Recent results suggest that EMT is not a complete transition from epithelial to mesenchymal state but a transition state between the two, and this state is reversible (44), that is, EMT and its reverse process, mesenchymal-epithelial transition (MET), are both dynamic. The mesenchymal cancer cells are likely to undergo MET transiently and subsequently re-undergo EMT to restart the metastatic process. Cells in this state have a high metastatic potential. They can effectively invade blood vessels to enter the systemic circulation and easily colonize the distant organs (45), which means such bidirectional transitions between epithelial and mesenchymal cells are involved in cancer development. EMT is a potential therapeutic target for TNBC. Specific anti-EMT drugs may be effective in preventing tumor metastasis in the future.

Cluster 2: The Effect of Neoadjuvant Chemotherapy on TNBC

Currently, neoadjuvant chemotherapy (NACT) is a conventional treatment for early-stage TNBC. Compared with adjuvant chemotherapy, NACT reduces the tumor burden before surgery. It also allows for further assessment of the prognosis of the tumor and its response to chemotherapy for subsequent adjuvant chemotherapy plans accordingly. Despite the substantial high rate of recurrence, TNBC patients have a more pronounced response to NACT compared with other subtypes of breast cancer patients, which is known as the "TNBC paradox" (46). Thus, the risk of tumor recurrence is high without chemotherapy, but the benefit is greater after treatment.

Currently, the standard regimen of NACT is still a combination of anthracyclines and taxanes. Approximately 30–40% of early-stage TNBC patients prescribed this therapy can achieve pathological complete response (PCR) (47, 48). Additionally, platinum-based compounds (as DNA damage agents) show better efficacy when tumor cells have DNA repair defects (such as the *BRCA 1/2* gene mutations). Incidentally, the mutation frequency in germline *BRCA* in TNBC patients is higher than that of other subtypes. Thus, platinum drugs exhibit promising clinical results in TNBC patients (7). Many trials have explored the effects of platinum in neoadjuvant settings, and the current consensus is that the application of platinum-based on standard chemotherapy will enhance the PCR of TNBC patients at the cost of noticeably increased chances of level 3/4 hematological AEs (49, 50). Therefore, exploration of new adjuvant chemotherapy regimens with platinum as the main component, which has less toxicity and side effects, has broad prospects and should be further investigated.

Cluster 3: The Application of Paclitaxel in TNBC Treatments

Paclitaxel (PTX) is an antimitotic chemotherapy drug widely used in a variety of cancers. By stabilizing microtubules, the cell cycle stops at the G2 and M stages, leading to subsequent apoptosis (51). Paclitaxel is widely used in many cancers and is currently the first-line chemotherapy drug for TNBC. Although traditional paclitaxel has good efficacy, its further clinical use is limited due to its poor solubility and toxic side effects (mainly peripheral neuropathy).

However, albumin-bound paclitaxel (nab-PTX) solves this problem considerably. Nab-paclitaxel is a modified structure based on traditional paclitaxel. By utilizing albumin nanoparticles as carriers, the safety and efficacy of paclitaxel are improved (52). Furthermore, cancer cells often overexpress albumin binding glycoprotein SPARC (an acid-secreting protein rich in cysteine), which promotes the release of drugs in tumor regions for a better targeted antitumor effect (53). Moreover, compared with solvent-based paclitaxel, nab-paclitaxel does not need a co-solvent; thus, the related hypersensitivity reactions are avoided. Problems such as preventive application of glucocorticoids and excessively long infusion time are also solved. It is predicted that nab-paclitaxel will become a research hotspot in the future.

Cluster 4: PARP Inhibitors in TNBC Treatments

The lack of suitable therapeutic alternatives for TNBC in the past is largely due to the lack of therapeutic targets. *BRCA* is currently the most vital tumor suppressor gene related to the occurrence and development of breast cancer, which plays a crucial role in repairing damaged DNA and maintaining genomic stability. The incidence of the *BRCA1/2* pathogenic mutation in TNBC patients is 10–20% (54, 55). In addition to *BRCA1/2*, DNA single-strand breaks (i.e., DNA damage) are repaired by PARP. When PARP inhibitors are used upon an existing *BRCA1/2* mutation, DNA repair in tumor cells is further limited, thereby resulting in lethality, i.e., the "synthetic lethality" phenomenon, which is the treatment principle of PARP inhibitors (56).

The OlympiAD clinical trial phase III showed that when compared with the chemotherapy group, the progression-free survival (PFS) of HER2-negative breast cancer patients (with *gBRCA* mutations) was prolonged by 2.8 months following administration of PARP inhibitors, olaparib. The clinical efficacy and safety of olaparib have been confirmed (57, 58). Based on this study, the FDA approved olaparib as treatment for these patients.

It is necessary to employ *BRCA* gene detection screenings among the appropriate population for early identification of those patients sensitive to PARP inhibitors. Precision therapy based on molecular characteristics of TNBC patients is the future direction of therapeutic development.

Cluster 5: A Tumor Stem Cell Study of TNBC

Reya et al. first proposed the term "tumor stem cells" in 2001, suggesting that malignant tumor tissues have a small number of cell subsets that retain stem cell features. These cells are called tumor stem cells (59). They have the ability of self-renewal, infinite proliferation, and multidirectional differentiation, which are related to tumor occurrence and recurrence.

Currently, most solid tumors, including breast cancer, are believed to be stem cell diseases (60). Compared with other subtypes, a high proportion of tumor stem cells in TNBC is considered an important factor of adverse outcomes. It is usually assumed that the recurrence of malignant tumors after a series of conventional treatments is due to the surviving tumor stem cells. During chemotherapy, surviving tumor stem cells are selectively enriched in the residual tumor, which differentiate into fast proliferating cells insensitive to drugs. Thus, they supplement the tumor cells lost during chemotherapy, resulting in chemotherapy resistance and tumor progression (61). Moreover, breast cancer stem cells (BCSCs) have strong migration abilities. Compared with other solid tumors, BCSCs are more likely to break away from the primary site, migrate, and invade lymphatics or blood vessels, causing breast cancer metastasis (62). Additionally, CSCs are highly tumorigenic. Previous trials have shown that 100 breast cancer tumor cells with stem cell phenotypes transplanted into non-obese diabetic/severe combined immunodeficiency mice can regenerate tumors,

and the new tumor has all the histopathological characteristics of the original tumor (63).

Accumulative studies have shown that TNBC cells show CSCs signatures at functional, molecular, and transcriptional levels. For example, the CD44+/CD24- phenotype and high ALDH activity have become the "golden standard" signature for BCSCs after research of Al-Hajj et al. and Ginestier et al. (61, 64). Interestingly, histopathological analyses revealed that TNBC tissues had more enriched CD44+/CD24- and ALDH1 expression signatures compared to non-TNBC tissues (65, 66), suggesting that the TNBC phenotype is highly like the CSC phenotype. In addition, the EMT signature, which can ultimately facilitate tumor cell migration, is consistently observed in both TNBC and CSCs cells (67). These data collectively indicated that BCSCs are enriched in TNBC, which may contribute to the propensity of TNBC for tumor metastasis and chemotherapy resistance, providing a different insight into the aggressive nature of TNBC. In the future, CSCs in research will focus on the role of CSCs in the tumor biology of TNBC to develop new, effective targeted therapies and improve prognosis of TNBC patients.

Cluster 6: The Special Tumor Microenvironment of TNBC

In 1889, Stephen Paget first proposed the "seed and soil" theory, wherein cancer cells were "seeds" and the microenvironment was the "soil" for their occurrence and metastasis. The tumor microenvironment (TME) is composed of vascular endothelial cells, mesenchymal stem cells (MSCs), tumor-associated fibroblasts (CAFs), immune cells, and extracellular matrix, which induce tumor proliferation, inhibit cell apoptosis, stimulate angiogenesis, and tumor immunosuppression, thereby blocking the antitumor response of TNBC and promoting its occurrence and development (68).

The excessive proliferation of tumor cells and abnormal vascular structure may lead to a hypoxic microenvironment. Consequently, endothelial cells are stimulated to generate new branch vessels that provide oxygen and nutrients, as well as a pathway for tumor metastasis. CAFs account for the highest proportion of stromal cells in TME. When activated by tumor cells, CAFs secrete various growth factors and chemokines. The former promotes growth and metastasis of tumor cells, while the latter guides recruitment of various types of extracellular matrix cells (69). In the TME, immune surveillance and immune escape mechanisms of tumor cells and the human immune system work against each other. Immune cells from various families show antitumor and tumorigenesis manifestations upon receiving environmental signals in the TME (70). It has been found that cytotoxic CD^{8+} T lymphocytes and CD4+ T lymphocytes in the TME induce antitumor immunity and are independent and favorable prognostic factors (71). However, most tumor-associated macrophages (TAMs) in the TME have the M2 phenotype, supporting tumor angiogenesis and metastasis.

Current research on TME is limited. TNBC has a unique immune microenvironment, and research on therapeutic targets of TME will contribute to early diagnosis and effective treatment of TNBC. Unfortunately, there is no standard treatment strategy for TME-specific components in TNBC patients.

Cluster 7: The Molecular Subtypes of TNBC

TNBC is especially characterized by extensive genomic, cellular, and phenotypic heterogeneity. There is no unified standard for molecular typing of TNBC, and the Lehmann classification system is the earliest and most mature TNBC typing system at present. In 2011, Lehmann's team conducted a detailed analysis of breast cancer gene expression profiles, revealing that the so-called "triple negative" cancer was just a common manifestation of a complex heterogeneity of multiple types of TNBC. Thus, TNBC could be specifically divided into six subtypes as follows: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) (23). BL-1 TNBC is primarily characterized by the lack of cell cycle regulation and impaired DNA damage repair machinery, and this subtype is highly sensitive to platinum chemotherapy drugs and presents the best prognosis. In the BL-2 subtype, the growth factor signaling pathway is abnormally active, and both the basal subtypes show high expression levels of proliferation-related genes. The M and MSL subtypes are related to cell movements and show high expression levels of EMT and stem cell-associated genes. The LAR TNBC subtype is associated with a high mutation burden and poor prognosis. This type of cell line depends on androgen growth and is sensitive to androgen receptor inhibitors such as bicalutamide and enzalutamide. Patients with the IM subtype exhibit high levels of immune signaling and checkpoint gene expressions, and they are most likely to benefit from treatment with checkpoint inhibitors.

In 2016, Lehmann et al. discovered that the gene expression profile characteristics of the IM and SLM subtypes were related to tumor-infiltrating lymphocytes and surrounding stromal cells, respectively. Thus, TNBC subtypes were grouped into four categories: BL-1, BL-2, M, and LAR. It is confirmed that different subtypes have significant heterogeneity in several aspects, including the age of onset, degree of malignancy, treatment sensitivity, and prognosis (72).

Based on different detection methods and purposes, other common subtypes include Burstein subtypes and Fudan subtypes (73, 74). In addition, a plethora of high-dimensional technologies, such as single cell RNA sequencing and spatial transcriptomics, has provided new insights into the understanding of subclonal diversity of TNBC (75). Single cell RNA-seq allows the assessment of gene expression patterns at an individual cell level and may provide stronger power to identify tumor cell subpopulations that drive poor prognosis. For instance, using a new single-cell, single-molecule DNA-sequencing method called acoustic cell tagmentation, Minussi et al. observed that there was a period of transient genomic instability followed by ongoing copy number evolution during expansion of primary tumor mass after early evolutionary events including clonal *TP53* mutations, genome doubling and extensive loss-of-heterozygosity events. Furthermore, by expanding single daughter cells *in vitro*, they found that TNBCs quickly rediversify their genomes into multiple subclones and do not retain isogenic properties. These results suggested that during primary expansion of TNBC, the chromosomal aberrations occur continuously and TNBC cells maintain a reservoir of subclonal diversity (76).

Karaayvaz1 et al. used single cell RNA-seq and found a single subpopulation which was associated with several signatures of metastasis and treatment resistance. This subpopulation was characterized functionally by activation of glycosphingolipid metabolism and associated innate immunity pathways (77). Moreover, the Lindeman group described three epithelial subsets including luminal progenitor, basal stem/progenitor, and mature luminal cells from precancerous breast tissues of individuals heterozygous for a *BRCA1* mutation and normal mammary tissues. The $BRCA1^{mut/+}$ tissue harbored an aberrant luminal progenitor population which showed a markedly higher *in vitro* clonogenic activity compared with normal breast tissues. Besides, breast tissues heterozygous for a *BRCA1* mutation and basal breast tumors were more similar to normal luminal progenitor cells in gene expression profile than any other subset, including the stem cell-enriched population, indicating that the basal-like subclass of breast tumors might be progressed from luminal progenitor (78). In the future, studies may focus on uncovering additional cell subpopulations and elucidating how they govern tumor behavior, particularly with respect to non-malignant compartments.

Keyword bursts may indicate the frontier topics or emerging trends in a certain field. In the selected years, the research on pattern, *Brca1*, phenotype and estrogen receptor showed a strong burst at the beginning (2010), and then several keywords, such as growth factor receptor and reoccurrence, showed a citation burst during 2012-2017, yet the bursts were not strong. In the recent 3 years, the keywords immunotherapy and target showed a prominent burst, which were the keywords we were particularly interested in. We have discussed the immunotherapy for TNBC in cluster 0. "Target" is a very generic word which usually refers to therapeutic target in the context of TNBC research. From the summary of keywords with high frequency in TNBC study (**Table 3**) and keyword burst detection results (**Figure 6**), therapeutic target has always been a research focus since keywords such as EGFR, PARP inhibitor, PD-L1 all had high frequency, and they showed a burst at different beginning year during 2010-2020. These suggested that identification of therapeutic targets for TNBC management is throughout the TNC studies, and therefore, it seems necessary to continue to investigate this issue.

However, there are some limitations in our study. First, we only retrieved publications from the WoS Core Collection. Therefore, not all relevant publications were included in this study. Second, although the database is constantly updated, we only included publications from January 2010 to October 2020, which may cause exclusion of some latest research results.

CONCLUSION

In this article, we summarized knowledge on TNBC from a visualization and bibliometric perspective. We focused on eight hotspots in TNBC research, which were summarized using bibliometric analysis. At the core of the hotspots, the NACT and paclitaxel therapy for TNBC treatment, as well as the molecular subtypes of TNBC are relatively mature. However, immunotherapy of TNBC, PARP inhibitors, and other targeted therapies are not yet mature, making them a future trend of this research field. Furthermore, "migration", "protein", and "receptors" are still very popular TNBC burst words among researchers and will continue to be the research direction in the future. Further studies on these topics may help improve our understanding of the pathogenesis of TNBC and guide its 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 author.

AUTHOR CONTRIBUTIONS

K-jH and XJ conceived of the study and designed the study. W-tD, K-jH, and XJ analyzed data and wrote the initial draft 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.2021.689553/full#supplementary-material

Supplementary Figure 1 | The core author and intellectual basis of TNBC Research Field. **(A)** The visualization network of co-cited authors; **(B)** The visualization network of co-cited articles.

Supplementary Figure 2 | The strategic diagram based on results of biclustering analysis and co-word matrix.

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REVIEW
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Potential Predictive and Prognostic Value of Biomarkers Related to Immune Checkpoint Inhibitor Therapy of Triple-Negative Breast Cancer

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As an aggressive subtype of breast cancer, triple-negative breast cancer (TNBC) is associated with poor prognosis and lack of effective therapy, except chemotherapy. In recent years, immunotherapy based on immune checkpoint (IC) inhibition has emerged as a promising therapeutic strategy in TNBC. TNBC has more tumor-infiltrating lymphocytes (TILs) and higher rate of mutation and programmed cell death ligand-1 (PD-L1) expression than other subtypes of breast cancer have. However, previous studies have shown that monotherapy has little efficacy and only some TNBC patients can benefit from immunotherapy. Therefore, it is important to identify biomarkers that can predict the efficacy of IC inhibitors (ICIs) in TNBC. Recently, various biomarkers have been extensively explored, such as PD-L1, TILs and tumor mutational burden (TMB). Clinical trials have shown that PD-L1-positive patients with advanced TNBC benefit from ICIs plus chemotherapy. However, in patients with early TNBC receiving neoadjuvant therapy, PD-L1 cannot predict the efficacy of ICIs. These inconsistent conclusions suggest that PD-L1 is the best to date but an imperfect predictive biomarker for efficacy of ICIs. Other studies have shown that advanced TNBC patients with TMB ≥10 mutations/Mb can achieve clinical benefits from pembrolizumab. TILs also have potential predictive value in TNBC. Here, we select some biomarkers related to ICIs and discuss their potential predictive and prognostic value in TNBC. We hope these biomarkers could help to identify suitable patients and realize precision immunotherapy.

Keywords: predictive biomarkers, immunotherapy, triple-negative breast cancer, immune checkpoint inhibitors, prognostic biomarker

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1 INTRODUCTION

Among women, breast cancer (BC) is the malignant tumor with the highest morbidity and the second highest mortality (1–4). BC can be divided into four subtypes on the basis of expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 as follows: luminal A, luminal B, HER2-enriched, and triple-negative (TN).

TNBC accounts for 12%–17% of BC, and compared with other subtypes, has specific characteristics including earlier onset age, higher metastatic potential, and worse prognosis (5, 6). As a heterogeneous disease, TNBC can be classified into multiple subtypes by different detection methods. According to transcriptome data from the Chinese population, TNBC can be divided into four subtypes: immunomodulatory (IM), luminal androgen receptor, mesenchymal-like, and basal-like and immune suppressed (7, 8). Because TNBC lacks expression of ER and PR and has little or no HER2 expression, it has become the most refractory BC, and chemotherapy is still the most important treatment regimen. Once the tumor has progressed, TNBC is often incurable and the overall survival (OS) is only 10–13 months (9, 10). Therefore, to extend the survival of TNBC patients, a novel treatment strategy is urgently needed.

Recently, immunotherapy has been the focus of investigation in tumor therapy. At present, immunotherapy has shown strong activity against some tumor types such as melanoma and non-small cell lung cancer (NSCLC). Tumor immunotherapy, because of its reliable efficacy and tolerable safety, is regarded as the most promising treatment after surgery, chemotherapy, radiotherapy and targeted therapy (11). The most frequently used immunotherapeutic drugs are immune checkpoint inhibitors (ICIs), such as programmed cell death protein 1 (PD-1)/PD ligand 1 (PD-L1) inhibitors and anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) agents. ICIs can increase lymphocytic cytotoxicity and proliferation by interrupting the binding of IC receptors and ligands to exert antitumor effects. Compared with other subtypes of BC, TNBC has higher frequency of copy number changes, genetic instability, and structural rearrangements, which contribute to its high mutation rate (7, 12). The high mutation rate in TNBC is associated with high lymphocyte infiltration and increased PD-L1 expression (13–15). Both immune cells and immunostimulators are enriched in the IM subtype of TNBC (7). These indicate that patients with TNBC, especially IM subtype, may benefit from ICIs. Therefore, increasing numbers of clinical trials have investigated the efficacy of ICIs for treatment of TNBC, and have shown promising results (16, 17). In the IMpassion 130 study, patients with metastatic or locally advanced unresectable TNBC treated with atezolizumab plus nab-paclitaxel had longer progression-free survival (PFS) in the intention-to-treat population and PD-L1-positive subgroup compared with patients treated with placebo plus nab-paclitaxel. Clinically meaningful prolonged OS was observed in the PD-L1-positive metastatic TNBC (mTNBC) subgroup treated with atezolizumab plus nab-paclitaxel (17, 18). Similar results were seen in the KEYNOTE-355 study, indicating that chemotherapy plus pembrolizumab significantly improved PFS compared with chemotherapy alone for PD-L1-positive patients with metastatic or locally advanced unresectable TNBC (19). Based on these results, pembrolizumab plus chemotherapy are strongly recommended by version 1.2021 of the National Comprehensive Cancer Network (NCCN) guidelines for BC as a first-line regimen in patients with locally advanced or mTNBC with PD-L1 expression.

However, not all TNBC patients can benefit from ICIs. The KEYNOTE-119 study showed that pembrolizumab did not prolong OS significantly in previously treated mTNBC patients, compared with chemotherapy (20). Similarly, in the IMpassion 131 study, paclitaxel combined with atezolizumab did not significantly prolong PFS or OS in the intention-to-treat population (21). These results question the efficacy of ICIs in TNBC. Therefore, it is necessary to identify biomarkers for the efficacy of ICIs to help select patients who could benefit from immunotherapy, and to guide the rational application of such drugs in clinical practice. Besides, some of these biomarkers also have potential prognostic value in TNBC. This review aims to summarize the recent development of the most-discussed biomarkers, which might help to predict the efficacy of immunotherapy and prognosis in TNBC patients.

2 POTENTIAL PREDICTIVE AND PROGNOSTIC VALUE OF THE BIOMARKERS RELATED TO ICIs in TNBC

Currently, the most studied biomarkers related to the efficacy of ICIs in TNBC are TILs, TMB, and PD-L1 expression status [**Table 1** (16, 18–38)]. PD-L1 as the target of anti-PD-L1 treatment is a potential predictive biomarker for the efficacy of PD-1/PD-L1 inhibitors and prognosis of TNBC (16, 19, 22, 39–41). However, the predictive value of PD-L1 is still questionable (23, 40, 42, 43). As mentioned above, TNBC patients have high levels of TILs and TMB. Previous studies have analyzed the predictive values of TILs and TMB for ICIs and have shown that they are associated with better efficacy in TNBC (24–26, 44–52). However, other studies have not confirmed the potential predictive value of TMB (53, 54). Myeloid-derived suppressor cells (MDSCs) and CTLA-4 are related to the increase of TNBC neoantigens, immunosuppression, and immune microenvironment; therefore, their value in IC inhibition cannot be ignored (55–59). Some studies have found that cytokines may predict the efficacy of ICIs and prognosis of BC, but there is a lack of consensus for TNBC. The following is an overview of the potential predictive and prognostic values (**Figure 1**), existing problems, and future application prospects of these biomarkers.

2.1 ICs

2.1.1 PD-L1

PD-L1 is the ligand of PD-1 and is related to immunosuppression. Under normal circumstances, the immune system reacts to foreign antigens in the lymph nodes or spleen by promoting activation of antigen-specific cytotoxic T cells (such as $CD8^+$ T cells). PD-1 combined with PD-L1 can transmit inhibitory signals and reduce the proliferation of $CD8^+$ T cells in lymph nodes, which leads to immune escape of tumor cells. The PD-1/PD-L1 inhibitors interrupt binding of PD-L1 to PD-1, and in this way, tumor cells cannot transmit inhibitory signals to T cells, and T cells recognize and destroy cancer cells. About 20% of TNBC cells express PD-L1 (15, 60). Several studies have explored the predictive value of PD-L1 for immunotherapy in TNBC (16, 19, 22, 39–41). However, inconsistent results have been shown in different studies (23, 40, 42, 43).

TABLE 1 | Summary of clinical trials evaluating the predictive value of the biomarkers for ICIs in TNBC.

Biomarkers	Application	Trials	Treatment	N	Group	Key Data
PD-L1	Early TNBC	KEYNOTE-522 (29)	Pembro /placebo +chemotherapy	602	• PD-L1+	• pCR: 68.9% vs 54.9%
					PD-L1-	• pCR: 45.3% vs 30.3%
		Impassion031 (23)	Atezo /placebo +chemotherapy	333	• PD-L1+	• pCR: 69% (95% CI, 57-79) vs 49% (95% CI, 38-61)
					• PD-L1-	• pCR: 48% vs 34%
	Advanced TNBC	Impassion130 (16, 18)	Atezo /placebo + nab-paclitaxel	902	• PD-L1 +	• m PFS: 7.5 (95%CI, 6.7-9.2) mo vs 5.0 (95%CI, 3.8-5.6) mo; HR=0.64 (0.51-0.80) • m OS: 25.4 (95% CI, 19.6-30.7) mo vs 17.9 (95% CI, 13.6-20.3)mo; HR=0.67 (0.53-0.86)
					• PD-L1-	• m PFS: 5.6 mo vs 5.6 mo; HR=0.95 (0.79-1.15) • m OS: 19.7 mo vs 19.7 mo; HR=1.05 (0.87-1.28)
		KEYNOTE-012 (30)	single-agent pembro	111	• PD-L1+	• m PFS: 1.9 (95% CI, 1.7-5.5) mo • m OS: 11.2 (95% CI, 5.3- (not reached)) mo
		KEYNOTE-086 (31, 32)	single-agent pembro	Cohort A:170 B:84	• Cohort A (PD-L1+vs PD-L1-)	• m PFS: 2.0 (95%CI, 1.9-2.1) mo vs 1.9 (95%CI, 1.7-2.0) mo • m OS: 8.8 (95%CI, 7.1-11.2) mo vs 9.7 (95%CI, 6.2-12.6) mo
					• Cohort B (PD-L1+)	• m PFS: 2.1 (95%CI, 2.0-2.2) mo • m OS: 18.0 (95%CI, 12.9, 23.0) mo
PD-L1	Advanced TNBC	KEYNOTE-119 (20)	Pembro/ chemotherapy[i]	1098	• CPS ≥1	• m OS: 10.7 (95% CI, 9.3-12.5) mo vs 10.2 (95% CI, 7.9-12.6) mo; HR=0.86(0.69-1.06)
					• CPS ≥10	• m OS: 12.7(95% CI, 9.9-16.3) mo vs 11.6 (95% CI, 8.3-13.7) mo; HR=0.78(0.57-1.06)
					• CPS ≥20	• m OS: 14.9 mo vs 12.5 mo; HR=0.58(0.38-0.88)
		KEYNOTE-355 (19, 33)	Pembro /placebo+ chemotherapy	847	• CPS ≥1	• m PFS: 7.6 (95% CI, 6.6-8.0) mo vs 5.6 (95% CI, 5.4-7.4) mo; HR=0.75 (0.62-0.91) • m OS: 17.6(95% CI, 15.5-19.5) mo vs 16.0 (95% CI, 12.8-17.4) mo; HR=0.86 (0.72-1.04)
					• CPS ≥10	• m PFS: 9.7 (95% CI, 7.6-11.3) mo vs 5.6 (95% CI, 5.3-7.5) mo; HR=0.66 (0.50-0.88) • m OS: 23.0(95% CI, 19.0-26.3) mo vs 16.1 (95% CI, 12.6-18.8) mo; HR=0.73(0.55-0.95)
		JAVELIN (22)	single-agent avelumab	168 (58 was TNBC)	• TNBC (PD-L1+ vs PD-L1-)	• ORR: 22.2% vs. 2.6%
					• ≥1% TC (PD-L1+ vsPD-L1-)	• mPFS:5.9(95%CI, 5.7-6.0)weeks vs 6.0(95% CI, 5.9-6.0) weeks; HR=1.183 (0.815-1.716) • m OS: 6.5 (95% CI, 3.7-9.2) mo vs 8.3 (95% CI 6.3, ne) mo; HR=1.331 (0.815-2.174)
					• ≥5% TC (PD-L1+ vsPD-L1-)	• mPFS:6.0(95% CI, 5.7-7.1)weeks vs 5.9(95%CI, 5.9-6.0) weeks; HR=0.782 (0.473-1.290) • m OS: 6.5 (95% CI, 2.2-ne) mo vs 7.1 (95% CI, 5.1-11.3) mo; HR=1.057 (0.556-2.010)
					• ≥25% TC (PD-L1+ vsPD-L1-)	• mPFS:6.0(95% CI 5.4- ne)weeks vs 5.9(95% CI 5.9- 6.0) weeks; HR=0.695 (0.172-2.813) • m OS: 9.2 (95% CI, ne-ne) mo vs 6.8 (95% CI, 4.9-10.8) mo; HR=0.441 (0.061-3.177)
					• ≥10% IC c (PD-L1+ vsPD-L1-)	• mPFS:6.1(95%CI, 2.3-24,1)weeks vs 5.9(95%CI, 5.9-6.0)weeks; HR=0.656 (0.341-1.263) • m OS: 11.3 (95% CI, 1.4-ne) mo vs 6.8 (95% CI, 4.7-9.2) mo; HR=0.620 (0.250-1.541)
		KEYNOTE-150 (34)	Eribulin +pembro	107	• PD-L1+	• m PFS: 4.1 (95%CI, 2.1-4.8) mo
					• PD-L1-	• m PFS: 4.1 (95%CI, 2.3-6.3)mo
		Impassion131 (21)	Atezo/ placebo +paclitaxel	651	• PD-L1 +	• m PFS: 6.0 (95% CI 5.6-7.4) mo vs 5.7 (95% CI 5.4-7.2) mo; HR=0.82 (0.60-1.12) • Final OS: 22.1(95%CI 19.2-30.5) mo vs 28.3 (95% CI 19.1-NE) mo; HR=1.11(0.76-1.64)
TILs	Early TNBC	KEYNOTE-173 (35)	Pembro + chemotherapy	60	• Available pre-treatment sTILs date of ypT0/Tis ypN0	• pCR : 60% vs 40% [a]
					• Available on-treatment sTILs date of ypT0/Tis ypN0	• pCR : 57% vs 43% [b]
					• Available pre-treatment sTILs date of ypT0 /ypN0	• pCR: 58% vs 42% [c]
					• Available on-treatment sTILs date of ypT0 /ypN0	• pCR: 53% vs 47%[d]
		GeparNuevo (28)	Durva / placebo+ chemotherapy	174	• Durvalumab-arm (sTILs)[e]	• OR: 1.23 (95%CI, 1.04-1.6)

(Continued)

TABLE 1 | Continued

Biomarkers	Application	Trials	Treatment	N	Group	Key Data
					• Durvalumab-arm (iTILs)[e]	• OR: 1.58 (95%CI, 0.85-2.97)
					• Durvalumab-arm (iTILs post-pre)[f]	• OR: 5.15 (95%CI, 1.1-24.05)
					• Placebo-arm (sTILs) [e]	• OR: 1.39 (95%CI, 1.12-1.74)
					• Placebo-arm (iTILs) [e]	• OR: 0.94 (95%CI, 0.73-1.22)
					• Placebo-arm (iTILs post-pre)[f]	• OR: 1.19 (95%CI, 0.65-2.17)
	Advanced TNBC	KEYNOTE-086 (27, 31, 32)	single-agent pembro	•Cohort A: 147 B:46	• Cohort A	• ORR: 6% vs 2%[g]
					• Cohort B	• ORR: 39% vs 9%[h]
TILs	Advanced TNBC	Impassion130 (24, 36, 37)	Atezo/ placebo + nab-paclitaxel	902	• Any PD-L1, sTILs<10%	•m PFS: 5.6 mo vs 5.4 mo; HR=0.86 (0.73-1.02) •m OS: 19.2 mo vs 18.1 mo; HR=0.88 (0.72-1.08)
					• Any PD-L1, sTILs≥10%	•m PFS: 8.3 mo vs 6.1 mo; HR=0.64 (0.50-0.84) •m OS: 25.0 mo vs 20.0 mo; HR=0.75 (0.54-1.03)
					• PD-L1 ≥1%, sTILs<10%	•m PFS: 6.4 mo vs 4.7 mo; HR=0.80 (0.59-1.10) •m OS: 19.1 mo vs 17.6 mo; HR=0.74 (0.50-1.10)
					• PD-L1 ≥1%, sTILs≥10%	•m PFS: 9.0 mo vs 5.4 mo; HR=0.54 (0.39-0.75) •m OS: 30.0 mo vs 18.2 mo; HR=0.54 (0.39-0.75)
					• PD-L1 <1%, sTILs<10%	•m PFS: 5.6 mo vs 5.5 mo; HR=0.90 (0.73-1.10) •m OS: 19.3 mo vs 18.2 mo; HR=0.95 (0.75-1.20)
					• PD-L1 <1%, sTILs≥10%	•m PFS: 7.2 mo vs 9.0 mo; HR=0.92 (0.59-1.44) •m OS: 23.7 mo vs 24.5 mo; HR=1.04 (0.59-1.82)
					• Any PD-L1, CD8 <0.5%	•m PFS: 5.6 mo vs 5.6 mo; HR=0.86 (0.65 to 1.14) •m OS: 16.3 mo vs 22.3 mo; HR=1.16 (0.81 to 1.65)
					• Any PD-L1, CD8 ≥0.5%	•m PFS:7.4 mo vs 5.5 mo; HR=0.75 (0.62 to 0.91) •m OS: 22.6 mo vs 18.1 mo; HR=0.69 (95%CI, 0.54-0.81)
					• PD-L1 ≥1%,CD8<0.5%	•m PFS: 9.2 mo vs3.8 mo; HR=0.33 (0.13 to 0.83) •m OS:30.7 mo vs19.4 mo; HR=0.22 (0.06 to 0.90)
					• PD-L1 ≥1%,CD8 ≥0.5%	•m PFS: 7.7 mo vs 5.3 mo; HR=0.64 (0.49 to 0.83) •m OS: 28.6 mo vs 17.7 mo; HR=0.63 (0.46 to 0.86)
					• PD-L1<1%,CD8 <0.5%	•m PFS: 5.6 mo vs 5.7 mo; HR=1.00 (0.73 to 1.37) • m OS: 15.5 mo vs 22.3 mo; HR=1.39 (0.95 to 2.03)
					• PD-L1<1%,CD8 ≥0.5%	•m PFS: 6.5 mo vs 7.2 mo; HR=0.91 (0.68 to 1.21) •m OS: 21.0 mo vs 19.6 mo; HR=0.78 (0.56 to 1.10)
TMB	Early TNBC	GeparNuevo (26)	Durva / placebo+ chemotherapy	149[j]	• Durvalumab-arm (TMB≥2.05 muts/mb vs <2.05 muts/mb)	• pCR: 63% vs 40%[k]
					• Placebo-arm (TMB≥2.05 muts/mb vs <2.05 muts/mb)	•pCR: 52% vs 37%[l]
	Advanced TNBC	KEYNOTE-119 (25)	Pembro/ chemotherapy	253[i]	• TMB ≥10	•ORR: 14.3% (95%CI, 4.0-39.9) vs 8.3% (95%CI, 0.4-35.4)
					• TMB<10	•ORR: 12.7% (95%CI, 7.9-19.9) vs 12.8% (95%CI, 7.8-20.4)
IL-8	Advanced TNBC	A phase II trial (38, 129)	Camrelizumab +apatinib	28[m]	• Responder vs non-respondern	•Levels of IL-8: 0 pg/ml vs 2.15 pg/ml[O]

N, number of patients; TC, tumor cells; IC, immune Cells; m PFS, median PFS; mo, months; m OS, median OS; HR, hazard ratio, HR(95%CI); Pembro, Pembrolizumab; Atezo, Atezolizumab; OR, odds ratio; Durva, durvalumab.

[a]: Levels of TILs: Median (IQR): 42% (95% CI,10-74) vs 10% (95% CI,5-25); [b]: Levels of TILs: Median (IQR): 65% (95% CI,5-89) vs 25% (95% CI,2-48); [c]: Levels of TILs: Median (IQR): 40% (95% CI,10-75) vs 10% (95% CI,5-38); [d]: Levels of TILs: Median (IQR): 65% (95% CI,5-86) vs 25% (95% CI,3-60); [e]: pre-therapeutic; [f]: difference of iTIL between post-window and pretherapeutic biopsy; [g]: Levels of TILs: Median (IQR): 10% (95% CI,7.5-25) vs 5% (95% CI,1-10); [h]: Levels of TILs: Median (IQR): 50% (95% CI,5-70) vs 15% (95% CI,5-37.5); [i]: TMB data were available for 253/601 (42.1%) treated patients (pembro, n = 132; chemo, n = 121); [j]: both whole exome sequencing and RNA-Seq data can be got from pretreatment samples of 149 TNBC of GeparNuevo; [k]: P=0.028; [l]: P=0.232; [m]: 28 Patients had biopsies and blood collected; [n]: responders (partial response); non-responders (stable disease or progressive disease); [o]: P = 0.001.

Some studies have provided evidence about the predictive value of PD-L1 for efficacy of ICIs in TNBC [**Table 2** (18–21–23, 29–34, 37)]. PD-L1 has been shown to predict the efficacy of PD-1/PD-L1 inhibitors in mTNBC, whether in monotherapy or combination therapy (22, 39). Atezolizumab-treated patients with advanced TNBC in the PD-L1-positive population had a higher objective response rate (ORR) compared with the PD-L1-negative population (22.2% vs 2.6%) (39). Similarly, the

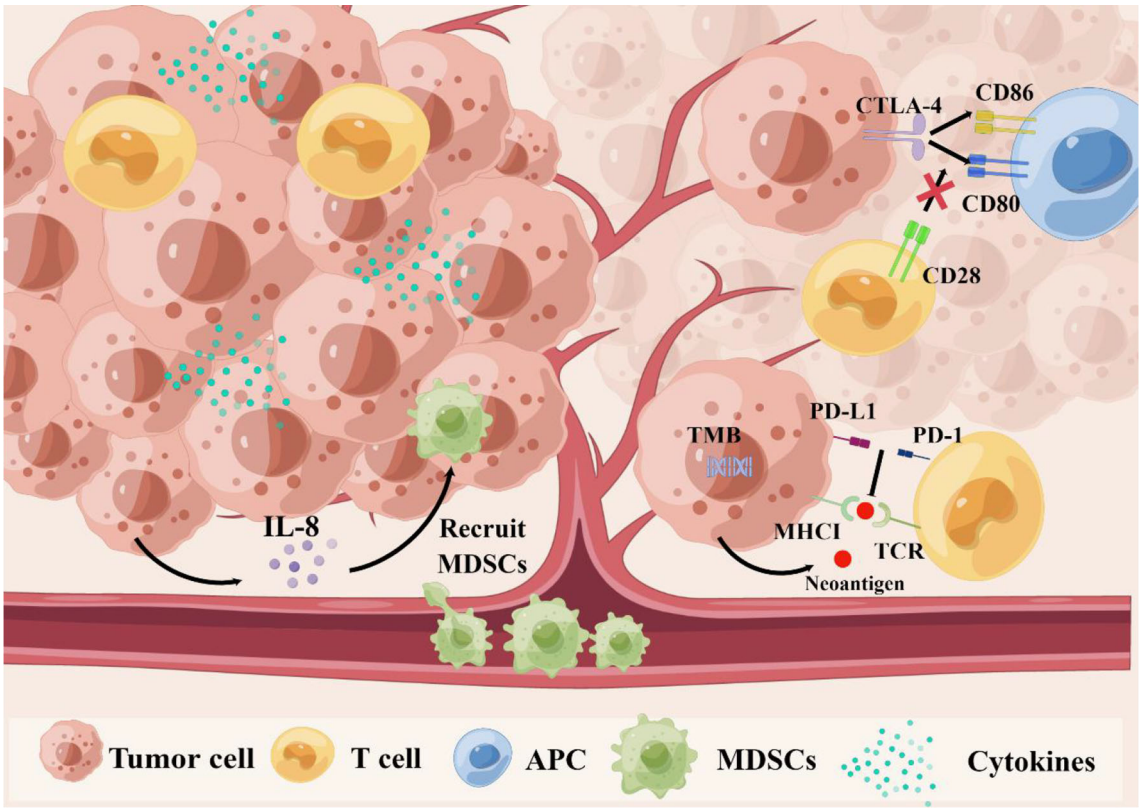

FIGURE 1 | The relationship between biomarkers and immune resistance. First, TMB might lead to new antigens and enhance immunogenicity. Second, the PD-1 combined with PD-L1 can transmit inhibitory signals and reduce immune activation, which leads to the immune escape of tumor cells. Third, CTLA-4 can compete with CD28 to bind to CD80 and CD86 on antigen-presenting cells (APC), and inhibit the activation signal. Fourthly, cytokines can regulate proliferation, differentiation and function of immune cells, tumor microenvironment, and even affect migration of cancer cells. Especially, tumor cells secrete IL-8 to recruit MDSCs into the tumor microenvironment to induce immunosuppression, and promote tumor progression. CTLA-4, cytotoxic T lymphocyte antigen-4; IL-8, interleukin-8; MDSCs, myeloid-derived suppressor cells PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1 TMB, tumor mutational burden.

JAVELIN study reported that mTNBC patients with higher PD-L1 expression had better efficacy of atezolizumab (22). The IMpassion 130 and KEYNOTE-355 studies indicated that PD-L1 positivity was related to better efficacy of PD-1/PD-L1 inhibitors in mTNBC (16, 17, 19). These studies have suggested that PD-L1 expression can identify patients who will benefit from ICIs.

Conversely, other clinical trials have not supported PD-L1 as a predictor of the efficacy of ICIs (23, 42). The KEYNOTE-522 and IMpassion 031 studies in early TNBC patients showed that, irrespective of positive or negative PD-L1 expression, PD-1/PD-L1 inhibitors combined with chemotherapy had a higher pathological complete remission (pCR) than placebo combined with chemotherapy (23, 42). Notably, the patients in these studies had early rather than advanced TNBC. The different results between early and advanced TNBC patients suggest that PD-is not an ideal biomarker and its predictive value varies according to individual immune function and/or disease setting. However, the potential mechanism underlying these results remains unclear.

The potential prognostic value of PD-L1 in TNBC remains contentious. Some studies have provided evidence that PD-L1-positive may be associated with better prognosis (40, 41, 61, 62). A meta–analysis reported that PD-L1-positive on tumor cells was related to poor prognosis, whereas PD-L1-positive on TILs was associated with better survival (41). Li et al. found that PD-L1 expression on TILs suggested better disease-free survival (DFS) in TNBC (61). However, Barrett et al. found that PD-L1-positive on tumor cells was associated with prolonged OS in patients with TNBC (40). Similarly, Botti et al. showed that PD-L1-positive on tumor cells was associated with better DFS in TNBC (62). However, other studies questioned the potential prognostic value of PD-L1 in TNBC (63, 64). A meta-analysis exploring the relationship between PD-L1 and prognosis in TNBC found no significant association between PD-L1 expression and OS (64). Thus, the prognostic value of PD-L1 in TNBC remains unclear and further studies are required.

The inconsistency of these studies suggests that the PD-L1 expression is affected by factors such as complex immune environment and different detection methods. First, expression

TABLE 2 | The predictive value of PD-L1 for PD-1/PD-L1 inhibitors in TNBC.

Application	Agents	Study	Combined Drug	N	Scoring Criteria	Group	Results
Early TNBC	Pembro	KEYNOTE-522 (29)	Pembro/placebo +chemotherapy[g]	602	CPS:1	•PD-L1+ •PD-L1-	• pCR: 68.9% vs 54.9% • pCR: 45.3% vs 30.3%
	Atezo	Impassion031 (23)	Atezo/placebo +chemotherapy[h]	333	PD-L1 in IC: 1%	• PD-L1 + • PD-L1 -	• pCR: 69% (95% CI, 57-79) vs 49% (95% CI, 38-61) • pCR: 48% vs 34%
Advanced TNBC	Atezo	Impassion130 (18, 37)	Atezo/placebo + nab-paclitaxel	902	PD-L1 in IC: 1%	• PD-L1 + • PD-L1 -	• ORR: 58.9% (51.5-66.1) vs 42.6% (35.4-50.1) HR=1.96 (1.29-2.98) • m PFS: 7.5 (95%CI, 6.7-9.2) mo vs 5.0 (95% CI, 3.8-5.6) mo HR=0.64 (0.51-0.80) • m OS: 25.4 (95% CI, 19.6-30.7)mo vs 17.9 (95%CI, 13.6-20.3)mo HR=0.67 (0.53-0.86) • m PFS: 5.6 mo vs 5.6 mo HR=0.95 (0.79-1.15) • m OS: 19.7 mo vs 19.7 mo HR=1.05 (0.87-1.28)
	Atezo	Impassion131 (21)	Atezo/ placebo +paclitaxel	651	PD-L1 in IC: 1%	• PD-L1 +	• m PFS: 6.0 (95% CI 5.6-7.4) mo vs 5.7 (95% CI 5.4-7.2) mo HR=0.82 (0.60-1.12) • Final OS: 22.1 (95% CI 19.2-30.5) mo vs 28.3 (95% CI 19.1-NE) mo HR=1.11(0.76-1.64)
Advanced TNBC	Pembro	KEYNOTE-012 (30)	single-agent pembro	111	PD-L1 in IC: 1%	• PD-L1+	• ORR: 18.5% (95% CI, 6.3-38.1) • m PFS: 1.9 (95% CI, 1.7-5.5) mo • m OS: 11.2 (95% CI, 5.3- (not reached)) mo
	Pembro	KEYNOTE-086 (31, 32)	single-agent pembro	Cohort A:170 B:84	CPS: 1	• Cohort A (PD-L1+) • Cohort A (PD-L1-) • Cohort B (PD-L1+)	• ORR: 5.7% (95%CI, 2.4-12.2) • m PFS: 2.0 (95%CI, 1.9-2.1) mo • m OS: 8.8 (95%CI, 7.1-11.2) mo • ORR: 4.7% (95%CI, 1.1-13.4) • m PFS: 1.9 (95%CI, 1.7-2.0) mo • m OS: 9.7 (95%CI, 6.2-12.6) mo • ORR: 21.4% (95%CI, 13.9-31.4) • m PFS: 2.1 (95%CI, 2.0-2.2) mo • m OS: 18.0 (95%CI, 12.9, 23.0) mo
	Pembro	KEYNOTE-150 (34)	Eribulin +pembro	107	CPS: 1	•PD-L1+ •PD-L1-	•ORR: 25.7% (95%, 12.9-40.8) •m PFS: 4.1 (95%CI, 2.1-4.8) mo •ORR: 25.0% (95%, 12.5-39.8) •m PFS: 4.1 (95%CI, 2.3-6.3)mo
Advanced TNBC	Pembro	KEYNOTE-119 (20)	Pembro/ chemotherapy[i]	1098	CPS: 1, 10, 20	•CPS ≥1 •CPS ≥10 •CPS ≥20	•ORR: 12.3% (95%CI, 8.1-17.6) vs9.4% (95% CI, 5.8-14.3) •m OS: 10.7 (95% CI, 9.3-12.5) mo vs 10.2 (95% CI, 7.9-12.6) mo HR=0.86(0.69-1.06) •ORR: 17.7% (95%CI, 10.7-26.8) vs9.2% (95% CI, 4.3-16.7) •m OS: 12.7(95% CI, 9.9-16.3) mo vs 11.6 (95% CI, 8.3-13.7) mo HR=0.78(0.57-1.06) •ORR: 26.0% vs12.0% •m OS: 14.9 mo vs 12.5 mo HR=0.58(0.38-0.88)
	Pembro	KEYNOTE-355 (19, 33)	Pembro/ placebo + chemotherapy[j]	847	CPS: 1 and 10	•CPS ≥1 •CPS ≥10	•ORR: 44.9% (95% CI, 40.1-49.8) vs 38.9% (95% CI, 32.2-45.8) •m PFS: 7.6 (95% CI, 6.6-8.0) mo vs 5.6 (95% CI, 5.4-7.4) mo HR=0.75 (0.62-0.91) •m OS: 17.6(95% CI, 15.5-19.5) mo vs 16.0 (95% CI, 12.8-17.4) mo HR=0.86 (0.72-1.04) •ORR: 52.7% (95% CI, 45.9-59.5) vs 40.8% (95% CI, 31.2-50.9)

(Continued)

TABLE 2 | Continued

Application	Agents	Study	Combined Drug	N	Scoring Criteria	Group	Results
							•m PFS: 9.7 (95% CI, 7.6-11.3) mo vs 5.6 (95% CI, 5.3-7.5) mo HR=0.66 (0.50-0.88) •m OS: 23.0(95% CI, 19.0-26.3) mo vs 16.1 (95% CI, 12.6-18.8) mo HR=0.73(0.55-0.95)
Advanced Breast cancer	Avelumab	JAVELIN (22)	single-agent avelumab	168 (58 was TNBC)	•PD-L1 in TC[a]: 1, 5 and 25% •PD-L1 in IC[b]: 10%	• TNBC[c] (PD-L1+ vs PD-L1-)	•ORR: 22.2% vs. 2.6%
						• ≥1% TC[d] (PD-L1+ vsPD-L1-)	•ORR: 3.4% (95% CI, 0.3-8.2) vs 3.9% (95% CI, 0.5-13.5) •m PFS:5.9 (95% CI, 5.7-6.0) weeks vs 6.0 (95% CI, 5.9-6.0) weeks HR=1.183 (0.815-1.716) •m OS: 6.5 (95% CI, 3.7-9.2) mo vs 8.3 (95% CI 6.3, ne) mo HR=1.331 (0.815-2.174)
						• ≥5% TC[e] (PD-L1+ vsPD-L1-)	•ORR: 4.3% (95% CI, 0.1, 21.9) vs 2.7% (95% CI, 0.6-7.6) •m PFS:6.0 (95% CI, 5.7-7.1) weeks vs 5.9 (95% CI, 5.9-6.0) weeks HR=0.782 (0.473-1.290) •m OS: 6.5 (95% CI, 2.2-ne) mo vs 7.1 (95% CI, 5.1-11.3) mo HR=1.057 (0.556-2.010)
						• ≥25% TC [f] (PD-L1+ vsPD-L1-)	•ORR: 0 (95% CI, 0-70.8) vs 3 (95% CI, 0.8-7.5) •m PFS:6.0 (95% CI 5.4- ne) weeks vs 5.9 (95% CI 5.9- 6.0) weeks HR=0.695 (0.172-2.813) •m OS: 9.2 (95% CI, ne-ne) mo vs 6.8 (95% CI, 4.9-10.8) mo HR=0.441 (0.061-3.177)
						• ≥10% IC [c] (PD-L1+ vsPD-L1-)	•ORR: 16.7 (95% CI, 2.1-48.4) vs 1.6 (95% CI, 0.2-5.7) •m PFS:6.1 (95% CI, 2.3-24,1) weeks vs 5.9 (95% CI, 5.9-6.0) weeks HR=0.656 (0.341-1.263) •m OS: 11.3 (95% CI, 1.4-ne) mo vs 6.8 (95% CI, 4.7-9.2) mo HR=0.620 (0.250-1.541)

N, number of patients; TC, tumor cells; IC, immune Cells; m PFS, median PFS; mo, months; m OS, median OS; HR, hazard ratio, HR (95%CI); Pembro, Pembrolizumab; Atezo, Atezolizumab.

[a]: the percentages of tumor cells expressing PD-L1: 1 and 5% thresholds with any staining intensity and a 25% threshold with moderate to high staining; [b]: 10% of immune cells expressing PD-L1 at any staining intensity in tumor tissue; [c]: ITT population, PD-L1+: PD-L1 expression≥10% immune cells; [d]: ITT population, PD-L1+: PD-L1 expression≥1% tumor cells; [e]: ITT population, PD-L1+: PD-L1 expression≥5% tumor cells; [f]: ITT population, PD-L1+:PD-L1 expression≥25% tumor cells; [g]: paclitaxel+carboplatin; [h]: nab-paclitaxel + doxorubicin+ cyclophosphamide; [i]: received investigator-choice (capecitabine, eribulin, gemcitabine, or vinorelbine); [j]: nab-paclitaxel, paclitaxel, or gemcitabine-carboplatin.

of PD-L1 is regulated by various mechanisms including the signal transducer and activator of transcription 3 and nuclear factor-κB pathways (65). Additionally, the function of PD-L1 is influenced by ubiquitination, glycosylation, phosphorylation and methylation (65). Therefore, expression of PD-L1 may be altered over time and be induced by other therapies. Second, the different antibodies or detection methods may have affected the results of PD-L1 expression in many studies (66–68). Antibodies for the detection of PD-L1, such as 28-8, 22C3, SP263 and SP142, have been approved as companion/complementary diagnostics to nivolumab, pembrolizumab, durvalumab and atezolizumab, respectively (69). In clinical application, the major differences among the four antibodies are mainly stained cells and scoring criteria of PD-L1. The Blueprint Project showed that 28-8, 22C3 and SP263 mainly stained tumor cells, and the test results were similar. SP142 stained immune cells more prominently than the other antibodies did. Compared with tumor cell staining, immune cell staining was more heterogeneous (70, 71). At present, there are four scoring criteria of PD-L1: combined positive score (CPS), tumor proportion score, immune cell score and tumor cell score (72, 73). Because of the different scoring criteria, the definitions of PD-L1-positive tumors were different. For example, PD-L1-positive tumors in the IMpassion 130 study were defined as staining of any intensity in immune cells occupying ≥1% of the tumor area tested by SP142 (16). In the

KEYNOTE-355 study, PD-L1-positive tumors were defined as CPS ≥1 and CPS ≥10, where CPS was the ratio of PD-L1-positive cells (tumor cells, lymphocytes and macrophages) to the total number of tumor cells tested by 22C3, multiplied by 100 (19). However, these four evaluation methods have not been comprehensively compared; therefore, which method can better reflect the expression level and predictive value of PD-L1 requires further study. Additionally, there were temporal and spatial differences in PD-L1 expression between primary and metastatic lesions (74). Expression of PD-L1 in the metastatic site was significantly lower compared with the primary site (75, 76). Factors such as the empirical judgment of pathologists, heterogeneity of PD-L1, and the effect of drugs might also interfere with PD-L1 expression (65, 70, 77–79).

In summary, the potential predictive and prognostic values of PD-L1 in TNBC remain controversial. Understanding of the tumor, microenvironment, and host factors that influence response to ICIs may contribute to identifying more reliable biomarkers (80). Accurate methods are needed to detect PD-L1 expression and guide precision medicine (81). At present, identifying patients who can benefit from ICIs partly relies on immunohistochemical assays used in clinical trials (82). However, it is difficult to detect the dynamic change in PD-L1 expression, and some factors can interfere with the results. Therefore, the determination of the optimal assay will require further rigorous studies. Scoring systems and thresholds for PD-L1 positivity lack standardization, and this may affect the judgment of PD-L1 positivity. Fortunately, this study area is rapidly developing and PD-L1 as a potential prognostic and predictive biomarker will be fully optimized for TNBC in the future.

2.1.2 CTLA-4

CTLA-4 is one of the immunoglobulin superfamily and a signal receptor on the T-cell membrane (83). It is homologous to CD28 on the surface of T cells and competes with CD28 to bind to B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells, although it has a stronger affinity for B7-1 and B7-2 (84). When B7 binds to CD28, it initiates an activation signal, which is inhibited when CTLA-4 binds to B7 (85). Normally, CTLA-4 participates in negative immunoregulation. However, tumors can also participate in these immunoregulatory pathways by expressing CTLA-4, which decreases immune cell functions (86, 87). Some studies have found that the high levels of CTLA-4 correlate with better efficacy of anti-CTLA-4 therapies in melanoma (88, 89). However, there is a lack of data from clinical trials about its predictive value for ICIs in TNBC. In addition to the above, the potential prognostic value of CTLA-4 in BC has been reported (55, 56). Yu et al. analyzed tissue samples from 130 BC patients who underwent surgery. They found that more interstitial CTLA-4$^+$ lymphocytes were related to longer DFS and OS, whereas more CTLA-4$^+$ tumor cells were related to shorter DFS and OS (55). Lu et al. analyzed an RNA-sequencing dataset and found that BC patients with high CTLA-4 expression had a significantly elevated risk of death compared with those with low CTLA-4 expression (56).

Thus, CTLA-4 expression in BC may be a potential prognostic biomarker. However, whether these results can be applied to TNBC is worthy of further investigation. The potential predictive value of CTLA-4 for efficacy of ICIs in TNBC has not been clarified. Relevant research should be carried out in the future to explore the potential prognostic and predictive value of CTLA-4 in TNBC.

2.2 Immune Cells

2.2.1 TILs

TILs are heterogeneous lymphocyte groups that exist in tumor nests and interstitial cells. They are dominated by different degrees of monocyte and lymphocyte infiltration. The percentage of TILs is higher in TNBC than in luminal type and HER2-enriched BC (46, 90). Some studies have reported that the quantity of TILs in TNBC has predictive value for efficacy of ICIs [**Table 3** (27, 31, 32, 35)]. In the KEYNOTE-086 study, Sherene et al. found that high ORR for mTNBC patients treated with pembrolizumab was associated with high level of TILs (27). Similar findings were reported in the KEYNOTE-173 study, where a high level of TILs was significantly related to better pCR or ORR for TNBC patients treated with pembrolizumab (44). Loi et al. found that stromal TILs ≥5% predicted the response to pembrolizumab monotherapy (45). The biomarker analyses of the GeparNuevo trial showed that higher level of stromal TILs was associated with pCR in the overall cohort but did not predict the efficacy of durvalumab (28). The increased level of intratumoral TILs from before to after treatment was predictive for pCR specifically in the durvalumab arm (28). An increase in TILs in early TNBC patients after neoadjuvant therapy was associated with improved DFS and OS (46, 47). A phase III trial reported an approximately 15% reduction in death and recurrence for every 10% increase in TILs (47). At present, several studies have demonstrated the predictive value of TILs, but there is a lack of high-quality evidence. Therefore, the predictive value of TILs for efficacy of ICIs in TNBC remains contentious.

Most of the above studies have focused on the predictive value of the level of TILs rather than TIL subsets for efficacy of ICIs in TNBC (27, 44–47). TIL subsets with different immune cell compositions represent different immune responses and prognosis (48, 91). On the one hand, TIL subsets can predict the efficacy of ICIs for TNBC. For example, mTNBC patients who received atezolizumab as monotherapy with intratumoral CD8$^+$ T cells >1.35% prior to treatment presented trends toward higher ORR and longer OS (92). An exploratory analysis of the IMpassion 130 study reported that the percentage of CD8$^+$ T cells (≥0.5%) was predictive for the efficacy of atezolizumab plus nab-paclitaxel in mTNBC (24). Similarly, Jiang et al. found that a high CD8 immunohistochemical score was associated with better efficacy of immunotherapy in the IM subtype of TNBC (93, 94). On the other hand, TIL subsets might be associated with worse prognosis in BC (50, 95). For example, a high enrichment score of immature DCs and eosinophils is associated with poor OS (95). Additionally, lymphocytes with positive expression of fork head box protein 3 in tumor tissues are significantly associated with poor prognosis in BC (50).

Taken together, the potential predictive value of TILs in TNBC needs further exploration, and TILs may have potential prognostic value in TNBC. The 17th St Gallen International

TABLE 3 | Results of the exploratory studies of TILs in TNBC patients treated with PD-1/PD-L1 inhibitors.

Application	Agents	Study	Combined Drug	N	Group	Results	Levels of TILs
Early TNBC	Pembro	KEYNOTE-173 (35)	Pembro + chemotherapy[a]	60[b]	•Available pre-treatment sTILs date of ypT0/Tis ypN0	•pCR :60% vs 40% [h]	•Median (IQR): 42% (95% CI,10-74) vs 10% (95% CI,5-25) [c] $
					•Available on-treatment sTILs date of ypT0/Tis ypN0	•pCR :57% vs 43% [h]	•Median (IQR): 65% (95% CI,5-89) vs 25% (95% CI,2-48) [e] $
					•Available pre-treatment sTILs date of ypT0 /ypN0	•pCR :58% vs 42% [h]	•Median (IQR): 40% (95% CI,10-75) vs 10% (95% CI,5-38) [d] $
					•Available on-treatment sTILs date of ypT0 /ypN0	•pCR :53% vs 47% [h]	•Median (IQR): 65% (95% CI,5-86) vs 25% (95% CI,3-60) [f] $
Advanced TNBC	Pembro	KEYNOTE-086 (27, 31, 32)	single-agent pembro	•Cohort [i]	•Cohort A	•ORR :6% vs 2%[j]	•Median (IQR): 10% (95% CI,7.5-25) vs 5% (95% CI,1-10) [k] $
				A: 147 B:46	•Cohort B	•ORR :39% vs 9%[j]	•Median (IQR): 50% (95% CI,5-70) vs 15% (95% CI,5-37.5) [k] $
Advanced TNBC	Atezo	Impassion130 (24, 36, 37)	Atezo/ placebo + nab-paclitaxel	902	•Any PD-L1, sTILs<10%	•m PFS: 5.6 mo vs 5.4 mo[l] HR=0.86 (0.73-1.02) •m OS: 19.2 mo vs 18.1 mo[l] HR=0.88 (0.72-1.08)	•sTILs<10%, any CD8
					•Any PD-L1, sTILs≥10%	•m PFS: 8.3 mo vs 6.1 mo[l] HR=0.64 (0.50-0.84) $ •m OS: 25.0 mo vs 20.0 mo[l] HR=0.75 (0.54-1.03)	•sTILs≥10%, any CD8
					PD-L1 ≥1%, sTILs<10%	•m PFS: 6.4 mo vs 4.7 mo[l] HR=0.80 (0.59-1.10) •m OS: 19.1 mo vs 17.6 mo •HR=0.74 (0.50-1.10)	•sTILs<10%, any CD8
					PD-L1 ≥1%, sTILs≥10%	•m PFS: 9.0 mo vs 5.4 mo[l] HR=0.54 (0.39-0.75) $ •m OS: 30.0 mo vs 18.2 mo[l] •HR=0.54 (0.39-0.75) $	•sTILs≥10%, any CD8
					PD-L1 <1%, sTILs<10%	•m PFS: 5.6 mo vs 5.5 mo[l] HR=0.90 (0.73-1.10) •m OS: 19.3 mo vs 18.2 mo[l] HR=0.95 (0.75-1.20)	•sTILs<10%, any CD8
					PD-L1 <1%, sTILs≥10%	•m PFS: 7.2 mo vs 9.0 mo[l] HR=0.92 (0.59-1.44) •m OS: 23.7 mo vs 24.5 mo[l] HR=1.04 (0.59-1.82)	•sTILs≥10%, any CD8

N, number of patients; TC, tumor cells; IC, immune Cells; IQR, interquartile range; $, indicates statistical significance; pCR, pathological complete response.

[a]: Pembro + taxane with or without carboplatin, and then doxorubicin and cyclophosphamide before surgery; [b]: 53 patients have pre-treatment sTILs data and 49 patients have on-treatment sTILs data; [c]: Median (IQR) TIL level in responders vs non-responders, P= 0.0059, AUROC (90% CI) 0.653 (0.527-0.779); [d]: Median (IQR) TIL level in responders vs non-responders, P= 0.0091, AUROC (90% CI) 0.638 (0.512-0.764); [e]: Median (IQR) TIL level in responders vs non-responders, P= 0.0085, AUROC (90% CI) 0.690 (0.564-0.817); [f]: Median (IQR) TIL level in responders vs non-responders, P= 0.0097, AUROC (90% CI) 0.676 (0.547-0.806); [g]: DCR (CR + PR + SD ≥ 24 weeks; [h]: Number of responders/number vs Number of no-responders/number, and patients not assessable for pCR were considered non-responders; [i]: 193 patients had evaluable tumor samples: 147 from cohort A, 46 from cohort B; [j]: ORR in patients with TIL level ≥vs<median; [k]: Median (IQR) TIL level in responders vs non-responders, and patients without response data were counted as non-responders. Response data included complete response or partial response; [l]: Atezo + nab-paclitaxel vs placebo + nab-paclitaxel.

Breast Cancer Consensus Conference has shown that TILs may serve as a prognostic biomarker in TNBC (96). The following factors may affect the predictive effect of TILs. First, the evaluation of TILs is still mainly dependent on pathologists, who may obtain different results. Because the composition of TILs is complex, it is a challenge for researchers to distinguish the functions of different cells and their predictive values. Restrictions among TILs and the influence of cytokines on their functions can also influence their predictive value. Fortunately, testing standards have been formed for TILs, and artificial intelligence has gradually been applied to case interpretation (97, 98). Therefore, TILs can be detected more

objectively. In the future, the clinical application of TILs will have broad prospects in TNBC, but currently, we should not use TILs to select individual patients for ICIs in clinical practice.

2.2.2 MDSCs

Myelopoiesis is a tightly regulated process that is altered in cancer, leading to the expansion of immature myeloid cells, now called MDSCs (99). Tumor cells secrete interleukin (IL)-8 to recruit MDSCs into the tumor microenvironment; inhibit T-cell activation by consuming and limiting cysteine and other essential amino acids, such as cysteine, for T-cell activation; induce immunosuppression; and promote tumor progression (100–104). MDSCs can be divided into two major groups: polymorphonuclear and monocytic MDSCs (105). Some studies have shown that the subsets of MDSCs are associated with the efficacy of ICIs in NSCLC and melanoma (106–108). However, there is no evidence whether the MDSCs are related to the efficacy of ICIs in TNBC. Therefore, the predictive value of MDSCs for efficacy of ICIs in TNBC is not clear.

Other studies have shown that higher levels of MDSCs are associated with worse prognosis in patients with solid tumors such as advanced BC (57, 58, 109, 110). Furthermore, advanced BC patients with circulating MDSCs >3.17% at baseline had poorer median OS than patients with circulating MDSCs ≤3.17% (5.5 vs 19.32 months) (57). In support of the prognostic value of MDSCs, Bergenfelz et al. observed 54 patients with metastatic BC and found that higher MDSC count was associated with worse PFS and OS (58).

As mentioned above, MDSCs may have potential prognostic value in BC, although no similar study has focused on TNBC. The potential predictive value of MDSCs for efficacy of ICIs in BC has not yet been clarified. Some studies have reported that ICIs reduce the number of circulating MDSCs, which implies that ICIs might have an MDSC-inhibiting effect (111, 112). Therefore, the detection of circulating MDSCs may contribute to a better understanding of the predictive value of MDSCs for efficacy of ICIs in TNBC. In the future, MDSCs are worth further exploration, especially for the potential predictive value of ICIs and prognostic value in TNBC.

2.3 TMB

Tumor formation and progression are accompanied by the acquisition and accumulation of mutations. TMB refers to the total number of base substitutions, somatic gene coding errors, and gene deletion or insertion errors detected per million bases (113). Exogenous DNA damage and DNA repair pathway defects can cause mutations. These mutations might lead to new antigens that are identified as foreign by the immune system, leading to activation of the immune microenvironment (114). Correspondingly, an activated immune microenvironment is favorable for tumor shrinkage by PD-1/PD-L1 inhibitors (115). In the Chinese population, the rate of TMB-high (TMB-H) in BC is higher than that reported by The Cancer Genome Atlas (116). Among the various subtypes of BC, TNBC has the highest TMB, followed by HER2-positive BC (117–119). Some trials reported that TMB-H was related to the better efficacy of immunotherapy in TNBC (25, 26, 51, 52). The KEYNOTE-119 study reported that ORR was significantly increased by single-agent pembrolizumab in mTNBC patients with TMB ≥10 mutations/Mb, while no significant difference was demonstrated in the ORR between chemotherapy and pembrolizumab in patients with TMB <10 mutations/Mb (25). The results of genome sequencing and whole exome sequencing from 3,369 BC patients also showed that patients with TMB ≥10 mutations/Mb might benefit from ICI treatment (51). Karn et al. performed whole exome sequencing in patients with early TNBC and obtained RNA data from pretreatment samples of patients treated with neoadjuvant ICIs (26). They found that TMB-H was associated with the efficacy of ICIs, and the pCR of patients with TMB-H and TMB-low in the durvalumab treatment arm was 63% and 40%, respectively (26). Barroso et al. analyzed 62 mTNBC patients who had previously been treated with ICIs alone or combined with another therapy (52). They found that TMB-H was associated with longer PFS among patients with mTNBC treated with anti-PD-1/PD-L1 therapies.

However, the predictive value of TMB in BC was questioned by other studies (53, 54). An analysis of 10,000 cases showed that patients with TMB-H BC treated with ICIs had worse efficacy than those who received other antitumor treatments (53). Additionally, Adams et al. found no relationship between PFS and TMB in patients with metastatic BC with TMB-H treated with pembrolizumab monotherapy (54). These results suggest that TMB-H may not have predictive value for efficacy of ICIs in BC. However, these trials did not report the BC subtypes, and whether these conclusions can be applied to TNBC requires further study.

The potential predictive value of TMB for efficacy of ICIs and its potential prognostic value in TNBC are unclear. TMB-H (≥10 mutations/Mb) is useful in certain circumstances to help define which BC patients can appropriately receive pembrolizumab, based on version 1.2021 of the NCCN guidelines for BC. However, there are still some unresolved issues for TMB in TNBC. First, the cutoff point of TMB-H is still uncertain and it has differed among trials. Even if the US Food and Drug Administration defines TMB-H as TMB ≥10 mutations/Mb, this definition is still controversial (120). Therefore, one of the challenges for the future application of TMB is to standardize the cutoff point of TMB. Not all TMB-H patients were positively correlated with a good therapeutic effect of ICIs. In some cases, tumor cells develop drug resistance because of TMB-H (121). For instance, as one of the forms of TMB, the deletion mutation of PTEN can promote tumor resistance to ICIs (122, 123). Therefore, clarifying correlations between mutation type and efficacy of ICIs in TNBC is important.

2.4 Cytokines

Cytokines are a class of soluble low-molecular weight proteins secreted by immune and nonimmune cells, including interleukins, tumor necrosis factors, interferons, colony-stimulating factors and transforming growth factors (124). Through the autocrine and paracrine pathways, cytokines can regulate proliferation, differentiation and function of immune cells, tumor microenvironment, and even affect the migration of cancer cells (124, 125). Recent studies have explored the relationship between cytokines and the efficacy of immunotherapy and prognosis in

tumors (125, 126). Cytokines may be related to the efficacy of ICIs in solid tumors, such as NSCLC and melanoma (127–131). Schalper et al. found that patients with melanoma or NSCLC with high IL-8 levels derived limited benefit from nivolumab and/or ipilimumab (100). Patients with TNBC with low plasma IL-8 levels are more likely to respond to camrelizumab combined with apatinib (129). However, there is insufficient evidence to support the predictive value of IL-8 levels for efficacy of ICIs in TNBC. Other studies have suggested that IL-8 may have prognostic value in TNBC (132–134). Deng et al. found that IL-8 induced TNBC cell migration and tumor growth by multiple signaling pathways (132). Through bioinformatic analysis, Kim et al. and Malone et al. found that high IL-8 expression was associated with poor prognosis compared with low IL-8 expression in TNBC (133, 134).

In summary, there is a lack of consensus whether cytokines can be used to evaluate the efficacy of ICIs and prognosis in TNBC. Because of the complexity of the tumor microenvironment and interaction among cytokines, further exploration of cytokines may be difficult. Compared with invasive examinations such as needle biopsy, cytokines provide another noninvasive examination that can be dynamically detected. At present, cytokine therapy is important for some cancers and has achieved good clinical efficacy in melanoma (135), prostate cancer (136) and colorectal cancer (137). In the future, how to expand the clinical application of cytokines in TNBC is still a challenge.

3 CONCLUSIONS AND FUTURE PERSPECTIVES

ICIs are a promising treatment approach for TNBC. Several clinical trials have shown that ICIs improve the treatment outcomes of TNBC patients (16, 19, 22, 39–41). However, some patients do not respond to ICIs and may suffer immune-related adverse events. Therefore, it is important to evaluate biomarkers in TNBC to identify patients that might benefit from immunotherapy. In this review, we discussed different biomarkers related to the efficacy of ICIs and their potential prognostic value in TNBC, including TILs, PD-L1, cytokines and TMB. Among them, PD-L1 and TMB-H are regarded as criteria for screening BC patients who are suitable for pembrolizumab according to versions 1.2020 and 1.2021 of the NCCN guidelines for BC.

Although many studies of biomarkers for ICIs are underway, there are still some unresolved issues. First, some trials collect samples at a single time point, which lack basic information regarding the dynamic responses to ICIs. This can be overcome by collecting longitudinal tumor samples. Compared with the collection of tumor tissues, peripheral blood testing has the advantages of easy sample collection and causing little harm to patients. Therefore, liquid biopsy may have promise in clinical translational studies. Second, there is no unified detection method or standard for biomarkers such as PD-L1 or TILs. Different studies may obtain different conclusions when using the same biomarker. Third, new immunotherapeutic combinations are gradually emerging, and whether these predictive biomarkers are suitable for new regimens needs to be explored further. Fourth, some patients develop drug resistance in the course of receiving ICIs. Therefore, studies of biomarkers should not only focus on the prognosis and efficacy for ICIs in TNBC, but also the role of biomarkers in the mechanisms related to drug resistance. Finally, a single factor cannot accurately predict the prognosis and efficacy of ICIs in TNBC. In the future, the predictive value of composite biomarkers should be further explored.

In summary, many biomarkers are emerging as potential predictive markers for ICIs and prognostic biomarkers in TNBC, which still need further validation. New detection methods, such as high-throughput sequencing (138), single-cell sequencing technology (139) and magnetic resonance imaging computer-aided detection (a technology used to identify the TILs level) (140), are being applied to biomarker research. These methods will help identify new biomarkers and facilitate more convenient and accurate use of them in the clinic.

First, TMB might lead to new antigens and enhance immunogenicity. Second, the PD-1 combined with PD-L1 can transmit inhibitory signals and reduce immune activation, which leads to the immune escape of tumor cells. Third, CTLA-4 can compete with CD28 to bind to CD80 and CD86 on antigen-presenting cells (APC), and inhibit the activation signal. Fourthly, cytokines can regulate proliferation, differentiation and function of immune cells, tumor microenvironment, and even affect migration of cancer cells. Especially, tumor cells secrete IL-8 to recruit MDSCs into the tumor microenvironment to induce immunosuppression, and promote tumor progression. CTLA-4, cytotoxic T lymphocyte antigen-4; IL-8, interleukin-8; MDSCs, myeloid-derived suppressor cells PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1 TMB, tumor mutational burden.

AUTHOR CONTRIBUTIONS

Conceptualization: HL. Article collection and analysis: SY, DZ, YC, and XM. Manuscript writing: QT and SY. All authors contributed to the article and approved the submitted version.

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