EARLY DIAGNOSES AND TREATMENTS OF UNCOMMON BREAST CANCERS

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Editorial: Early diagnoses and treatments of uncommon breast cancers

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Editorial on the Research Topic
Early diagnoses and treatments of uncommon breast cancers

Breast cancer (BC) is the most frequently diagnosed malignant disease in women and the second cause of cancer-related death globally (1). Despite the enormous progress of the last 30 years, which allowed to improve considerably the clinical management of BC patients, mortality at 5 years after first diagnosis still affects nearly 13% of women. In the vast majority of cases, metastatic evolution is one of the main most important factors implicated in mortality; in addition, disease relapse may be associated with higher mortality rates.

The categorization of BCs into three major molecular subtypes, based on the presence of the estrogen receptor (ER) and progesterone receptor (PR) (ER-positive BCs), epidermal growth factor 2 receptor (HER2-positive BCs), or the lack thereof (Triple Negative Breast cancers-TNBCs) has allowed to get an immediately reliable tool to predict prognostic profiles and delineate treatment strategies. However, additional research efforts need to be undertaken to better detect populations at risk, develop improved tools for early diagnosis and treatment, and identify efficient prognostic and predictive biomarkers, particularly for uncommon forms of BC.

This Research Topic includes 3 case reports and 9 original articles focusing on certain novel molecular, biological and clinical features, together with epidemiological aspects that might be helpful in the early diagnosis and treatment of uncommon forms of BC.

Although BC is usually less frequently diagnosed in younger women (< 40 years), the disease is more likely to present in a more aggressive and advanced form compared to older women (> 40 years). By analyzing the clinicopathological characteristics and survival data of a large cohort of young women with early stage BC, Liu et al. have developed a predictive

nomogram to identify potential risk factors of cancer-specific survival, that would help the clinicians in decision-making processes. Similar efforts have been made by the study of Tang et al. in which through a novel nomogram based on several independent prognostic factors the authors highlight that both radiation and adjuvant chemotherapy are significantly associated with favorable long-term overall survival (OS) and cancer-specific survival (CSS) probability in elderly primary operable TNBC patients. Another way to effectively stratify the high-risk and low-risk BC patients has been advanced by Min et al. establishing an autophagy-related 4-gene signature as significantly associated with an early relapse. As lymph node negative BC can coexist with distant metastasis Min et al. proposed a novel nomogram to better stratify patients who are at high-risk for developing distant metastasis. Moreover, by using a multiple databases and bioinformatic tools Wu et al. found SPINT1/2 (serine proteases acting as HGF activator inhibitors) as potential prognostic biomarkers for patients with BC.

As the majority of patients with early ER-positive BC will not experience a recurrence when treated with 5 years of adjuvant endocrine therapy, identifying those patients that can safely be excluded from additional adjuvant chemotherapy and/or extended adjuvant endocrine therapy has been a high priority over recent years and has led to the development of several commercial multiparameter genomic tests, including the 21-gene recurrence score (RS). Chen et al. conducted a study to assess the impact of the 21-gene RS in early BC patients confirming that the result was due to the estrogen module regardless of age.

Enhanced invasive behavior is frequently observed in primary small cell breast carcinomas (SCBCs). Zhu et al. perform the largest population-based study of SCBC, to gather information on incidence, clinic-pathological features, and prognostic factors. The authors confirm that SCBC is an infrequent and aggressive neoplasm with characteristics of poor differentiation; in addition, chemotherapy, surgery and stage were identified as important predictors of disease-specific survival (DSS) and OS.

It should be mentioned that the molecular profiling of uncommon forms of BC may also have a prognostic significance and may lead the decision-making processes. This is the case for metaplastic BC, which represents a rare and aggressive form of BC with uncertain clinical outcome due to the lack of specific treatment options. In this regard, Hu et al. clarified the prognostic role of HER2 in metaplastic BC, by performing a propensity score-matched analysis in almost 3000 patients over a 28-months' time. Data show that despite ER status and HER2 status have no impact on DSS, HER2 positive status and post-mastectomy radiotherapy are associated with better prognosis. Further dissecting the possible therapeutic options for metaplastic BC, another case report by Fu et al. shows that a 58 years old woman was unresponsive to standard adjuvant chemotherapy provided as first-line treatment, and chemotherapy combined with anti-angiogenic treatment administered as a second-line therapy. However, a partial response was achieved after treatment with immunotherapy (toripalimab) in combination with anti-angiogenic therapy (anlotinib). These findings highlight the need to gather more data on metaplastic BC for better stratifying and treating patients.

Gao et al. presented a case report of a peritoneal metastasis from an uncommon invasive lobular carcinoma (ILC) of the breast with resistance to therapy due to acquired ESR1 and PI3KCA mutations revealed through whole exome sequencing (WES). For this reason, the authors propose WSE as a supplementary technique for early diagnosis of metastatic BC patients.

Rarely BCs can appear as phyllodes tumors giving rise to hypoglycemia due to the production of tumor-derived high molecular weight form of insulin like growth factor 2 (IGF-2). This is the case reported by Zhao et al. in which the surgical resection of the tumor successfully resolved the hypoglycemia associated symptoms.

For improving early diagnosis of invasive BCs, Zhao et al. developed a clinical prediction tool using molecular classification, tumor size and Cooper's ligament status to predict the probability to have an axillary lymph node tumor burden in addition to the sentinel lymph node biopsy.

As a result of ongoing breakthroughs in prognostic and predictive biomarkers as well as in cancer therapy, cancer patients' survival rates have grown considerably. However, further efforts may help to convert BC research into clinical practice in the future for early detection and improvement of cancer survival. Moreover, new biomarkers are warranted to individualize treatment. This will provide health professionals with a powerful decision-making tool that can be used to better manage BC patients.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CASE REPORT
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A Giant Borderline Phyllodes Tumor of Breast With Skin Ulceration Leading to Non-Insular Tumorigenic Hypoglycemia: A Case Report and Literature Review

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Phyllodes tumor (PT) is a special type of breast tumors, including three types: malignant, borderline, and benign. Most of these tumors form unilateral disease and can rapidly increase in size. The occurrence of axillary lymph node metastasis is rare. Tumor-associated hypoglycemia can be divided into non-islet cell tumor and insulinoma. In non-islet cell tumor hypoglycemia (NICTH), a considerable high molecular weight form of insulin like growth factor 2 (IGF-2) is formed, which abnormally binds to insulin receptors in the tissues and causes hypoglycemia. Breast phyllodes tumors with NICTH are rare and first reported in 1983. Surgical resection is the main treatment and hypoglycemia symptoms usually resolve after surgery. Nevertheless, prior to surgery, intravenous glucose infusion is used to maintain blood glucose levels. A female patient presented with a rapidly growing breast mass and was diagnosed with a phyllodes tumor with NICTH at our hospital in August 2020; she was successfully treated through surgical resection. We reviewed the relevant literature to investigate and analyze the relationship between NICTH and phyllodes tumors, as well as optimize its diagnosis and treatment.

Keywords: giant borderline phyllodes tumors, breast, skin ulceration, non-islet cell tumor hypoglycemia, insulin-like growth factor-II

INTRODUCTION

Among the various forms of breast tumors, phyllodes tumor (PT) has the lower incidence rate (1/100,000 individuals) (1). Moreover, the total risk of malignancy is approximately 2.1/1 million individuals (2). According to WHO data, this type includes malignant, borderline and benign tumors (3). This is mostly unilateral disease (4), and the probability of axillary lymph node metastasis is extremely low (4, 5). Hypoglycemia is a common endocrine emergency, usually associated with diabetes and endocrine disorders (6). Certain tumors may lead to hypoglycemia which are known as tumor-associated hypoglycemia. Tumor-associated hypoglycemia can be classified into two categories according to the mechanism underlying its development. The most

common mechanism is high secretion of insulin by insulinomas. The second mechanism, termed non-islet cell tumor hypoglycemia (NICTH), involves the formation of a certain high molecular weight form of insulin like growth factor 2 (IGF-2), which abnormally binds to insulin receptors in the tissues, leads to increased glucose utilization, and causes hypoglycemia (7). NITCH was first reported on primary hepatocellular carcinoma in 1929 (8). Clinically, breast PTs with NITCH are rare and first reported in 1983 (9). We investigated and detected a type of NICTH initiation method, which is mainly caused by giant borderline PT. Preoperative hypoglycemia may be affected by IGF-2 produced by PTs.

CASE DESCRIPTION

A female patient aged 45 years was admitted to the hospital with a primary complaint of a left breast mass present for 6 months. The patient was a farmer without family history and didn't receive other treatment before. The mass increased rapidly in the past 3 months, reaching the size of a basketball with local skin ulceration and infection. The mass (25 cm in diameter) made the breasts asymmetric and was observed in the left breast with tough texture and poor mobility. The surface of the mass is uneven with skin ulceration. Bilateral axillary lymph nodes were not touched. Examination by magnetic resonance imaging showed that the solid mass had not invaded the chest wall muscle (**Figure 1**). On the second and third day of admission, she experienced fasting hypoglycemia associated with fatigue, cold sweats, and confusion. Blood examination showed severe hypoglycemia (0.78 mmol/L), hypoinsulinemia (<1.39 pmol/L), and C peptide level (0.01 nmol/L). The serum insulin antibody was negative and the patient didn't take sulfonylureas previously. The level of carcinoembryonic antigen (CEA), alpha fetoprotein (AFP) and carbohydrate antigen153 (CA153) were within the normal range. The unenhanced magnetic resonance scan showed a regular pancreas and liver contour. Hence, the presence of islet cell tumor and hepatocellular carcinoma (hepatocellular carcinoma often lead to NICTH) were ruled out, and symptomatic treatments (e.g., intravenous infusion of 10% glucose solution) were administered to regulate the concentration of blood glucose. After symptomatic treatments, the symptoms above completely relieved. A comprehensive assessment of patient signs, clinical manifestations, medical history, auxiliary examinations, and preoperative puncture pathology considered the possibility of PTs. On August 27, 2020, the patient underwent left mastectomy. Intraoperative frozen section diagnosis revealed a fibroepithelial tumor, and the possibility of PT was not excluded. The upper, lower, inner, and outer edges of skin samples were submitted for examination, and there were no tumor cells found. The breast incision flap was sutured without axillary lymph node dissection. Immunohistochemical stains with antibodies against IGF-2 (IGF-2 Rabbit pAb A2086, dilution 1:300), CKpan (RAB-0050, dilution 1:200), SMA (alpha smooth muscle actin Rabbit pAb A7248, dilution 1:200) and Desmin (DES Rabbit pAb A0699, dilution 1:200) were performed. Postoperative immunohistochemical staining results (**Figure 2**) were as follows: IGF-2(+), CKpan (+), SMA (+), Desmin (+). Postoperative paraffin pathology analysis (left breast) showed that the mass was borderline PT, part of the epithelium was squamous, and surface skin ulcers were formed (tumor diameter: 25 cm). The patient recovered well after the operation, and blood glucose and insulin levels returned to normal. No adjuvant therapy was administered after surgery and

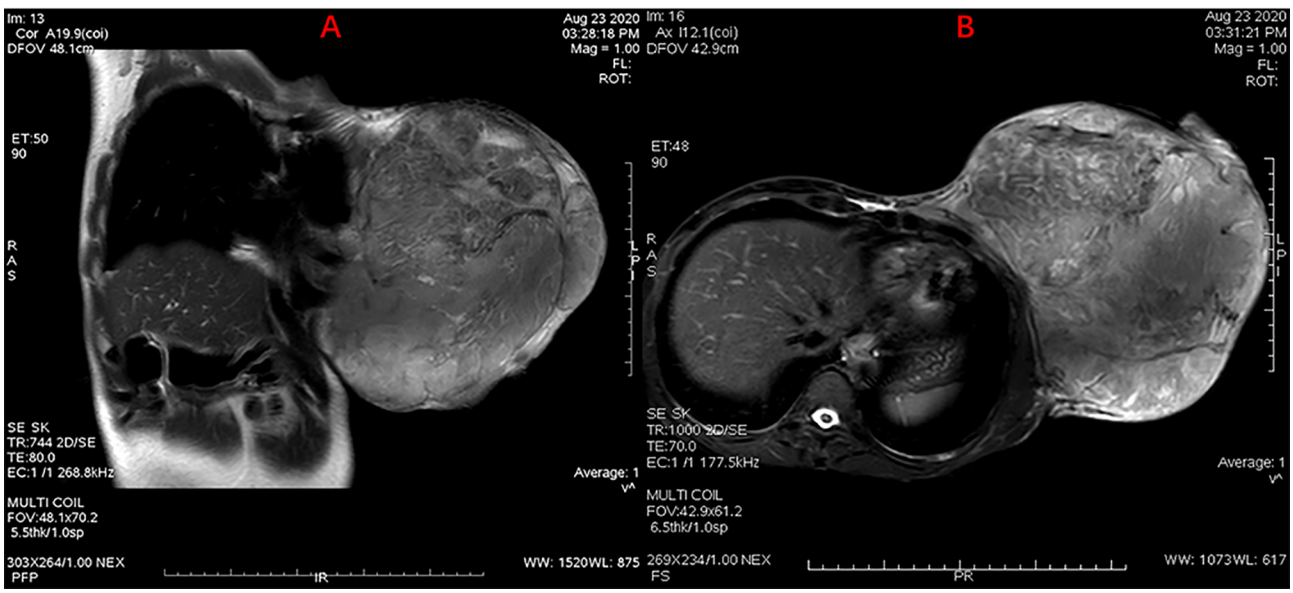

FIGURE 1 | MRI scan showing a giant mass in the left breast with skin ulceration. Without chest wall infiltration or axillary lymph node metastasis were observed. **(A)** Sagittal MR scanning, **(B)** Horizontal MR scanning.

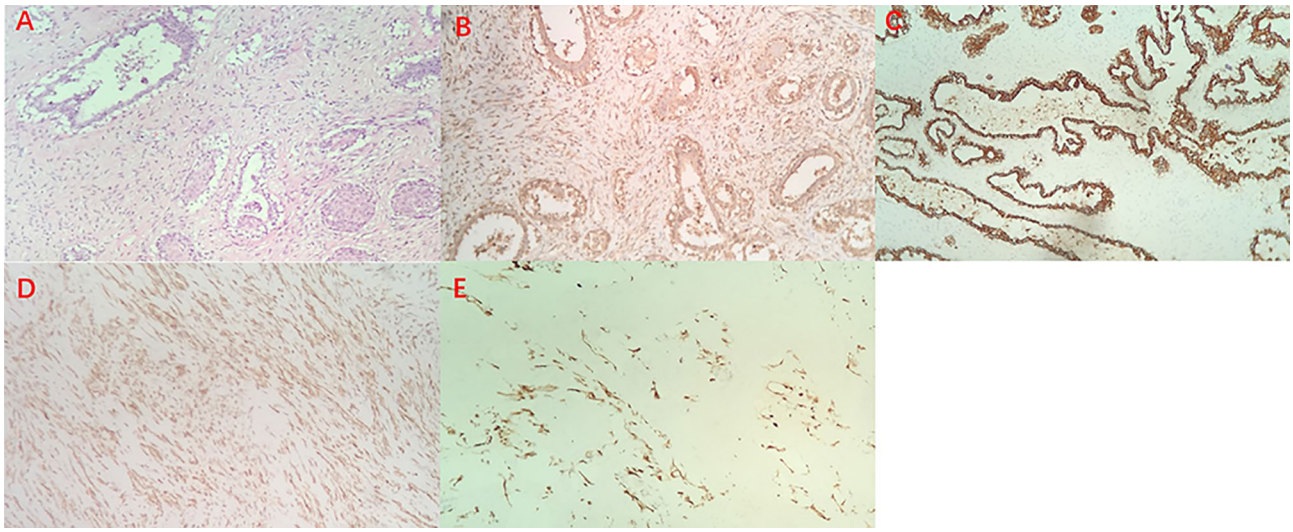

FIGURE 2 | Hematoxylin and eosin staining of the borderline Phyllodes Tumor of Breast (original magnification ×100) **(A)**, Immunohistochemistry staining revealed positive expression of IGF-2 **(B)**, CK **(C)**, SMA **(D)** and Desmin **(E)**.

there was no tumor recurrence or metastasis detected at 3-month follow-up. The patient's family has signed the written consent.

DISCUSSION

Typically, borderline or malignant PT do not involve multiple lesions, and most of them form unilateral disease. The total tumor size of surgical specimens ranges from no residue after biopsy to 38 cm, while the size of PT is larger for malignant PT (4). This patient had a unilateral giant borderline PT; the tumor grew rapidly within 3 months, which made the skin of the left breast tight and thin. The huge mass caused ischemia and formed ischemic ulcers. In addition, the patient developed severe hypoglycemia prior to surgery. Pancreatic magnetic resonance imaging and serological examinations ruled out the possibility of insulinomas. One day after surgery, the blood glucose and insulin levels of the patient rapidly returned to normal. Postoperative immunohistochemical staining results were as follows: IGF-2(+), CKpan (+), SMA (+), Desmin (+). Thus, the patient's preoperative hypoglycemia may have been non-islet cell tumor hypoglycemia (NICTH).

A study analyzed IGF-2 of 44 patients with NICTH, found that 31 patients had high levels of big IGF-2. Furthermore, the study was performed to determine the presence of IGF-2 in the tumors of 20 NICTH patients. Of those, high levels of big IGF-2 were found in the serum of 18 patients (10). These data indicate that hypoglycemia is affected by high molecular weight IGF-2, leading to the development of NITCH. The symptoms of preoperative hypoglycemia in this case may have been caused by IGF-2 in tumor cells.

In addition, another study had found (11) that IGF-2 played a certain role in the diagnosis of the large breast borderline PTs with hypoglycemia. IGF-2 was detected in the tumor and corresponding normal tissues and the measured high serum IGF-2 level led to a preoperative diagnosis. Following mastectomy, the postoperative levels of IGF-2 in the serum gradually returned to normal. Masahiro Hikichi et al. (7) measured the high molecular weight IGF-2 in the serum and tumor tissues of a giant borderline PTs with hypoglycemia through western blotting and immunohistochemical analysis. The western blotting showed that significant levels of high molecular weight IGF-2 were accumulated in tumors at the preoperative stage, but not in the serum obtained 3 days after tumor resection. This confirmed that the core factor of hypoglycemia is caused by the high molecular weight IGF-2. Therefore, for patients with breast PTs with severe hypoglycemia in whom high levels of IGF-2 are detected in tumor cells or serum, the diagnosis of NICTH can be considered. Nevertheless, Jannin A et al. (12) considered that an IGF-2/IGF-1 ratio > 10 was much more useful than the measurement of Big IGF-2, they measured the IGF-2/IGF-1 ratio in six patients with NICTH, found that all of the ratio are more than 10 and the median is 31.8. Unfortunately, we were unable to detect the level of IGF-2 and IGF-1 in serum before and after surgery in our study. However, we excluded the possibility of islet cell tumor by the preoperative examination. According to the perioperative blood glucose changes, the postoperative immunohistochemical staining results and the relevant literature reports. We inferred that preoperative hypoglycemia was most likely caused by the high expression of IGF-2 in the phyllodes tumor. It provides experience for the diagnosis of NICTH.

The first-choice treatment of breast PT is surgical resection (13). Without adjuvant treatment, patients with borderline and malignant PT do not experience recurrence after local tissue resection, and the two conditions are similar (14). The PTs that caused NITCH will no longer produce IGF-2 after surgical resection, the symptoms of NICTH will resolve, and blood glucose levels will return to normal (7, 12, 15–17). Of note, hypoglycemia caused by NICHT is serious, and intravenous glucose infusion is used prior to surgery to regulate blood glucose levels (7, 12, 15–17). Surgical resection requires negative margins.

Many studies have confirmed this view. A study of 164 cases of PTs (18) showed that the length of the margin is not associated with local recurrence, whereas a positive margin is related to recurrence. Another study of 183 cases of PTs (19) also suggested that the size of the surgical margin is not linked to local recurrence. Noordman PCW et al. (13) stated that the primary treatment for borderline and malignant PTs is extensive local excision with tumor-negative resection margins. In addition, if the skin cannot be preserved, it can be filled with the method of rectus abdominis musculocutaneous or latissimus dorsi, which is important for reconstruction after mastectomy (20). Considering that the risk of axillary lymph node metastasis is extremely low, routine lymph node dissection is not recommended (4, 5). In our present case, there was no axillary lymph node enlargement found in the preoperative imaging. Therefore, this patient did not undergo axillary lymph node dissection during left breast resection, and the results of the intraoperative frozen section diagnosis showed negative margins. As a result, the patient recovered well after operation and no recurrence occurred during 3 months follow-up, which proved our treatment was effective for PTs with NICTH.

CONCLUSION

This report presents a case of NITCH caused by a giant borderline PT of the breast. However, it was a pity that we were unable to measure the serum IGF-2 and IGF-1 levels before and after surgery. After excluding the possibility of islet cell tumor by the preoperative examination. And then combining with relevant literature and perioperative blood glucose changes and the postoperative immunohistochemical staining, we inferred that the hypoglycemia symptoms were most likely caused by the high expression of IGF-2 in the phyllodes tumor. The disease and related clinical manifestations are extremely rare in clinical practice. To avoid missed diagnosis, it is necessary for clinicians to consider that PTs with hypoglycemia may be caused by NICTH, monitor the blood glucose levels during the perioperative period, and select the most appropriate treatment to avoid delays in diagnosis and treatment. This article can provide useful guidelines for clinical practice.

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 Ethics Committee of the Fourth Affiliated Hospital of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JZ and MG completed the surgery of the patient. JZ had the subject of the case and provided financial support. YR collected relevant literature and wrote the manuscript. JZ reviewed all related literature and revised the manuscript in this study. HW and RG performed postoperative immunohistochemical staining. SC provided the relevant imaging data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Comprehensive Analysis of the Expression and Prognostic Value of SPINT1/2 in Breast Carcinoma

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Background: Hepatocyte growth factor (HGF) signaling plays a plethora of roles in tumorigenesis and progression in many cancer types. As HGF activator inhibitors, serine protease inhibitor, Kunitz types 1 and 2 (SPINT1 and SPINT2) have been reported to be differentially expressed in breast cancer, but their prognostic significance and functioning mechanism remain unclear.

Methods: In our study, multiple databases and bioinformatics tools were used to investigate SPINT1/2 expression profiles, prognostic significance, genetic alteration, methylation, and regulatory network in breast carcinoma.

Results: SPINT1/2 expression was upregulated in breast cancer, and was relatively higher in human epidermal growth factor receptor 2 (HER2) and node positive patients. Elevated SPINT1/2 expression was significantly correlated with a poorer prognosis. Genetic alterations and SPINT1/2 hypomethylation were observed. In breast carcinoma, SPINT1/2 were reciprocally correlated and shared common co-expressed genes. Gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that their common co-expressed genes were primarily involved in regulating cell attachment and migration.

Conclusions: Our study identified the expression profiles, prognostic significance and potential roles of SPINT1/2 in breast carcinoma. These study results showed that the SPINT1/2 were potential prognostic biomarker for patients with breast cancer.

Keywords: SPINT1, SPINT2, prognosis, functions, breast cancer

INTRODUCTION

Breast carcinoma is the most common malignancy and the second leading cause of cancer-related mortality in women worldwide (1). According to statistics published in 2020, breast cancer alone accounted for approximately 30% of all new cancer cases and 15% of all cancer-related deaths in women in the United States (1). Despite advances in early diagnosis and treatment, almost 5–10% of

patients have metastatic lesions when diagnosed with breast cancer, of which only 20% can survive over 5 years (2). As a disease with high heterogeneity, current methods of prognostic prediction and management are still sub-optimal. Therefore, it is crucial to identify novel reliable prognostic biomarkers and treatment targets.

Signaling transduction by HGF and mesenchymal–epithelial transition tyrosine kinase receptor (MET) is aberrantly activated in many types of cancers, and experimental evidence suggests that the activation of the HGF/MET pathway facilitates cancer cell proliferation, therapy resistance, metastasis, and adaptive response to adverse microenvironments (3–5). HGF, a paracrine factor in the extracellular matrix, is activated by serine proteases mediated proteolysis after being synthesized and secreted by stromal cells as an inactive precursor, proHGF (6). HGF activator (HGFA) and matriptase, two dominant activators of proHGF, can be blocked by several endogenous inhibitors, particularly, SPINT1 and SPINT2 (7). These two protease inhibitors have been reported to be aberrantly expressed in many types of cancer and represent one of the mechanisms of HGF/MET signaling.

Previous studies have shown that HGF and MET expression are elevated and correlated with progression and poorer prognosis in breast cancer patients (8–10). Ectopic expression of SPINT1 or SPINT2 in fibroblasts induced a reduction in HGF levels, thus ablating the HGF-mediated metastatic influence on MDA-MB-231 breast cancer cells (11). Therefore, SPINT1/2 were theoretically supposed to be downregulated in breast carcinoma. However, Parr et al. reported that HGF and MET expression and the HGFA, SPINT1, and SPINT2 levels were relatively higher in breast cancer tissues by immunohistochemical investigations (12). Currently, there are few studies on the expression and functions of SPINT1 and SPINT2 in breast cancer. As crucial regulators of a key transmembrane signaling in mammary malignancies, SPINT1 and SPINT2 should be scrutinized thoroughly. This study aimed to investigate the expression and roles of SPINT1/2 in breast cancer using bioinformatics approaches.

MATERIALS AND METHODS

Analysis of The SPINT1/2 Expression

The expression of SPINT1/2 was examined with Oncomine, the Cancer Genome Atlas (TCGA), and the Human Protein Atlas (HPA) databases. The Oncomine database (https://www.oncomine.org/resource/login.html) consists of abundant microarray data across 35 cancer types and advanced analytical tools to facilitate data mining in cancer research (13). The inclusion threshold for the published SPINT1/2 expression datasets was as follows: P <1E−4, fold change higher or less than 2, and gene rank of the top 10%. Moreover, HTseq-FPKM data for breast cancer samples were downloaded from TCGA (https://portal.gdc.cancer.gov/) and converted to TMP with R package 'zFPKM', Those data were arranged using R and normalized with 'DESeq2' package. The differentially expressed genes were identified using 'limma' package. The differential expression of SPINT1/2 and hub genes were visualized with 'ggplot2'. We analyzed the protein expression of SPINT1/2 in normal and breast cancer tissues using HPA database (https://www.proteinatlas.org/), which contains a large compendium of transcriptomic data and over 10 million images showing immunohistochemistry and immunocytochemistry staining of human proteins spanning 17 cancer types (14). SPINT1/2 expression profiles in cancer patients with different molecular subtypes and node status were explored with Breast Cancer Gene-Expression Miner v4.5 (http://bcgenex.centregauducheau.fr/) (15, 16).

Patients and Samples

A total of 21 human breast cancer specimens, including 8 HER2+ and 13 HER2- samples, were obtained from the First Affiliated Hospital of Chongqing Medical University. All patients (41–72 years old) underwent mammary resection for breast cancer at the First Affiliated Hospital of Chongqing Medical University between May 2020 and May 2021. The status of hormone receptors and HER2 were determined according to the results of immunohistochemistry (IHC) by the Department of Pathology of Chongqing Medical University. The study was approved by the Ethics Committee of Chongqing Medical University. Written informed consent was obtained from all patients.

IHC

The samples were fixed with 4% formaldehyde buffer. Deparaffinized tissues were then sectioned to into 4-μm-thick slices. Following antigen repair and endogenous peroxidase blocking, the sections were incubated with specific rabbit primary antibodies against SPINT1 (1:100; cat. no. FNab08182; FineTest) and SPINT2 (1:400; cat. no. bs-10062R; Bioss) overnight at 4°C. Next, the slices were treated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:300; cat. no. TA140003; OriGene) for 30 min at room temperature. Protein expression was detected with 3,3′-diaminobenzidine (OriGene) and hematoxylin staining and images were captured under Nikon Eclipse 80i microscope (magnification, ×200; Nikon Corporation). The mean optical density (MOD) in five randomly selected areas was calculated with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). SPINT1 and SPINT2 staining intensities (I) were scored as: 0 (no staining), 1 (weak staining), 2 (intermediate staining), 3 (strong staining) and 4 (very strong staining). The percentage of the positively stained area (A) was scored as: 1 (0–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). The results were scored by adding up the intensity and percentage scores (I + A).

Analysis of The Prognostic Significance of SPINT1/2

The prognostic significance of SPINT1/2 was evaluated using Kaplan–Meier plotter and PrognoScan. The Kaplan–Meier plotter database (https://kmplot.com/analysis/) contains expression and clinical prognosis data for more than 54,000 genes from the Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and TCGA cohorts and

provides useful tools to analyze the effect of queried genes on cancer patient prognosis (17). The PrognoScan database (http://dna00.bio.kyutech.ac.jp/PrognoScan/) is an online platform used to analyze the prognostic value of queried genes in publicly available microarray datasets across 13 types of cancer (18).

Genetic Alteration Analysis

The genetic mutation of SPINT1/2 and its correlation with patient survival were investigated using the cBioportal (http://www.cbioportal.org/). The cBioportal database integrates multidimensional cancer genomics data with interactive analyzing modules for research on gene alteration, co-expression profiles, survival, and pathways (19). Moreover, the Catalogue Of Somatic Mutations In Cancer (COSMIC) database (https://cancer.sanger.ac.uk/cosmic/) was used to mine the distributions of genetic alteration of SPINT1/2. The COSMIC database contains comprehensive somatic mutation data of human cancers and offers access to genetic alteration profiles in different contexts (20, 21).

Methylation Analysis

The methylation differences between SPINT1/2 promoters and gene bodies were investigated using DiseaseMeth 2.0 (http://bio-bigdata.hrbmu.edu.cn/diseasemeth/). This database provides direct access to high-throughput methylome data of 679,602 samples and visualization tools (22). The relationship between methylation and expression of SPINT1/2 was examined using the cBioportal database. We looked into the prognostic values of methylated sites in breast cancer with MethSurv (https://biit.cs.ut.ee/methsurv/), and the 'single CPG' module was used to draw the survival plots and violin plots. The MethSurv database uses DNA methylation data from the Genome Data Analysis Center Firehose (http://gdac.broadinstitute.org/) across 25 types of cancers and provides mining solutions to facilitate methylation studies (23).

Identification of Co-Expressed Genes and Enrichment Analysis

Using the LinkedOmics database (http://www.linkedomics.org/) (24), we screened the co-expressed genes of SPINT1/2 in TCGA breast cancer RNA-seq data. Pearson correlation test was applied, and the top 500 correlated genes (ranked by the absolute value of the correlation score) of SPINT1 and SPINT2 were identified. LinkInterpreter module and Gene Set Enrichment Analysis (GSEA) were used to investigate the enriched biological process (BP) and KEGG pathways. Additionally, common co-expressed genes were obtained by cross-referencing the respective top 500 co-expressed genes of SPINT1/2, and the protein–protein interaction (PPI) network of the common co-expressed genes was constructed with String database (https://string-db.org/) (25). The CluGO plugin in Cytoscape v3.8.2 was employed to perform the BP and KEGG pathway enrichment analyses of the common co-expressed genes. Core nodes and hub genes of the PPI network were identified with Cytoscape plugins MCODE and cytoHubba, respectively. The selection criteria in MCODE were as follows: MCODE score >5 points, degree cut-off = 2, node score cut-off = 0.2, Max depth = 100, and k-Score = 2.

Statistical Analysis

Inclusion criteria for Oncomine datasets were set as follows: P <1E −4, fold change higher or lesser than 2, and gene rank of the top 10%. The student's t-test was applied to compare the expression differences in Oncomine datasets. The Wilcoxon signed-rank test was performed to assess the expression of SPINT1/2 and hub genes in normal, and breast cancer tissues with R 3.6.3. For comparisons between two groups in the Breast Cancer Gene-Expression Miner v4.5 analysis, student's t-test was applied, and one-way ANOVA followed by Dunnett's multiple comparisons was performed when three groups were compared. The log-rank test was conducted for P-value in Kaplan–Meier plotter and cBioportal. The Cox P values were presented in PrognoScan. In the survival plots of MethSurv, a likelihood-ratio test was applied. Pearson's correlation coefficient was used to measure the linear dependence between variables in the cBioportal and LinkedOmics. P <0.05 was considered to be statistically significant (*, P <.05; **, P <.01; ***, P <.001).

RESULTS

Expression of SPINT1 and SPINT2 in Breast Cancer

First, we analyzed SPINT1/2 mRNA expression in normal and breast cancer tissues using the Oncomine database. Results showed one out of 45 datasets for SPINT1 and 12 out of 53 analyzes for SPINT2 reported mRNA upregulation in breast cancer tissues (**Figure 1A**). The complete transcriptional profiles of SPINT1/2 are shown in **Table S1**. Moreover, analysis of the RNA-Seq data of 1,222 TCGA breast cancer samples showed that SPINT1/2 were overexpressed in non- and paired cancer tissues compared to normal controls (**Figures 1B–E**). To further evaluate SPINT1/2 protein expression in normal versus breast cancer tissues, we explored the HPA database. The results showed the high intensity of SPINT1 staining in normal and cancer tissues (**Figure 1F**). Meanwhile, the SPINT2 intensity was medium in normal and robust in malignant samples, respectively, when incubated with the HPA006903 antibody (**Figure 1G**). Moreover, the normal tissues showed weak staining, but breast cancer tissues were moderately stained with CAB018969 antibody incubation (**Figure 1H**).

Correlation Between SPINT1/2 Expression and Molecular Subtypes

We explored the bc-GenExMiner v4.5 for the relationship between SPINT1/2 expression and molecular subtypes of breast cancer. In this analysis, RNA-seq data from TCGA and Sweden Cancerome Analysis Network-Breast database (SCAN-B) were selected. The results showed that SPINT1 expression did not correlate with the status of estrogen receptor (ER) (P = 0.2679) or progesterone receptor (PR) (P = 0.6177) (**Figures 2A, B**), but was significantly related to HER2 status. Patients with positive HER2

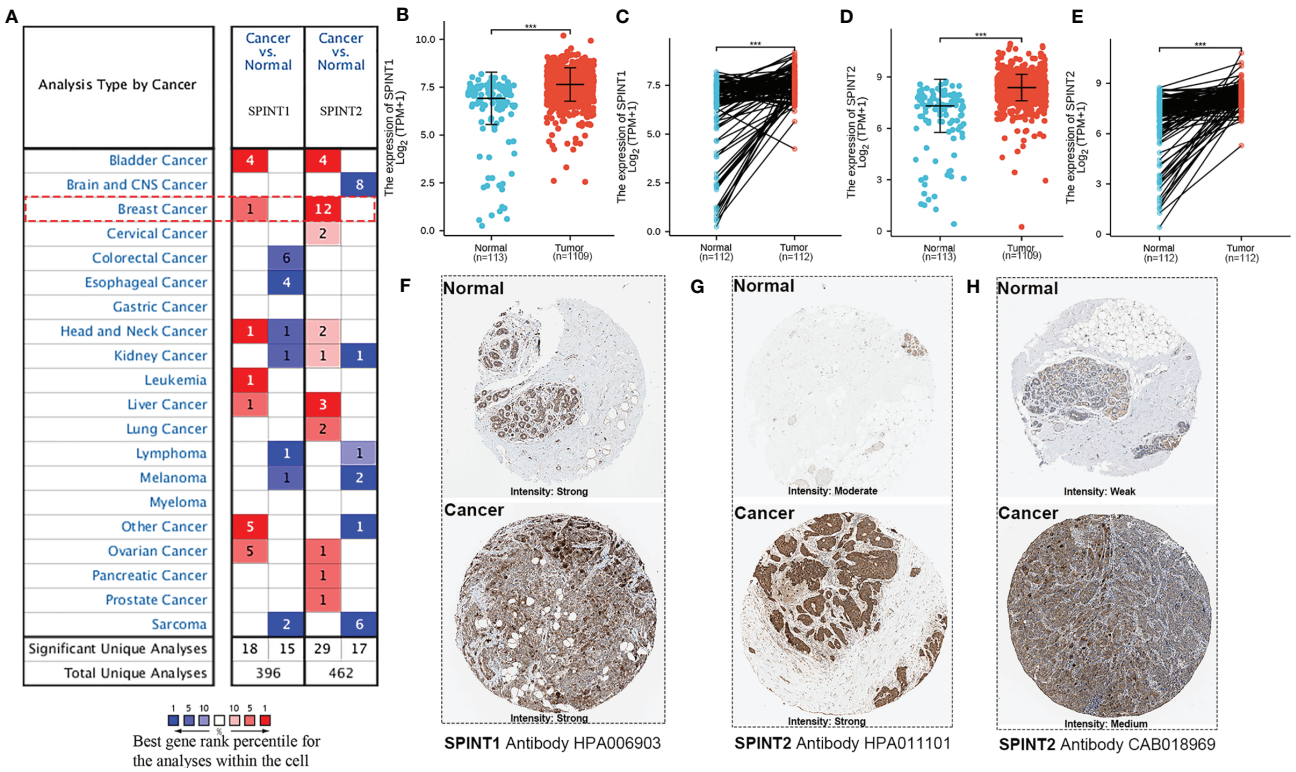

FIGURE 1 | SPINT1/2 were overexpressed in breast cancer. **(A)** datasets reporting the upregulated expression of SPINT1/2 in Oncomine. The differential expression of SPINT1 mRNA in non- **(B)** and paired **(C)** breast cancer tissues. The mRNA expression of SPINT2 in non- **(D)** and paired **(E)** breast cancer samples. The protein expression of SPINT1 **(F)** and SPINT2 **(G, H)** in normal and cancer tissues from HPA database. *P <.05; **P <.01; ***P <.001.

lesions had a higher expression of SPINT1 ($P = 0.0001$) (**Figure 2C**). HER2-expression patients showed the highest SPINT1 level, and the basal-like group presented the lowest abundance (P <0.0001) (**Figure 2D**). Moreover, SPINT1 expression was significantly correlated with node status, and patients with node involvement had a relatively higher SPINT1 expression (P <0.0001) (**Figure 2E**).

In terms of SPINT2, its expression was not related to ER ($P = 0.2411$) or PR ($P = 0.3451$) status (**Figures 2F, G**), but was significantly correlated with HER2 status. SPINT2 expression was higher in HER2 positive versus negative patients ($P = 0.0034$) (**Figure 2H**). Likewise, the HER2 expression group showed the highest expression of SPINT2, and the basal-like group had the lowest abundance (P <0.0001) (**Figure 2I**). In addition, SPINT2 expression was higher in node-positive patients than in node-free patients ($P = 0.0098$) (**Figure 2J**).

Elevated SPINT1/2 Expression in HER2+ Breast Cancer

As SPINT1/2 mRNA expression were found to be upregulated in HER2+ breast cancer, we further appraised their protein level in 21 breast cancer specimens *via* IHC. Representative images are presented in **Figures 3A, B**. Semiquantitative analyses revealed that SPINT1/2 expression were significantly increased in HER2+ breast cancer tissues than that in HER2- samples (P <0.05) (**Figures 3C, D**). These data suggested an upregulated expression of SPINT1/2 in HER2+ breast cancer, which was consistent with the bioinformatic analyses and substantiated the correlation between the status of HER2 and SPINT1/2 expression.

SPINT1/2 Expression Correlated With Prognosis in Breast Cancer Patients

We employed Kaplan–Meier plotter to examine the correlation between SPINT1/2 expression and patient prognosis, specifically overall survival (OS), relapse-free survival (RFS)), and disease metastasis-free survival (DMFS). The results showed that higher expression of SPINT1 was correlated with poorer OS (HR = 1.53, 95% CI = 1.22–1.92, $P = 0.00019$), RFS (HR = 1.21, 95% CI = 1.07–1.37, $P = 0.002$), and DMFS (HR = 1.42, 95% CI = 1.15–1.74, $P = 0.00094$) in breast cancer patients (**Figures 4A–C**). Similarly, the elevated expression of SPINT2 was related to worse OS (HR = 1.34, 95% CI = 1.07–1.68, $P = 0.011$) and RFS (HR = 1.12, 95% CI = 1.01–1.25, $P = 0.035$) (**Figures 4D, E**). However, there was no significant impact of SPINT2 on DMFS (HR = 1.19, 95% CI = 0.95–1.46, $P = 0.11$) (**Figure 4F**).

We further investigated the prognostic correlations of SPINT1/2 in breast cancer using the PrognoScan database. The results of two independent microarray datasets GSE9893 and GSE1378, respectively, showed that the upregulated expression

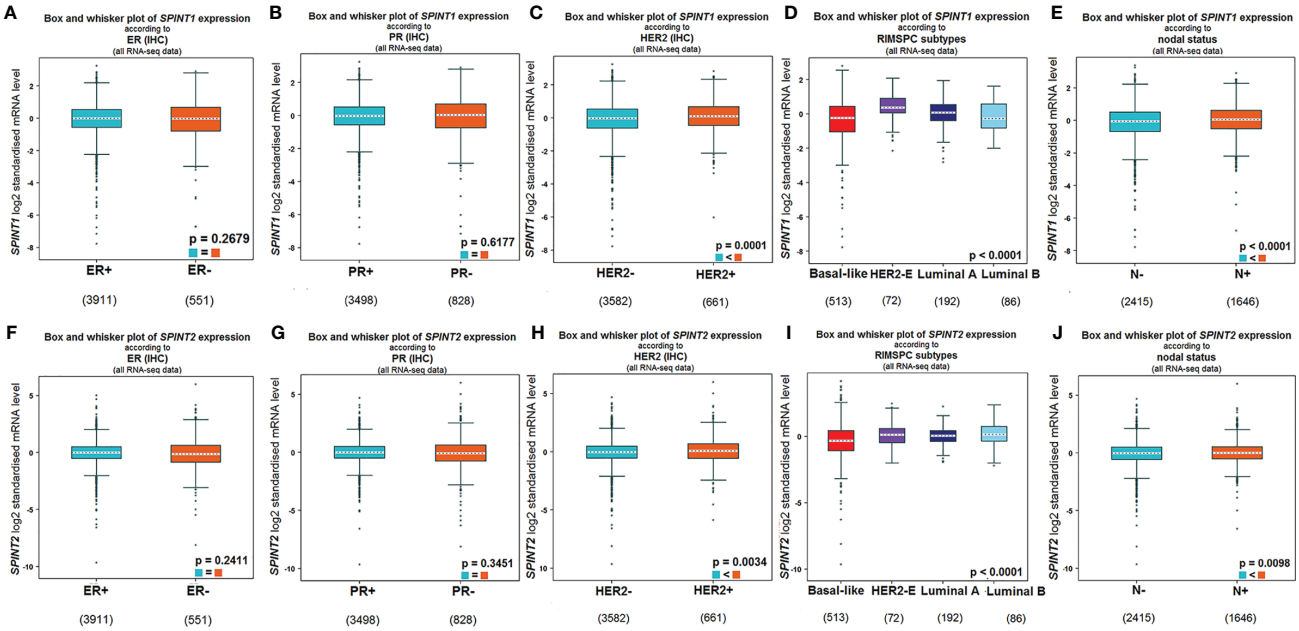

FIGURE 2 | SPINT1/2 expression correlated with HER2 and node status. Relationship between SPINT1 expression with ER **(A)**, PR **(B)**, HER2 **(C)**, molecular subtypes **(D)** and node status **(E)**. Relationship between SPINT2 expression with ER **(F)**, PR **(G)**, HER2 **(H)**, molecular subtypes **(I)** and node status **(J)** from bc-GenExMiner v4.5.

of SPINT1 resulted in poorer OS (HR = 1.39, Cox $P = 0.005$) and RFS (HR = 2.33, Cox $P = 0.017$) (**Figures 4G, H**). In addition, the results of the GSE4922 and GSE3494 cohorts showed significant correlations between SPINT1 expression and disease-free survival (DFS) (HR = 1.14, Cox $P = 0.04$) and disease-specific survival (DSS) (HR = 1.63, Cox $P = 0.024$) (**Figures 4I, J**). Meanwhile, the elevated expression of SPINT2 was found to be correlated with a worse OS (HR = 1.17, Cox $P = 3.3e{-}04$) and RFS (HR = 1.76, Cox $P = 0.013$) in breast cancer (**Figures 4K, L**). These findings indicate that SPINT1 and SPINT2 are valuable biomarkers for prognosis in breast cancer patients.

Correlation Between SPINT1/2 Expression and Clinicopathological Characteristics in Breast Cancer

Using Kaplan–Meier plotter, we also examined the correlation between SPINT1/2 expression and patient prognosis with restricted clinicopathological characteristics. The results showed that SPINT1 was significantly correlated with OS, RFS, and DMFS and with the TP53 status and molecular subtypes, except luminal A. Higher SPINT1 expression was correlated with poorer prognosis in ER+ and ER− subgroups. The expression of SPINT1 was significantly associated with DMFS irrespective of ER status, molecular subtypes, or grades (**Table 1**). Moreover, the highest HR was detected in the correlation between SPINT1 expression and DMFS in HER2+ patients (HR = 7.74, $P = 0.0011$), suggesting that SPINT1 influenced patient clinical outcomes, possibly by affecting tumor metastasis, particularly in the HER2+ subgroup.

In addition, we found that SPINT2 expression was significantly correlated with OS, RFS, and DMFS in patients with ER+, luminal B, and grade 2 subgroups (**Table 2**). However, SPINT2 was only significantly associated with DMFS in the ER+, HER2-, luminal B, grade 2, and TP53 wild type groups, indicating that SPINT2 was possibly involved in metastasis in particular subtypes.

Genetic Alteration Frequency of SPINT1/2 Was Low and Not Related to the Prognosis

Mutations in protein-encoding genes induce expression changes in cancer (26), therefore, we investigated the genetic alteration of SPINT1/2 in breast cancer with the cBioportal database. The breast invasive carcinoma case set (TCGA, Firehose Legacy) containing 963 samples was selected, and the results showed that the mutation of SPINT1/2 was detected in 1.9% (18/963) and 3% (31/963) patients, respectively (**Figure 5A**). To unveil the mutation distributions of SPINT1/2 in breast carcinoma, we searched COSMIC database. The results showed that the alteration types of SPINT1 included nonsense substitution, missense substitution, synonymous substitution, and frameshift deletion. Missense substitution was the most common mutation for SPINT1, accounting for about 40% (**Figure 5B**). Moreover, various nucleotide changes in the substitution mutations were observed, of which C > T and G > A accounted for the largest proportion (**Figure 5C**). Similarly, missense substitutions, synonymous substitutions, and nonsense substitutions were detected in the SPINT2 mutation. The missense substitutions constituted the biggest percentage, which were approximately

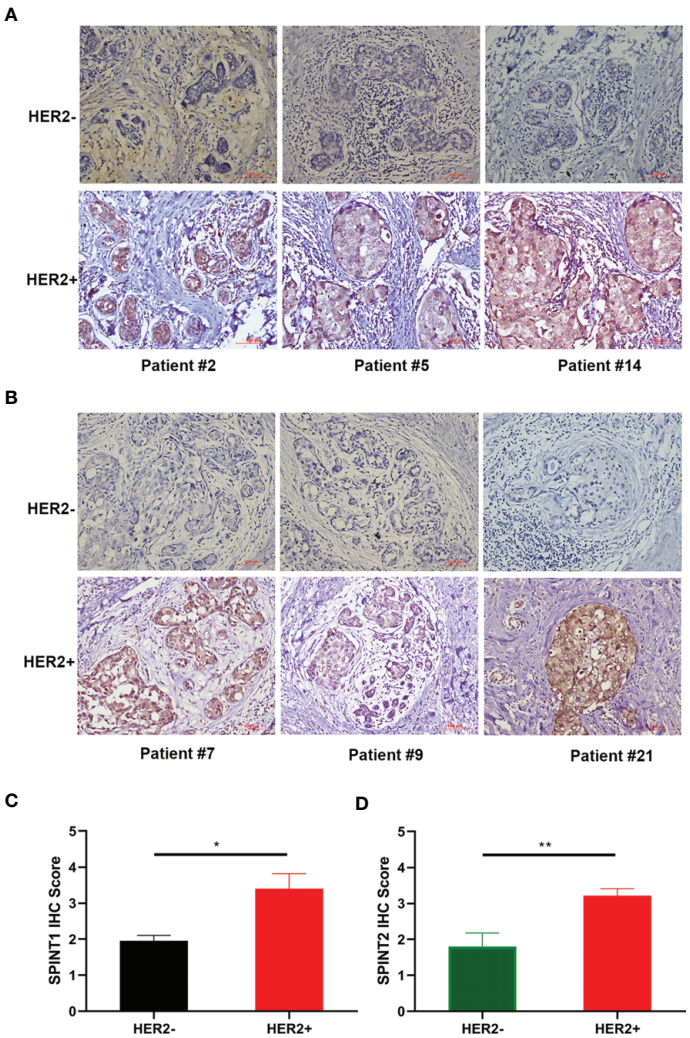

FIGURE 3 | Protein expression of SPINT1/2 in HER2+ breast cancer. Representative images of SPINT1 **(A)** and SPINT2 **(B)** expression in cancer tissues. Semiquantitative results of SPINT1 **(C)** and **(D)** expression in HER2± breast cancer. *$P < .05$ and **$P < .01$.

29% of the 308 samples (**Figure 5D**). The nucleotide changes in observed mutations included C > A, C > T, C > G, G > A, and G > C. C > T was the most frequent change accounting for about 30% of the change (**Figure 5E**).

Subsequently, cBioportal was used to investigate the correlation between SPINT1/2 alteration and prognosis. Survival plots showed that SPINT1 genetic alteration was not significantly correlated with patient OS (log-rank $P = 0.777$) or DFS (log-rank $P = 0.170$) (**Figures 5F, G**). The mutation of SPINT2 had no impact on OS (log-rank $P = 0.340$) or DFS (log-rank P = 0.126) in breast cancer patients (**Figures 5H, I**).

Methylation of SPINT1/2 Was Correlated With Prognosis in Breast Cancer

We explored the DiseaseMeth2.0 database for the SPINT1/2 DNA methylation status and found hypomethylation of SPINT1/2 in the promoter and the gene body (**Figures S1A–D**). The relationship between SPINT1/2 methylation and expression was further investigated using the cBioportal database. The results showed that SPINT1/2 methylation was negatively correlated with its expression in breast cancer (**Figures S1E, F**).

Next, we used MethSurv to identify which methylation sites in SPINT1/2 were significantly correlated with breast cancer prognosis. According to the University of California Santa Cruz Genome Browser (UCSC) database, the CpG sites were grouped into six gene subregions: 'TSS200', 'TSS1500', 'first exon', '5' UTR', 'body' and '3' UTR' (23). The heat maps evaluating the relationship of SPINT1/2 methylation levels with the available patient characteristics and gene subregions were plotted with 'Gene Visualization' (**Figures 6A, B**). By analyzing all 13 methylation sites of SPINT1 in breast cancer patients, we found three hypomethylated sites (TSS200; 5'-UTR-cg11701759, 3'-UTR-Open_Sea-cg04519327, and TSS200; 5'-UTR-Island-cg27510007) were significantly correlated with a

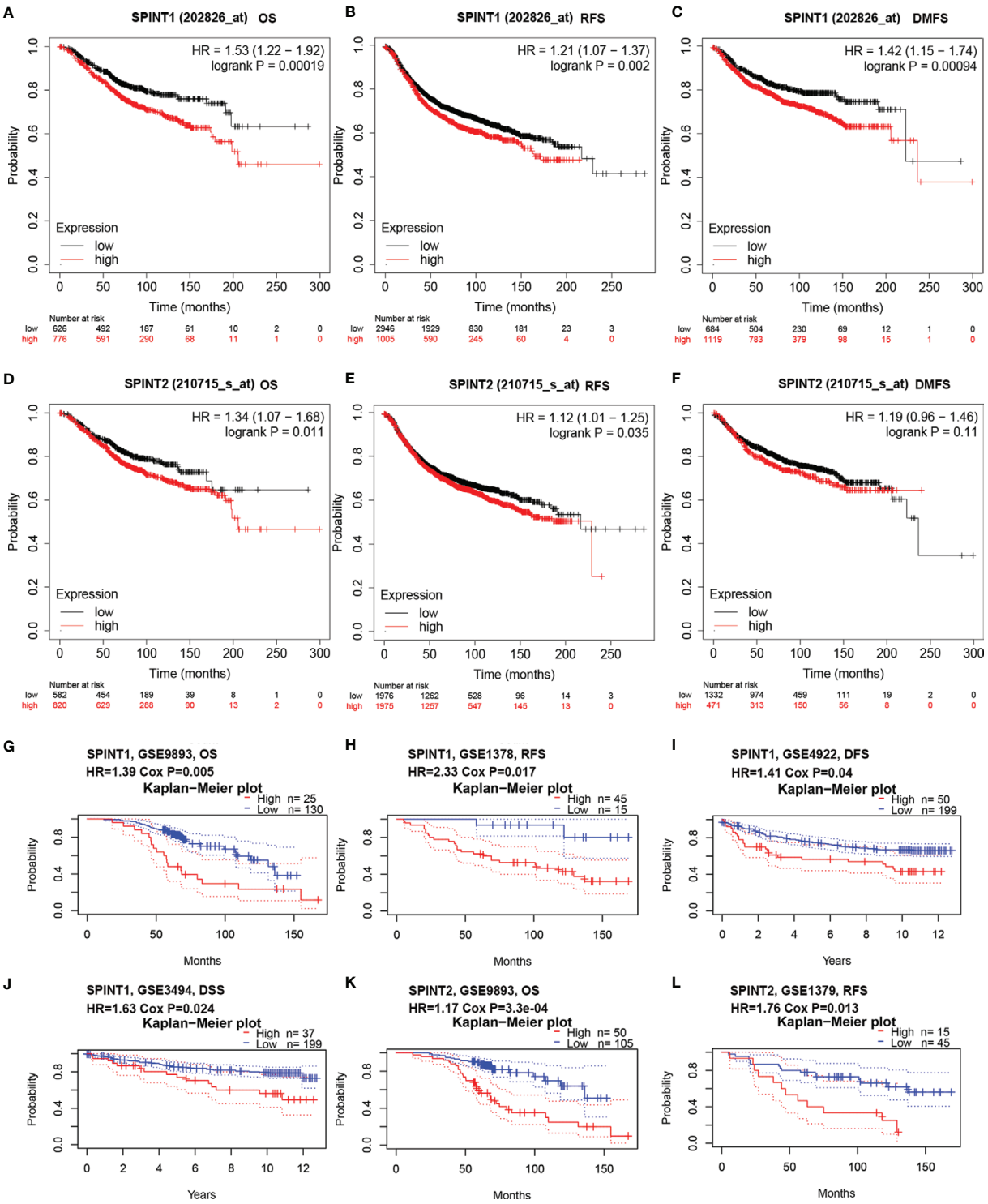

FIGURE 4 | SPINT1/2 were correlated with patient prognosis. Correlation between SPINT1 expression and OS **(A)**, RFS **(B)** and DMFS **(C)** from Kaplan–Meier plotter. Association between SPINT2 expression and OS **(D)**, RFS **(E)** and DMFS **(F)** Kaplan–Meier plotter. The correlation between SPINT1 expression and the OS from GSE9893 **(G)**, RFS from GSE1378 **(H)**, DFS from GSE4922 **(I)** and DSS from GSE3494 **(J)**. The correlation between SPINT2 expression and the OS from GSE9893 **(K)** and the RFS from GSE1379 **(L)** on PrognoScan server.

TABLE 1 | The correlation between SPINT1 expression and prognosis in breast cancer patients with different clinicopathological parameters.

Clinicopathological characteristics	OS (n = 1,402)			RFS (n = 3,951)			DMFS (n = 1,803)		
	N	Hazard ratio	*P* value	N	Hazard ratio	*P* value	N	Hazard ratio	*P* value
ER status									
ER positive	548	1.51 (1–2.28)	0.048	2,061	0.94 (0.8–1.11)	0.4582	664	1.63 (1.16–2.29)	0.0049
ER negative	251	1.73 (1.1–2.74)	0.0173	801	1.26 (0.99–1.59)	0.0588	275	2.12 (1.35–3.34)	9e-04
PR status									
PR positive	83	0.22 (0.05–0.88)	0.0193	589	1.48 (0.98–2.24)	0.0602	191	0.48 (0.21–1.11)	0.0796
PR negative	89	0.51 (0.15–1.75)	0.2721	549	1.2 (0.9–1.61)	0.216	154	1.99 (0.93–4.28)	0.0711
HER2 Status									
HER2 positive	129	0.53 (0.27–1.07)	0.072	252	0.7 (0.42–1.17)	0.1665	126	0.59 (0.26–1.34)	0.2048
HER2 negative	130	0.58 (0.24–1.37)	0.2048	800	0.77 (0.58–1.01)	0.054	150	1.6 (0.65–3.97)	0.3054
Intrinsic type									
Luminal A	611	1.39 (0.97–1.99)	0.745	1,933	1.2 (1–1.45)	0.0543	968	1.6 (1.18–2.16)	0.0024
Luminal B	433	2.31 (1.47–3.63)	0.0002	1,149	1.34 (1.09–1.66)	0.0062	449	1.74 (1.14–2.67)	0.0093
HER2+	117	2.54 (1.66–5.55)	0.0157	251	1.71 (1.14–2.55)	0.0082	125	7.74 (1.8–30.98)	0.0011
Basal	241	1.82 (1.09–3.01)	0.0191	618	1.61 (1.25–2.07)	0.0002	261	2.45 (1.38–4.36)	0.0016
Lymph node status									
Lymph node positive	313	1.41 (0.95–2.09)	0.0826	1,133	0.76 (0.61–0.94)	0.0103	382	1.45 (0.9–2.34)	0.1261
Lymph node negative	594	1.75 (1.19–2.58)	0.0042	2,020	1.15 (0.97–1.36)	0.1186	988	1.39 (1.05–1.85)	0.0227
Grade									
1	161	0.62 (0.23–3.26)	0.3335	345	1.62 (0.96–2.72)	0.0683	188	2.07 (0.89–4.79)	0.0826
2	387	2.04 (1.28–3.26)	0.0022	901	1.43 (1.12–1.82)	0.0045	546	1.5 (1.4–2.17)	0.0297
3	503	1.76 (1.27–2.45)	0.0006	903	1.37 (1.1–1.71)	0.0042	458	2.28 (1.46–3.56)	0.0002
TP53 status									
Muted	111	2.66 (1.24–5.71)	0.0087	188	3.24 (1.55–6.78)	0.0009	83	3.04 (1.16–7.97)	0.0173
Wild type	130	1.53 (1.1–7.26)	0.0243	272	1.72 (1.05–2.8)	0.0293	150	1.6 (0.65–3.97)	0.0711

OS, overall survival; RFS, relapse-free survival; DMFS, disease metastasis-free survival.

TABLE 1 SPINT1 expression was correlated with prognosis in breast cancer patients with different characteristics from Kaplan–Meier plotter.

TABLE 2 | The correlation between SPINT2 expression and prognosis in breast cancer patients with different clinicopathological parameters.

Clinicopathological characteristics	OS (n = 1,402)			RFS (n = 3,951)			DMFS (n = 1,803)		
	N	Hazard ratio	*P* value	N	Hazard ratio	*P* value	N	Hazard ratio	*P* value
ER status									
ER positive	548	1.47 (1.03–2.11)	0.033	2,061	1.25 (1.05–1.48)	0.0098	664	1.74 (1.16–2.63)	0.0072
ER negative	251	1.67 (0.99–2.82)	0.05	801	1.21 (0.97–1.52)	0.093	275	1.42 (0.92–2.18)	0.1093
PR ststus									
PR positive	83	4.16 (0.86–20.11)	0.055	589	1.29 (0.89–1.86)	0.1793	192	2.13 (0.87–5.19)	0.0887
PR negative	89	3.05 (0.88–10.54)	0.0637	549	0.82 (0.6–1.13)	0.2276	192	1.58 (0.87–2.88)	0.1285
HER2 Status									
HER2 positive	129	2.25 (0.96–5.26)	0.0551	252	0.57 (0.36–0.9)	0.0135	126	1.54 (0.79–3.01)	0.2042
HER2 negative	130	3.82 (1.57–9.28)	0.0015	800	1.27 (0.95–1.69)	0.1004	129	2.87 (1.05–7.84)	0.0314
Intrinsic type									
Luminal A	611	1.6 (1.13–2.29)	0.0078	1,933	1.22 (1.02–1.46)	0.027	918	1.35 (0.99–1.83)	0.057
Luminal B	433	1.51 (1.01–2.27)	0.0449	1,149	1.32 (1.08–1.62)	0.007	449	1.67 (1.15–2.43)	0.0063
HER2+	117	1.77 (0.9–3.48)	0.093	251	0.7 (0.45–1.08)	0.1035	125	0.62 (0.32–1.18)	0.1434
Basal	241	1.29 (0.77–2.17)	0.3286	618	1.2 (0.93–1.55)	0.1641	261	0.81 (0.48–1.36)	0.4301
Lymph node status									
Lymph node positive	311	1.69 (1.14–2.5)	0.0078	1,133	1.32 (1.08–1.61)	0.0059	382	0.66 (0.43–1.02)	0.0593
Lymph node negative	382	1.34 (0.86–2.09)	0.1986	2,020	1.15 (0.95–1.38)	0.1442	988	1.28 (0.96–1.71)	0.095
Grade									
1	161	2.19 (0.63–7.56)	0.2039	345	1.37 (0.78–2.41)	0.2768	188	1.73 (0.75–3.99)	0.1938
2	387	1.77 (1.15–2.73)	0.0086	901	1.45 (1.14–1.86)	0.0028	546	1.88 (1.31–2.69)	0.0005
3	503	1.54 (1.05–2.26)	0.0271	903	1.15 (0.92–1.44)	0.2068	458	1.38 (0.94–2.01)	0.0956
TP53 status									
Muted	111	1.64 (0.73–3.65)	0.2231	188	1.79 (1.09–2.92)	0.019	83	2.37 (0.91–6.17)	0.0695
Wild type	187	1.77 (0.92–3.41)	0.0854	273	1.74 (1.13–2.68)	0.0114	109	2.56 (1.18–5.56)	0.0137

OS, overall survival; RFS, relapse-free survival; DMFS, disease metastasis-free survival.

TABLE 2 SPINT2 expression was associated with prognosis in breast cancer patients with different characteristics.

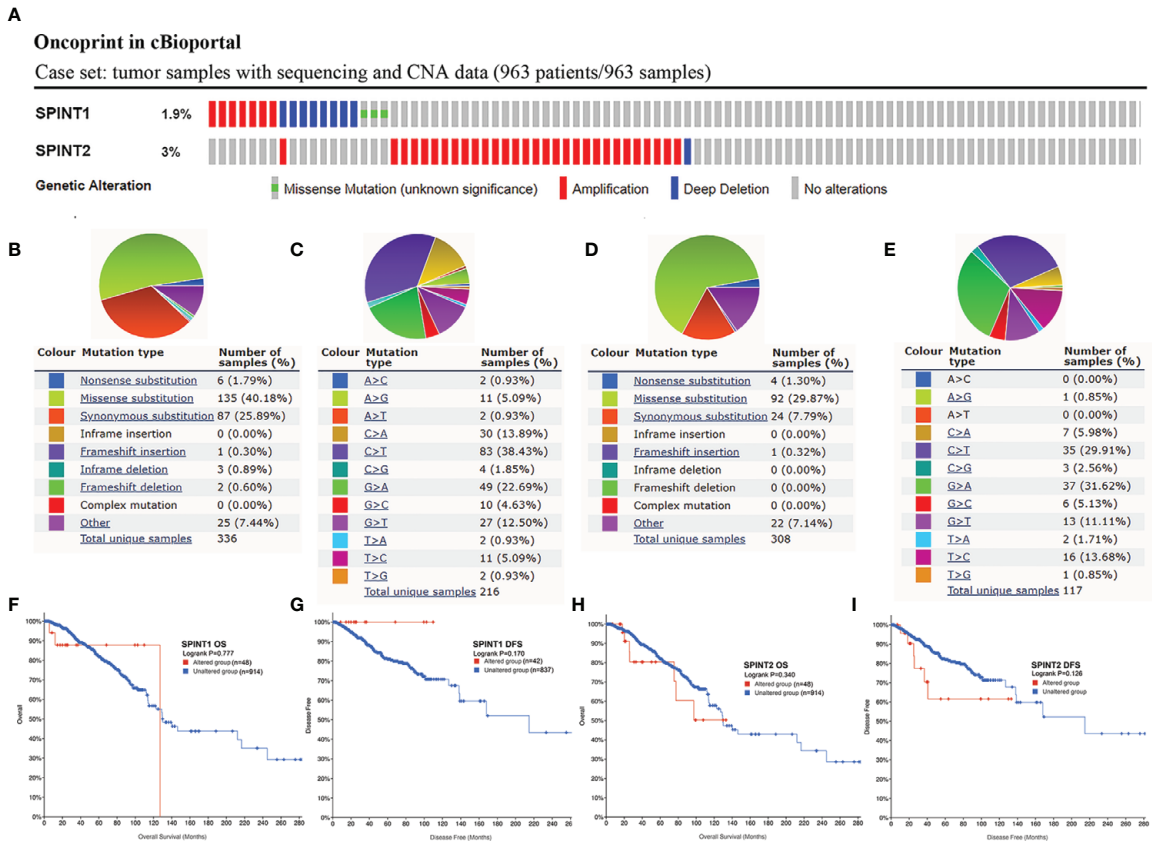

FIGURE 5 | Genetic alteration frequency of SPINT1/2 was low and not related to prognosis. Genetic mutations in SPINT1/2 **(A)**, mutation subtypes distribution of SPINT1 **(B)**, nucleotide changes in SPINT1 **(C)**. Alteration subtypes of SPINT2 **(D)**, nucleotide changes of SPINT2 **(E)**. The correlation between SPINT1 gene alteration and OS **(F)** and DFS (**G**). The correlation between SPINT2 gene alteration and OS **(H)** and DFS **(I)**.

poorer OS (**Figures 6C–E**). Of the 12 methylation sites in SPINT2, only two hypomethylated sites (TSS1500-Island-cg10154122 and TS1500-N_Shore-cg22522066) were associated with poorer OS (**Figures 6F, G**).

Identification and Enrichment Analysis of SPINT1/2 Co-Expressed Genes

The Linkedomics database was used to identify co-expressed genes of SPINT1 and SPINT2 in TCGA. The results showed that 3,499 genes (red dots) were positively and 6,420 genes (green dots) were negatively correlated with SPINT1 in breast cancer (FDR <0.01) (**Figure 7A** and **Table S2: Sheet 1**). Of note, SPINT2 (red rectangle) was among the top 50 positively correlated SPINT1 genes (**Figure 7B**). The top 50 negatively correlated genes of SPINT1 are shown in **Figure 7C**. Meanwhile, 4,411 genes (red dots) were positively, and 6,545 genes (green dots) were negatively associated with SPINT2 in breast cancer (FDR <0.01) (**Figure 7D** and **Table S2: Sheet 2**). SPINT1 was in the top 50 positively correlated genes of SPINT2 (**Figure 7E**), and the top 50 negatively correlated genes are shown in **Figure 7F**.

We investigated the functions of SPINT1/2 co-expressed genes using the LinkedOmics LinkInterpreter module. GSEA was applied for GO BP and KEGG enrichment of the respective correlated genes of SPINT1 and SPINT2. The results showed that the SPINT1 co-expressed genes were involved in mitochondrial gene expression, mitochondrial transport, precursor metabolites, and energy generation of precursor metabolites and energy. Moreover, BP terms, such as positive regulation of cell adhesion and immune responses were negatively enriched (**Figure 7G**). KEGG pathway enrichment revealed that the co-expressed genes were involved in the ribosome, proteasome, endoplasmic reticulum protein processing, and metabolic signaling (**Figure 7H**). Meanwhile, the SPINT2 co-expressed genes were primarily involved in mitochondrial gene expression, the generation of precursor metabolites and energy, protein targeting, and protein folding (**Figure 7I**). Notably, biological processes, such as cell-substrate adhesion and T-cell activation are inversely regulated. KEGG pathway enrichment indicated that the co-expressed genes of SPINT2 played a role in signaling the ribosome, proteasome, metabolic pathways, and protein processing in the endoplasmic reticulum (**Figure 7J**).

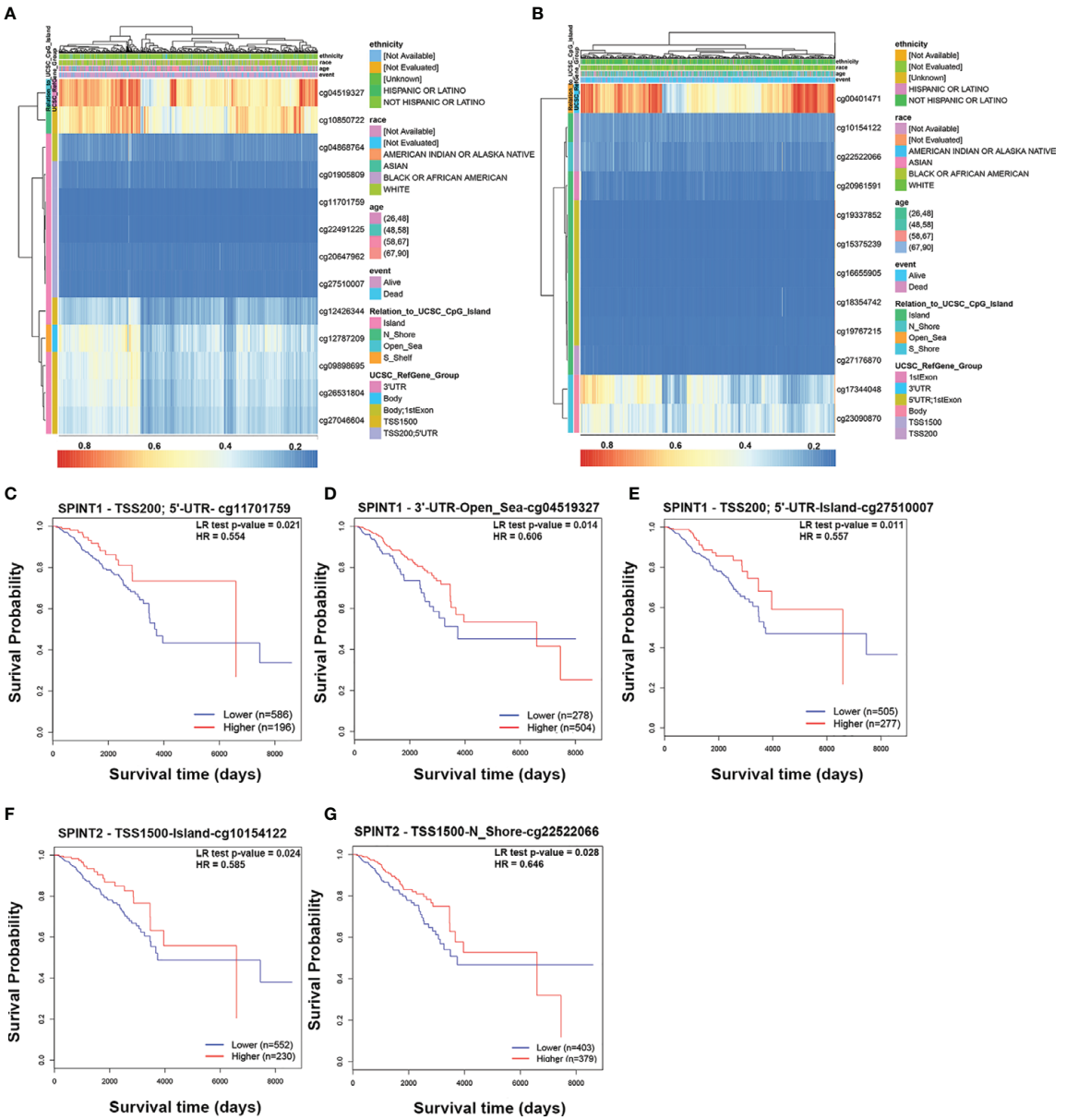

FIGURE 6 | Methylation of SPINT1/2 was correlated with prognosis in breast cancer from MethSurv. Heatmaps of the association between methylation level of SPINT1 **(A)** and SPINT2 **(B)** and patient characteristics and genomic subregions. Association of methylation at TSS200; 5'UTR-cg11701759 **(C)**, 3'UTR-Open_Sea-cg04519327 **(D)** and TSS200; 5'UTR-Island-cg27510007 **(E)** in SPINT1 with patient OS. Correlation between methylation at TSS1500-Island-cg10154122 **(F)** and TSS1500-N_Shore-cg22522066 **(G)** in SPINT2 and OS.

Enrichment Analysis and PPI Network of the Common Co-Expressed Genes

The Linkedomics results showed that SPINT1 and SPINT2 were reciprocally correlated in breast cancer (Pearson correlation = 0.437, P = 2.103e−52) (**Figure S1G**), which was close to the correlation statistics from cBioportal (TCGA, Firehose Legacy) (Pearson correlation = 0.42, P = 4.68e−83) (**Figure S1H**). To further investigate the roles SPINT1 and SPINT2 jointly played in breast cancer, the common co-expressed genes were screened first by cross-referencing the respective top 500 correlated genes of SPINT1 and SPINT2 (ranking by the absolute value of Pearson correlation) (**Table S3: Sheet 1, Sheet 2**), and a total of 201 common co-expressed genes were obtained (**Figure 8A** and **Table S4**).

We further constructed the PPI network of the 201 common co-expressed genes using the String database. The results were

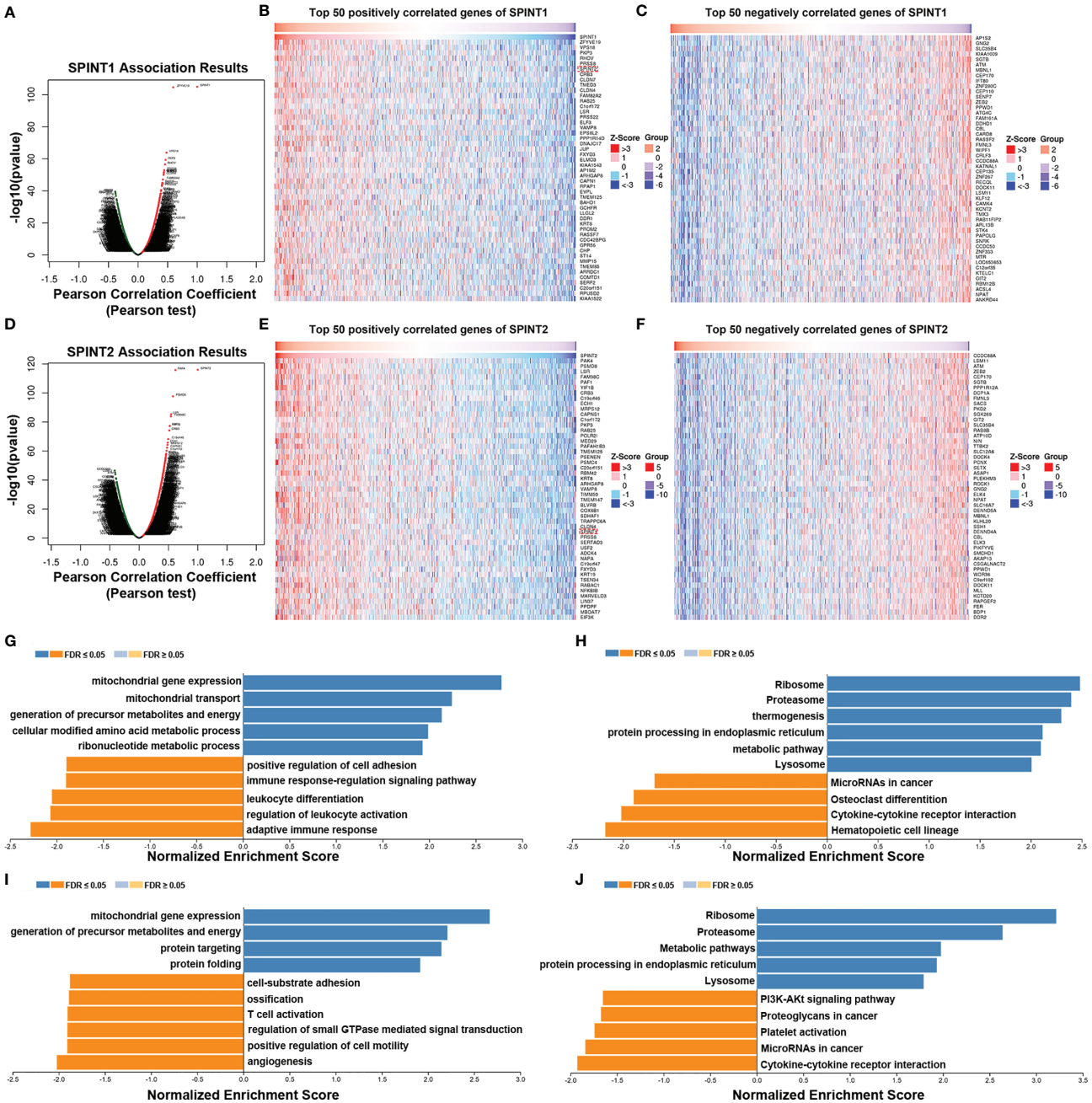

FIGURE 7 | Screening and enrichment analysis of SPINT1/2 co-expressed genes by Linkedomics. Volcano plot of SPINT1 co-expressed genes **(A)**. Top 50 positively **(B)** and top 50 negatively **(C)** correlated genes of SPINT1. Volcano plot of SPINT2 co-expressed genes **(D)**. Top 50 positively **(E)** and top 50 negatively **(F)** correlated genes of SPINT2. Biological process **(G)** and KEGG pathway **(H)** enrichment of the SPINT1 co-expressed genes. Biological process **(I)** and KEGG pathway **(J)** enrichment of the SPINT2 co-expressed genes.

then imported to Cytoscape and refined after removing disconnected nodes. Using the MCODE plugin, the top eight most important modules, including PKP3, KRT8, KRT18, KRT19, CLDN3, CLDN4, CLDN7, and EPCAM (amber circles) were screened (**Figure 8B**). We filtered out the top 10 hub genes in the PPI network based on the MCC scores with the CytoHubba plugin, they were CLDN7, CLDN3, CLDN4, EPCAM, PKP3, KRT19, KRT8, KRT18, PRSS8, and RAB25 (**Figure 8C** and **Table S5**).

Subsequently, we investigated the biological clustering of the common co-expressed genes of SPINT1/2 using ClueGO in Cytoscape for BP and KEGG enrichment. ClueGO

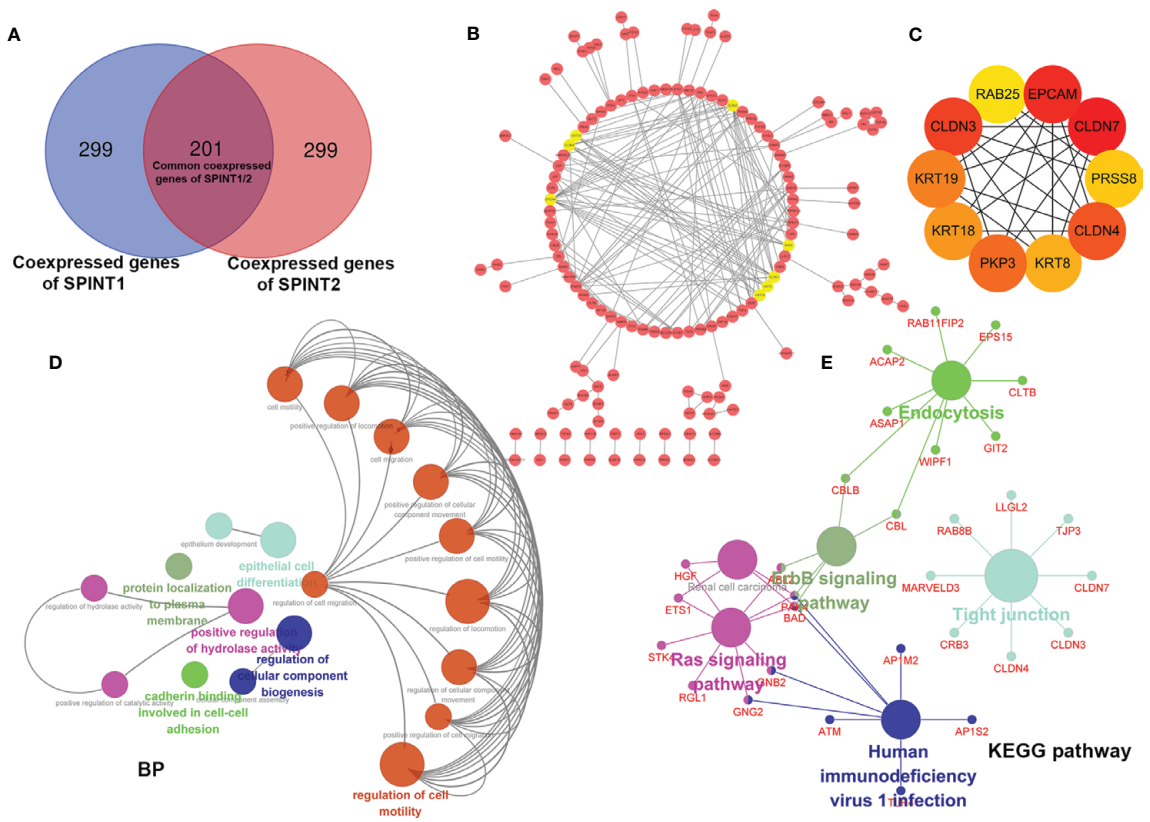

FIGURE 8 | Enrichment analysis and PPI network construction of SPINT1/2 common co-expressed genes with Cytoscape. Cross-reference of respective top 500 co-expressed genes of SPINT1/2 **(A)**, PPI network of SPINT1/2 common co-expressed genes **(B)** and hub genes **(C)** in the PPI network. BP terms **(D)** and KEGG pathways **(E)** enriched of SPINT1/2 common co-expressed genes.

incorporates GO terms and KEGG pathways and creates functionally organized GO/pathway term networks (27). In this analysis, medium network specificity and yFiles Radial Layout were applied, and other parameters were default. The results showed that 19 terms (circles in various colors) including regulation of cell migration, cadherin binding involved in cell–cell adhesion, and regulation of cellular component biogenesis were significantly enriched (P <0.05) (**Figure 8D**). KEGG pathway enrichment indicated that these common co-expressed genes were involved in tight junction, Ras signaling pathway, erbB signaling pathway, endocytosis, and human immunodeficiency virus 1 infection (**Figure 8E**). These results suggest that SPINT1 and SPINT2 may be jointly responsible for cell adhesion in breast cancer.

Expression and Prognostic Significance of Hub Genes

We examined the differential expression of the hub genes in breast cancer patients from TCGA cohorts. The results showed that all 10 hub genes were overexpressed in cancer tissues (**Figures 9A, B**). We further analyzed the prognostic significance of the hub genes in breast cancer patients using Kaplan–Meier plotter and found that the upregulated expressions of CLDN7, CLDN3, EPCAM, KRT8, KRT18, RAB25, and KRT19 were significantly correlated with DMFS in breast cancer (**Figures 9C–I**), but PRSS8, PKP3, and CLDN4 expression was not (**Figures 9J–L**).

Additionally, we investigated the correlation between the expression of 10 hug genes and the OS in breast cancer. The results showed that higher levels of CLDN4, CLDN7, CLDN3, EPCAM, PKP3, KRT8, and RAB25 were correlated with the poorer OS (**Figures S2A–G**). However, this correlation was not found for KRT18, KRT19, and PRSS8 (**Figures S2H–J**).

DISCUSSION

Although most studies involving SPINT1 or SPINT2 reported reduced expression in cancers (28–32), we observed a paradoxical upregulated expression of SPINT1/2 in breast cancer. Due to the high heterogeneity, the histopathological characteristics and clinical manifestations of breast cancer are subtype-dependent. Accordingly, we discovered that SPINT1/2 were significantly related to HER2 status and node status, but not to ER or PR status. Specifically, patients with HER2+ and node involvement had a relatively higher expression of SPINT1/2 than node-free patients.

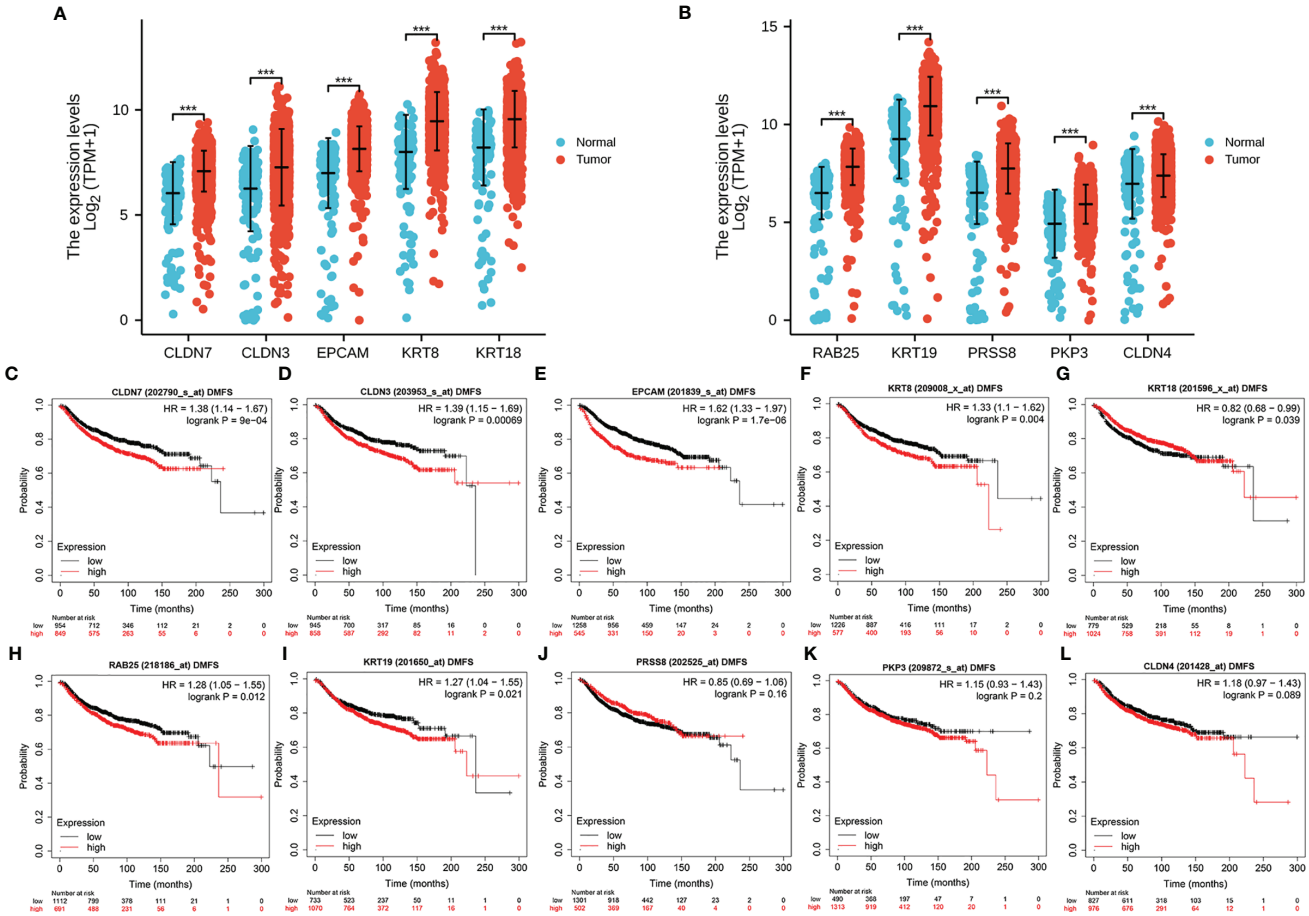

FIGURE 9 | Expression and prognostic significance of the hub genes in TCGA. Differential expression of the 10 hub genes **(A, B)** in breast cancer. The correlation between patient DMFS and the expression of CLDN7 **(C)**, CLDN3 **(D)**, EPCAM **(E)**, KRT8 **(F)**, KRT18 **(G)**, RAB25 **(H)**, KRT19 **(I)**, PRSS8 **(J)**, PKP3 **(K)** and CLDN4 **(L)**. ***$P < .001$.

We found that SPINT1/2 expression was significantly correlated with prognosis in breast cancer. SPINT1 was correlated with OS, RFS, and DMFS in patients with different molecular subtypes, except for luminal A. The highest HR was observed in the correlation between SPINT1 expression and DMFS in HER2+ patients. HER2, a transmembrane tyrosine kinase receptor, is considered a strong predictive biomarker of regional and distant metastasis, leading to an increased malignancy and poor prognosis (33, 34). These results suggest that SPINT1 may influence patient clinical outcomes by facilitating tumor dissemination. Targeting SPINT1 may be a promising strategy for breast cancer patients, particularly for the HER2+ subgroup.

Besides, SPINT2 upregulation was correlated with unfavorable OS and RFS in breast cancer. It was, however, not related to the DMFS. However, in the subgroup analysis, SPINT2 was correlated with poorer DMFS in particular types, specifically in patients with luminal A, luminal B, and HER2–, which implied that SPINT2 was possibly involved in metastasis in particular subtypes, thus rendering adverse outcomes in breast cancer patients.

Although genetic alterations were observed in SPINT1/2, they were less frequent and did not affect patient prognosis. SPINT2 has been reported to be hypermethylated in many other cancers (28, 35). However, we found decreased DNA methylation of SPINT2 in breast cancer. Additionally, SPINT1 methylation is decreased in breast cancer, representing the same status as in hepatocellular carcinoma (36). Moreover, several hypomethylated sites in the SPINT1/2 genes correlated with patient prognosis have also been identified, representing ideal aberrantly demethylated sites of SPINT1/2 in breast cancer.

To further investigate the independent and combined functions of SPINT1 and SPINT2, we screened and conducted enrichment analysis of their respective co-expressed genes and common co-expressed genes. We discovered that SPINT1 and SPINT2 have different roles and overlapping functions in breast cancer biology. In our study, some BP and KEGG pathways modulated by SPINT1 or SPINT2 were not enriched by the other. Previous studies also identified that their functions in matriptase trafficking were cell-type dependent (37–40), and their immunoreactivity locations were different (7, 41). Meanwhile, SPINT1 and SPINT2 are well-

documented upstream HGF precursors regulators and modulate epithelial integrity (42, 43). Their common co-expressed genes were jointly involved in regulating cell migration, cadherin binding in cell–cell adhesion, and cellular component biogenesis regulation. Moreover, in the KEGG enrichment analysis, two metastasis-related pathways, Ras signaling and erbB signaling were significantly enriched. Ras signaling is a crucial determinant of breast cancer distant dissemination and positively correlated with HER2+ subtypes (44, 45). erbB signaling is widely involved in regulating breast cancer cell proliferation, epithelial-to-mesenchymal transition, metastasis, and drug resistance (46–48). More studies are needed to clarify the detailed direct relationship between SPINT1/2 and these pathways in the future.

SPINT1 and SPINT2 expression were reciprocally correlated in breast cancer, which was in accordance with reports that they were frequently co-expressed in the same cell (39, 41). By cross-referencing, 201 out of the top 500 co-expressed genes of SPINT1/2 were significantly correlated with both SPINT1 and SPINT2. We screened out eight core nodes and 10 hub genes in the PPI network of the common co-expressed genes, and the results showed that the core nodes and hub genes were primarily overlapped. CLDN7, CLDN3, and CLDN4, members of the claudin family, are integral membrane proteins of tight junctions (49). Dysregulation of the claudin family proteins plays an oncogenic role in some malignancies (50, 51). EPCAM is known for its role in preventing cell-cell adhesion, cell signaling, migration, proliferation, and differentiation (52). PKP3 is a member of the armadillo protein family, which plays a central role in tumorigenesis by regulating cell adhesion (53). Moreover, keratin families, such as KRT8, KRT18, and KRT19, were also screened. It is widely reported that the keratin family regulates intermediate filaments, which trigger cancer progression and metastasis (54, 55). All these results indicated that SPINT1 and SPINT2 jointly regulated cell attachment and metastasis in breast cancer.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was performed in line with the principles of the Declaration of Helsinki. The studies involving human participants were reviewed and approved by the Ethics Committee of Chongqing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and JLu conceived the project and reviewed the manuscript. QW and GY participated data analysis and wrote the manuscript. YZ, TA, JT, YJ, and JLe participated in discussion. 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/fendo.2021.665666/full#supplementary-material

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CASE REPORT
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Peritoneal Metastasis After Treated With Abemaciclib Plus Fulvestrant for Metastatic Invasive Lobular Breast Cancer: A Case Report and Review of the Literature

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Peritoneal metastases from invasive lobular carcinoma (ILC) of breast are uncommon and usually related to poor prognosis due to difficulty of detection in clinical practice and drug resistance. Therefore, recognizing the entities of peritoneal metastases of ILC and the potential mechanism of drug resistance is of great significance for early detection and providing accurate management. We herein report a case of a 60-year-old female who presented with nausea and vomiting as the first manifestation after treated with abemaciclib (a CDK4/6 inhibitor) plus fulvestrant for 23 months due to bone metastasis of ILC. Exploratory laparotomy found multiple nodules in the peritoneum and omentum, and immunohistochemistry confirmed that the peritoneal metastatic lesions were consistent with ILC. Palliative therapy was initiated, but the patient died two months later due to disease progression with malignant ascites. Whole exome sequencing (WES) was used to detect the tumor samples and showed the peritoneal metastatic lesions had acquired ESR1 and PI3KCA mutations, potentially explaining the mechanism of endocrine therapy resistance. We argue that early diagnosis of peritoneal metastasis from breast cancer is crucial for prompt and adequate treatment and WES might be an effective supplementary technique for detection of potential gene mutations and providing accurate treatment for metastatic breast cancer patients.

Keywords: breast, lobular carcinoma, neoplasm metastasis, peritoneum, whole exome sequencing

INTRODUCTION

Invasive breast cancer is a histologically diverse disease that has several defined histological subtypes. Invasive breast carcinoma of no special type (IBC-NST), which presents in 70%-75% of the cases, is the most common histologic subtype of breast cancer, followed by invasive lobular carcinoma (ILC), which accounts for only 5%-15% of invasive mammary carcinomas (1, 2). ILC was

more likely estrogen receptor positive, HER-2 negative and had a lower proliferative index compared to IBC-NST (3).

ILC, with the hallmark loss of E-cadherin expression, is characterized by its infiltrating growth behavior, which invades the surrounding tissue with a single-file pattern at histologic examination (3, 4). Compared with IBC-NST, ILC displays a predilection for distant metastasis to uncommon sites such as gastrointestinal (GI) tract, peritoneum and genitourinary system (5, 6), and has slightly worse prognosis (7). Peritoneal metastases of breast cancer are challenging for clinicians to diagnose promptly, and recognition of the entities is of great significance for early detection and providing accurate management. As for HR-positive metastatic breast cancer (MBC), hormonal therapy represents the backbone of treatment. Recently, a new class of molecular drug, cyclin-dependent kinase 4/6 (CDK4/6) inhibitors, has been proved to improve efficacy of the first- or second-line treatment of HR positive, HER2-negative MBC (8–13). Herein, we present a rare case of metastatic ILC with peritoneal metastases causing bowel obstruction during the treatment with abemaciclib (a CDK4/6 inhibitor) plus fulvestrant.

CASE PRESENTATION

A 60-year-old female with no family history of cancer underwent left mastectomy with axillary lymph node dissection in November 2012 for stage IIIC (cT2N3M0) invasive lobular carcinoma. Histopathological examination demonstrated an invasive lobular carcinoma with positive estrogen receptor (ER+) and progesterone receptor (PgR+), negative human epidermal growth factor receptor 2 (HER2-), lymphovascular invasion and metastases to axillary lymph nodes (11/21). The pathological stage was pT2N3M0. She completed 4 cycles of epirubicin and cyclophosphamide (EC) followed by 4 cycles of paclitaxel. Then the patient underwent adjuvant radiotherapy; specifically, the left chest wall, infraclavicular and supraclavicular region, and internal mammary nodes were irradiated at a dose of 50.4 Gy in 5 weeks with a 1.8 Gy daily fraction. Meanwhile, she received once-daily regimen of letrozole 2.5 mg regularly. Follow-up was arranged every 3 months for 2 years in the breast clinic and there was no distinct evidence of recurrence. Then, the patient was followed up every 6 months in the next 3 years.

In December 2017 (5 years after surgery), bone scan detected solitary bone metastasis in the left ischium. She received intravenous zoledronic acid injections every month. She was subjected to abemaciclib plus fulvestrant with stable disease until her current presentation.

In November 2019, the patient complained of nausea. A contrasted abdominal Computed Tomography (CT) showed no distinct abnormalities. The patient took some prescribed medication and felt better thereafter. However, vomiting after meals occurred in December 2019. The patient came to our hospital for further treatment. She presented with jaundice and mild tenderness in the upper abdomen on admission. Blood chemistry tests showed elevated bilirubin and liver enzymes. The laboratory workup showed CA153 186.8U/ml, CA199 203.5U/ml, CA125 66.27U/ml, and CEA 13.2U/ml. An upper gastrointestinal X-ray (**Figure 1A**)

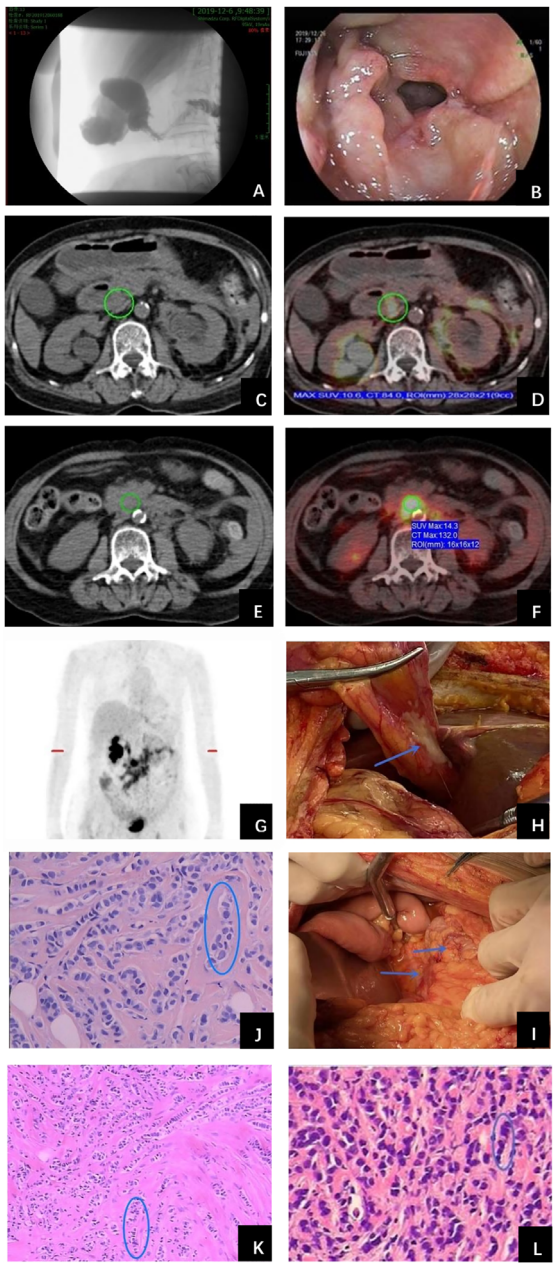

FIGURE 1 | Examinations and histopathological results during work-up of the patient. **(A)** Upper gastrointestinal X-ray showed a stricture in the second portion of the duodenum. **(B)** Upper gastrointestinal endoscopy detected a stricture with circumferential edematous friable mucosa, extending from the duodenal bulb to the second portion of the duodenum. **(C)** PET/CT revealed duodenal wall was thickened and identified as metabolically active lesions (SUVmax=10.6) **(D)**. **(E)** Thickened peritoneum and mesenteries and slightly larger lymph nodes in the mesenteries were found with intense FDG uptake (SUVmax=14.3) **(F)**. **(G)** Holistic view of PET/CT: metabolic lesions in the duodenum, peritoneum and mesenteries. Exploratory laparotomy showed three metastatic nodules in the peritoneal cavity, including one nodule on the ligamentum teres hepatis **(H)** and the other two on the omentum **(I)** (arrows). Histopathological examination of primary breast cancer **(J)**, metastatic axillary lymph node **(K)** and metastatic peritoneal nodule **(L)** all revealed single-file strands of infiltrating small tumor cells dispersed in the fibrous matrix (circle).

and upper gastrointestinal endoscopy (**Figure 1B**) showed a stricture in the horizontal part of duodenum which had poor distension. A biopsy obtained from the duodenum did not detect any malignant cell. Positron Emission Tomography/Computed Tomography (PET/CT) showed thickening of duodenal wall, peritoneum and mesenteries, slightly larger lymph nodes in the mesenteric area, and varying degrees of increase in glucose metabolism (**Figures 1C–G**); combined with perirenal, duodenal and bladder lesions, peritonitis was highly suspected, while tuberculous peritonitis was supposed to be excluded. Thereafter, a decision was made to perform an exploratory laparotomy. In the operation, approximately 1 liter of yellow-brown ascitic fluid was drained and three nodules were seen in the peritoneal cavity, including one nodule on the ligamentum teres hepatis (**Figure 1H**) and the other two on the omentum (**Figure 1I**). Similar to the primary breast cancer (**Figure 1J**) and metastatic axially lymph node (**Figure 1K**), histological examination of the peritoneal nodule showed single-file strands of infiltrating tumor cells throughout the fibrous matrix, which were consistent with ILC (**Figure 1L**). The immunohistochemical (IHC) studies revealed the tumor cells were highly positive for gross cystic disease fluid protein-15 (GCDFP-15), Cytokeratin 7 (CK7), GATA-3 and ER, but negative for E-cadherin, PR and HER2 status; and the Ki67 index was 20%. The results of IHC staining were consistent with a diagnosis of peritoneal metastases from ILC. During the process of diagnosis, the patient manifested with severer nausea and vomiting and even abdominal distention, and she was received parenteral nutrition instead of oral feeding. Due to the poor condition of the patient, aggressive treatment such as chemotherapy was not considered for her, and finally palliative therapy was initiated. Unfortunately, the patient died two months later due to disease progression with malignant ascites (**Figure 2**).

In order to know the patient's genetic information and investigate the possible mechanisms of resistance to endocrine therapy (ET), we utilized whole exome sequencing (WES) to detect the tumor samples from primary lesion, regional lymph nodes and peritoneal metastatic lesions. The 3-way Venn Diagram showed that 47 common mutations were detected among primary lesion, lymph nodes and peritoneal metastatic lesions (**Figure 3A**); combined with somatic mutation heatmap (**Figure 3B**) and variation frequency (VAF) distribution (**Figure 3C**), it was implied that the three tumor samples may have the same origin. Mutation analysis of signal transduction enrichment showed that the PI3K-AKT signaling pathway was significantly enriched in the peritoneal metastatic lesions. All three tumor samples carried PIK3CA p.H1047R, which is one of the most common mutation of PIK3CA in breast cancer. What' more, the sample of metastatic lesion was found to have acquired PIK3CA p.D959N (**Figure 4A**) and ESR1 p.E380Q mutation (**Figure 4B**), which were not detected in neither primary lesion nor lymph node.

DISCUSSION

The patient in this case was found to have peritoneal metastasis from ILC after diagnostic work-up for the presence of nausea and vomiting. Peritoneal carcinomatosis secondary to breast cancer has been reported in literatures (**Table 1**) (14–21), and some studies showed nearly 3% of ILC patients had peritoneal metastasis, which was higher than those of IBC-NST patients (22, 23).

Clinical manifestations of peritoneal metastasis from ILC are variable and non-specific. Patients usually do not have any symptoms until later, even several days before death. Metastasis to the peritoneum or retroperitoneum leads to thickening and sclerosis of the surrounding tissues. A common finding of peritoneum metastasis from breast cancer is stenosis, frequently with presentation of abdominal pain, early satiety and obstructive symptoms. These patients might be misdiagnosed with a primary GI tumor or even not diagnosed with malignancy at all (24, 25). A case series of 12, 001 patients found 11% of the patients were not diagnosed with GI metastasis from breast carcinoma until an exploratory laparotomy was performed, as was the patient in our case (6). As the clinical presentation of peritoneal metastases is usually non-specific, histopathological and immunohistochemical examinations are the definitive diagnostic methods. Microscopically, single-file strands of infiltrating tumor cells invading the surrounding tissue can frequently be seen in metastases from ILC as observed in our

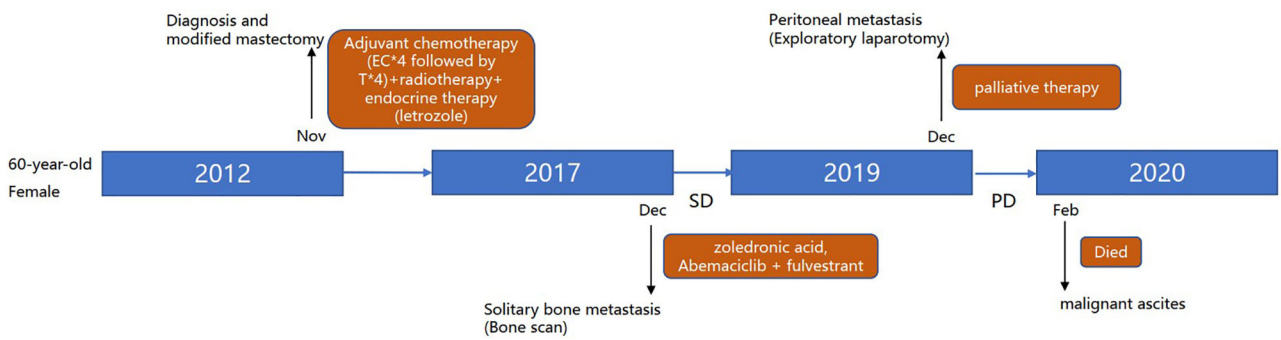

FIGURE 2 | Review of treatment process of the patient (from November 2012 to February 2020).

FIGURE 3 | Whole exome sequencing (WES) of tumor samples from primary lesion, regional lymph nodes and peritoneal metastatic lesions. **(A)** 3-way Venn Diagram showed the mutational overlaps in the three samples. There were 47 common mutations in the three samples, while another 21 common mutations between lymph node and metastatic site, and another 54 common mutations between lymph node and primary site. **(B)** Somatic mutation heatmap. The mark "*" means that there are 2 or more mutations in the same gene, which was labelled with gene or amino acid changes. Yellow means there is variation, while blue means there is no variation. **(C)** Variation frequency (VAF) distribution. The mark "*" means that there are 2 or more mutations in the same gene, which was labelled with gene or amino acid changes.

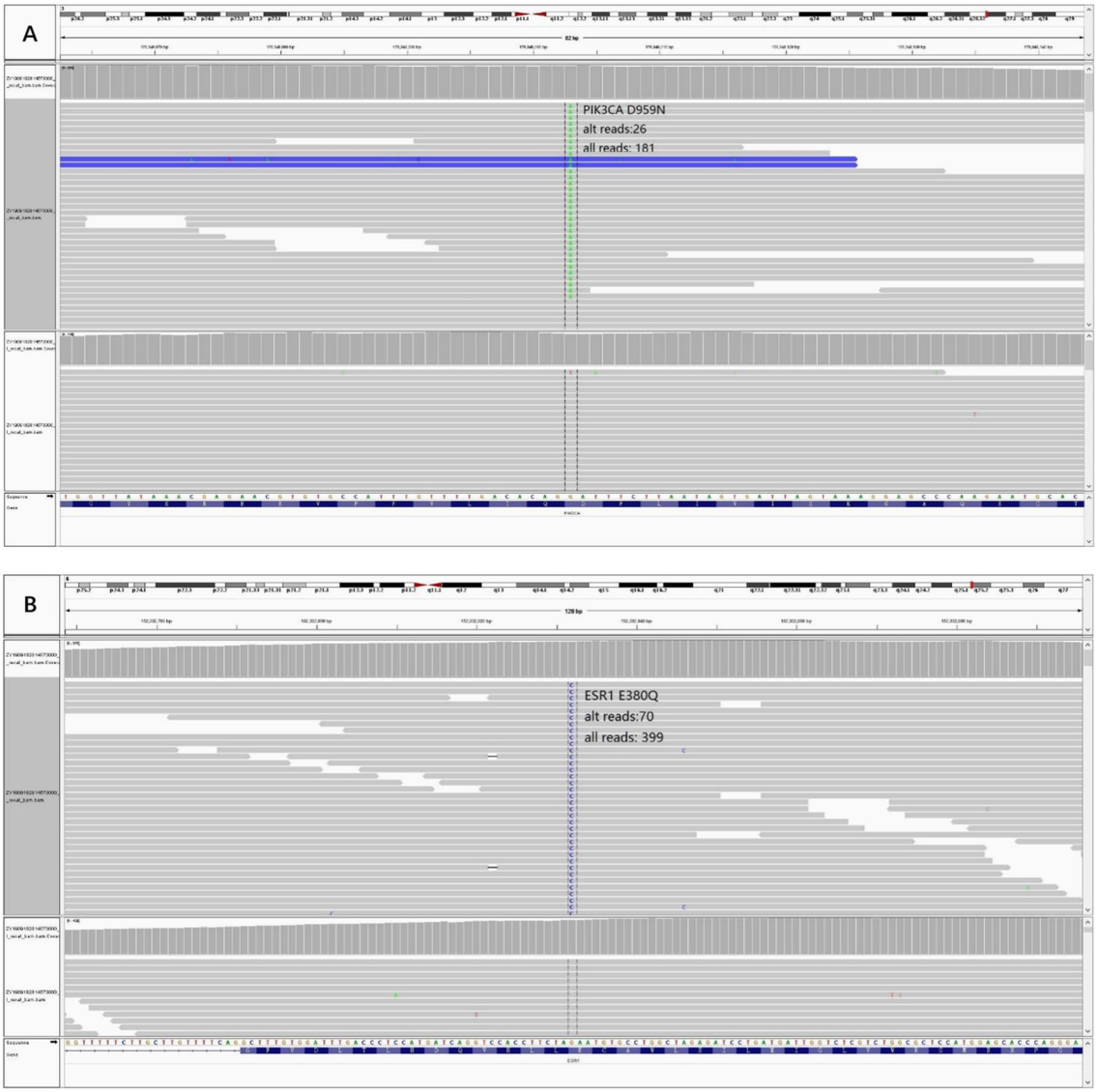

FIGURE 4 | Acquired mutations were detected in the sample of peritoneal metastatic lesion and visualized through Integrative Genomics Viewer (IGV). **(A)** Variant PIK3CA p.D959N IGV plot (all reads: 181, alternative allele supported reads: 26). **(B)** Variant ESR1 p.E380Q IGV plot (all reads: 399, alternative allele supported reads: 70).

study (26, 27). However, it is still quite challenging to come up with the definite diagnosis *via* histological examination because signet ring cell carcinoma can indeed arise from any tissue. Immunohistochemical markers are crucial for diagnosing metastatic lobular carcinoma of the breast. The most important markers for ILC are Cytokeratin 7 (CK7), GATA-3, gross cystic disease fluid protein-15 (GCDFP-15), ER and PR, all of which but not PR were highly positive in the biopsy specimens of peritoneal metastatic lesions in our patient (28, 29). There was also negative of HER2. Samples from distant sites often show features similar to that of primary breast cancer which is most commonly an ILC. The availability of IHC studies allowed clinicians to accurately diagnose metastatic lobular breast carcinoma (30, 31).

There is no consensus for the treatment of peritoneal carcinomatosis secondary to breast cancer, as there have not yet been any large-scale studies that compared the efficacy of different managements (30, 31). Palliative surgery is necessary in the treatment of patients with symptomatic obstruction, bleeding or perforation, even though no survival benefit may ensue (24, 30, 32). A few studies have been published describing the combination of surgical debulking and hyperthermic intraperitoneal chemotherapy (HIPEC) for patients with secondary peritoneal carcinomatosis due to breast cancer as well as other primary diseases, which showed improvement in morbidity and mortality (33, 34). A retrospective study (6), which included 73 breast cancer patients with GI or peritoneal metastasis, reported palliative surgical intervention

TABLE 1 | Review of literature: characteristics and outcomes of breast cancer patients with peritoneal metastasis.

First Authors	Age (years)	TNMstage	Molecular Type	Surgery	Adjuvant Therapies	DFS*	Clinical Characteristics of Metastasis	Metastasis Site	Diagnosis and treatment	Outcomes
J.A. Mosiun (14)	51	T2N2M0	ER(-), PR (-), and equivocal for HER2	Mastectomy and axillary dissection	3*FEC-3*taxotere; radiotherapy	2 years	Have a right iliac fossa mass on abdominal examination.	Terminal ileum wall, multiple peritoneal nodules and enlarged intraabdominal lymph nodes	Right hemicolectomy with creation of double barrel stoma; oral letrozole and intravenous zoledronic acid injections	Disease progression with malignant ascites
Yasuhiro Nihon-yanagi (15)	57	T2N1M0	ER(+), PgR (+), HER2(-)	Modified radical mastectomy with axillary and infraclavicular lymph-node dissection	8*paclitaxel; tamoxifen	17 months	Nausea, vomiting and jaundice	Duodenum, Peritoneum	Pancreatoduodenectomy	Bilateral hydronephrosis; subsequently died
R. Syed (16)	63	T2N0M0	ER(+), PgR (-)	Mastectomy with axillary node sampling	Local radiotherapy; tamoxifen	5 years	abdominal pain, distension and diarrhea	Omentum, peritoneum and pelvis	Ascitic drainage, tissue biopsy of an omental deposit	Unknown
Kobayashi T (17)	56	T4bN1M0	ER(+)	Modified radical mastectomy	Unknown	Unknown	Abdominal distension, vomiting and epigastric pain	Peritoneum and retroperitoneum	Gastrojejunostomy; cisplatin, 5-fluorouracil, doxifluridine, and TS1	Unknown
Foluso O. Ademuyiwa (18)	81	Unknown	Unknown	Mastectomy	Unknown	41 years	Fatigue, nausea, vomiting, weight loss, and a right lower quadrant abdominal mass	Left periaortic soft tissue, right intraabdominal soft tissue, falciform ligament and bilateral perinephric fat	CT-guided biopsy of the periaortic mass; weekly paclitaxel and zoledronic acid	Unknown
Osaku (19)	69	/	/	/	/	/	Constipation	Abdominal cavity, rectum and ileocecum	Exploratory laparotomy; hormone therapy and taxane- and anthracycline-based drugs	Died four years later
I. Mylonas (20)	76	Unknown	Unknown	Mastectomy and axillary lymphadenectomy	No	30 years	loss of appetite, nausea, vomiting and abdominal enlargement without weight gain	Greater epiploon, peritoneum, ileum and uterus	Biopsies of the greater epiploon	Unknown
Aurello (21)	73	T2N1M0	ER(+), PgR (+)	Mastectomy with axillary node dissection	Chemotherapy	14 years	Vomiting, epigastric pain and weight loss	Angulus, Peritoneum	Subtotal gastrectomy with D1-lymphadenectomy and stapled gastrojejununi anastomosis, chemotherapy	Free from disease until March 2004 when she revealed a peritoneal carcinosis

**DFS, disease-free survival.*

conferred no survival benefit while systemic chemotherapy or hormone therapy might have improved survival of the patients. Late presentation of signs and symptoms of peritoneal metastasis was related to poor prognosis. However, there is no enough data in the best treatment and precise prognosis for those patients due to the limited number of case reports. Treatment should be tailored to the patient and their projected performance status along with quality-of-life consideration (35, 36).

In this case, ILC metastasized to the peritoneum and omentum in association with spread of many small nodules. It remains unclear concerning the mechanism of these metastatic patterns. Previous study (37) revealed that most of ILC lack cohesiveness because the E-cadherin was inactivated, which was a cell-to-cell adhesion protein. WES showed the patient had gene mutations in ESR1 and PIK3CA at the metastatic lesions, which was thought to be acquired due to chronic exposure of CDK4/6 inhibitor plus ET (38). As the most common mechanism of resistance to ET in MBC, acquired ESR1 mutations may have been existing in primary tumors and become enriched only when metastasis occurs (39). By enhancing coactivator recruitment, ESR1 mutations with altered structure conferred distinct mechanism of resistance to ER antagonists such as tamoxifen (40, 41). Previous studies demonstrated that MBC patients with ESR1 mutation are resistant to standard ET and have worse overall survival (42, 43), as seen in our patient. Currently, the best treatment for MBC patients with ESR1 mutations is fulvestrant combined with CDK4/6 inhibitor, which conferred significantly improved PFS in patients with ESR1 mutations (39). In this case, the patient was treated with abemaciclib plus fulvestrant after solitary bone metastasis, and the PFS was 23 months. Besides to ESR1 mutation, the PIK3CA mutation was also found in the metastatic lesions. Some studies (44, 45) demonstrated that the PI3K/mTOR pathway was upregulated in response to long-term use of CDK4/6 inhibitor, which drove cell cycle progression *via* upregulating cyclin D. Therefore, PIK3CA mutation and the subsequently activated PI3K-AKT signaling pathway might mediate resistance to CDK4/6 inhibitor for this patient. The SOLAR-1 trial (46) showed patients with PIK3CA mutation had double PFS after receiving PIK3CA inhibitor alpelisib plus fulvestrant compared with those receiving fulvestrant plus placebo (11.0 months and 5.7 months, respectively). In the subgroup of patients who had been treated with CDK4/6 inhibitors previously, receiving alpelisib reduced 52% risk in PFS compared with placebo (47). Hence, PIK3CA inhibitors may be used to overcome resistance to CDK4/6 inhibitor for MBC patients. However, PIK3CA inhibitors including alpelisib are not available in the mainland of China up to now. Meanwhile, ILC patients with peritoneal metastasis usually progress quickly and are easily misdiagnosed, which makes it difficult for these patients to acquire timely and effective treatment. Therefore, it is crucial that more efforts need to be put into early detection of ILC patients with peritoneal metastasis and availability of new drugs like PIK3CA inhibitors.

By using WES to detect the tumor samples, it is available for us to get access to the genomic information of this patient and investigate the possible mechanism of endocrine therapy resistance. WES showed the peritoneal metastatic lesions had acquired ESR1 and PI3KCA mutations, potentially explaining the mechanism of endocrine therapy resistance. Therefore, we argue that early diagnosis of peritoneal metastasis from breast cancer is crucial for prompt and adequate treatment and WES might be an effective supplementary technique for detection of potential gene mutations and providing accurate treatment for metastatic breast cancer patients.

CONCLUSION

All clinicians should realize that there is an unusual pattern of peritoneal metastasis from ILC. For patients with vomiting and previous history of ILC, it is necessary to highly suspect peritoneum metastasis. Early diagnosis is vital in ensuring prompt and adequate treatment. Our results suggest that ESR1 and PIK3CA mutations are acquired resistance mechanism of CDK4/6 inhibitor plus endocrine therapy and WES might be an effective supplementary technique for detection of potential gene mutations for MBC patients with drug resistance, thus ensuring timely and accurate salvage treatment. Nevertheless, further studies need to be conducted to investigate the mechanism and predictive factors of peritoneal metastasis of ILC.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

H-FG and J-SZ was mainly responsible for the article writing. Q-ZZ, and FJ was mainly responsible for the gene analysis. KW and GN were in charge of all study procedures. TZ, C-QY, L-LZ and MY were responsible for patient's clinical data and analysis. J-QL and M-YC were responsible for consent from the patient and ethics committee. All authors gave final approval of the manuscript to be submitted and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.659537/full#supplementary-material

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Impact of Different Modules of 21-Gene Assay in Early Breast Cancer Patients

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Background: The 21-gene assay recurrence score (RS) provides additional information on recurrence risk of breast cancer patients and prediction of chemotherapy benefit. Previous studies that examined the contribution of the individual genes and gene modules of RS were conducted mostly in postmenopausal patients. We aimed to evaluate the gene modules of RS in patients of different ages.

Methods: A total of 1,078 estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative breast cancer patients diagnosed between January 2009 and March 2017 from Shanghai Jiao Tong University Breast Cancer Data Base were included. All patients were divided into three subgroups: Group A, ≤40 years and premenopausal (n = 97); Group B, >40 years and premenopausal (n = 284); Group C, postmenopausal (n = 697). The estrogen, proliferation, invasion, and HER2 module scores from RS were used to characterize the respective molecular features. Spearman correlation and analysis of the variance tests were conducted for RS and its constituent modules.

Results: In patients >40 years, RS had a strong negative correlation with its estrogen module (ρ = −0.76 and −0.79 in Groups B and C) and a weak positive correlation with its invasion module (ρ = 0.29 and 0.25 in Groups B and C). The proliferation module mostly contributed to the variance in young patients (37.3%) while the ER module contributed most in old patients (54.1% and 53.4% in Groups B and C). In the genetic high-risk (RS >25) group, the proliferation module was the leading driver in all patients (ρ = 0.38, 0.53, and 0.52 in Groups A, B, and C) while the estrogen module had a weaker correlation with RS. The impact of ER module on RS was stronger in clinical low-risk patients while the effect of the proliferation module was stronger in clinical high-risk patients. The association between the RS and estrogen module was weaker among younger patients, especially in genetic low-risk patients.

Conclusions: RS was primarily driven by the estrogen module regardless of age, but the proliferation module had a stronger impact on RS in younger patients. The impact of modules varied in patients with different genetic and clinical risks.

Keywords: breast cancer, hormone receptor positive, recurrence score, 21-gene assay, adjuvant therapies

BACKGROUND

Estrogen receptor (ER) is one of the most significant biomarkers of breast cancer, and the ER-positive (ER+) subtype constitutes about 70% of invasive breast cancers (1). Endocrine therapy is essential for all ER+ breast cancer patients, while chemotherapy can improve the prognosis of only a part of this group (2). Several multi-parameter molecular profiling assays were developed to identify ER+ breast cancer patients who can benefit from chemotherapy. The 21-gene recurrence score (RS) is the most widely used assay, which concludes 16 cancer-related genes and 5 reference genes (3). Using fixed coefficients predefined by the regression analysis of gene expression and patient prognosis in the three training studies, patients can be categorized into low-, intermediate-, or high-risk groups. With the results of RS, clinicians can have a clearer understanding about individual patient prognosis and make personalized adjuvant treatment decisions.

Young breast cancer patients (≤40 years old) account for approximately 2%–6% of patient population in RS-related clinical trials (4–6). Previous studies suggested that clinicopathological features in young ER+ breast cancer patients were more aggressive when compared to those in old patients (7–9) and young patients were more likely to benefit from chemotherapy (10). Accordingly, RS was found to show different values when predicting the benefit of chemotherapy in patients of different ages. In the TAILORx trial, researches refined RS groups as low risk (<11), intermediate risk (11-25), or high risk (>25) and discovered that for the majority of patients with RS <25, endocrine therapy alone was noninferior to combined chemo-endocrine therapy. Of note, the interaction between age and RS was significant. For patients <50 years, RS 11-25 might predict some benefit derived from chemotherapy, whereas in patients ≥50 years with a RS 11–25, chemotherapy-derived benefit was absent (11).

The refined ranges of RS can provide more accurate prognosis information and allow certain groups of patients to avoid chemotherapy as well as the side effects along with it. Thus, it is important to understand the biological features as well as molecular drivers behind RS. A previous study discovered that in contrast to the weight of coefficient for calculating RS, the leading molecular driver of RS was actually the estrogen module instead of the proliferation module in the postmenopausal patients (12). However, a similar study in young women was absent. Given the predictive value of RS among different age groups, it is valuable to explore the molecular mechanisms of RS, especially in younger patients.

In this study, we aim to explore the association of RS with its modules and identify the discordance of molecular drivers in patients of different ages.

PATIENTS AND METHODS

Patients

Clinical data of a total of 1,078 unilateral ER-positive and human epidermal growth factor receptor 2 (HER2)-negative female breast cancer patients diagnosed between January 2009 and March 2017 was derived from the prospectively-maintained Shanghai Jiao Tong University Breast Cancer Data Base (SJTU-BCDB). The use of data was approved by SJTU-BCDB for clinical research. Patient information would be collected if it met all of the following criteria: (1) ER positivity with ≥1% immunoreactive tumor cell nuclei determined by immunohistochemical (IHC) staining test (13); (2) HER2 negativity defined as IHC score 0, 1+, or 2+ and/or non-amplified HER2 gene on fluorescence *in situ* hybridization (HER2/centromeric probe for chromosome 17 ratio < 2.0 with average HER2 gene copy number <6.0 signals/cell, or average HER2 gene copy number <4.0 signals/cell regardless of the ratio) (14); (3) intact 21-gene test report. Menopause was determined if: (1) prior bilateral oophorectomy; (2) age ≥60 years old; or (3) age <60 years old, amenorrheic for 12 or more months and the follicle-stimulating hormone and estradiol in the postmenopausal range.

The 21-Gene RS Assay

The 21-gene tests were performed on formalin-fixed, paraffin-embedded tissue. Hematoxylin and eosin-stained slides were deparaffinized into two 10-μm unstained sections using xylene followed by ethanol as we described in our previous study (15). RNA was extracted and purified using the RNeasy FFPE kit (QIAGEN, Hilden, Germany). Gene-specific reverse transcription was conducted using Omniscript RT kit (Qiagen, 205111, Germany). Standardized quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in 96-well plates with Applied Biosystems (Foster City, CA, USA) 7500 Real-Time PCR system. RT-PCR was carried out with the Omniscript RT kit (Qiagen, Valencia, CA, USA). Expression of each gene was measured in triplicate, and normalized relative to a set of five reference genes.

Genetic and Clinical Risk Stratification

As defined in the TAILORx trial (11), we categorized patients into genetic high-risk *versus* low-risk with a cutoff RS value of 25. In addition, patients with tumors of (1) ≤3 cm and Grade I; (2) ≤2 cm and Grade II; (2) ≤1 cm and Grade III were classified as clinical low-risk while others were considered clinical high-risk (4, 11).

Statistical Analysis

Spearman's rank correlation was performed to analyze the correlation of RS and its modules. The variance of components of RS was studied in Groups A, B, and C. p-value < 0.05 was considered to indicate a statistically significant difference. All tests were performed using R Studio version 1.2.5019 based on R version 4.0.3.

RESULTS

Baseline Characteristics

According to the 4th International Consensus Conference for Breast Cancer in Young Women (BCY4) international consensus guidelines (16) as well as patients' menopausal status, we divided patients into three subgroups: (1) Group A, ≤40 years and premenopausal; (2) Group B, >40 years and premenopausal; (3) Group C, postmenopausal. Among 1,078 cases included in this study, 9.0%, 26.3%, and 64.7% fit into Groups A, B, and C, respectively. The median age was 37 (range 27–40), 47 (range 41–56), and 63 (range 45–93), respectively, in the three subgroups. A total of 31.5% patients had luminal-A tumors (17) and the invasive ductal cancer was the most common histology type (86.4%). Approximately half of the patients had grade II tumors. When using the 8th AJCC staging, 67.9% of tumors were pT1 and 93.4% were node-negative. Among all patients, 638 (59.2%) had RS ≤25 and 440 (40.8%) had RS >25. Forty-nine percent *vs.* 50.5% of the patients had a clinical high-risk *vs.* low-risk. All patients received endocrine treatment. More than half (51.2%) of the patients received chemotherapy (72.2%, 54.2%, 47.1% in Groups A, B, and C, respectively). For premenopausal women, 37.1% of patients ≤40 years and 4.0% of patients >40 years received ovarian function suppression. The distribution of clinicopathologic features in each subgroup was summarized in **Table 1**.

Correlation Between RS and Individual Modules

We analyzed the relationship between RS and its constituent modules (**Figure 1**). For the HER2 and proliferation module, the thresholds of 8 and 6.5 were applied. For the estrogen module, it had a stronger negative correlation with RS in patients >40 years (ρ = −0.76 and −0.79 in Groups B and C) than in patients ≤40 years (ρ = −0.64 in Group A). In contrast, the positive correlation

TABLE 1 | Basic features of HR+/HER2- early breast cancer patients from SJTU-BCDC.

Characteristics	Total (%) *n* = 1,078	Premenopausal ≤40 years *n* = 97	Premenopausal >40 years *n* = 284	Postmenopausal *n* = 697
Median age	58 (24–93)	37 (27–40)	47 (41–56)	63 (45–93)
Subtype				
Luminal-A	340 (31.5)	28 (28.8)	101 (35.6)	211 (30.3)
Luminal-B(HER2-)	738 (68.5)	69 (71.2)	183 (64.4)	486 (69.7)
Pathology				
IDC	932 (86.4)	88 (90.7)	243 (85.6)	601 (86.3)
ILC	46 (4.3)	2 (2.1)	13 (4.6)	31 (4.4)
Others	100 (9.3)	7 (7.2)	28 (9.8)	65 (9.3)
Histologic grade				
1	103 (9.6)	8 (8.2)	31 (10.9)	64 (9.2)
2	559 (51.9)	51 (52.6)	161 (56.7)	398 (57.1)
3	224 (20.8)	28 (28.9)	53 (18.7)	143 (20.5)
Undifferentiated	141 (13.1)	10 (10.3)	39 (13.7)	92 (13.2)
pT				
1	732 (67.9)	64 (66.0)	212 (74.6)	456 (65.4)
2	335 (31.1)	29 (29.9)	71 (26.1)	235 (33.7)
3	11 (0.1)	4 (4.1)	1 (0.3)	6 (0.8)
pN				
0	1,007 (93.4)	94 (96.9)	278 (97.9)	635 (91.1)
1	71 (6.6)	3 (3.1)	6 (2.1)	62 (8.9)
RS score				
≤25	638 (59.2)	58 (59.8)	179 (63.0)	401 (57.5)
>25	440 (40.8)	39 (40.2)	105 (37.0)	296 (42.5)
Clinical Risk				
Low	544 (50.5)	42 (43.3)	165 (58.1)	350 (50.2)
High	534 (49.5)	55 (56.7)	119 (41.9)	347 (49.8)
Chemotherapy				
Yes	552 (51.2)	70 (72.2)	154 (54.2)	328 (47.1)
No	526 (48.8)	27 (27.8)	130 (45.8)	369 (52.9)
OFS				
Yes	47 (4.4)	36 (37.1)	11 (4.0)	0 (0)
No	1031 (95.6)	61 (62.9)	273 (96.0)	697 (100)

HR, hormone receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal cancer; ILC, invasive lobular cancer; RS, recurrence score; OFS, ovarian function suppression.

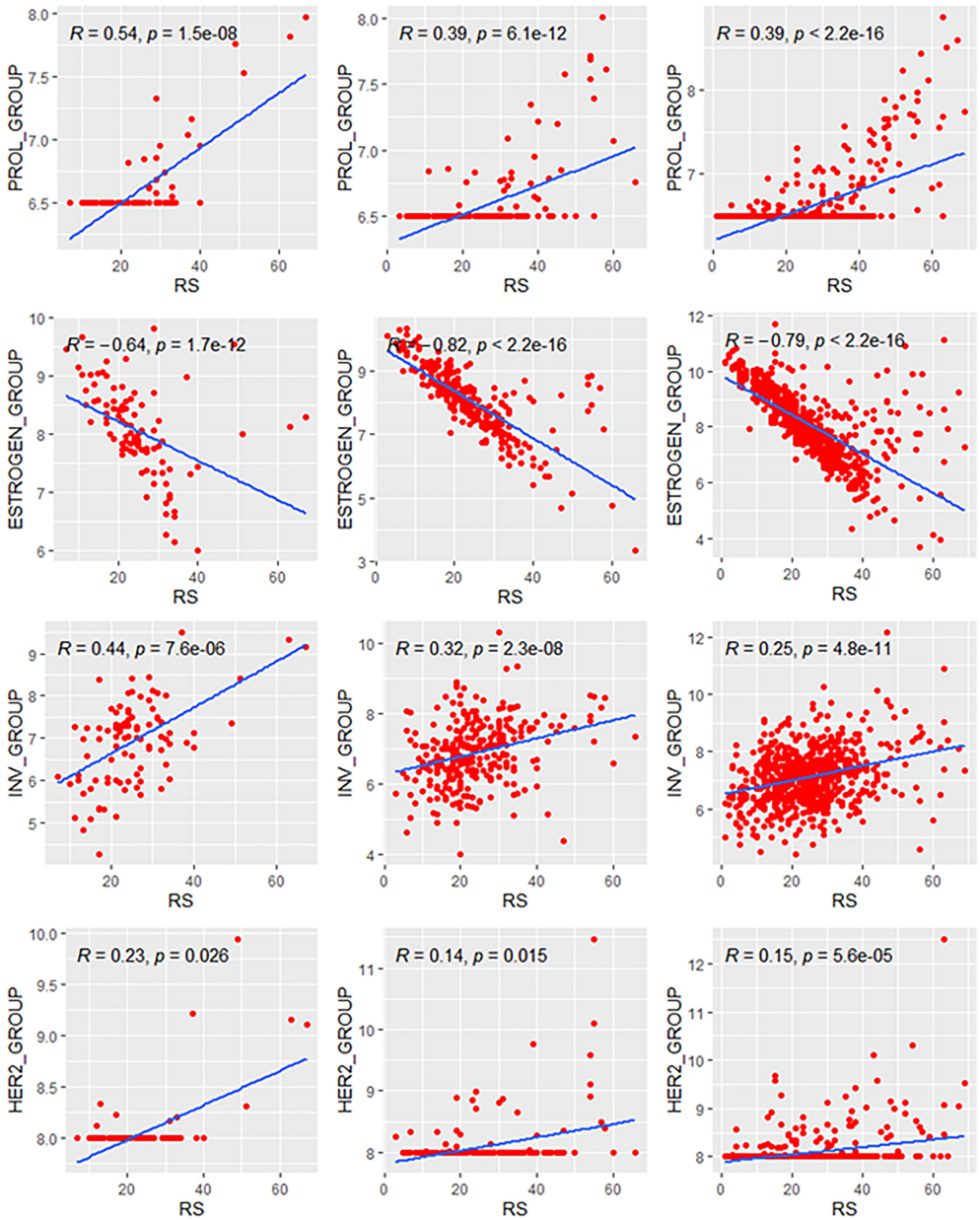

FIGURE 1 | Relationships of the RS with its proliferation module and estrogen module. Groups A, B, C were presented from left to right. RS, recurrence score.

between RS and the invasion module was weaker in patients >40 years (ρ = 0.29 and 0.25 in Groups B and C) than in patients ≤40 years (ρ = 0.44 in Group A). The coefficients of the HER2 module also showed difference between patients >40 years (ρ = 0.14 and 0.15 in Groups B and C) and patients ≤40 years (ρ = 0.23 in Group A). For the proliferation module, the impact of RS was similar in premenopausal patients (ρ = 0.54 and 0.56 in Group A and B), while it was slightly weaker in postmenopausal patients (ρ = 0.39 in Group C). A total of 15.1% patients in our study had the unthresholded proliferation module (19.6%, 12.3%, and 15.6% in Groups A, B, and C).

Contribution of Individual Modules to the Variance of RS

The variance analysis was applied to evaluate the ratio of each module contributing to the variance of RS. The distribution of the variance of Groups B and C was similar and showed a different pattern compared with that of Group A (**Figure 2**). In patients <40 years, the variance (37.3% in Group A) of RS mostly derived from the proliferation module. Meanwhile, the estrogen module contributed most variance of RS in the elder patients (54.1% and 53.4% in Groups B and C). In all three groups,

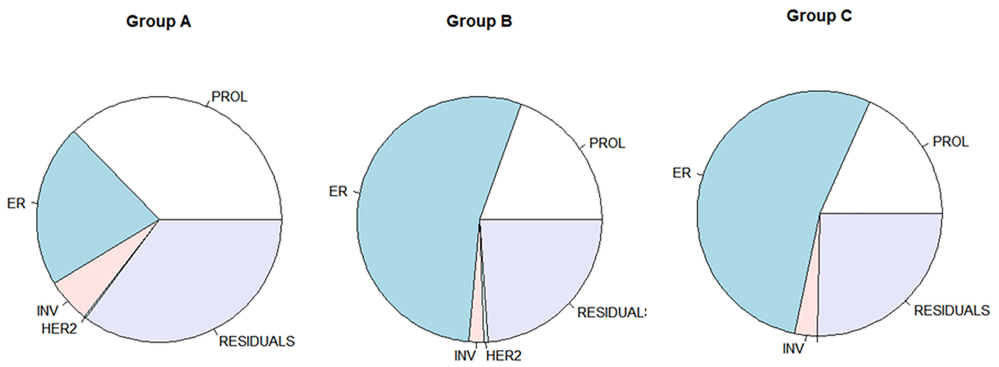

FIGURE 2 | The variance of RS scores as accounted for by individual modules. RS, recurrence score.

the invasion and HER2 module explained little in the variance of RS (shown in **Table 2**).

Correlations in Genetic High-Risk and Low-Risk Subgroups

We explored the correlation of RS with its modules in genetic high-risk and low-risk subgroups (RS>25 and RS ≤ 25, **Figures 3**–**5**). For the estrogen module, its negative impact was much stronger in genetic low-risk patients compared to its high-risk counterparts. Its impact in genetic low-risk subgroup was also stronger in elder patients (ρ = −0.68, −0.77, and −0.84 in Groups A, B, and C). For the proliferation module, its positive impact only occurred in genetic high-risk subgroups. Different from the tendency in the whole population (ρ = 0.54, 0.56, and 0.39 in Groups A, B, and C), the correlation of the proliferation module with RS reversed between the young and elder patients (ρ = 0.38, 0.53, and 0.52 in Groups A, B, and C). For the invasion module, the coefficient was the highest in the genetic low-risk <40-year patients (ρ = 0.55) while the difference was not obvious in other patients.

Correlations in Clinical High-Risk and Low-Risk Subgroups

We further compared the correlations between patients with different clinical risks. The tendency of the correlations between RS and its individual modules was similar between clinical high-risk and low-risk subgroups while some small difference was observed. As for the estrogen module, its negative impact on RS was stronger in patients with low clinical risk compared with high risk (**Figure 6**). For the proliferation module, the positive impact on RS was stronger in high-risk patients regardless of age (**Figure 7**). For the invasion module, the coefficient was stronger in patients ≤40 years old (**Figure 8**). The relationships between RS and its estrogen/proliferation module are summarized in **Figure 9**.

DISCUSSION

The 21-gene RS was a vital tool to help clinicians predict patient prognostic outcomes and assist treatment decisions. Clinical data showed that patients with the same RS but different ages derived different benefit from adjuvant chemotherapy (11). Thus, it was necessary to understand the internal molecular drivers of RS. A recent study uncovered the discordance of the primary coefficient in the Cox model of RS and the unique molecular features of RS in postmenopausal patients (12). However, data in premenopausal women were insufficient. Here, we made a comparison of the molecular drivers of RS between young and old patients. We found that RS was primarily driven by the estrogen module in patients regardless of age, while the proliferation module had a more substantial impact on RS in patients ≤40 years than in those >40 years.

As reported, patients with the same RS but of different ages might respond differently to the addition of chemotherapy.

TABLE 2 | The variance of RS as accounted for by individual modules.

RS modules	Group A		Group B		Group C	
	Sum of Squares	Variance Explained (%)	Sum of Squares	Variance Explained (%)	Sum of Squares	Variance Explained (%)
Proliferation (unthresholded)	3,430	37.3	6,125	19.5	16,681	18.2
ER	1,968	21.4	17,025	54.1	48,958	53.4
Invasion	541	5.9	614	2.0	2,779	3.0
HER2 (unthresholded)	24	0.3	170	0.5	81	0
Residuals	3,235	35.2	7,541	23.9	23,113	25.2

RS, recurrence score; ER, hormone receptor; HER2, human epidermal growth factor receptor 2.

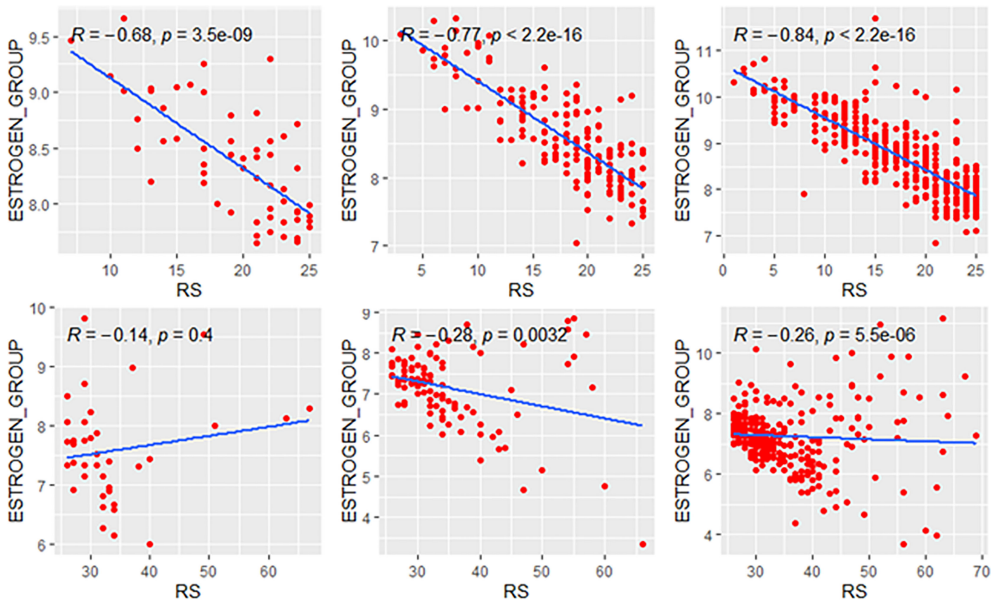

FIGURE 3 | Relationships of the RS with its estrogen module. The upside and downside ranks showed the relationship in patients with RS ≤25 and RS >25, respectively. Groups A, B, and C were presented from left to right. RS, recurrence score.

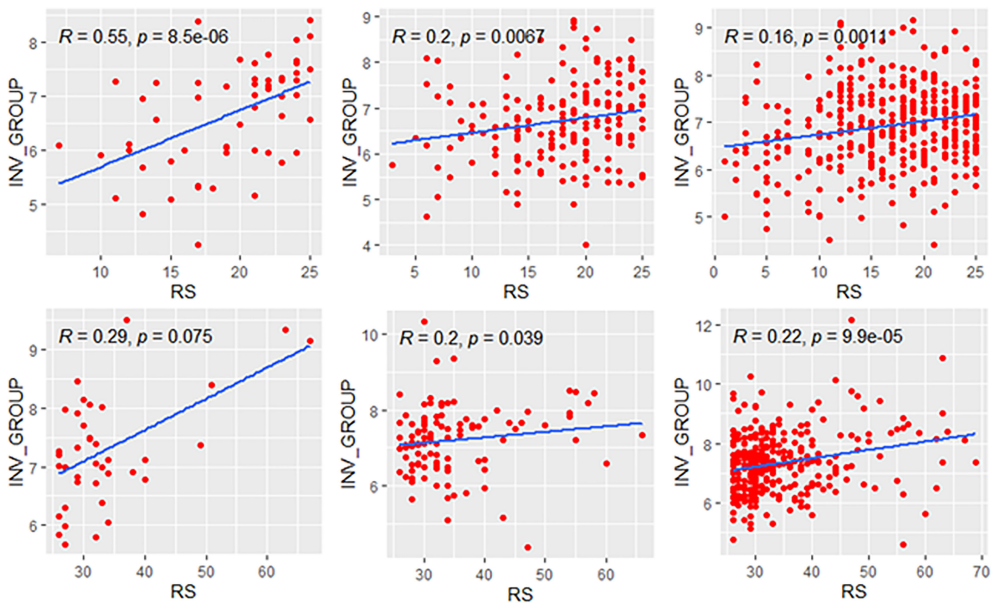

FIGURE 4 | Relationships of the RS with its proliferation module. The upside and downside ranks showed the relationship in patients with RS ≤25 and RS >25, respectively. Groups A, B, and C were presented from left to right. RS, recurrence score; PROL, proliferation.

The result of the TAILORx (11) and the RxPONDER (18) trial suggested that premenopausal patients with RS ≤25 gained a survival improvement from the addition of chemotherapy while the postmenopausal counterparts did not. Likewise, the MINDACT trial (4) showed that for clinical high-risk and genetic low-risk patients, a 5.4% absolute risk reduction of distant metastasis achieved by chemotherapy was observed in patients ≤50 years but not in those >50 years. Based on these results, we divided the patients according to their menopausal status. To explore the mechanisms of RS in patients with

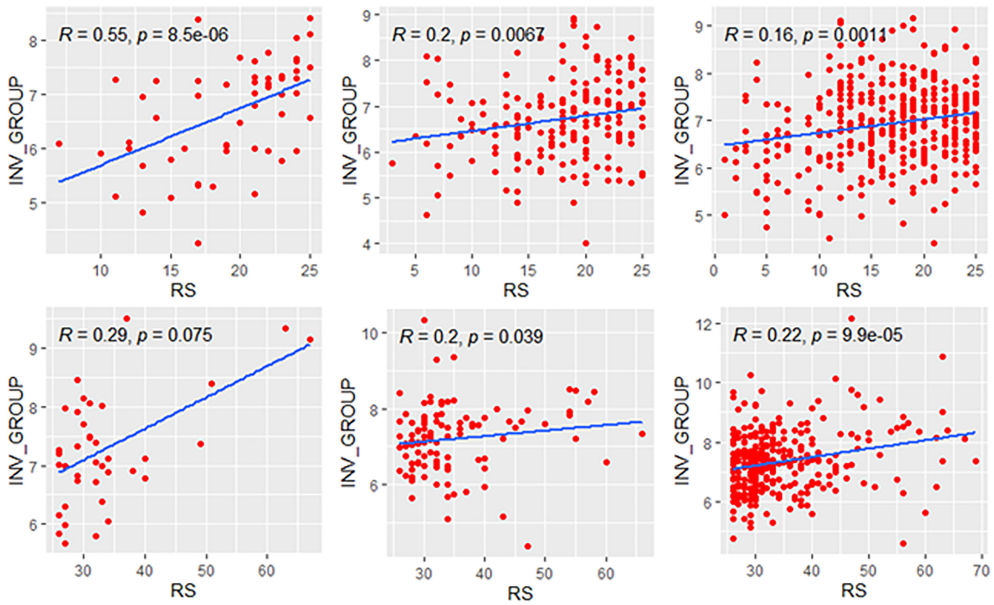

FIGURE 5 | Relationships of the RS with its invasion module. The upside and downside ranks showed the relationship in patients with RS ≤25 and RS >25, respectively. Groups A, B, and C were presented from left to right. RS, recurrence score; INV, invasion.

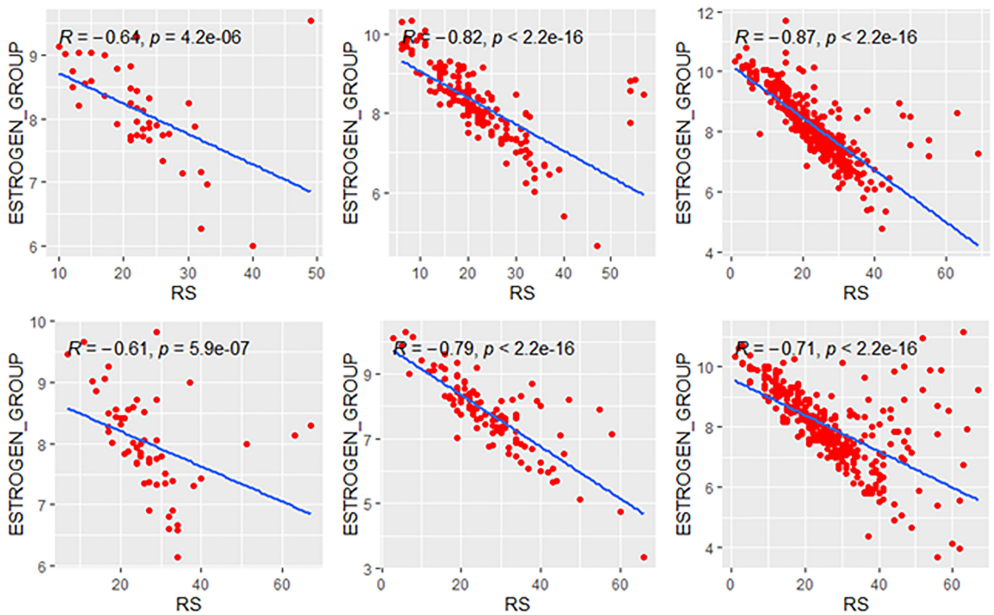

FIGURE 6 | Relationships of the RS with its estrogen module. The upside and downside ranks showed the relationship in patients with low and high clinical risk respectively. Groups A, B, and C were presented from left to right. RS, recurrence score.

different ages, we further categorized patients as young or aged by a cutoff of 40 years old according to BCY4 guidelines.

The results of our study were consistent with the recent study based on patients from the ATAC trial (12). In the ATAC trial, RS was found to be mainly driven by estrogen-related features in postmenopausal women. Our study confirmed that the estrogen module also played a leading role in premenopausal patients >40 years. However, in patients ≤40 years, the link between the estrogen module and RS became weak. Instead, the proliferation module had a strong impact on RS and explained

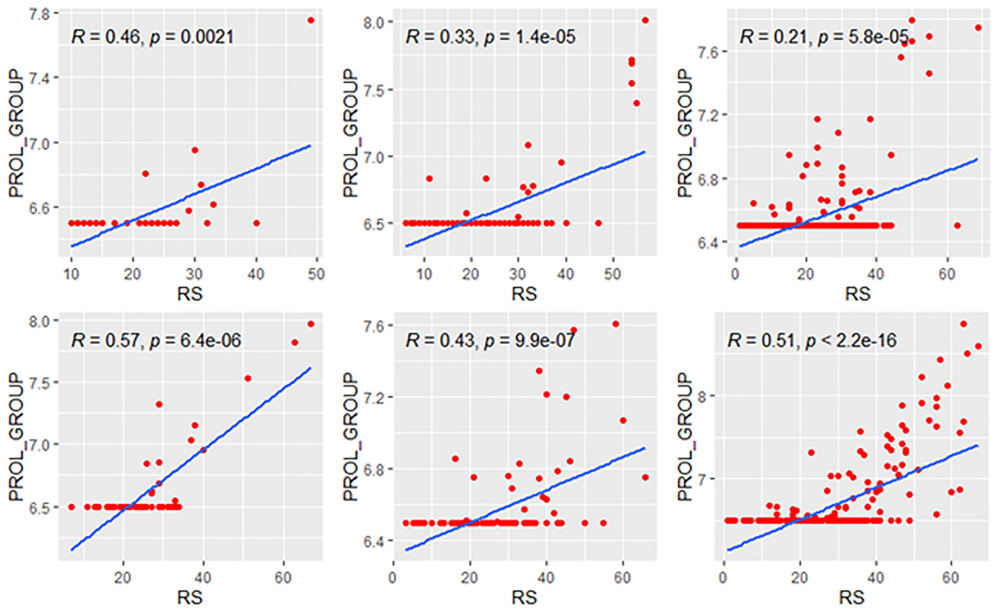

FIGURE 7 | Relationships of the RS with its proliferation module. The upside and downside ranks showed the relationship in patients with low and high clinical risk respectively. Groups A, B, and C were presented from left to right. RS, recurrence score; PROL, proliferation.

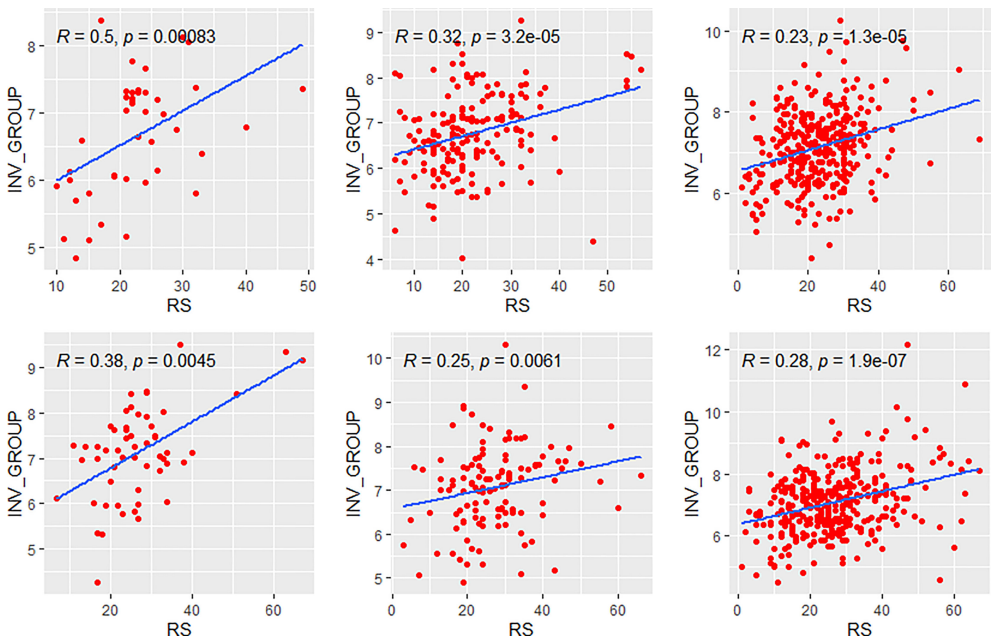

FIGURE 8 | Relationships of the RS with its invasion module. The upside and downside ranks showed the relationship in patients with low and high clinical risk respectively. Groups A, B, and C were presented from left to right. RS, recurrence score; INV, invasion.

most of RS variance. Given the increased impact of the estrogen module on RS, we assumed that the loss of prediction value of RS after 5 years (19) could be attributed to the strong impact of estrogen module on RS in patients >40 years, because most of them received only 5 years of endocrine therapy. Second, in patients ≤40 years, the weak impact of the estrogen module might be due to relatively lower expressions of ER-related genes. As for the proliferation module, its strong correlation with RS in

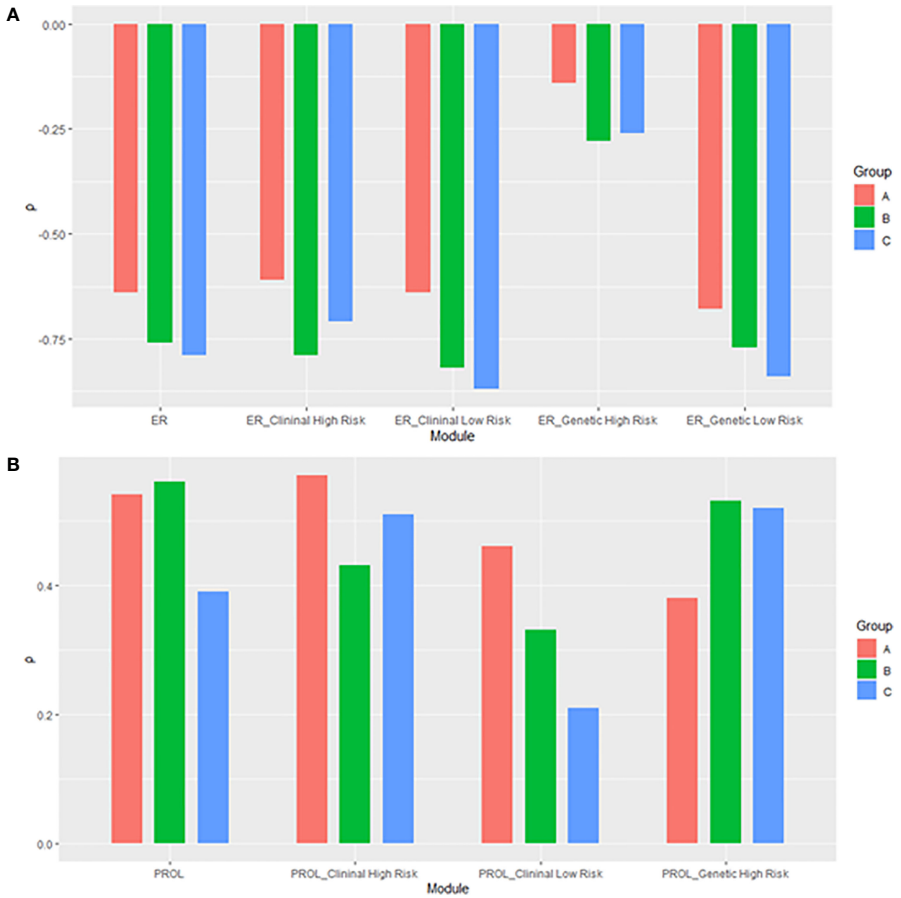

FIGURE 9 | The histogram of relationships of the RS with its **(A)** estrogen and **(B)** proliferation module. The subgroup of proliferation module with genetic low risk was omitted due to non-significance. ER, estrogen receptor; PROL, proliferation.

young patients was in accordance with the previous retrospective studies that young patients were more likely to have tumors with higher grades (9) and higher expression of proliferation related genes (20). In our study, a larger proportion of patients ≤40 years (19.6%) had unthresholded high proliferation module scores than those patients who were >40 years (12.3% and 15.6% in Groups B and C). In fact, the application of threshold distinctly narrowed the gap of proliferation modules' contribution to RS between patients <40 years and ≥40 years.

In our exploratory analysis, in subgroups with different genetic risks, the association between the RS and its estrogen module was weaker among younger patients, especially in low genetic risk groups. In terms of proliferation-related features, no statistically significant relationship was found between RS and its proliferation module in patients with RS <25, suggesting that proliferation-related features might affect very little in patients with low-to-immediate gene risk. Evidence from TAILORx showed that patients with a mild RS of 11 to 25 could benefit from chemotherapy if they were 41–50 years of age (11). Correspondingly, in our study, RS strongly correlated with the ER module in premenopausal patients who were 40 years or older, while no significant association between RS and the proliferation module was observed. Therefore, a probable presumption was that the chemotherapy benefit for patients 41–50 years old with moderate genetic risk was mainly derived from chemotherapy-induced amenorrhea (CIA), which was common in women 40 years of age or older (21). Over 80% of experts acknowledged the importance of CIA at the 17th St. International Breast Cancer Conference. For these patients, endocrine therapy plus ovarian function suppression might be an alternative option for chemotherapy (22, 23).

Clinicopathological features were traditional important prognostic factors (24). Thus, we investigated the molecular drivers in subgroups with different clinical risks. The negative impact of ER-related features on RS was stronger in clinical low-risk patients. On the other hand, the impact of the proliferation module was stronger in clinical high-risk patients. Our results aligned with previous evidence and suggested that the internal molecular mechanisms might differ even with the same RS. For instance, for a 60-year postmenopausal low clinical risk patient, an RS of 30 might be driven primarily by the strong impact of the estrogen module. Meanwhile, for a similar patient with high clinical risk, an RS of 30 might be attributed to the proliferation-related gene expression. Our results supported the conclusion of

the secondary analyses of TAILORx (21). We reconfirmed that clinical-risk stratification (based on tumor size and tumor grade) combined with RS could provide better prognostic information. Additionally, it also explained the better performance of RSClin tool (25) than that of RS alone.

Our study has several strengths. First, we explored the molecular drivers of RS in young patients and compared them with those in elder patients, which had rarely been illuminated before. Second, previous studies were based on samples from the ATAC trial. In the ATAC trial, the majority of patients were clinical low-risk and able to receive tamoxifen or anastrozole alone (26). Instead, patients studied in our study derived from real-world data thus might be more representative of clinical practice. Thirdly, we used a cutoff age of 40 years instead of 50 years to divide customized risk groups. We found distinct patterns of molecular drivers between patients ≤40 years and those >40 years. Thus, it might be necessary to further categorize the ranges of ages in addition to the cutoff of 50 years used by the TAILORx trial and recommended by the ASCO Clinical Practice Guideline (27) and NCCN (28) guideline.

In conclusion, our study confirmed that RS was primarily driven by the estrogen module in patients regardless of age. The proliferation module had a stronger impact on RS in patients ≤40 years than in those >40 years. In RS ≤25 groups, the proliferation module had no apparent association with RS, and thus the chemo-related benefit in young patients might be primarily derived from CIA. In RS >25 groups, the proliferation module became the leading driver, while the estrogen module had a weaker association with RS. The impact of the ER module on RS was stronger in clinical low-risk patients while the effect of the proliferation module was stronger in clinical high-risk patients. Further analysis might pay more attention to the difference between patients ≤40 years and >40 years when using RS to determine the addition of chemotherapy to endocrine therapy.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LZ, JW, and MC made the study design. DL, WlC, WgC, and KS participated in data acquisition. JW and MC conducted statistical analysis and manuscript preparation. KS and LZ helped to review the manuscript. All authors contributed to the article and approved the submitted version.

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Risk Factors, Prognostic Factors, and Nomogram for Distant Metastasis in Breast Cancer Patients Without Lymph Node Metastasis

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Background: Lymph node negative (N0) breast cancer can be found coexisting with distant metastasis (DM), which might consequently make clinicians underestimate the risk of relapse and insufficient treatment for this subpopulation.

Methods: The clinicopathological characteristics of N0 breast cancer patients from the Surveillance, Epidemiology, and End Results (SEER) database between January 2010 and December 2015 were retrospectively reviewed. Multivariate logistic and Cox analyses were used to identify independent risk factors in promoting DM and the 1-, 3-, and 5- year cancer-specific survival (CSS) in this subpopulation.

Result: Seven factors including age (<40 years), tumor size (>10 mm), race (Black), location (central), grade (poor differentiation), histology (invasive lobular carcinoma), and subtype (luminal B and Her-2 enriched) were associated with DM, and the area under curve (AUC) was 0.776 (95% CI: 0.763–0.790). Moreover, T1-3N0M1 patients with age >60 years at diagnosis, Black race, triple-negative breast cancer subtype, no surgery performed, and multiple DMs presented a worse 1-, 3-, and 5-year CSS. The areas under the ROC for 1-, 3-, and 5- year CSS in the training cohort were 0.772, 0.741, and 0.762, respectively, and 0.725, 0.695, and 0.699 in the validation cohort.

Conclusion: The clinicopathological characteristics associated with the risk of DM and the prognosis of female breast cancer patients without lymph node metastasis but with DM are determined. A novel nomogram for predicting 1-, 3-, 5- year CSS in T1-3N0M1 patients is also well established and validated, which could help clinicians better stratify patients who are at a high-risk level for receiving relatively aggressive management.

Keywords: N0 breast cancer, distant metastasis, risk factor, nomogram, cancer-specific survival

Abbreviations: DM, distant metastasis; CSS, cancer-specific survival; N0, lymph node negative; ROC, receiver operating characteristic curves; CTCs, circulating tumor cells; IDL, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; IDLC, infiltrating ductal mixed lobular carcinoma; OS, overall survival; CSS, cancer-specific survival; ER, estrogen receptor; PR, progesterone receptor.

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INTRODUCTION

Breast cancer is currently the most frequent malignancy and one of the leading causes of cancer death in the United States (estimated 279,100 new cases and 42,690 death) (1) and China mainland (estimated 304,000 new cases and 70,000 deaths) (2). Although the long-term survival of patients with breast cancer has been significantly increased in the past years with the application of targeted therapy (3), endocrine therapy (4), and even immunotherapy (5, 6), distant metastasis (DM), as the most common form of recurrence and the main cause (approximately 90%) of death, could reverse this favorable outcome (7, 8). Historically, the "Halsted" hypothesis indicated that the processing steps of breast cancer metastasis were mechanized and orderly, including primary focus enlargement, invasion to the regional lymph nodes, and further metastasis to distant organs *via* the bloodstream. However, subsequent studies on the biological characteristics of breast cancer metastasis have shown that the DM in breast cancer was a non-random process as it allowed circulating tumor cells (CTC) to seed at specific distant tissues, which suggested the metastasis did not require circulation through the lymph system but directly invade the distant organs *via* the bloodstream. Consequently, the CTC analysis technique has become a novel utility tool for predicting the prognosis of breast cancer patients, which could provide better treatment guidance for clinicians (9, 10).

Indeed, as a key component of tumor stage classification, the status of the regional lymph nodes plays an important role in predicting the biological aggressiveness and propensity to spread in patients with breast cancer (11, 12). Some scholars believe that regional nodal disease may precede metastatic dissemination (11). Therefore, after surgery, patients with negative lymph node status could remain a favorable outcome, and only a small fraction of them need adjuvant therapy during the postoperative follow-up (11). Additionally, reviewing the recent literature, negative lymph node status was frequently referred to as the "control group" in the study when scholars aimed to explore the risk factors of DM (13–16). Patients with negative lymph node status were more likely to be assigned to the low-risk group. However, one thing that cannot be ignored was that there were still a considerable proportion of patients screened out having DM but negative lymph node status (17). The insufficient adjuvant therapy and management for this population might increase the risk of relapse in those lymph-node-negative (N0) patients with multiple risk factors. And clinicians may underestimate the risk of relapse and make insufficient treatment for N0 patients with breast cancer.

Therefore, it is equally important to identify the independent risk factors of DM in this particular subpopulation, which would not only help oncologists to begin tailoring treatment strategies to patients but also encourage researchers to investigate the underlying molecular mechanisms in breast cancer metastasis. Although some scholars have made efforts on evaluating the DM in lymph node negative primary breast cancer *via* evaluating the gene expression profiles and the integration of proliferation and immunity (17, 18), whether there was a different clinical pattern between DM and non-DM patients without lymph node involvement was still unclear.

In the present study, we aimed to extract the potential risk clinicopathological factors in promoting DM of N0 primary breast cancer, which would fill the gap in identifying high-risk subgroups. Besides, we also evaluated the cancer-specific survival (CSS) in this subpopulation and further developed a novel predictive model to provide quantitative predictions on the outcome for N0 patients with DM. More aggressive treatment modalities and active surveillance may be justified in high-risk subgroups of patients.

MATERIALS AND METHODS

Data Source

This is an observational retrospective cohort study. As a result, the data we analyzed were extracted from a large population-based (Surveillance, Epidemiology, and End Results, SEER, derived from the 18 cancer registries) research program, which included approximately 28% of the U.S. population and various ethnic groups. The medical records collection and analysis were performed by two study researchers, working independently to decrease the selection bias. The reporting of this study followed the guidelines of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (19).

Patients who met the following criteria were included: (1) female patients with histological confirmed invasive breast cancer; (2) aged at diagnosis between 18 and 79 years; (3) pathological confirmed negative lymph node status; (4) diagnosed between 2010 and 2015 years; (5) the histology types of breast cancer were infiltrating ductal carcinoma (IDL), infiltrating lobular carcinoma (ILC), and infiltrating ductal mixed lobular carcinoma (IDLC). Patients with T4 (invasion to the chest wall/skin and inflammatory carcinoma) primary site, no regional nodes examined, coexisting with one or more cancers, lost to follow-up, or incomplete medical records were excluded during the patients' selection process (**Figure 1**).

Variable Evaluation and Definition

According to the requirement of establishing sample size of multivariate linear regression equation, the sample size in the present study should be at least 10 times of the number of independent variables in the equation. Thus, after excluding the unqualified cases, there were 79,746 female patients with invasive breast cancer enrolled in this study. They were assigned to explore the risk factors in promoting the DM in N0 breast cancer. Besides, for predicting 1-, 3-, and 5-year CSS, the N0 patients with DM between 2010 and 2015 years were randomly divided into a training group and validation group at a ratio of 7:3 *via* the "R" program.

We selected the variables on the basis of their associations with the outcomes of interest. Specifically, the following clinicopathological characteristics were collected and transformed into categorical variables: age (≥20 and <40 years; ≥40 and <60 years; ≥60 and <80 years), race (White, Black, Asian or Pacific Islander, and American Indian/Alaska Native), laterality (right and left origin of primary), stage (I, II, IV derived from AJCC staging system 7th edition), grade (well differentiated, moderately differentiated, poorly differentiated,

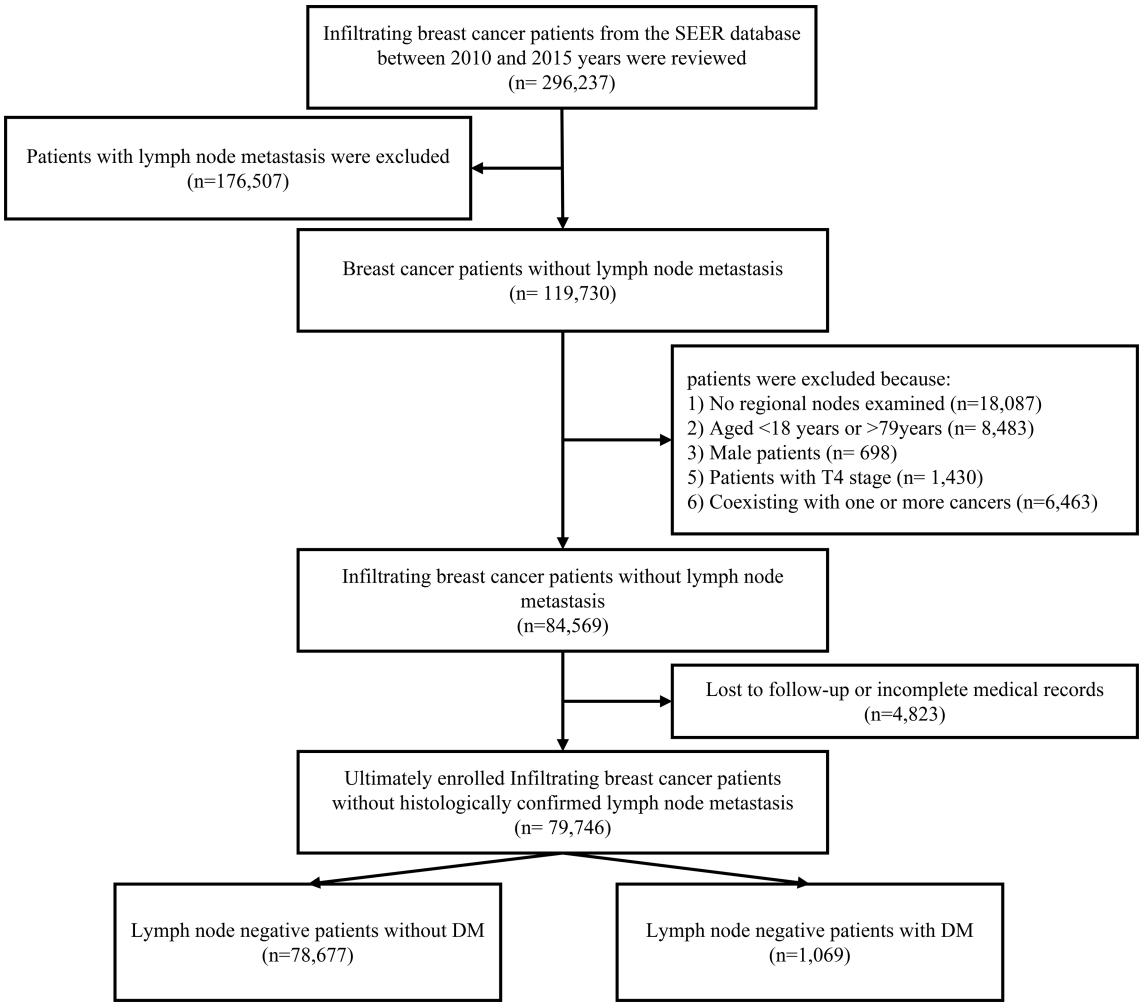

FIGURE 1 | The patients' selection processing. T4, invasion to the chest wall/skin and inflammatory carcinoma; DM, distant metastasis.

and undifferentiated), location (central, outer, inner, overlapping, and axillary of breast), histological type (IDC, ILC, and IDLC), ICD-O-3 codes (8500/3, 8520/3, 8521/3, and 8522/3), breast cancer subtype [Luminal A: hormonal receptor (HR)+/HER2−, Luminal B: HR+/HER2+, Triple-negative: HR−/HER2−, Her-2 enriched: HR−/HER2+], primary tumor size (T_{1mic}: >0 and ≤1 mm; T_{1a}: >1 and ≤5 mm; T_{1b}: >5 and ≤10 mm; T_{1c}: >10 and ≤20 mm; T_2: >20 and ≤50 mm; T_3: >50 mm), DM at meet (bone, liver, lung, brain, and multiple DM); surgery; cause-specific death, and 60 survival months (more than 0 days of survival).

Statistical Analysis

The primary endpoint of this study was DM and 1-, 3-, and 5-year CSS probability. The univariate and multivariate logistic analyses were used for identifying the potential independent clinical risk factors in promoting DM of lymph node negative patients. And the univariate and multivariate Cox regression analyses were performed to find out the prognostic factors of CSS in patients with DM. The analyses were conducted *via* IBM SPSS (version 25.0). A two-tailed P-value of <0.05 was defined as the criterion for variable deletion when performing backward stepwise selection. The nomogram, calibration curve, and Kaplan-Meier analysis were constructed and plotted based on the results of the multivariate Cox regression analysis *via* using the "survival," "rms," "survminer," and "foreign" packages of the R software (R Foundation, Vienna, Austria, version 3.5.2, http://www.r-project.org). Harrell's C-index is calculated to assess the discrimination performance of the present nomogram.

RESULT

Clinicopathological Characteristics of Patients With Negative Lymph Node Status

Generally, between the years 2010 and 2015, a total of 79,746 female patients with invasive breast cancer were enrolled in this study with a median age of 61 years (range: 20–79 years) at diagnosis and a median follow-up time of 51 months (range: 0–95

months). There were 1,069 cases (1.34%) identified coexisting with DM in the N0 patients, in which 748 cases were observed in the training cohort and 321 cases were in the validation cohort (**Table 1**). Specifically, the most frequent metastasis site was bone, which made up 327 cases (43.72%) and 150 cases (46.73%) of the DM patients in the training and validation cohorts. Notably, 385 (36.01%) patients suffered from multiple DMs. And almost 70.63% (755/1,069 cases) of patients with DM did not receive surgery for the primary tumor.

Univariate and Multivariate Logistic Analyses of the Risk Factors of DM

To investigate the potential clinical factors associated with the risk of DM in female breast cancer with negative lymph node status,

TABLE 1 | Clinicopathological characteristics of female patients with negative lymph node status but distant metastasis in training and validation cohorts.

Characteristics	No. (%) of patients		
	Initial cohort (n = 1,069)	Training cohort (n = 748)	Validation cohort (n = 321)
Age			
≥20 and <40	78 (7.3)	53 (7.09)	25 (7.79)
≥40 and <60	416 (38.91)	289 (38.64)	127 (39.56)
≥60 and <80	575 (53.79)	406 (54.28)	169 (52.65)
Race			
White	866 (81.01)	605 (80.88)	261 (81.31)
Black	140 (13.10)	99 (13.24)	41 (12.77)
※Other	63 (5.89)	44 (5.88)	19 (5.92)
Location			
Nipple	4 (0.37)	3 (0.40)	1 (0.31)
Central	75 (7.01)	50 (6.68)	25 (7.79)
Upper-inner	144 (13.47)	93 (12.43)	51 (15.89)
Lower-inner	61 (5.71)	42 (5.61)	19 (5.92)
Upper-outer	385 (36.01)	268 (35.83)	117 (36.45)
Lower-outer	97 (9.07)	71 (9.49)	26 (8.10)
Axillary	11 (3.43)	8 (1.07)	3 (0.93)
Overlapping	292 (28.34)	213 (28.48)	79 (24.61)
&Grade			
I	138 (12.91)	96 (12.83)	42 (13.08)
II	535 (50.04)	386 (51.60)	149 (46.42)
III/IV	396 (37.04)	266 (35.56)	130 (40.50)
Laterality			
Right	496 (46.40)	356 (47.59)	140 (43.61)
Left	573 (53.60)	392 (52.41)	181 (56.39)
Histology			
IDC	870 (81.39)	610 (81.55)	260 (81.00)
ILC	142 (13.28)	98 (13.10)	44 (13.71)
IDLC	57 (5.33)	40 (5.35)	17 (5.29)
Tumor size			
T_{1mic}	1 (0.01)	0 (0.00)	1 (0.31)
T_{1a}	20 (1.89)	15 (2.00)	5 (1.56)
T_{1b}	67 (6.27)	47 (6.28)	20 (6.23)
T_{1c}	240 (22.45)	164 (21.92)	76 (23.68)
T_2	605 (56.59)	436 (58.30)	169 (52.65)
T_3	136 (12.72)	86 (11.50)	50 (15.58)
M status			
M_1-bone	477 (44.62)	327 (43.72)	150 (46.73)
M_1-liver	95 (8.89)	67 (8.96)	28 (8.72)
M_1-lung	102 (9.54)	73 (9.76)	29 (9.03)
M_1-brain	10 (0.94)	7 (0.94)	3 (0.93)
M_1-multiple	385 (36.01)	247 (33.02)	111 (34.58)
Subtype			
Luminal A	693 (64.83)	493 (65.91)	200 (62.30)
Luminal B	175 (16.37)	118 (15.78)	57 (17.76)
TNBC	134 (12.54)	92 (12.30)	42 (13.08)
Her-2 enriched	67 (6.27)	45 (6.02)	22 (6.85)
Surgery			
Not performed	755 (70.63)	534 (71.39)	221 (68.85)
Performed	314 (29.37)	214 (28.61)	100 (31.15)

※Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native; &Grade: I, well differentiated; II, moderately differentiated; III/IV, poorly differentiated and undifferentiated. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; TNBC, triple-negative breast cancer; Her-2, human epidermal growth factor receptor-2.

the logistics analysis was performed. During the univariate logistic analysis, age at diagnosis (p<0.0001), tumor size (p<0.0001), race (p<0.0001), tumor location (p<0.0001), grade (p<0.0001), histology (p<0.0001), and subtype (p<0.0001) were identified to be significantly associated with DM. Thus, we incorporated seven clinicopathological factors into the multivariate logistic analysis and further obtained a good AUC of 0.776 (95% CI: 0.763–0.790) (**Supplementary Figure S1**) in predicting the risk of DM in female patients with negative lymph node status. Specifically, the results presented that tumor size >10 mm [>10 maximum diameter ≤20 mm: hazard ratio (HR)= 2.28, 95% confidence interval (CI): 1.78–2.92; >20 maximum diameter ≤50mm: HR=9.46, 95% CI: 7.51–11.92; maximum diameter >50 mm: HR= 19.12, 95% CI: 14.44–25.33; <0.0001], Black race (HR=1.12, 95% CI: 0.93–1.35, p<0.0001), moderate grade (HR=1.57, 95% CI: 1.29–1.90, p<0.0001), ILC (HR=1.31, 95% CI: 1.08–1.58, p<0.0001), and subtype (luminal B: HR=1.54, 95% CI: 1.29–1.84; Her-2 enriched: HR=1.46, 95% CI: 1.12–1.92; p<0.0001). On the contrary, elderly age (≥40 age <60 years: HR=0.70; 95% CI: 0.54–0.89; ≥60 age <80 years: HR= 0.84; 95% CI: 0.65–1.08; p=0.002), Asian/Pacific Islander and American Indian/Alaska Native race (HR=0.52, 95% CI: 0.40–0.67, p<0.0001), and tumor location (inner location: HR=0.57, 95%CI: 0.44–0.75; outer location: HR= 0.64, 95% CI: 0.50–0.82; axillary and overlapping location: HR= 0.71, 95% CI: 0.55–0.92; p<0.0001, respectively) were determined to be the protective factors in DM (**Table 2**).

Univariate and Multivariate Cox Analyses of the Risk Factors of CSS

To identify the independent risk factors of 1-, 3-, and 5-year CSS in women with negative lymph node status but DM during the follow-up, only significant factors from univariate Cox regression analysis were further applied into multivariate Cox regression analysis. During the univariate Cox regression analysis, age (p=0.017), race (p<0.0001), grade (p=0.004), subtype (p<0.0001), tumor size (p=0.027), surgery (p<0.0001), and metastasis site (p<0.0001) were identified to be the predictive factors. Additionally, elderly age (≥60 age <80 years: HR= 1.58; 95% CI: 1.03–2.44; p=0.015), black race (HR=1.64, 95% CI: 1.24–2.16, p=0.002), TNBC (HR=2.77, 95% CI: 2.02–3.80, p<0.0001), and metastasis site (liver: HR=2.01, 95% CI: 1.36–2.90; multiple sites: HR=2.13, 95% CI: 1.69–2.68, p<0.0001) were regarded as the independent risk factors of CSS in this subpopulation (**Table 3**). However, the tumor size (p=0.123) and differentiation grade (p=0.101) were not determined to be statistically significant.

Furthermore, to actuarially estimate the survival probability and cumulative hazard in patients with different variables, five factors (p ≤ 0.05) from multivariate analysis in Cox proportional

TABLE 2 | Univariate and multivariate logistic regression analyses of clinical variables correlated with distant metastasis in female breast cancer with negative lymph node status.

Variables	Subgroup	Univariable		Multivariable	
		Hazard ratio	*P*	Hazard ratio	*P*
Age (year)	≥20 and <40	Reference	**<0.0001**	Reference	**0.002**
	≥40 and <60	0.49 (0.38–0.63)		0.70 (0.54–0.89)	
	≥60 and <80	0.52 (0.41–0.67)		0.84 (0.65–1.08)	
Tumor size (mm)	>0 and ≤10	Reference	**<0.0001**	Reference	**<0.0001**
	>10 and ≤20	2.37 (1.86–3.03)		2.28 (1.78–2.92)	
	>20 and ≤50	10.09 (8.06–12.63)		9.46 (7.51–11.92)	
	>50	21.79 (16.60–28.61)		19.12 (14.44–25.33)	
Race	White	Reference	**<0.0001**	Reference	**<0.0001**
	Black	1.31 (1.09–1.57)		1.12 (0.93–1.35)	
	[※]Other	0.57 (0.44–0.74)		0.52 (0.40–0.67)	
Location	[&]Central	Reference	**<0.0001**	Reference	**<0.0001**
	Inner	0.47 (0.36–0.61)		0.57 (0.44–0.75)	
	Outer	0.55 (0.43–0.70)		0.64 (0.50–0.82)	
	[¶]Other	0.62 (0.48–0.80)		0.71 (0.55–0.92)	
Grade	Well	Reference	**<0.0001**	Reference	
	Moderate	2.34 (1.94–2.83)		1.57 (1.29–1.90)	**<0.0001**
	Poor	2.68 (2.21–3.26)		1.22 (0.98–1.52)	
Laterality	Right	Reference	0.068	/	
	Left	1.11 (0.99–1.26)			
Histology	IDC	Reference	**<0.0001**	Reference	**<0.0001**
	ILC	1.71 (1.43–2.05)		1.31 (1.08–1.58)	
	IDLC	1.07 (0.82–1.41)		0.99 (0.75–1.31)	
Subtype	Luminal A	Reference		Reference	
	Luminal B	1.96 (1.66–2.32)	**<0.0001**	1.54 (1.29–1.84)	**<0.0001**
	TNBC	1.30 (1.08–1.56)		0.89 (0.72–1.10)	
	Her-2	1.99 (1.54–2.56)		1.46 (1.12–1.92)	

[※]Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native; [&]Central: central portion of breast combined with nipple; [¶]Other: axillary and overlapping of the breast. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; TNBC, triple-negative breast cancer; Her-2, human epidermal growth factor receptor-2.

Bold values indicate statistical significance (p < 0.05).

TABLE 3 | Univariate and multivariate Cox regression analyses of predictive variables correlated with CSS in IV stage female breast cancer with negative-lymph node status.

Variables	Subgroup	Univariable		Multivariable	
		Hazard ratio	*P*	Hazard ratio	*P*
Age (year)	≥20 and <40	Reference	**0.017**	Reference	**0.015**
	≥40 and <60	1.29 (0.84–2.00)		1.21 (0.78–1.88)	
	≥60 and <80	1.63 (1.07–2.48)		1.58 (1.03–2.440	
Race	White	Reference	**<0.0001**	Reference	**0.002**
	Black	1.72 (1.31–2.24)		1.64 (1.24–2.16)	
	[※]Other	0.86 (0.54–1.36)		0.89 (0.56–1.44)	
Location	[&]Central	Reference	0.991	/	
	Inner	1.06 (0.67–1.65)			
	Outer	1.02 (0.68–1.54)			
	[¶]Other	1.01 (0.66–1.56)			
Grade	I	Reference	**0.004**	Reference	0.101
	II	1.30 (0.92–1.82)		1.35 (0.95–1.91)	
	III/IV	1.68 (1.19–2.38)		1.52 (1.03–2.23)	
Histology	IDC	Reference	0.821	/	
	ILC	1.07 (0.80–1.43)			
	IDLC	0.92 (0.58–1.44)			
Laterality	Right	Reference	0.816	/	
	Left	1.00 (0.82–1.21)			
Subtype	Luminal A	Reference	**<0.0001**	Reference	**<0.0001**
	Luminal B	0.92 (0.69–1.23)		0.79 (0.58–1.07)	
	TNBC	2.52 (1.92–3.30)		2.77 (2.02–3.80)	
	HER2	1.37 (0.90–2.06)		1.29 (0.82–2.01)	
Tumor size (mm)	>0 and ≤10	Reference	**0.027**	Reference	0.123
	>10 and ≤20	0.66 (0.46–0.96)		0.78 (0.54–1.14)	
	>20 and ≤50	0.63 (0.45–0.88)		0.72 (0.51–1.00)	
	>50	0.54 (0.34–0.83)		0.602 (0.38–0.93)	
Surgery	No	Reference	**<0.0001**	Reference	**<0.0001**
	Yes	0.51 (0.40–0.64)		0.39 (0.30–0.51)	
M status	Bone	Reference	**<0.0001**	Reference	**<0.0001**
	Liver	2.20 (1.55–3.12)		2.01 (1.396–2.90)	
	Lung	1.34 (0.92–1.95)		1.06 (0.72–1.57)	
	Brain	1.84 (0.68–4.97)		1.44 (0.528–3.93)	
	*Multiple	2.31 (1.85–2.89)		2.13 (1.69–2.68)	

*[※]Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native; [&]Central: central portion of breast combined with nipple; [¶]Other: axillary and overlapping of the breast; *Multiple: two or more distant metastasis sites.*

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor-2.

Bold values indicate statistical significance ($p < 0.05$).

hazard model were used to plot the Kaplan-Meier survival curves; namely, a significant decrease in survival probability was observed in patients without surgery performed (1-, 3-, and 5-year CSS rate: 73.8, 48.9, and 28.8%, respectively, **Figure 2A**), age ≥60 (1-, 3-, and 5-year CSS rate: 74.2, 52.5, and 32.1%, respectively, **Figure 2B**), TNBC subtype (1-, 3-, and 5-year CSS: 48.7, 21.4, and 18.7%, respectively, **Figure 2C**), and DM to multiple sites (1-, 3-, and 5-year CSS: 66.6, 40.1, and 24.4%, respectively, **Figure 2D**), as well as Black race (1-, 3-, and 5-year CSS: 61.5, 35.8, and 24.6%, respectively, **Supplementary Figure S2**) were all associated with the survival probability.

Predictive Nomogram Construction and Validation

Based on the multivariate Cox regression analysis, five variables including age at diagnosis, race, surgery performed, distant metastasis site, and tumor subtype were extracted for constructing the nomogram for predicting the 1-, 3-, and 5-ear CSS in patients with negative lymph node status but DM at meet (**Figure 3**). Each factor represented a score on the points scale, and the total point could be calculated by adding up all the specific values from an individualized patient. The C-index of the nomogram reached 0.694, which represented relatively favorable discrimination (the specific value of each variable was calculated in **Table 4**). In the training cohort, the AUC of each 1-, 3-, and 5-year CSS ROC was 0.772, 0.741, and 0.762 with a cutoff value of 185, 191, and 151, respectively, which indicated a satisfying prediction ability (**Figures 4A–C**). Moreover, the established nomogram was validated by an internal validation cohort with 321 cases. The results in the validation cohort also presented good discrimination with an AUC of 0.725 in predicting the 1-year CSS (**Figure 4D**), an AUC of 0.695 in predicting the 3-year

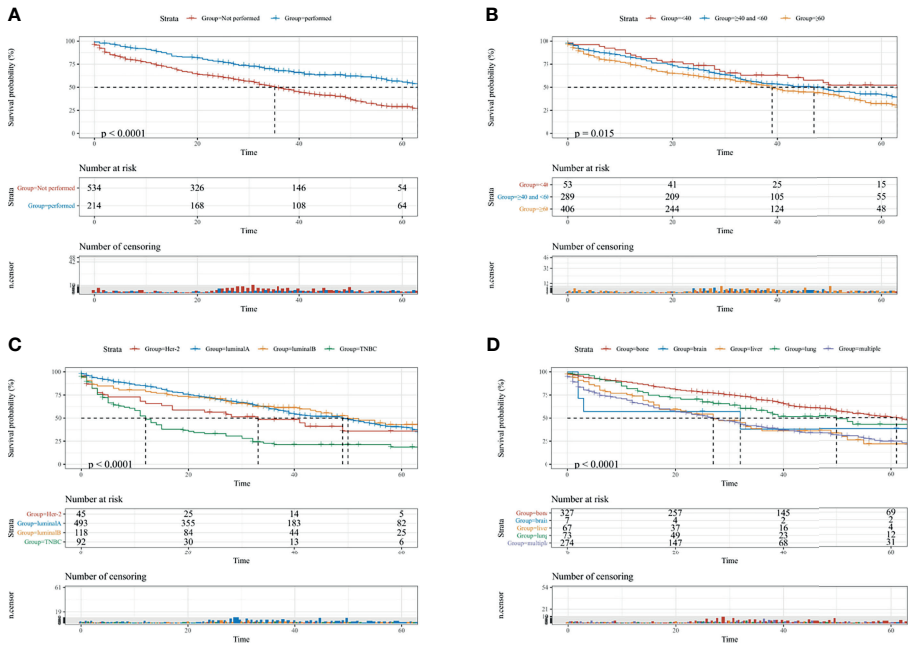

FIGURE 2 | The KM survival curves for predicting the CSS of lymph-node-negative women with DM. **(A)** Surgical intervention; **(B)** age at diagnosis; **(C)** breast subtype; **(D)** metastasis sites. CSS, cancer-specific survival; Multiple, two or more distant metastasis sites.

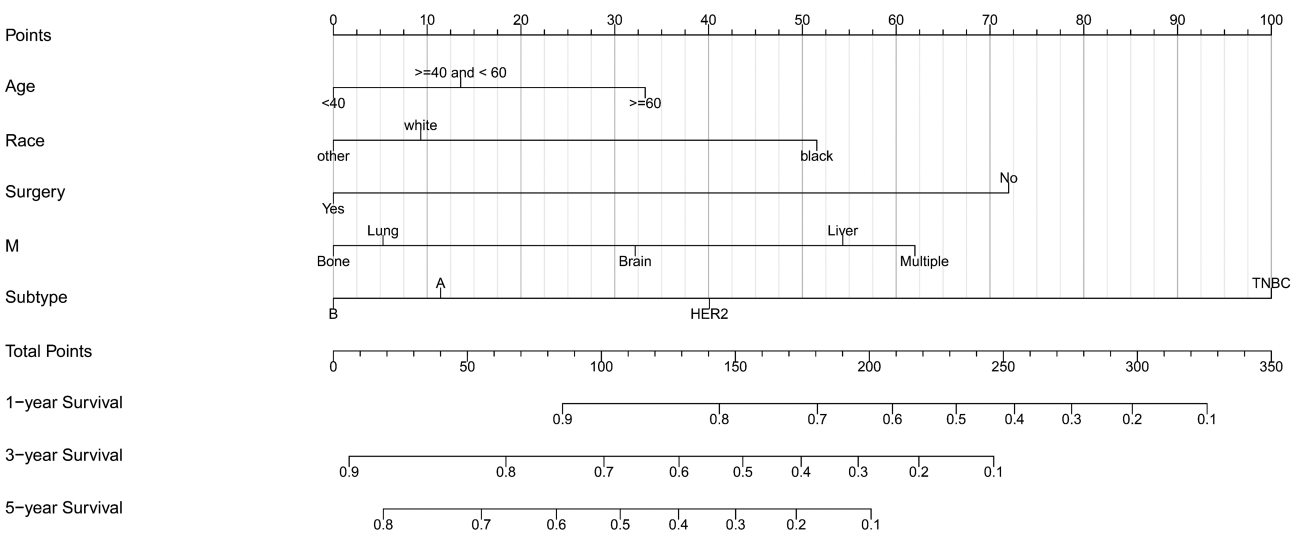

FIGURE 3 | Nomogram for predicting the 1-, 3-, and 5-year CSS in lymph-node-negative women with DM. Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native. CSS, cancer-specific survival; A, Luminal A; B, Luminal B; TNBC, triple-negative breast cancer; HER2, Her-2 enriched.

CSS (**Figure 4E**), and an AUC of 0.699 in predicting the 5-year CSS (**Figure 4F**), respectively. Besides, to examine the discrimination of the proposed nomogram, the patients in the training set were categorized into four groups based on the total points obtained from the nomogram. The KM curve presented good discrimination in identifying the high-risk population (**Supplementary Figure S3**). To further evaluate the accuracy of the nomogram, the calibration curves for the probability of CSS presented a high agreement between 1-, 3-, and 5-year predictions of the nomogram (**Figure 5**).

TABLE 4 | The specific value of clinicopathological factors in the nomogram in the training cohort.

Characteristics	Score
Age	
≥20 and <40	0
≥40 and <60	14
≥60 and <80	33
Race	
White	9
Black	52
※Other	0
M status	
M_1-bone	0
M_1-liver	54
M_1-lung	5
M_1-brain	32
M_1-&multiple	62
Subtype	
Luminal A	11
Luminal B	0
TNBC	100
Her-2 enriched	40
Surgery	
No	72
Yes	0
Total point for 1-year CSS	
0.1	326
0.2	298
0.3	275
0.4	254
0.5	232
0.6	209
0.7	181
0.8	144
0.9	86
Total point for 3-year CSS	
0.1	246
0.2	218
0.3	196
0.4	175
0.5	153
0.6	129
0.7	101
0.8	64
0.9	6
Total point for 5-year CSS	
0.1	201
0.2	173
0.3	150
0.4	129
0.5	107
0.6	83
0.7	55
0.8	19

※*Other: defined as the non-Hispanic Asian/Pacific Islander and American Indian/Alaska Native;* &*Multiple: two or more distant metastasis sites.*
TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor-2; CSS, cancer-specific survival.

DISCUSSION

Nowadays, breast cancer has become the most frequent malignancy among women worldwide (1, 2, 20). While the overall survival (OS) rate in breast cancer patients has improved with the help of early-detection and multiple treatment modalities, patients who were diagnosed with DM at presentation still underwent a worse prognosis. In the last decades, great advances have been achieved in understanding and detecting breast cancer metastasis. Breast cancer was no longer regarded as a locoregional but systemic disease with an inherent feature of metastasis (8). There is no doubt that regional lymph node involvement is one of the important predictive factors in breast cancer DM. Even some scholars suggested and validated that regional node metastasis could precede metastatic dissemination (11). Notably, a considerable number of N0 patients were observed occurring *de novo* DM. With the wide application of circulating tumor cell (CTC) analysis, many scholars recognized that the DM was considered triggered by hematogenous spread of CTCs, rather than by lymphatic or direct intracavitary spread, which possibly occurred by a different mechanism. For this reason, breast cancer patients without regional lymph node metastasis but distant organ invasion would be the objects for exploring the underlying mechanisms.

However, only a few previous studies could be reviewed in predicting the risk factors and the prognosis of N0 patients (17, 18, 21). Herein, we provided a new insight in exploring whether there was a significant difference between N0 patients with DM or not, and the prognosis of those patients with DM was also evaluated. In this study, the incidence rate of DM in N0 patients was about 1.34% (1,069/79,746). Several clinicopathological factors including age at diagnosis, tumor size, race, tumor location, differentiation grade, histology, and subtype were significantly associated with DM. Younger patients (especially <40 years) have nearly twice the risk of DM than elderly patients, which was in accordance with Sabiani's report (22). Consistent with previous studies on evaluating the risk factors of DM in patients with invasive breast cancer, patients with tumor size (>10 mm), ILC, estrogen receptor (ER), and progesterone receptor (PR) as well as Her-2-positive subtype, and Black race (**Supplementary Figure S2**) presented a higher risk of DM (12, 14, 21). In terms of the tumor location, it has been determined that tumor location was significantly associated with the regional lymph node metastasis, especially when the tumor originated from the nipple and central location as well as overlapping of the breast (23–26). We took it a step further that the nipple and central tumor locations were identified had a higher risk of DM ($p<0.0001$) in N0 women. Despite that we have discovered seven independent risk factors associated with DM in N0 patients, further studies are needed to verify the underlying molecular mechanisms in promoting this complex process.

Notably, some researchers have conducted to explore the risk factors of DM and the prognosis of patients with DM at presentation (7, 13, 15, 16, 27). For instance, Rosa Mendoza determined that tumor stage, primary tumor size, and lymph node involvement were the major predictors of DM in adult breast cancer (14). Besides, the Black race and Her-2-enriched subtype were also identified as the risk factors of DM in a recent study (28–30). In the present study, we explored the prognostic factors of 1-, 3-, and 5-year CSS among 748 N0 patients with DM. Although the N0 women at a young age were more likely to have DM, compared with elderly women, the young population, however, had better long-term outcomes than the elderly

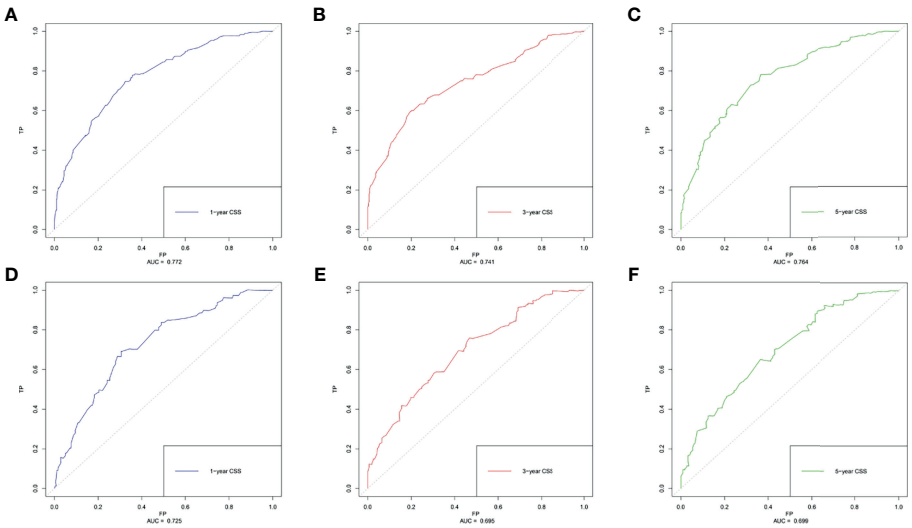

FIGURE 4 | The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC). **(A)** Predicting 1-year CSS in the training cohort; **(B)** predicting 3-year CSS in the training cohort; **(C)** predicting 5-year CSS in the training cohort; **(D)** predicting 1-year CSS in the validation cohort; **(E)** predicting 3-year CSS in the validation cohort; **(F)** predicting 5-year CSS in the validation cohort.

population (HR= 1:1.58; 95% CI: 1.03–2.44). This result was consistent with one recent large population-based epidemiological study in Brazil that young women had a lower rate of modality (31). On the contrary, in another study by Sabiani and colleagues, they concluded that patients at a young age (<35 years) had the lower estimated disease-free survival (DFS) and OS rate (22). These discrepancies might be due to the differences in sample size and patient inclusion criteria. For example, all included patients in their study were under 50 years old, while the patients in ours were at the age between 20 and 79 years old combined with negative lymph node status. Additionally, we determined five independent risk factors in the poor CSS probability of N0 patients with DM.

Moreover, the role of surgical treatment for the primary focus is regarded as a palliative surgery for patients with DM, and whether patients with DM can benefit from it remains controversial (32–35). One meta-analysis derived from two randomized controlled trials presented that there was no final conclusion about the role of surgery performed in breast cancer patients with DM at presentation (35). With further exploration, some studies, including the present study, found that locoregional surgery would improve the CSS and OS outcomes of metastatic breast cancer (15, 32, 34, 36). Indeed, there were still many questions on the discussion of the timing, type, and extension of the surgical procedures, which needed to be addressed in future works (33). Noticeably, compared with the previous study on evaluating the prognostic factors for patients with DM, primary tumor size (p=0.123) and grade differentiation (p=0.101) were not significantly associated with the CSS in the N0 population. In a similar studied population, Yu and his colleagues (29) determined that the larger tumor size was non-linear with the DM in N0 patients. They consequently believed the primary tumor biological features rather than the accumulated metastatic ability during tumor evolution likely determined the potential of distant dissemination, which indicated the indolent biological characteristics of the tumor. Accordingly, our results support this hypothesis but need further evaluation.

To visualize and more intuitively present the prognostic factors we determined for clinical use, the nomogram model was subsequently plotted. Markedly, in the nomogram, the breast cancer subtype accounted for a major part of the scoring system. Referencing similar nomograms for evaluating the prognostic of breast cancer

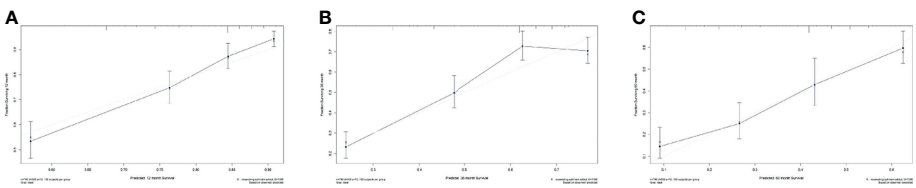

FIGURE 5 | Calibration curves for evaluating the accuracy of the nomogram. **(A)** 1-year CSS in lymph-node-negative women with DM, **(B)** 3-year CSS in lymph-node-negative women with DM, and **(C)** 5-year CSS in lymph-node-negative women with DM. The solid black line represents the performance of the nomogram, of which the closer fit to the gray line represents the better prediction of the nomogram we constructed. CSS, cancer-specific survival.

(37, 38), the TNBC subtype was determined to yield the highest score. Consequently, the clinicians could obtain the risk coefficient in 1-, 3-, and 5-year CSS probability. Compared with other recent works on evaluating the 3- and 5-year CSS in breast cancer women with bone metastasis, the C-index of the present nomogram was 0.694, which was higher than Liu's (0.660) (16) and very close to the C-index of nomograms developed by Wang (0.705) (15) and Zhao (0.723) (37), confirming the promising discrimination of our model. To evaluate the accuracy of the nomogram, an independent cohort was subsequently used for validation. Expectedly, the AUC of the 1-, 3-, and 5-year CSS predicting ROC in the validation cohort reached 0.725, 0.695, and 0.699, respectively, which further proved the utility of our model to be applied to access the long-term CSS in this subpopulation. Besides, compared with the study of Wang and colleagues (17), the number of N0 patients in the training cohort of the present study was considerably large (748 *vs* 286). Different from previous studies of N0 patients with a focus on gene expression profiles (17, 18), we provide a new insight in accessing the individual risk of DM and long-term CSS probability in N0 patients based on the clinicopathological characteristics. For instance, a 65-year-old black woman was diagnosed with HER2+ tumor, with only bone involvement. This patient would have a total of 125 points and an estimated 1-year, 3-year, and 5-year CSS of 84, 62, and 41% probability after surgery.

Alternatively, this study has some limitations that have to be addressed in the future works. First, this is a retrospective study in which selection bias inevitably exists. Second, while the SEER database contains approximately 28% population-based cancer registration data, some significant confounding prognostic factors including but not limited to Ki-67 index (39), BRCA1- and BRCA2-related mutation (40, 41), as well as high 21-Gene Recurrence Score (21-GRS) (42), which have been proved to be related to worse survival in patients with breast cancer, are unavailable in the SEER database. Third, further information about adjuvant management of these patients was not reported in the present study, as these data were limited in the SEER database. Consequently, future works are supposed to fill this gap to get robust clinical evidence. Besides, with the technical advances in multidisciplinary management, the CSS in patients with breast cancer would increase in the future, which could influence the predictive ability of the model. Lastly, another weakness of this study is the lack of an external validation cohort, which limits further enforcing the reliability and clinical application of the nomogram. Thus, more external validation cohorts from multicenter and countries are urgently demanded to further evaluate the feasibility of our nomogram.

CONCLUSION

In summary, this study first identified the potential risk clinicopathological characteristics of DM in N0 patients and the prognostic factors in patients with DM at presentation. N0 patients with younger age at diagnosis, larger tumor size, central tumor location, Black race, poorer differentiated grade, ILC, and luminal B subtype have the highest risk of DM, which could help clinicians to avoid underestimating the risk of DM and subsequent undertreatment in N0 patients. However, DM patients with elderly age at diagnosis, TNBC subtype, and multiple metastasis sites have the worst prognosis. Besides, the novel validated nomogram could help clinicians to better stratify patients who are at high risk of cancer-specific death for receiving relatively aggressive treatment and management. Meanwhile, we propose more external validation to further strengthen our findings.

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

Ethical approval was waived by the local Ethics Committee of the Chongqing Medical University in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

AUTHOR CONTRIBUTIONS

All authors contributed to conception and design of the study. YM, XL, and HC organized the database. YM, YF, JC, KX, GY, and YH performed the statistical analysis. All authors wrote the first draft of the manuscript. All authors wrote sections of 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/fendo.2021.771226/full#supplementary-material

Supplementary Figure 1 | The ROC curve and AUC of the risk factors in promoting distant metastasis in lymph-node-negative women.

Supplementary Figure 2 | The KM survival curves for predicting the CSS of lymph-node-negative women with DM according to race.

Supplementary Figure 3 | The KM survival curves for predicting the CSS in the different risk population, based on the risk stratification of the nomogram.

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Cancer-Specific Survival Outcome in Early-Stage Young Breast Cancer: Evidence From the SEER Database Analysis

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Background: Young women with breast cancer are determined to present poorer survival compare with elderly patients. Therefore, identifying the clinical prognostic factors in young women with early-stage ($T_{1-2}N_{0-1}M_0$) breast cancer is pivotal for surgeons to make better postoperative management.

Methods: The clinicopathological characteristics of female patients with early-stage breast cancer from the Surveillance, Epidemiology, and End Results program between Jan 2010 and Dec 2015 were retrospectively reviewed and analyzed. Univariate and multivariate Cox regression analyses were used to determine the potential risk factors of cancer-specific survival in young women with early-stage breast cancer. The nomogram was constructed and further evaluated by an internal validation cohort. The Kaplan-Meier survival curves were used to estimate cancer-specific survival probability and the cumulative incidence.

Results: Six variables including race, tumor location, grade, regional lymph node status, tumor subtype, and size were identified to be significantly associated with the prognosis of young women with early-stage breast cancer during the postoperative follow-up. A nomogram for predicting the 3-, 5- year cancer-specific survival probability in this subpopulation group was established with a favorable concordance index of 0.783, supported by an internal validation cohort with the AUC of 0.722 and 0.696 in 3-, 5- year cancer-specific survival probability, respectively.

Conclusions: The first predictive nomogram containing favorable discrimination is successfully established and validated for predicting the 3-, 5- year cancer-specific survival probability in young women with early-stage breast cancer during the postoperative follow-up. This model would help clinicians to make accurate treatment decisions in different clinical risk population.

Keywords: breast cancer, nomogram, cancer-specific survival, early-stage, SEER

INTRODUCTION

Breast cancer has become the most frequently diagnosed malignancy and one of the leading causes of cancer-specific death in China and around the world (1–4), with a female predominance. Although young women (usually refers to <40 years) made up only a limited proportion of breast cancer, a similar increasing prevalence was also observed in this subpopulation (5, 6). Among adolescents and young adults, the overall cancer mortality declined over the past few decades by 1% annually across age and sex groups. However, the rates were stable in young female patients aged between 30-39 years because of a flattening of declines in female breast cancer (5). According to the latest report from the American Cancer Society (5), the breast cancer incidence rate was 0.1, 5.7, and 46.6 per 100,000 population among young women aged between 15-19 years, 20-29 years, and 30-39 years, respectively. Furthermore, approximately 43% and 7% of young women were diagnosed at the regional or distant stage and the breast cancer-specific death reached 22% among women aged 15-39 years.

Compared with elderly women, young patients, accounting for a relatively small number of breast cancer, were more likely to present aggressive subtypes and advanced disease, and the survival and outcomes were worse (5-year relative survival rate comparison: 86% vs 91% in elderly women) (5). Consequently, the strategies of treatment and prevention for young breast cancer have gradually aroused wide attention (7–11). Notably, in one study from China, young patients with breast cancer were more likely to have larger tumor size, poorer differentiation grade, a higher proportion of triple-negative breast cancer (TNBC), and more advanced stage, when compared with elder patients (11).

Currently, only a small number of listed studies were focused on investigating the risk factors for predicting the clinical outcomes in young women with breast cancer (10, 12). For instance, based on the clinicopathological features of the large-scale population, Billena et al. (9) determined that tumor size, hormone receptor status, surgery, adjuvant therapies, lymph node status, and race were independent predictive factors for overall survival (OS) in young female breast cancer. To the best of our knowledge, however, neither systematic attempts have ever been made to explore the risk factors for predicting the cancer-specific survival (CSS) in young women with early-stage ($T_{1-2}N_{0-1}M_0$) breast cancer nor develop prognostic nomograms. Therefore, in this present study, we aim to investigate the independent prognostic factors for CSS of young women with early-stage breast cancer and further construct and validate a visualized predictive model for clinicians to identify the patients with high risk and make better-individualized management (relatively more aggressive treatment approaches) for these patients.

MATERIALS AND METHODS

Data Source

The data we analyzed were extracted from the Surveillance, Epidemiology, and End Results (SEER) 18 registry research database, which represented approximately 28% of the U.S. population and included various ethnic groups. For this study, we signed the SEER research data agreement to access SEER information with the reference of the username "10189-Nov2020". Data were collected following the approved guideline. Data analysis from this database is considered to be non-human subjects by the Office for Human Research Protection as part of the US Department of Health and Human Services, because patient data was anonymized and publicly available. For these reasons, the need for ethics approval was waived by The Second Affiliated Hospital of Chongqing Medical University Ethics Committee.

Patients who met the following criteria were included: 1) young female patients between the age of 18 and 40 years; 2) diagnosis year between 2010 and 2015; 3) the diagnosis of breast cancer was confirmed by histopathology; 4) TNM stage was derived from the AJCC staging system 7th edition. The excluding criteria: 1) No regional lymph node examined; 2) Patients were diagnosed with distant metastasis; 3) Patients coexisted with one or more cancers; 4) Incomplete medical records.

Variables Evaluation and Definition

After excluding the unqualified cases, there were 7203 young female patients with invasive breast cancer enrolled in this retrospective cohort study. The included patients were randomly divided into a training group and validating group at a ratio of 7:3. The following clinicopathological characteristics were collected and transformed into categorical variables: age (>18 and <40 years), race (Hispanic, non-Hispanic White, non-Hispanic Black, non-Hispanic Asian or Pacific Islander, and non-Hispanic American Indian/Alaska Native), laterality (right and left origin of primary), stage (I and II derived from AJCC staging system 7th edition), grade (well differentiated, moderately differentiated, poorly differentiated), location (central, outer, inner, overlapping and axillary of breast), histological type (infiltrating ductal carcinoma (IDC), infiltrating lobular carcinoma (ILC), and infiltrating ductal mixed lobular carcinoma (IDLC)), ICD-O-3 codes: 8500/3, 8503/3, 8507/3, 8500/3, 8520/3, 8521/3, and 8522/3), regional lymph node status (N0: no regional lymph node metastasis; N1: 1-3 axillary lymph nodes metastasis and/or internal mammary lymph node metastases), the number of regional nodes examined and positive nodes, breast cancer subtype (Luminal A: hormonal receptor (HR)+/Her-2-, Luminal B: HR+/Her-2+, Triple-negative: HR-/Her-2-, Her-2 enriched: HR-/Her-2 2+), tumor size (T_1: >0mm and ≤20mm, T_2: >20mm and ≤50mm), cause-specific death, and survival months (more than 0 days of survival).

Statistical Analysis

The primary endpoint of this study was breast cancer-specific death during the follow-up. A two-tail P-value of <0.05 was defined as the criterion for variable deletion when performing backward stepwise selection. The development and validation of the nomogram, calibration curve, and Kaplan-Meier analysis were based on the results of the multivariate Cox regression analysis using the "survival", "rms", "survminer", and "foreign" packages of the R software (R Foundation, Vienna, Austria, version 3.5.2,

http://www.r-project.org). The area under the receiver (AUC) operating characteristic (ROC) curve and the Harrell's C-index (an important indicator to estimate the discrimination capability of each prognostic model and to compare their prognostic performance) (13) are conducted to assess the feasibility of the present nomogram.

RESULTS

Clinicopathological Characteristics of Patients

From the SEER database, a total of 7203 young female patients aged between 18 and 40 years old with early-stage breast cancer were ultimately included in this study and further randomized into training (5042 cases) and validating (2161 cases) cohorts at a ratio of 7:3. A majority of patients were white which accounted for 53.6% in the training cohort and 54.4% in validating cohort, respectively. Besides, the foremost proportion of differentiated grade was in grade III (poorly differentiated) with a ratio of 57.4%, whereas grade I (well-differentiated) and II (moderately-differentiated) were presented in only 8.1% and 34.9% of patients. Notably, there were more than half of patients diagnosed with breast cancer at T_2 tumor size, and approximately 40% of patients suffered from regional lymph node metastasis. The median and average follow-up months in the training cohort were 52 and 48 months (a range of 0- 65 months), respectively. The specific demographic and clinical characteristics of the patients in the training and validation datasets were summarized in **Table 1**.

Univariate and Multivariate Analyses of the Risk Factors of CSS

To screen out the potential independent risk factors of CSS in young women with early-stage breast cancer during the postoperative follow-up, only significant factors from univariate Cox regression analysis were further applied into multivariate Cox

TABLE 1 | Clinicopathological characteristics of young women with early-stage breast cancer.

Variables	No. (%) of Patients		
	Initial Cohort (n = 7203)	Training Cohort (n = 5042)	Validating Cohort (n = 2161)
Age (years)			
Mean ± SD	34.8 ± 3.8	34.8 ± 3.7	34.8 ± 3.8
Race			
Hispanic	1369 (19.0)	951 (18.9)	418 (19.3)
White	3879 (53.8)	2704 (53.6)	1175 (54.4)
Black	962 (13.4)	687 (13.6)	275 (12.7)
※Other	993 (13.8)	700 (13.9)	293 (13.5)
Location			
central	288 (4.0)	209 (4.1)	79 (3.6)
outer	1548 (21.5)	1089 (21.6)	459 (21.3)
inner	3552 (49.3)	2509 (49.8)	1043 (48.3)
¶other	1815 (25.2)	1235 (24.5)	580 (26.8)
Grade			
well	584 (8.1)	387 (7.7)	197 (9.1)
moderate	2516 (34.9)	1762 (34.9)	754 (34.9)
poor	4103 (57.0)	2893 (57.4)	1210 (56.0)
Histology			
IDC	6786 (94.2)	4765 (94.5)	2021 (93.5)
ILC	157 (2.2)	108 (2.1)	49 (2.3)
IDLC	260 (3.6)	169 (3.4)	91 (4.2)
Laterality			
right	3604 (50.0)	2556 (50.7)	1048 (48.5)
left	3599 (50.0)	2486 (49.3)	1113 (51.5)
¥**Stage**			
IA	2472 (34.3)	1692 (33.6)	780 (36.1)
IB	241 (3.3)	180 (3.6)	61 (2.8)
IIA	2703 (37.5)	1924 (38.2)	779 (36.0)
IIB	1787 (24.8)	1246 (24.7)	541 (25.0)
Tumor size (mm)			
T_{1a}	299 (4.1)	217 (4.3)	82 (3.8)
T_{1b}	617 (8.6)	428 (8.5)	189 (8.7)
T_{1c}	2571 (34.9)	1771 (35.12)	800 (37.0)
T_2	3716 (51.6)	2626 (52.1)	1090 (50.4)
Lymph node status			
N_0	4401 (61.1)	3072 (60.9)	1329 (61.5)
N_1	2802 (38.9)	1970 (39.1)	832 (38.5)
ER status			

(Continued)

TABLE 1 | Continued

Variables	No. (%) of Patients		
	Initial Cohort (n = 7203)	Training Cohort (n = 5042)	Validating Cohort (n = 2161)
negative	1989 (27.6)	1405 (27.9)	584 (27.0)
positive	5214 (72.4)	3637 (72.1)	1577 (73.0)
PR status			
negative	2629 (36.5)	1856 (36.8)	773 (35.8)
positive	4574 (63.5)	3186 (63.2)	1385 (64.1)
Her-2 status			
negative	5473 (76)	3825 (75.9)	1648 (76.3)
positive	1730 (24)	1217 (24.1)	513 (23.7)
Subtype			
Luminal A	4026 (55.9)	2802 (55.6)	1224 (56.6)
Luminal B	1332 (18.5)	939 (18.6)	393 (18.2)
TNBC	1447 (20.1)	1023 (20.2)	424 (19.6)
Her-2 enriched	398 (5.5)	278 (5.5)	120 (5.5)

※Other: defined as the non-Hispanic Asian/Pacific Islander and American Indian/Alaska Native; ¶other: axillary and overlapping of the breast; ¥Stage: derived from the AJCC 7th guideline. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer; Her-2, human epidermal growth factor receptor-2; CSS, cancer-specific survival.

regression analysis (**Table 2**). During the univariate Cox regression analysis, race (p<0.001), tumor location (p=0.028), grade (p<0.0001), lymph node status (p<0.0001), subtype (p<0.0001), and tumor size (p<0.0001) were identified to be the independent predictive factors. The black race (hazard ratio (HR) =1.52, 95% confidence interval (CI): 1.05- 2.20, p=0.001), inner location (HR=2.36, 95%CI: 1.02- 5.46, p=0.003), moderately and poorly grade (HR=8.86, 95%CI: 1.22- 64.26, HR=14.04, 95%CI: 1.95- 101.08, respectively, p<0.0001), lymph node metastasis (HR=2.233, 95%CI: 1.81- 3.01, P<0.0001), TNBC (HR=2.28, 95%CI: 1.71- 3.03, p<0.0001), and T_2 (HR=2.09, 95%CI: 1.56- 2.81, p<0.0001) were regarded as the independent risk factors of CSS in these patients.

Furthermore, to actuarially estimate the survival probability and cumulative hazard in patients with different variables after surgery, we selected four factors ($p \leq 0.001$) from multivariate analysis in Cox proportional hazard models to plot the Kaplan–Meier survival curves and cumulative hazard.

TABLE 2 | Univariate and multivariate Cox regression analyses of predictive variables correlated with CSS in young women with early-stage breast cancer.

Variables	Subgroup	Univariable		Multivariable	
		Hazard Ratio	*P*	Hazard Ratio	*P*
Race	Hispanic	Reference	**<0.001**	Reference	0.001
	White	0.74 (0.54- 1.03)		0.89 (0.64- 1.24)	
	Black	1.60 (1.10- 2.31)		1.52 (1.05- 2.20)	
	※Other	0.52 (0.32- 0.87)		0.62 (0.37- 1.02)	
Location	&central	Reference	**0.028**	Reference	0.003
	inner	2.38 (1.03- 5.48)		2.36 (1.02- 5.46)	
	outer	1.63 (0.71- 3.70)		1.41 (0.62- 3.23)	
	¶other	1.97 (0.85- 4.55)		1.97 (0.85- 4.57)	
Grade	well	Reference	**<0.0001**	Reference	0.001
	moderately	10.90 (1.50- 78.94)		8.86 (1.22- 64.26)	
	poorly	27.89 (3.91- 198.95)		14.04 (1.95- 101.08)	
Lymph node status	N_0	Reference	**<0.0001**	Reference	<0.0001
	N_1	2.21 (1.72- 2.83)		2.233 (1.81- 3.01)	
Histology	IDC	Reference	0.431	/	
	ILC	0.54 (0.17- 1.68)			
	IDLC	0.74 (0.35- 1.57)			
Subtype	Luminal A	Reference	**<0.0001**	Reference	<0.0001
	Luminal B	0.49 (0.30- 0.81)		0.40 (0.24- 0.66)	
	TNBC	3.11 (2.40- 4.04)		2.28 (1.71- 3.03)	
	Her-2	1.25 (0.70- 2.23)		0.97 (0.54- 1.74)	
Size	T_1	Reference	**<0.0001**	Reference	<0.0001
	T_2	3.14 (2.35- 4.19)		2.09 (1.56- 2.81)	

※Other: defined as the non-Hispanic Asian/Pacific Islander and American Indian/Alaska Native; &central: central portion of breast combined with nipple; ¶other: axillary and overlapping of the breast. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; TNBC, triple-negative breast cancer; Her-2, human epidermal growth factor receptor-2; T_1, 0mm≥maximum diameter <20mm; T_2, 20mm≥maximum diameter <50mm. Bold values indicate statistical significance (p<0.05).

Specifically, a significant decrease in cumulative survival rate was observed in TNBC patients, compared with Luminal A, Luminal B, and Her-2 enriched subtypes (3- year CSS: 92.8% vs 98.8% vs 99.3% vs 96.9%; 5- year CSS: 85.9% vs 94.7% vs 97.3% vs 93.6%, p<0.0001, **Figure 1A**). Similarly, tumor size (T_1 vs T_2: 3- year CSS: 99.0% vs 96.3%; 5- year CSS: 96.7% vs 90.1%, p<0.0001, **Figure 1B**), differentiation grade (well vs moderately vs poorly: 3- year CSS: 99.6% vs 99.0% vs 96.4%; 5- year CSS: 99.6% vs 96.1% vs 90.9%, p<0.0001, **Figure 1C**), and regional lymph node status (N_0 vs N_1: 3- year CSS: 98.4% vs 96.3%; 5- year CSS: 95.5% vs 90.0%, p<0.0001, **Figure 1D**) were all associated with the cumulative survival probability. Moreover, the cumulative incidence of cancer-specific death increased to 0.15 in TNBC (**Figure 2A**), 0.1 in T_2 (**Figure 2B**), 0.1 in poorly differentiated grade (**Figure 2C**), and 0.11 in N_1 (**Figure 2D**), respectively.

Predictive Nomogram Construction and Validation

Based on the results of multivariate Cox regression analysis, six independent variables including race, tumor location, grade,

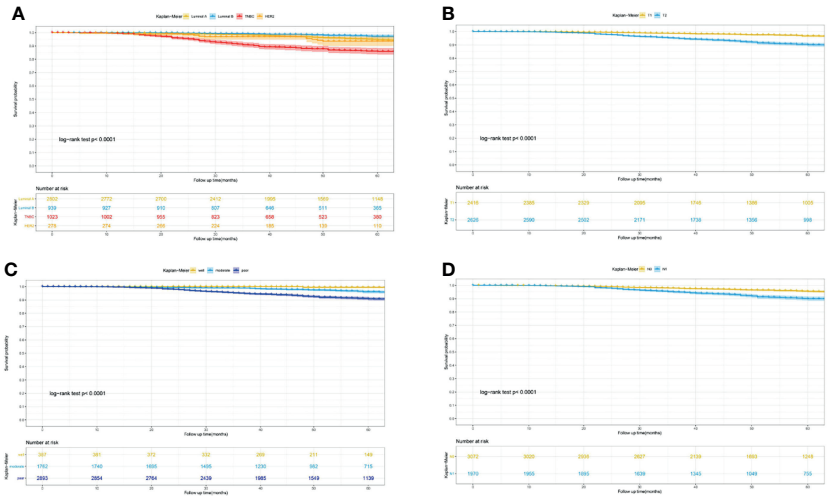

FIGURE 1 | Kaplan-Meier curves for predicting the 3-, 5- year CSS of young women with early-stage breast cancer. **(A)** different molecular subtype; **(B)** tumor size; **(C)** differentiation grade; **(D)** lymph node status. CSS, cancer-specific survival.

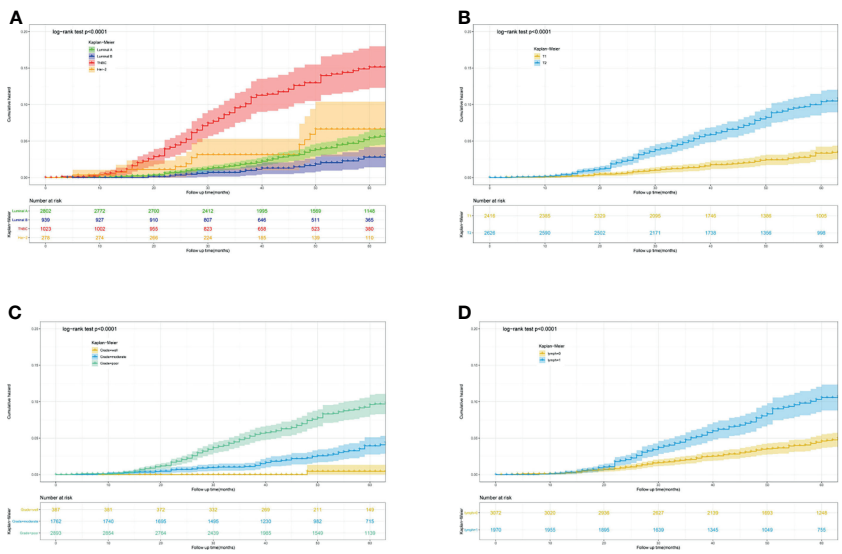

FIGURE 2 | Predicting the 3-, 5- year cumulative hazard of cancer-specific death risk in young women with early-stage breast cancer. **(A)** different molecular subtype; **(B)** tumor size; **(C)** differentiation grade; **(D)** lymph node status.

lymph node status, subtype, and tumor size were screened out for establishing a visualized nomogram to predict the 3- year and 5- year CSS in young women with early-stage breast cancer (**Figure 3**). The model contained a satisfying C-index of 0.783, combined with an AUC of 0.708 in predicting 3- year CSS (**Figure 4A**) and 0.703 in predicting 5- year CSS (**Figure 4B**), respectively. The specific value of each variable was calculated in **Table 3**. Thus, patients could obtain individualized total scores based on their clinicopathological characteristics (race, tumor location, differentiation grade, N stage, molecular subtype, and tumor size) and the corresponding 3-, 5-year CSS probability. Moreover, the accuracy of our nomogram was validated by an internal validation cohort with 2161 cases. The results in the validating cohort also presented good discrimination with an AUC of 0.722 in predicting 3- year CSS (**Figure 4C**) and an AUC of 0.696 in predicting 5- year CSS (**Figure 4D**), respectively. Furthermore, a calibration curve for evaluating the accuracy of the predictive ability in 3- year CSS (**Figure 5A**) and 5- year CSS of young women with early-stage breast cancer was also displayed (**Figure 5B**), which indicated a great agreement in the training data set.

DISCUSSION

Over the past years, with an increasing prevalence of breast cancer worldwide (1–4, 6), the comprehensive and individualized management for patients with this particular disease has gradually attracted much attention, especially in terms of young breast cancer (7, 9, 10, 14, 15). It was believed that age was an independent prognostic indicator among female breast cancer and younger age, especially under the age of 40 years, frequently presented a higher risk of locoregional metastasis, recurrence, and ultimately worse OS outcomes (7, 10, 12). Indeed, while surgery remained to be the first-line strategy in the management of breast cancer, great changes have taken place in terms of the surgical extension (8, 16), especially with the wide application of neoadjuvant chemotherapy (17, 18) and postoperative radiation therapy (19) for early-stage breast cancer. Some scholars even suggested that patients with clinical complete response (cCR) after neoadjuvant chemotherapy might be exempted from the subsequent surgery because there was no significant difference in 5- year OS between patients with cCR and those with pathological complete response (pCR) (20). Furthermore, recent studies have confirmed that breast-conserving surgery would not affect the OS or DFS in young women with early-stage breast cancer, even though it did not improve the long-term survival in this subpopulation like the old population, as compared with mastectomy (8, 16, 21). Additionally, identifying more risk clinical factors and their effects on the prognosis of young women with early-stage breast cancer was equally important for predicting the 3- and 5- CSS. Herein, we retrospectively evaluated the clinicopathological characteristics of young women with early-stage breast cancer from the SEER database.

In our study, six variables including race, tumor location, grade, lymph node status, molecular subtype, and primary tumor size were independent prognostic factors in predicting the CSS of early-stage breast cancer in young women. Specifically, in accordance with the results of previous epidemiological studies, we found young Black women had worse CSS and a higher risk of cumulative incidence, compared with other races. As showed in the results of *Cancer Statistics for Adolescents and Young Adults* (2020 version) (5), young Black women had higher incidence rates of breast cancer than White as well as higher death rates (25.9 vs 22.3 per 100,000 population, 3.9 vs 2.0 per 100,000 population during 2012-2016, respectively). Most recently, Walsh et al. (7) examined a large-scale population of young Black women with breast cancer and they confirmed the young Black women had worse disease-free survival (DFS) than old women, but OS was not significantly different. On the contrary,

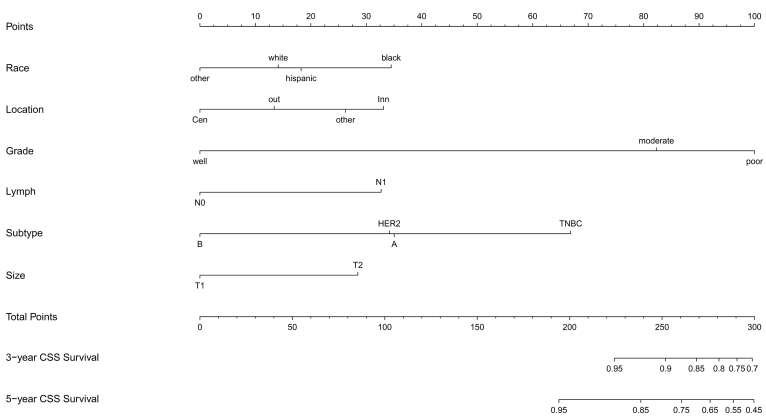

FIGURE 3 | Nomogram for predicting the 3-,5- year CSS in young women with early-stage breast cancer. Note: other: defined as the non-Hispanic Asian/Pacific Islander and American Indian/Alaska Native; CSS, cancer-specific survival; A, Luminal A; B, Luminal B; TNBC, triple-negative breast cancer; HER2, Her-2 enriched.

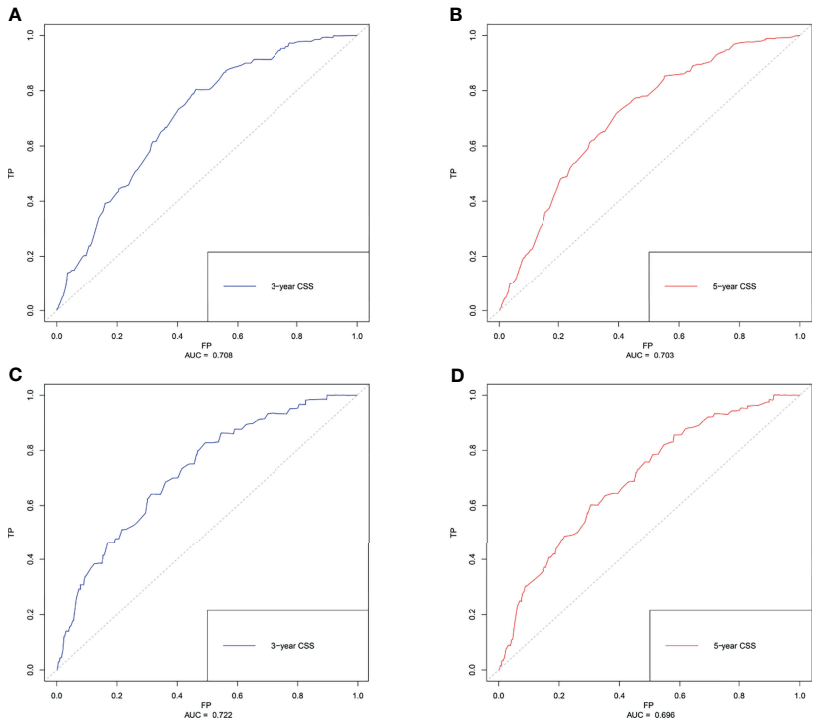

FIGURE 4 | The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC). **(A)** predicting 3- year CSS in the training cohort; **(B)** predicting 5- year CSS in the training cohort. **(C)** predicting 3- year CSS in the validating cohort. **(D)** predicting 5- year CSS in the validating cohort. CSS, cancer-specific survival.

TABLE 3 | The specific value of each variable in the nomogram derives from the multivariate Cox logistic regression results.

Characteristics	Score
Race	
Hispanic	18
White	14
Black	34
※Other	0
Tumor location	
&Central	0
Inner	33
Outer	13
¶other	26
Grade	
well	0
moderately	82
poorly	100
Lymph status	
N_0	0
N_1	33
Size	
T_1	0
T_2	28
Subtype	
Luminal A	35
Luminal B	0
TNBC	67
Her-2 enriched	34
Total point for 3-year CSS	
0.7	299
0.75	291
0.80	281
0.85	269
0.90	252
0.95	224
Total point for 5-year CSS	
0.45	300
0.50	294
0.55	289
0.60	283
0.65	276
0.70	269
0.75	260
0.80	251
0.85	239
0.90	222
0.95	194

(Continued)

TABLE 3 | Continued

※Other: defined as the non-Hispanic Asian/Pacific Islander and American Indian/Alaska Native; &central: central portion of breast combined with nipple; ¶other: axillary and overlapping of the breast. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IDLC, invasive ductal mixed with lobular carcinoma; TNBC, triple-negative breast cancer; Her-2, human epidermal growth factor receptor-2; T_1, 0mm≥maximum diameter <20mm; T_2, 20mm≥maximum diameter <50mm.

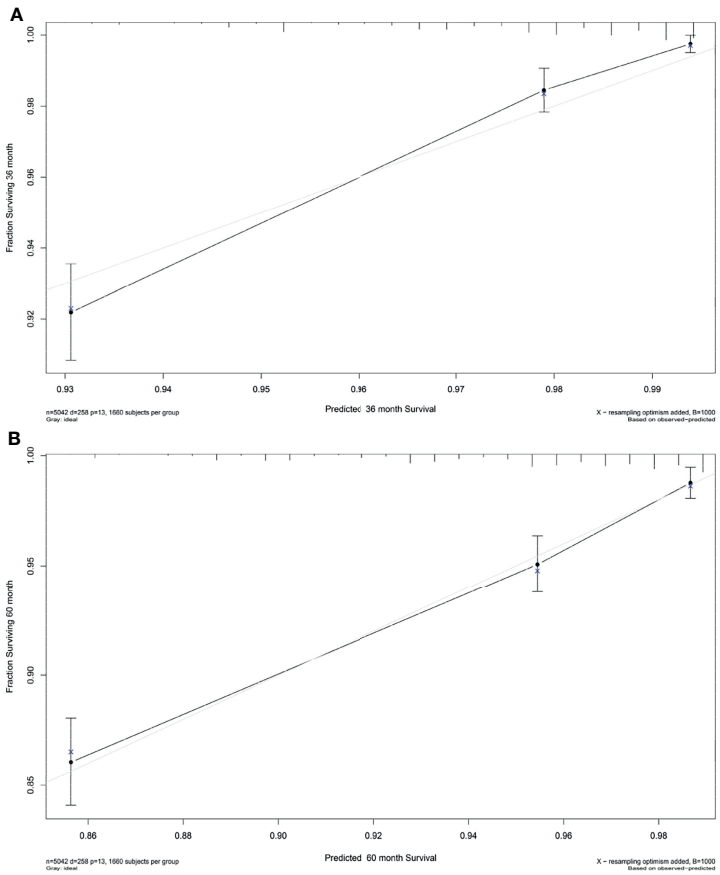

FIGURE 5 | Calibration plot for the prediction of **(A)** 3-year CSS and **(B)** 5-Year CSS in young women with early-stage breast cancer. The solid black line represented the performance of the nomogram, of which the closer fit to the gray line represents the better prediction of the nomogram we constructed. CSS, cancer-specific survival.

this divergence of OS or CSS in different races were disappeared among female patients with bone metastasis at presentation (p=0.282, p=0.413, respectively) (22). Interestingly, we determined that the inner location of the primary tumor was associated with relatively poor CSS in young women with early-stage breast cancer (p=0.003). Our finding was consistent with the conclusions of two recent studies from China (23, 24). Yang et al. (24) and Wu et al. (23) both highlighted that lower inner tumor location presented significantly lower DFS or recurrence-free survival (RFS) in young women with early-stage breast cancer. In contrast, central or nipple tumor location was regarded as the risk of axillary lymph node metastasis (25, 26) regardless of the stage of patients at presentation. On the other hand, poorer differentiation grade, triple-negative molecular subtype, lymph node metastasis, and large tumor size have frequently indicated the poor survival of breast cancer (22, 27–29). In our study, we determined similar findings to previous studies (**Table 2**), in which moderately or poorly grade, TNBC subtype, N_1 status, and T_2 tumor size all played a pivotal role. Notably, the pathological type in our study comprised a spectrum of IDC (94.2%), ILC (2.2%), and IDLC (3.6%). However, we did not find any significant difference among these subtypes (p=0.431).

Currently, nomograms have been used for conveniently predicting the outcomes of many kinds of cancers (30–33) which significantly improved the process of individualized medical decision-making. Regarding breast cancer, some previous studies have developed survival predicting models for those with N_3 status, HR-/Her-2-, or IV stage (22, 27, 29, 34). Although those models contained good predictive ability, yet the patients they enrolled have already been in a dangerous situation which should be recommended to elect relatively more aggressive treatment modalities. On the contrary, while young breast cancer often presents a worse prognosis than older patients, whether radical treatments should also be recommended for this subpopulation with early-stage breast cancer was rarely exemplified (28, 35). Therefore, we established the first nomogram for predicting the 3- year, 5- year CSS in young women with early-stage breast cancer. As expected, the nomogram showed sufficient discrimination ability with a C-index of 0.783. The AUC of 3- year CSS and 5- year CSS in both training and validating cohorts were close and even higher than 0.7, which indicated the satisfied prediction capacity.

Furthermore, the calibration plot also demonstrated the high agreement of this model.

Admittedly, there were still some limitations that have to be addressed in the future study. First, although the sample size of this study was considerable, the character of the retrospective design was inevitably flawed with bias. Second, some clinical risk factors like Ki-67 index (36, 37) and BRCA1- and BRCA2- related mutation (15, 38) as well as high 21-Gene Recurrence Score (21-GRS) (39) which have been proved to be related to worse OS in patients with breast cancer were unavailable in the SEER database. Third, the detailed information of hormone receptors (estrogen receptor and progesterone receptor) and Her-2 status were unavailable in the SEER database. Thus, further study should be performed to fill this gap and make more accurate calculations to better define the molecular subtype of patients in accordance with the latest guideline (40, 41). Furthermore, we did not investigate the role of adjuvant chemotherapy or radiotherapy in young women with early-stage breast cancer because of the unknown chemotherapy regimens and the scope of radiotherapy in these patients. Fourth, the information of insurance and socioeconomic status which were determined to be associated with the young breast cancer-specific survival in other studies (14, 42) was also missing. Those important variables should be considered in future research and further prospective randomized controlled studies are urgently needed to obtain more detailed strategies on the field.

CONCLUSION

In summary, we identified six variables including race, tumor location, differentiation grade, lymph node status, molecular subtype, and primary tumor size were independent prognostic factors in predicting the CSS of early-stage breast cancer in young women. Based on these variables, we successfully established the first nomogram for predicting the 3-, 5- year CSS in this particular group and could help clinicians better distinguish the young breast cancer patients at high risk.

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 STATEMMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

AUTHOR CONTRIBUTIONS

Conception and design: RL, HL, GY, YF. (II) Administrative support: YM, YF. Provision of study materials or patients: YM, YF. Collection and assembly of data: RL, ZX. Data analysis and interpretation: All authors. Manuscript writing: All authors. Final approval of manuscript: All authors.

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Identifying and Validating of an Autophagy-Related Gene Signature for the Prediction of Early Relapse in Breast Cancer

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Background: Compelling evidence has demonstrated the pivotal role of autophagy in the prognosis of breast cancer. Breast cancer (BC) patients with early relapse consistently exhibited worse survival.

Methods: The autophagy-related genes were derived from the Human Autophagy Database (HADb) and high-sequencing data were obtained from The Cancer Genome Atlas (TCGA). Discrepantly expressed autophagy genes (DEAGs) between early relapse and long-term survival groups were performed using the Linear Models for Microarray data (LIMMA) method. Lasso Cox regression analysis was conducted for the selection of the 4-gene autophagy-related gene signature. GSE42568 and GSE21653 databases were enrolled in this study for the external validation of the signature. Then patients were divided into high and low-risk groups based on the specific score formula. GSEA was used to discover the related signaling pathway. The Kaplan-Meier curves and the receiver operating characteristic (ROC) curves were used to evaluate the discrimination and accuracy of the 4-gene signature.

Results: A signature composed of four autophagy-related mRNA including APOL1, HSPA8, SIRT1, and TP73, was identified as significantly associated with the early relapse in BC patients. Time-dependent receiver-operating characteristic at 1 year suggested remarkable accuracy of the signature [area under the curve (AUC = 0.748)]. The risk score model based on the autophagy-related signature showed favorable predicting value in 1-, 2-, and 3-year relapse-free survival (RFS) in training and two validating cohorts. The GSEA displayed gene sets were remarkably enriched in carcinogenic activation pathways and autophagy-related pathways. The nomogram involving three variables (progesterone receptor status, T stage, and 4-gene signature) exhibited relatively good discrimination with a C-index of 0.766.

Conclusions: Our study establishes an autophagy-related 4-gene signature that can effectively stratify the high-risk and low-risk BC patients for early relapse. Combined with the clinicopathological variables, the signature could significantly help oncologists tailor more efficient treatment strategies for BC patients.

Keywords: breast cancer, early relapse, autophagy, signature, nomogram

INTRODUCTION

Breast cancer (BC) is currently the most frequent malignancy and one of the leading causes of cancer death in the United States (estimated 279,100 new cases and 42,690 death) (1) and China mainland (estimated 304,000 new cases and 70,000 death) (2). Although the long-term survival of patients with BC has been significantly increased in the past years with the application of targeted therapy (3), endocrine therapy (4), and even immunotherapy (5, 6), early relapse (2 years after initial treatment) with metastasis could reverse this favorable outcome (7, 8). Regardless of the prognosis, all women with BC are at risk for early recurrence. According to a recent review report, nearly 50% of early recurrences occur within 5 years of surgery, and they peak at 2 years after surgery in women treated with adjuvant tamoxifen (9). Besides, early relapse in BC patients is frequently associated with poor clinicopathological features [such as young age (10), late TNM stage, poor differentiation grade, and worse histopathological type (11, 12)] and resistance to adjuvant chemotherapy or endocrine therapy (13–16). Those cases who developed early relapse consistently tended to have poorer long-term survival rates. Notably, a recent study has demonstrated that BC patients experienced altered hormone receptor and HER2 status throughout tumor progression, which significantly influences survival (17). Thus, for the great heterogeneity of BC, the prognosis varies significantly in BC patients with the same stage and comparable clinicopathological features. For this reason, hall markers and other biological indicators could help to predict the recurrence of BC (18).

Autophagy is a routine physiological process associated with aging and human disease *via* guiding the degradation of damaged, denatured, or senescent proteins and organelles in lysosomes (19, 20). Accordingly, compelling evidence has demonstrated that autophagy plays a pivotal role in tumor growth, metastasis, and recurrence of BC, which could maintain the homeostasis and the survival of BC cells by removing dysfunctional or unnecessary substances (21–24). On the other hand, accumulating evidence showed that autophagy-related genes were significantly involved in the regulations of the autophagy process. For instance, recent two basic research demonstrated that MTA1 (metastasis-associated 1) (13) and long noncoding RNA H19 (14) were the regulators of autophagy in resistance to the endocrine therapy (tamoxifen). However, Marsh et al. (25) discovered autophagy could inhibit the metastasis of BC cells by accumulating the autophagy cargo receptor (ACR), neighbor to BRCA1. A number of coding RNA (mRNA) and non-coding RNAs (microRNA, lncRNA, and circRNA) signatures have been identified for predicting the proliferation and prognosis of BC patients (26–30). Nonetheless, most of these signatures focused on overall survival, there is still a lack of work on investigating the impact of mRNA on the relapse-free survival of BC, and none of the previous studies have concentrated on early relapse. Therefore, identifying autophagy-related mRNA signature could not only easily help oncologists classify the BC patients with a high risk of early relapse but also make more efficient therapeutic modalities at an earlier stage of a patient's treatment (31).

In the present study, we conducted an autophagy-related 4-gene signature to predict the early relapse of BC patients and construct a nomogram for predicting the 1-, 2-, and 3-year RFS probability during clinical practice.

MATERIALS AND METHODS

Data Source Collection

The messenger RNA (mRNA)-seq expression and clinicopathological characteristics of 1,025 BC patients were obtained from the TCGA program website (https://cancergenome.nih.gov/). Meanwhile, the autophagy-related genes were derived from the human autophagy database (HADb, http://www.autophagy.lu/). After excluding BC patients with incomplete clinicopathological medical records and patients initially diagnosed with metastasis, there were 785 BC patients included for further analysis. The data from the TCGA database were assigned as a training cohort (**Figure 1**). Moreover, two Gene Expression Omnibus (GEO) cohorts including the GSE42568 and GSE21653 datasets (detailed clinical information was summarized in **Table S1**) were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/) and assigned as the validation cohorts. All GEO datasets were produced by the Affymetrix HG-U133 plus 2.0. Raw microarray was normalized using Robust Multichip Average (32). When multiple probes were mapped to the same Entrez Gene ID, we used the mean value to represent its average expression level.

Ethics Approval

The protocol for this study was approved by Chongqing Medical University. Ethical approval was waived by the local Ethics Committee of the Chongqing Medical University in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

Identification of Autophagy-Related mRNA Signature for the Early Relapse of BC

Recurrence of BC patients was frequently occurred within 5 years after the initial treatment, while the first 2 years were the peak of recurrence (9). However, the definition of early relapse in BC patients is still ambiguous in recently published literature (33–36). Thus, early relapse in the present study was defined as the locoregional recurrence or distant metastasis within a short-term of 2 years follow-up after the initial primary resection. Samples in the training set were selected and divided into early relapse group and long-term survival group (no relapse at least 5 years follow-up). The calculations of differentially expressed genes (DEGs) between early relapse and long-term survival BC patients were conducted using the linear models for microarray data (LIMMA) method. The threshold for identification of DEGs was set as P value < 0.1. Besides, the LASSO Cox regression model (37) was used to select the most significantly relapse-associated mRNA of all the DEGs. A risk score model containing both coefficients and mRNA expression levels was established to generate the risk score for all BC patients in the training cohort. Based on the risk

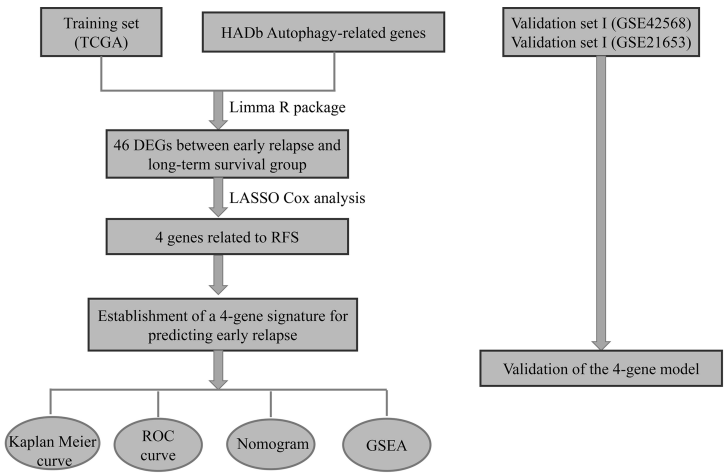

FIGURE 1 | The autophagy-related 4-gene signature selection and validation process.

score, patients were divided into high-risk and low-risk groups with the median risk score as the cut-off point.

Gene Set Enrichment Analysis

The Gene Set Enrichment Analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) was applied to evaluate differences between the low-risk and high-risk groups. Namely, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted to differentially expressed genes between these two groups. Normal P values <0.05 were regarded as statistically significantly enriched.

Validation Analysis

To further confirm the classification reliability and prognosis value of the 4-gene signature analyzed by TCGA, similar analyses were performed on GSE42568 and GSE216533 datasets to validate the prognostic significance of this autophagy-related signature.

Statistical Analysis

Survival differences between the low-risk and high-risk groups in each set were assessed by the Kaplan–Meier estimate and compared *via* the log-rank test. Baseline characteristics between low-risk and high-risk groups in each set were compared using the Pearson-chi square test (minimal expected value > 5). Multivariate Cox regression analysis and data stratification analysis were exploited to evaluate the independent prognostic significance of risk score and clinicopathological factors in predicting the RFS of BC patients. Time-dependent receiver-operating characteristic (ROC) analysis was used to investigate the prognostic and predictive accuracy of the signature. To access the probability of RFS survival in BC patients, a nomogram was subsequently developed based on the risk score and clinical features by using the "rms" R package. And the predictive feasibility of the nomogram was weighed by the Harrell concordance indexes (C-index) and calibration curves. All statistical analyses were performed with the use of R (version 4.0.3, www.r-project.org). All statistical tests were two-tailed, and P values < 0.05 were considered statistically significant.

RESULTS

Identification of an Autophagy-Related Gene Signature for the Early Relapse of BC

Generally, we take the intersection of mRNA from the TCGA database with 222 autophagy genes in HADb. 46 differentially expressed autophagy-related genes were identified between the early relapse group and long-term survival group by using the "limma" package in the R software. These genes were subsequently included for LASSO analysis (**Figure 2**). Based on the LASSO analysis, four genes including the APOL1, HSPA8, SIRT1, and TP73, were regarded as the independent prognostic factor in early relapse BC patients. A gene-based prognostic model was further established to evaluate the RFS risk for each patient. The results are as follows: Risk score= (-0.209* status of APOL1) +(0.387* status of HSPA8) +(-1.073* status of SIRT1) + (-0.233* status of TP73). Thus, BC patients were divided into high-risk and low-risk groups with the median risk score as the cut-off point (**Table 1**).

The Prognostic Value of 4-Gene Signature in Training and Validating Cohorts

Among the high-risk group and low-risk group, the distribution of risk score and relapse status of BC patients were displayed. The results showed that the higher the risk score, the higher the morbidity rate was observed in the training group and two validating groups (**Figure 3**). Similarly, the Kaplan-Meier

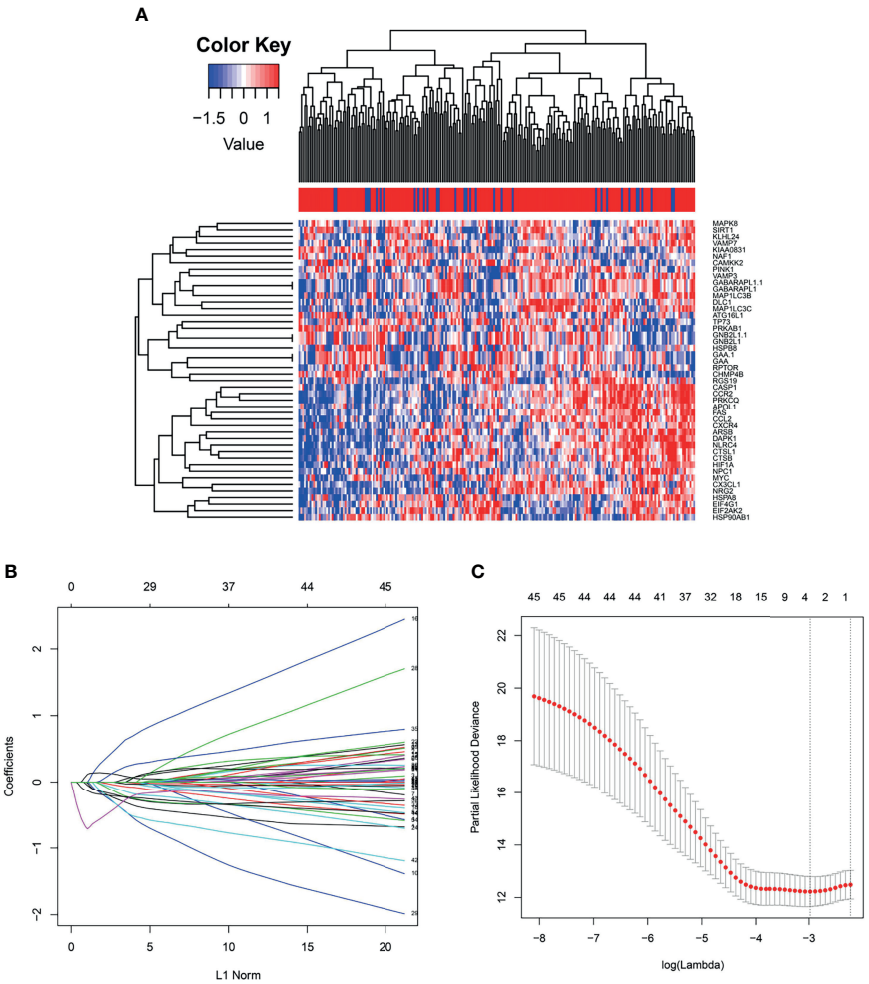

FIGURE 2 | **(A)** The heat map demonstrates forty-six differentially expressed mRNA in breast cancer among early relapse and long-term survival group both in training cohort; **(B)** LASSO coefficient profiles of the 4 early relapse-associated mRNA. A vertical line is drawn at the value chosen by 10-fold cross-validation; **(C)** X-tile analysis of the 4 selected GRGs.

survival analysis demonstrated that the RFS of the high-risk group was significantly inferior to those of the low-risk group in the training group and two validating groups (log-rank p=0.002 in the training set, log-rank p=0.013 in the validation set I, log-rank p=0.003 in the validation set II, respectively). Moreover, the time-dependent ROC analyses at 1-, 2-, and 3-year were also conducted to evaluate the accuracy of the 4-gene classifier. In the training cohort, the AUC of 1-, 2-, and 3-year was 0.748, 0.696, and 0.651, respectively. Accordingly, a relatively promising AUC value was also observed in the validating sets (validating set I: the AUC of 1-, 2-, and 3-year was 0.611, 0.649, and 0.655, respectively; validating set II: the AUC of 1-, 2-, and 3-year was 0.524, 0.640, and 0.654, respectively).

Establishment of a Predictive Nomogram

To access the independence and accuracy of the 4-gene signature in predicting the RFS of BC patients. The univariate and multivariate Cox analyses integrated with the clinicopathological characteristics were performed (**Table 2**). At univariate analysis, PR status, primary tumor size, regional lymph node status, and the 4-gene based signature were significantly associated with the early relapse of BC patients. During the multivariate analysis, larger tumor size (T2: HR=1.82, 95%CI: 0.51- 6.38, T3: HR=3.06, 95% CI: 0.50- 18.61, T4: HR=19.99, 95%CI: 3.88- 102.76, p=0.001) and high-risk BC patients derived from the 4-gene classifier (HR=5.73, 95%CI: 1.63- 20.16, p=0.006) were identified as the independent risk factors in promoting the early relapse of BC. PR status reached marginal significance (HR=2.34, 95%CI: 0.96- 5.73, p=0.063). A novel nomogram (**Figure 4**) was subsequently established with the three variables involvement (PR status, tumor size, and 4-gene signature). Optimally, the model contained a satisfying C-index of 0.766 (95%CI: 0.604-0.927). Moreover, three calibration curves for evaluating the accuracy of the predictive ability in short-term RFS were also performed *via* 1000 bootstrap repetitions (**Figure 4**). The curves (apparent, ideal, and bias-corrected lines) suggested a promising agreement in the training model.

TABLE 1 | The demographic characteristics of breast cancer patients among high-risk and low-risk groups according the autophagy-related 4-gene signature.

Variables	Subgroup	No. (%) of patients			
		Total (n=785)	High risk (n=392)	Low risk (n=393)	[a]*P*
Literality	**Left**	424 (54.0)	208 (53.1)	216 (55.0)	0.593
	Right	361 (46.0)	184 (46.9)	177 (45.0)	
Age	**<50**	221 (28.2)	123 (31.4)	98 (24.9)	**0.045**
	≥50	564 (71.8)	269 (68.6)	295 (75.1)	
ER	**Positive**	562 (71.6)	238 (60.7)	324 (82.4)	**<0.001**
	Negative	185 (23.6)	130 (33.2)	55 (14.0)	
	Other	38 (4.8)	24 (6.1)	14 (3.6)	
PR	**Positive**	496 (63.2)	205 (52.3)	291 (74.0)	**<0.001**
	Negative	248 (31.6)	162 (41.3)	86 (21.9)	
	Other	41 (5.2)	25 (6.4)	16 (4.1)	
HER2	**Positive**	117 (14.9)	68 (17.3)	49 (12.5)	0.350
	Negative	414 (52.7)	199 (50.8)	215 (54.7)	
	Other	254 (32.4)	125 (31.9)	129 (32.8)	
pT	**T1**	214 (27.3)	105 (26.8)	109 (27.7)	**0.003**
	T2	467 (59.5)	249 (63.5)	218 (55.5)	
	T3	85 (10.8)	27 (6.9)	58 (14.8)	
	T4	19 (2.4)	11 (2.8)	8 (2.0)	
pN	**N0**	399 (50.8)	201 (51.3)	198 (50.4)	0.333
	N1	256 (32.6)	125 (31.9)	131 (33.3)	
	N2	87 (11.1)	46 (11.7)	41 (10.4)	
	N3	43 (5.5)	20 (5.1)	23 (5.9)	
Surgery	**Lumpectomy**	178 (22.7)	105 (26.8)	73 (18.6)	**0.010**
	Mastectomy	359 (45.7)	162 (41.3)	197 (50.1)	
	Other	248 (31.6)	125 (31.9)	123 (31.3)	

ER, Estrogen receptor; PR, Progesterone receptor; HER-2, Human epidermal growth factor receptor-2; pT, pathologically diagnosed tumor size; pN, pathologically diagnosed lymph node status.
[a]*Pearson's Chi-squared test.*
Bold values indicate statistical significance (p<0.05).

Gene Set Enrichment Analysis

The KEGG pathway analysis was conducted to discover the associated biological signaling pathway of 4 autophagy-related mRNA sets. Notably, differentially expressed genes between high-risk and low-risk groups were determined. Namely, the GSEA results indicated that the genes enriched in the high-risk group were related to the regulation of homologous recombination, N-glycan biosynthesis, oxidative phosphorylation, protein export, and RNA polymerase (**Figure 5**). On the contrary, in the low-risk group, the autophagy-related gene sets were involved in pathways related to dilated cardiomyopathy, cardiomyopathy HCM, phosphatidylinositol signaling system, proximal tubule bicarbonate reclamation, and vascular smooth muscle contraction (**Figure 5**).

DISCUSSION

To date, BC has become the leading malignancy among women worldwide (1, 2, 38) with a promising relatively higher 5- year survival rate, compared with other invasive cancers. Nevertheless, survivors can experience early recurrence with resistance to the initial treatments paralleled by highly invasive metastasis (9, 35). TNM stage and immunohistochemical indicators like ER, PR, Her-2, and Ki-67 index were frequently used to access the prognosis of BC patients. Chen et al. (12) determined that the late-stage ($p< 0.001$), poor differentiated grade ($p = 0.002$), PR-negative status ($p = 0.014$), and HER2-negative status ($p = 0.033$) were significant associated with the early relapse of BC. However, Huang et al. (11) determined that the cancer TNM stage was significantly associated with the early-relapse in BC patients, while clinical variables including age, tumor location, ER status, PR status, or HER2 status were not. In addition, a different survival pattern has been observed in BC patients with a relatively similar condition during clinical practice. These results indicated that genetic biomarkers also played a pivotal role in regulating tumor cell cycle progression and metastasis.

Regarding the gene signatures, previous works highlighted that the imbalance of cell proliferation and apoptosis, as well as autophagy regulation disorder, might also be attributed to the occurrence and development of BC. Namely, autophagy is a pivotal process in control of cell fate and significantly correlates with apoptosis *via* inactivating the mammalian target of rapamycin (mTOR) signaling pathway or directly activates the initiation step of autophagy by phosphorylating unc-51-like autophagy activating kinase 1 (ULK1). In terms of cancer initiation, autophagy is considered tumor-suppressive due to its cytoprotective role (23, 25, 39, 40). Notably, Marsh et al. (25) discovered autophagy could inhibit the metastasis of BC cells by accumulating the autophagy cargo receptor (ACR), neighbor to BRCA1. Moreover, recent two basic research demonstrated that MTA1 (metastasis-associated 1) (13) and long noncoding RNA H19 (14) were the regulators of autophagy in resistance to the endocrine therapy (tamoxifen). On the contrary, several studies (22, 41) demonstrated the autophagy was positively associated with the tumor growth, metastasis, and recurrence of BC, which

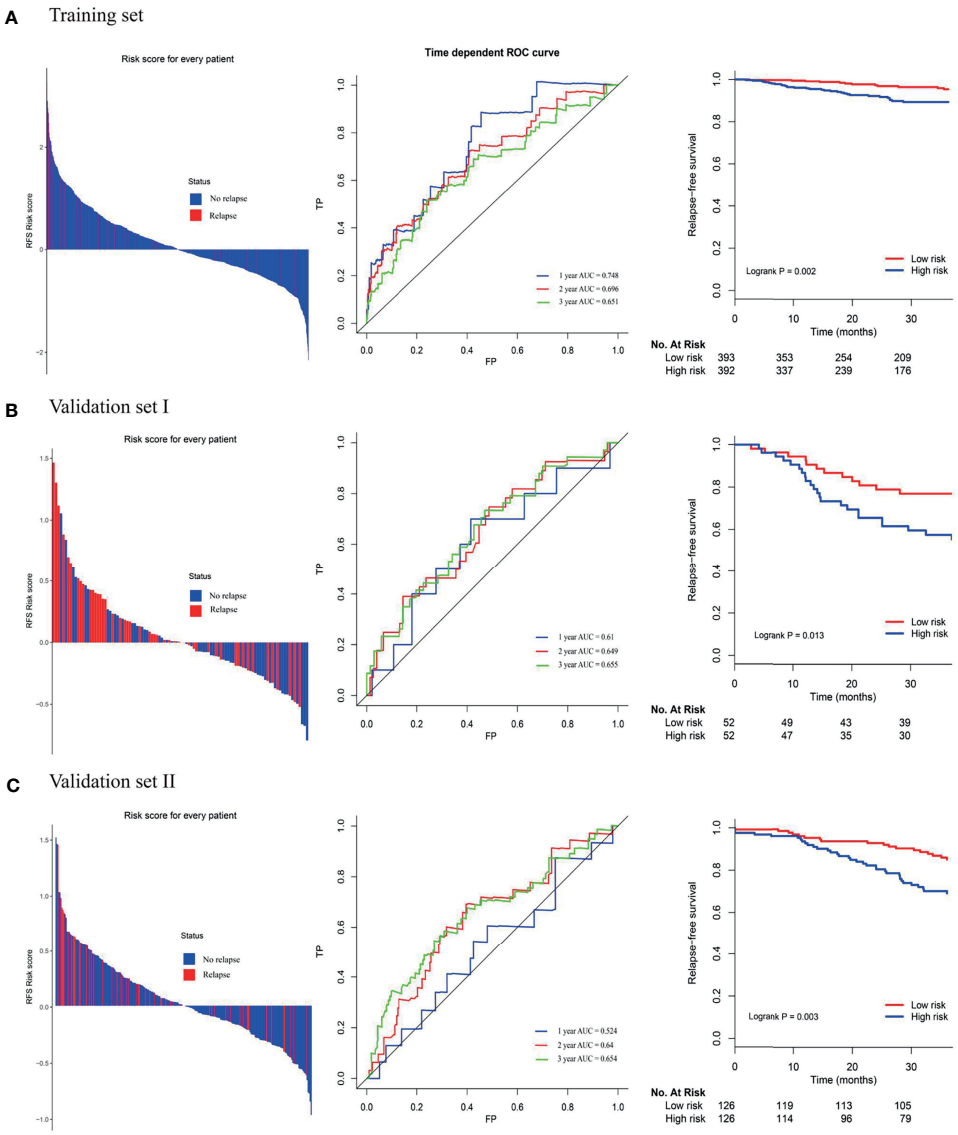

FIGURE 3 | **(A)** Distribution of risk score, time-dependent ROC curves at 1, 2, and 3 years and Kaplan–Meier survival analysis between patients at low and high risks of relapse in training cohort; **(B)** first external validation cohort; **(C)** second external validation cohort.

could maintain the homeostasis and the survival of BC cells by removing dysfunctional or unnecessary substances. Therefore, autophagy is a powerful but double-edged sword, which had an essential impact on the prognosis of BC (41).

In the present study, 4 autophagy-related mRNA including APOL1, HSPA8, SIRT1, and TP73 were pivotal genes in the RFS of BC. Of these genes, APOL1 (apolipoprotein-L1) has been observed significantly associated with kidney disease, especially in terms of HIV-related chronic renal disease (42, 43). In regulating the proliferation and metastasis of cancer cells, recent studies speculated the phenotype of APOLs was involved in several cancers' metastasis *via* the strong reduction of cellular adherence and increased in cell motility, together with an important reduction of the capacity for apoptosis (44–47). Besides, members of the heat-shock protein 70 (HSPA) family gained plenty of attention as a potential target for tumor therapy, which could promote cancer cell growth by different mechanisms (48–50). For instance, Rohde et al. (50) demonstrated the suppression role of HSPA in "HeLa" cells, namely, depletion of HSPA and HSPA2 arrested cancer cells in G2/M and G1, respectively. Regarding the SIRT1 (Sirtuin-1) mRNA, it significantly participated in gene regulation, genome stability maintenance, apoptosis, autophagy, and tumorigenesis (51). As recent studies reported, a downregulation of SIRT1 has already been described in gastric cancer (52) and breast cancer (53, 54). Zhang et al. demonstrated the activation of SIRT1 could suppress gastric cancer cells proliferation and metastasis *via* STAT3/MMP-13 signaling pathway (52). Meanwhile. Latifkar et al. (54) reported

TABLE 2 | The univariate and multivariate Cox regression analysis in the early relapse of breast cancer in the training group.

Variables	Subgroup	Univariable		Multivariable	
		Hazard ratio	*P*	Hazard ratio	*P*
Laterality	**Left**	1	0.285	/	
	Right	1.640 (0.662, 4.064)			
Age	**<50**	1	0.175	/	
	≥50	0.550 (0.231, 1.305)			
ER	**Positive**	1	0.041	1	0.469
	Negative	1.569 (1.018, 2.418)		0.643 (0.194, 2.125)	
PR	**Positive**	1	**0.006**	1	0.063
	Negative	1.856 (1.195, 2.883)		2.340 (0.955, 5.732)	
HER2	**Positive**	1	0.396	/	
	Negative	0.814 (0.507, 1.308)			
pT	**T1**	1	**0.001**	1	**0.001**
	T2	1.991 (0.567, 6.987)		1.820 (0.518, 6.389)	
	T3	1.818 (0.304, 10.881)		3.061 (0.503, 18.619)	
	T4	19.395 (3.876, 94.048)		19.992 (3.889, 102.767)	
pN	**N0**	1	**0.025**	1	0.192
	N1	0.357 (0.101, 1.265)		0.349 (0.097, 1.259)	
	N2	0.810 (0.181, 3.620)		0.854 (0.184, 3.956)	
	N3	3.477 (1.118, 10.816)		2.051 (0.568, 7.415)	
Surgery	**Lumpetomy**	1	0.762	/	
	Mastectomy	0.896 (0.306,2.621)			
	Other	0.660 (0.201, 2.162)			
Score	**Low risk**	1	**0.003**	1	**0.006**
	High risk	6.304 (1.857, 21.407)		5.737 (1.632, 20.167)	

ER, Estrogen receptor; PR, Progesterone receptor; HER-2, Human epidermal growth factor receptor-2; pT, pathologically diagnosed tumor size; pN, pathologically diagnosed lymph node status.

Bold values indicate statistical significance (p<0.05).

that inhibition of SIRT1 would impair the lysosomal function, resulting in the enhanced secretion of pro-tumorigenic exosomes which might reconstruct the extracellular matrix and enhance the invasive properties of cultured BC cells. Additionally, accumulating evidence has proved the dysfunction of TP73 (tumor protein p73) was associated with the proliferation and prognosis of different cancers (55). Notably, *in vitro* study, Sharif et al. (56) demonstrated that high expression levels of TP73 suppressed the proliferation of BC *via* enhanced autophagy and cell death. Alternatively, knockdown of TP73 decreased NAMPT (nicotinamide phosphoribosyl transferase) inhibition-induced autophagy and cell death.

Regarding the clinicopathological characteristics of BC patients, only primary tumor size was significantly associated with the early relapse of BC patients after stepwise multivariate analysis (adjust p=0.001). Interestingly, negative progesterone receptor status trended towards significantly increasing the risk of early relapse of BC patients (adjust p=0.063). Previously, compare to the prognostic value of estrogen receptor and HER2 status, progesterone receptor status was not so important. However, recent studies have demonstrated that progesterone receptor-negative tumors have generally been shown to have a poorer prognosis than progesterone receptor-positive tumors (57). Notably, evidence from one large population-based study, negative progesterone receptor status was associated with higher differentiation grade and subsequent recurrence score (58). Meanwhile, Zhang et al. demonstrated that the expression levels of progesterone receptor (cutoff point: 55%) played a pivotal role in predicting the relapse of hormone receptor-positive BC patients (59). However, the underlying mechanism and potential signaling pathway are still not clear but worth further investigation.

To our knowledge, we first discovered this autophagy-related 4-gene signature involved in the early relapse of BC. Based on the 4-gene signature, a risk score model was successfully established. And it was externally validated by two cohorts of GSE42568 and GSE21653, suggesting the favorable reproducibility of this signature in BC. However, the underlying molecular mechanism and signaling pathways of this signature are still inadequately clarified in BC. Nonetheless, the GSEA showed that the genes enriched in the high-risk group were related to the regulation of homologous recombination, N-glycan biosynthesis, oxidative phosphorylation, protein export, and RNA polymerase cancer-related signaling conduction. Alternatively, among the low-risk population, the autophagy-related gene sets were involved in pathways related to dilated cardiomyopathy, cardiomyopathy HCM, phosphatidylinositol signaling system, proximal tubule bicarbonate reclamation, and vascular smooth muscle contraction. Thus, further investigation of the underlying mechanisms may be meaningful. Additionally, constructing a convenient while reliable autophagy-related mRNA signature for identifying the risk biomarkers in promoting early relapse of BC would make up for the deficiency of clinicopathological classification, and further assist oncologists in formulating more efficient treatment modalities at an earlier stage of patients' management. For this reason, we constructed a nomogram combined with two clinicopathological prognostic factors to predict the 1-, 2-, and 3-year RFS of BC patients in an effective quantitative approach. An optimal C-index of 0.766 was achieved

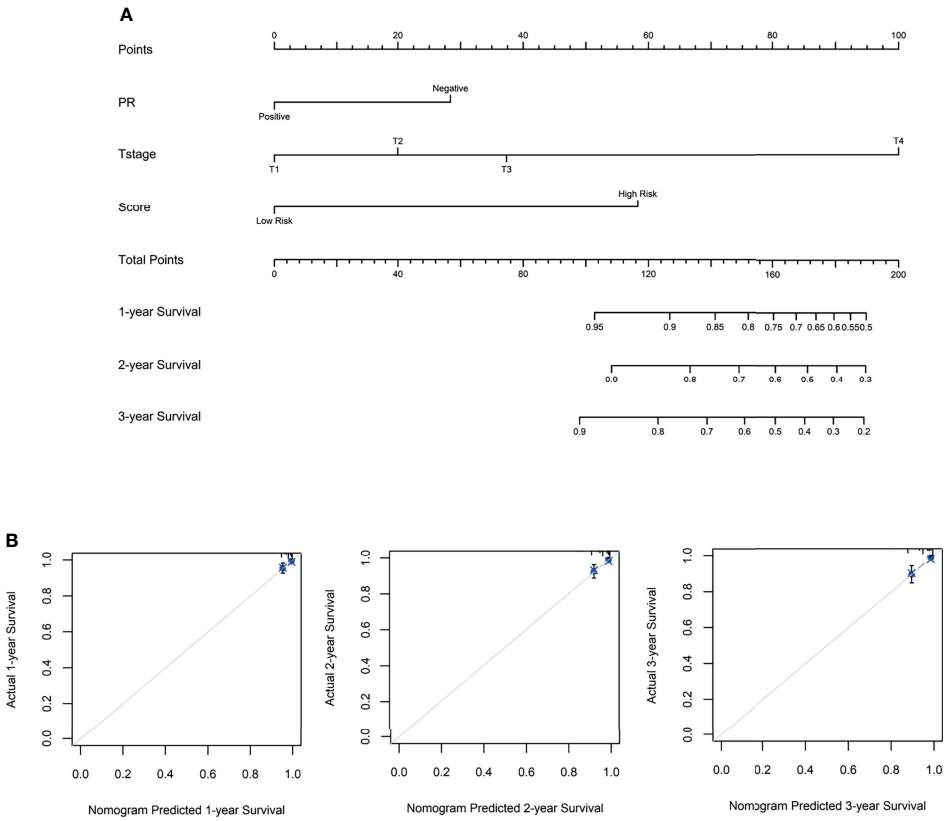

FIGURE 4 | **(A)** The nomogram for predicting the 1-, 2-, and 3- year relapse-free survival in breast cancer patients, based on the autophagy-related 4-gene signature selection and clinical factors. **(B)** The 1-, 2- and 3-year calibration curves were derived from the nomogram, respectively.

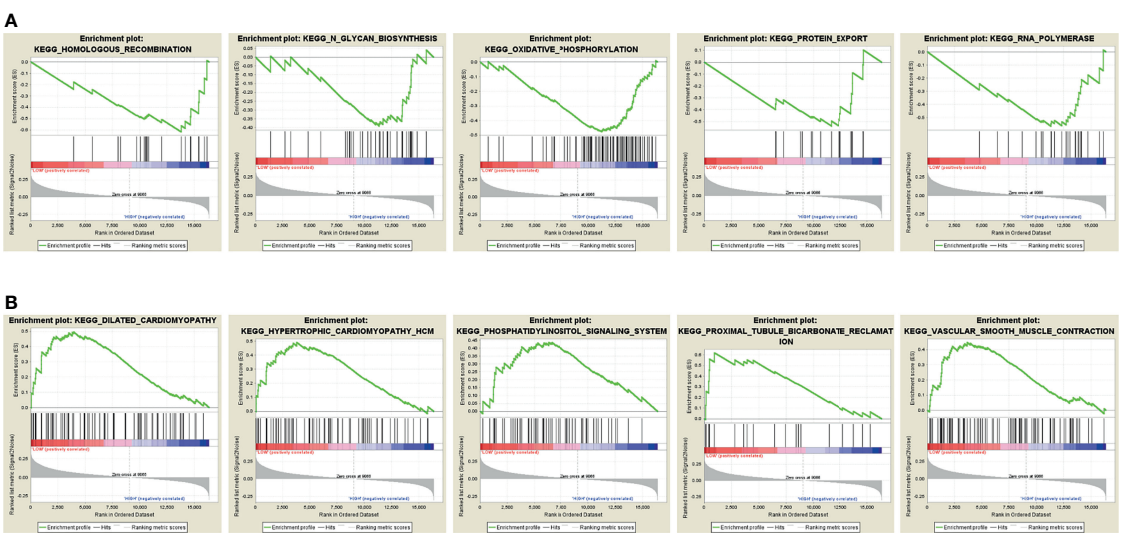

FIGURE 5 | Gene Set Enrichment Analysis (GSEA). **(A)** GSEA shows a significant enrichment of cancer-related pathways in the high-risk group based on the training cohort. **(B)** GSEA shows a significant enrichment of cancer-related pathways in the low-risk group based on the training cohort.

which indicated the feasibility of identifying the high-risk BC patients with early relapse during clinical practice.

Indeed, there are some limitations in the current study needed to be mentioned and addressed in future works. First, this is a retrospective-designed study, and all BC samples were identified from the public database which inevitably weakened the findings we determined. Second, further basic research in our department and other medical centers is merited to external validate our conclusions and elucidate the functional roles of autophagy-related mRNA signature involved in the early relapse of BC. Moreover, with a significant improvement of overall survival in BC patients, longer follow-up (like 10 years) time could better help oncologists predict the clinical outcome in these patients. Last, the risk score model and nomogram can only be applied to predict early relapse in BC patients, and its prognostic role in the different molecular subtypes of BC warrants further evaluation.

CONCLUSION

In summary, our works demonstrate that 4 autophagy-related mRNA (APOL1, HSPA8, SIRT1, and TP73) are significantly associated with the early relapse of breast cancer during the postoperative follow-up. Based on the autophagy-related mRNA signature risk score classifier, good discrimination in identifying the BC patients with a high risk of early relapse is achieved. Moreover, we successfully establish and validate a utility nomogram derived from the risk scores combining tumor size and PR status for clinically predicting the 1-, 2-,3-year RFS probability in BC patients after initial surgical intervention. Future prospective clinical trials could verify the clinical significance of our autophagy-related mRNA signature in stratifying early relapse in BC patients postoperatively. The mechanisms and underlying signaling of the identified genes on the early relapse of BC are also needed to be further explored.

AUTHOR'S NOTE

The software application generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

(I) Conception and design: YM and YF. (II) Administrative support: SF and GY. (III) Provision of study materials or patients: YF, XW, and YM. (IV) Collection and assembly of data: YM and HL. (V) Data analysis and interpretation: YM and XW. (VI) Manuscript writing: All authors. All authors contributed to the article and approved the submitted version.

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Prognostic Factors and Models for Elderly (≥70 Years Old) Primary Operable Triple-Negative Breast Cancer: Analysis From the National Cancer Database

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Background: Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer. In the elderly (≥70 years old) primary operable ($T_{1-3}N_{0-1}M_0$) TNBC, individualized treatment modalities for this population are pivotal and important, but limited studies are explored.

Methods: The clinicopathological features of elderly primary operable TNBC patients were retrospectively selected from the Surveillance, Epidemiology, and End Results (SEER) database between January 2010 and December 2015. Kaplan–Meier curves were used to show the survival patterns in the different subgroups. Multivariate Cox analysis was used to identify independent risk factors in the 3-, 5-, and 7- year overall survival (OS) and cancer-specific survival (CSS) in this subpopulation. The predictive model was further developed and validated for clinical use.

Result: Between 2010 and 2015 years, a total of 4,761 elderly primary operable TNBC patients were enrolled for the study, with a mean age of 76 years and a median follow-up of 56 months. The multivariate Cox analysis showed that age (increased per year: hazard ratio (HR) = 1.05), race (Asian/Pacific Islander and American Indian/Alaska Native, HR = 0.73), differentiation grade (grade II: HR = 2.01; grade III/IV: HR = 2.67), larger tumor size (T_{1c}: HR = 1.83; T_2: HR = 2.78; T_3: HR = 4.93), positive N stage (N_{1mi}: HR = 1.60; N_1: HR = 1.54), receiving radiation therapy (HR = 0.66), and receiving adjuvant chemotherapy (HR = 0.61) were the independent prognostic factors for OS, and a similar prognostic pattern was also determined in CSS. Besides, two nomograms for predicting the 3-, 5-, and 7-year OS and CSS in this population were developed with a favorable concordance index of 0.716 and 0.746, respectively.

Conclusion: The results highlight that both radiation and adjuvant chemotherapy are significantly associated with favorable long-term OS and CSS probability in elderly primary

operable TNBC patients. Based on the determined independent prognostic factors, the novel nomograms could assist the oncologists to make individualized clinical decisions for the subpopulation at different risks.

Keywords: triple-negative breast cancer, overall survival, retrospective study, nomogram, cancer-specific survival

INTRODUCTION

Nowadays, breast cancer has become the most frequently diagnosed malignancy and one of the leading causes of cancer-specific death in industrialized countries, with a female predominance (1–4). Nearly 40% of breast cancers occur in patients aged over 65 years and 25% in patients aged over 70 years. As the global population ages, the number of older patients with breast cancer will continue to increase (2, 4). Therefore, breast cancer in the elderly will represent a major public health issue during the next decades. Despite the biological invasive characteristics in older patients being less aggressive than younger breast cancer (5–7), outcomes for older patients with breast cancer are highly variable due to not only several biological factors but also potentially mutable factors (8–10). Thus, there is also a growing number of clinical treatment problems from these patient subgroups including but not limited to young, old, obese, and male breast cancer who often have unique clinical information and who are at high risk for disparate prognostic outcomes (6).

Triple-negative breast cancer (TNBC) accounts for 10%–15% of all breast cancer cases, which lack estrogen and progesterone receptors and express low levels of human epidermal growth factor 2 (Her-2) and therefore do not respond to hormonal or anti-HER2 therapies. Compelling evidence has demonstrated that TNBC frequently implies more aggressive biology and shows a worse prognosis that requires optimal treatment to reduce the future risk of recurrence and mortality (11, 12). For instance, based on the evidence from the large Epidemio-Strategy-Medical-Economical (ESME) metastatic breast cancer cohort, Gobbini et al. reported that there was no improvement in overall survival (OS) of metastatic TNBC patients over the past decades and yielded the need for new strategies in this unique molecular subtype (13). Also, with 390 cases involved, Gal et al. determined that women aged >75 years with TNBC had the highest recurrence rates, the shortest OS probability, and the subsequent worst clinical outcome (14).

Regarding this special subpopulation, however, there are limited data to make appropriate recommendations for those ≥70 years of age. Recently, many well-designed trials and comprehensive reviews demonstrated that adjuvant chemotherapy and radiotherapy are effective at reducing TNBC recurrences and associated with better cancer-specific survival (CSS) and OS in early-stage or younger TNBC patients (11, 15–19). Nevertheless, evidence-based data on the best treatment approach to the elderly patient group are mostly lacking, partly owing to the underrepresentation of elderly patients in clinical studies (9, 20, 21). Moreover, the favorable role of adjuvant chemotherapy in promoting postoperative survival in elderly TNBC patients is still in conflict, with most studies limited to subgroup analyses or small retrospective studies (21–23).

Hereby, the purpose of this study is to explore the impact of radiation therapy or chemotherapy after surgery on the long-term OS and CSS in the setting of elderly primary operable TNBC patients. Besides, we also aim to explore the independent prognostic factors for OS and CSS in this subpopulation and further establish a utility nomogram for oncologists to make tailored clinical decisions.

MATERIALS AND METHODS

Data Source

As an observational retrospective cohort study, patients' clinical information was extracted from one national cancer database (Surveillance, Epidemiology, and End Results, SEER, derived from the 18 cancer registries), which covered approximately 28% of the US population and is grouped in various races and ethnicities. In 2010, SEER registries began collecting Her-2 receptor status for breast cancer cases (24). Thus, the period of data collection was from 2010 to 2015 years. The reporting of this study has followed the guidelines of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (25).

TNBC was defined by the absence of estrogen receptor α (ERα), progesterone receptor (PR), and Her-2. The age cutoff for breast cancer in the elderly was assigned based on what has been used in previous studies (26–28). Patients who met the following criteria included the following: 1) female patients with postoperative histological confirmed TNBC; 2) age at diagnosis ≥70 years; and 3) the TNM stage classification limited to $T_{1-3}N_{0-1}M_0$. The excluding criteria were as follows: 1) patients with T_4 (invasion to the chest wall/skin and inflammatory carcinoma) primary site; 2) no regional nodes examined; and 3) lost to follow-up or incomplete medical records. The flow diagram was presented in the study (**Figure 1**).

To perform the multivariate Cox regression analysis, the sample size in this study should be at least 10 times the number of independent variables in the equation. Thus, after excluding the unqualified cases, there were 4,761 elderly female patients with primary operable TNBC enrolled in this study. Moreover, for predicting 3-, 5-, and 7- year OS and CSS probability, the original cohort was randomly divided into a training group and validating cohort at a ratio of 7:3 *via* the "R" program.

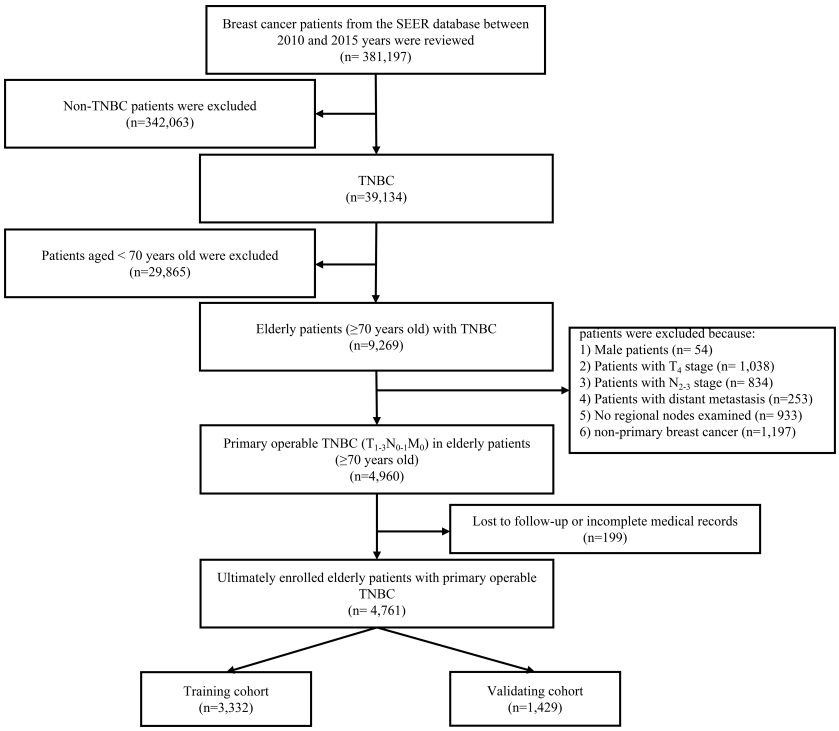

FIGURE 1 | The flow diagram of the patient selection process. TNBC, triple-negative breast cancer.

Ethical Approval

Data analysis from this database is considered to be non-human subjects by the Office for Human Research Protection as part of the US Department of Health and Human Services because patient data were anonymized and publicly available. For these reasons, the need for ethics approval was omitted by the Mianyang Central Hospital Ethics Committee.

Variable Evaluation and Definition

Variables were extracted based on their associations with the prognosis outcomes of interest. Namely, the following clinicopathological features were collected and transformed into categorical variables: race (White, Black, other including Asian or Pacific Islander, and American Indian/Alaska Native), laterality (right and left origin of primary), stage (IA, IB, IIA, IIB, and IIIA deriving from the adjusted AJCC staging system 7th edition), grade (I: well differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated), tumor location (nipple, central, outer, inner, overlapping and axillary of breast), histological subtype (IDC, ILC, and other kinds of subtypes), primary tumor stage (T_{1mi}: >0 and ≤1 mm, T_{1a}: >1 and ≤5 mm, T_{1b}: >5 and ≤10 mm, T_{1c}: >10 and ≤20 mm, T_2: >20 and ≤50 mm; T_3: >50 mm), lymph node stage (N_0: no regional lymph node metastasis identified or isolated tumor cell; N_{1micro}: micrometastases: approximately 200 cells, larger than 0.2 mm, but none larger than 2.0 mm; N_{1macro}: metastasis in 1–3 axillary lymph nodes, and/or in clinical negative internal mammary nodes with micrometastases or macrometastases by sentinel lymph node biopsy), primary surgical extension (partial/less than total mastectomy: includes segmental mastectomy, lumpectomy, quadrantectomy, tylectomy, wedge resection, nipple resection, excisional biopsy, or partial mastectomy; modified radical/total mastectomy), postoperative radiation record (performed or not), and chemotherapy recode (performed or not). The OS and CSS probability were calculated in months (more than 0 days of survival). The age at diagnosis was used as a continuous variable.

Statistical Analysis

The primary endpoint of this observational retrospective study was the 3-, 5-, and 7-year OS and CSS. The secondary endpoint was the efficacy of radiation and chemotherapy in the prognosis of the elderly primary operable TNBC patients. The univariate and multivariate Cox regression analyses were performed to find out the independent prognostic factors of OS and CSS in elderly primary operable TNBC patients. A two-tailed p-value of <0.05 was considered significant. Age, chemotherapy, radiation therapy, and factors significant in the univariate analysis are defined as the criterion for performing backward stepwise selection. The nomogram, decision curve analysis (DCA), calibration curve, and Kaplan–Meier analysis were constructed and plotted based on the results (availability, importance, and clinical relevance) derived from the multivariate Cox regression analysis *via* using the "survival," "rms," "survminer," and "foreign" packages of the R software (R Foundation, Vienna, Austria, version 4.0.3, http://www.r-project.org). Harrell's

C-index (29) and the time-dependent area under the receiver (AUC) operating characteristic (ROC) curve are conducted to assess the discrimination performance of the present nomogram.

RESULTS

Clinicopathological Characteristics of Elderly Primary Operable TNBC Patients

In total, from the SEER database between 2010 and 2015 years, 4,761 elderly primary operable TNBC patients were enrolled in this study with a mean age of 76.85 years at diagnosis and a median follow-up time of 56 months (range: 1–107 months). White race played a majority population in the present study (3,742 cases, 78.6%), whereas Asian or Pacific Islander and American Indian/Alaska Native only accounted for 3.4% of the whole population (340 cases). Based on the TNM stage classification, nearly half of the study population in the present study was at the IA stage (2,365 cases, 49.7%). The patients were subsequently randomized divided into training (3,332 cases) and validating (1,429 cases) cohorts for further Cox analysis and nomogram construction as well validation. The specific demographic and clinical characteristics of the elderly primary operable TNBC patients are shown in **Table 1**.

Kaplan–Meier Curves of Radiotherapy and Chemotherapy in OS and CSS

Among the whole study population, approximately 47.4% (2,256 cases) of patients were not elected to receive radiation therapy and a similar result was found in the chemotherapy record (2,686 cases, 56.4%). The KM curves presented that receiving radiation therapy could benefit the OS ($p < 0.0001$, **Figure 2A**) and CSS probability ($p < 0.0001$, **Figure 2C**) of the elderly primary operable TNBC patients during the follow-up, compared with patients not assigned to radiation therapy. On the other hand, patients who received chemotherapy had significantly higher 3-, 5-, and 7-year OS probability ($p < 0.0001$, **Figure 2B**), while it did not show any statistically significant CSS benefit ($p = 0.17$, **Figure 2D**). In the subgroup analysis, patients who did not receive any chemoradiotherapy had the worst survival outcome (OS: $p < 0.0001$, **Figure 3A**; CSS: $p < 0.0001$, **Figure 3B**).

Kaplan–Meier Curves of Clinicopathological Characteristics in OS and CSS

According to the KM curves, a significant decrease in cumulative OS probability was observed in patients with black or white race ($p = 0.0031$, **Figure 4A**), worse differentiation grade (III/IV, $p < 0.0001$, **Figure 4D**), larger primary tumor size (T_{1c}, T_2, and T_3; $p < 0.0001$, **Figure 4E**), late N stages (N_{1mi} and N_{1ma}; $p < 0.0001$, **Figure 4F**), and relatively aggressive surgical extension (modified radical/total mastectomy, $p < 0.0001$, **Figure 4G**). On the contrary, tumor subtype ($p = 0.800$, **Figure 4B**) and tumor location ($p = 0.410$, **Figure 4C**) were not associated with the OS in elderly primary operable TNBC patients. Regarding the CSS, a similar survival pattern was observed in the KM curves (**Figures 5A–G**).

Univariate and Multivariate Cox Analyses of the Prognostic Factors for OS

In terms of 3-, 5-, and 7- year OS, univariate Cox analysis showed that age (increased per year: hazard ratio (HR) = 1.08, 95% confident interval (CI): 1.07–1.09; $p \leq 0.001$), worse differentiation grade (grade II: HR = 2.31, 95% CI: 1.34–3.97; grade III/IV: HR = 3.37, 95% CI: 1.98–5.72, $p < 0.001$), larger tumor size (T_{1c}: HR = 1.87, 95% CI: 1.30–2.70; T_2: HR = 3.30, 95% CI: 2.31–4.73, T_3: HR = 6.81, 95% CI: 4.58–10.12, $p < 0.001$), positive N stage (N_{1mi}: HR = 1.65, 95% CI: 1.31–2.07; N_1: HR = 1.91, 95% CI: 1.65–2.21, $p < 0.001$), and radical surgical extension (HR = 1.76, 95% CI: 1.55–1.98) were the potential risk factors in impairing the long-term OS probability. On the contrary, Asian/Pacific Islander and American Indian/Alaska Native race (HR = 0.76, 95% CI: 0.58–1.01, $p = 0.025$), receiving radiation therapy (HR = 0.51, 95% CI: 0.45–0.58, $p < 0.001$), and receiving chemotherapy (HR = 0.56, 95% CI: 0.49–0.64, $p < 0.001$) were favorable prognostic factors for OS (**Table 2**).

In stepwise multivariate Cox analysis, seven factors including age (increased per year: HR = 1.05, 95% CI: 1.04–1.06, $p < 0.001$), Asian/Pacific Islander and American Indian/Alaska Native race (HR = 0.73, 95% CI: 0.55–0.96, $p = 0.029$), differentiation grade (grade II: HR = 2.01, 95% CI: 1.16–3.45; grade III/IV: HR = 2.67, 95% CI: 1.57–4.55, $p < 0.001$), larger tumor size (T_{1c}: HR = 1.83, 95% CI: 1.26–2.64; T_2: HR = 2.78, 95% CI: 1.93–4.02, T_3: HR = 4.94, 95% CI: 3.27–7.46, $p < 0.001$), positive N stage (N_{1mi}: HR = 1.60, 95% CI: 1.27–2.01; N_1: HR = 1.54, 95% CI: 1.32–1.79, $p < 0.001$), receiving radiation therapy (HR = 0.66, 95% CI: 0.56–0.77, $p < 0.001$), and receiving chemotherapy (HR = 0.61, 95% CI: 0.52–0.71, $p < 0.001$) were the independent prognostic factors for OS (**Table 2**).

Univariate and Multivariate Cox Analyses of the Prognostic Factors for CSS

In terms of 3-, 5-, and 7- year CSS, univariate Cox analysis showed that age (increased per year: HR = 1.06, 95% CI: 1.04–1.08; $p \leq 0.001$), worse differentiation grade (grade II: HR = 2.50, 95% CI: 1.01–6.16; grade III/IV: HR = 4.61, 95% CI: 1.91–11.14, $p < 0.001$), larger tumor size (T_{1c}: HR = 3.39, 95% CI:.58–7.26; T_2: HR = 7.89, 95% CI: 3.72–16.71, T_3: HR = 17.24, 95% CI: 7.89–37.69, $p < 0.001$), positive N stage (N_{1mi}: HR = 2.25, 95% CI: 1.65–3.07; N_1: HR = 2.86, 95% CI: 2.35–3.48, $p < 0.001$), and radical surgical extension (HR = 1.90, 95% CI: 1.59–2.27) were the potential risk factor in impairing the long-term CSS. By contrast, Asian/Pacific Islander and American Indian/Alaska Native race (HR = 0.48, 95% CI: 0.29–0.79, $p = 0.004$) and receiving radiation therapy (HR = 0.51, 95% CI: 0.43–0.62, $p < 0.001$) were favorable prognostic factors for CSS (**Table 3**). As for chemotherapy, there was a slight trend to become statistically significant (HR = 0.87, 95% CI: 0.73–1.05, $p = 0.161$).

In stepwise multivariate Cox analysis, seven variables including age (increased per year: HR = 1.03, 95% CI: 1.01–1.05, $p < 0.001$), Asian/Pacific Islander and American Indian/Alaska Native race (HR = 0.44, 95% CI: 0.26–0.73, $p = 0.004$), differentiation grade (grade III/IV: HR = 2.86, 95% CI: 1.17–6.88,

TABLE 1 | Clinicopathological characteristics of elderly primary operable TNBC patients (≥70 years old) in training and validation cohorts.

Characteristics	No. (%) of patients		
	Initial cohort (n = 4,761)	Training cohort (n = 3,332)	Validation cohort (n = 1,429)
Age	76.85 ± 5.54[c]	76.87 ± 5.64	76.81 ± 5.49
Race			
White	3,742 (78.6)	2,642 (79.3)	1,100 (77.0)
Black	705 (14.8)	471 (14.1)	234 (16.4)
Other[a]	314 (6.6)	219 (6.6)	95 (6.6)
Location			
Nipple	14 (0.3)	8 (0.2)	6 (0.4)
Central	218 (4.6)	156 (4.7)	62 (4.3)
Upper-inner	690 (14.5)	489 (14.7)	201 (14.1)
Lower-inner	326 (6.8)	216 (6.5)	110 (7.7)
Upper-outer	1,888 (39.7)	1,308 (39.2)	580 (40.6)
Lower-outer	398 (8.3)	289 (8.8)	109 (7.6)
Axillary	28 (0.6)	22 (0.6)	6 (0.4)
Overlapping	1,199 (25.2)	844 (25.3)	355 (24.8)
[&]Grade			
I	166 (3.5)	120 (3.6)	46 (3.2)
II	1,201 (25.2)	823 (24.7)	378 (26.5)
III/IV	3,394 (71.3)	2,389 (71.7)	1,005 (70.3)
Laterality			
Right	2,307 (48.5)	1,603 (48.1)	704 (49.3)
Left	2,454 (51.5)	1,729 (51.9)	725 (50.7)
Histology			
IDC	4,169 (87.6)	2,912 (87.4)	1,257 (88.0)
ILC	74 (1.5)	48 (1.4)	26 (1.8)
[#]Other	518 (10.9)	372 (11.2)	146 (10.2)
T stage			
T_{mi+1a}	288 (6.0)	208 (6.2)	80 (5.6)
T_{1b}	728 (15.3)	505 (15.2)	223 (15.6)
T_{1c}	1,689 (35.5)	1,176 (35.3)	513 (35.9)
T_2	1,811 (38.0)	1,265 (38.0)	546 (38.2)
T_3	245 (5.2)	178 (5.3)	67 (4.7)
N stage			
N_0	3,692 (77.5)	2,568 (77.1)	1,124 (78.7)
$N1_{mi}$	287 (6.1)	202 (6.1)	85 (5.9)
N_1	782 (16.4)	562 (16.8)	220 (15.4)
AJCC 7th stage			
IA	2,365 (49.7)	1,638 (49.2)	727 (50.9)
IB	77 (1.6)	60 (1.8)	17 (1.2)
IIA	1,531 (32.2)	1,071 (32.1)	460 (32.2)
IIB	687 (14.4)	487 (14.6)	200 (14.0)
IIIA	101 (2.1)	76 (2.3)	25 (1.7)
Surgical extension			
Less than total mastectomy	2,843 (59.7)	1,994 (60.0)	849 (59.4)
Modified radical/total mastectomy	1,918 (40.3)	1,338 (40.0)	580 (40.6)
Radiation			
Not performed	2,256 (47.4)	1,565 (47.0)	691 (48.4)
Performed	2,505 (52.6)	1,767 (53.0)	738 (51.6)
Chemotherapy			
Not performed	2,686 (56.4)	1,858 (55.8)	828 (57.9)
Performed	2,075 (43.6)	1,474 (44.2)	601 (42.1)
RLN harvested	4.95 ± 5.26[c]	4.91 ± 5.24	4.97 ± 5.40
RLN positive	0.33 ± 0.94[c]	0.32 ± 0.88	0.33 ± 1.05
Marital status			
Married	2,069 (43.5)	1,446 (43.4)	623 (43.6)
Divorce	456 (9.5)	310 (9.3)	146 (10.2)
Single	2,012 (42.3)	1,421 (42.6)	591 (41.4)
Unknown	224 (4.7)	155 (4.6)	69 (4.8)
Postoperative follow-up	56 [1–107][¶]	56 [1–107][¶]	56 [1–107][¶]

[a]*other: defined as the Asian/Pacific Islander and American Indian/Alaska Native.*

[&]*Grade: I: well differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated.*

[c]*Mean ± SD.*

[¶]*Median [range].*

TNBC, triple-negative breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; [#]other, other types of breast cancer; RLN, regional lymph node.

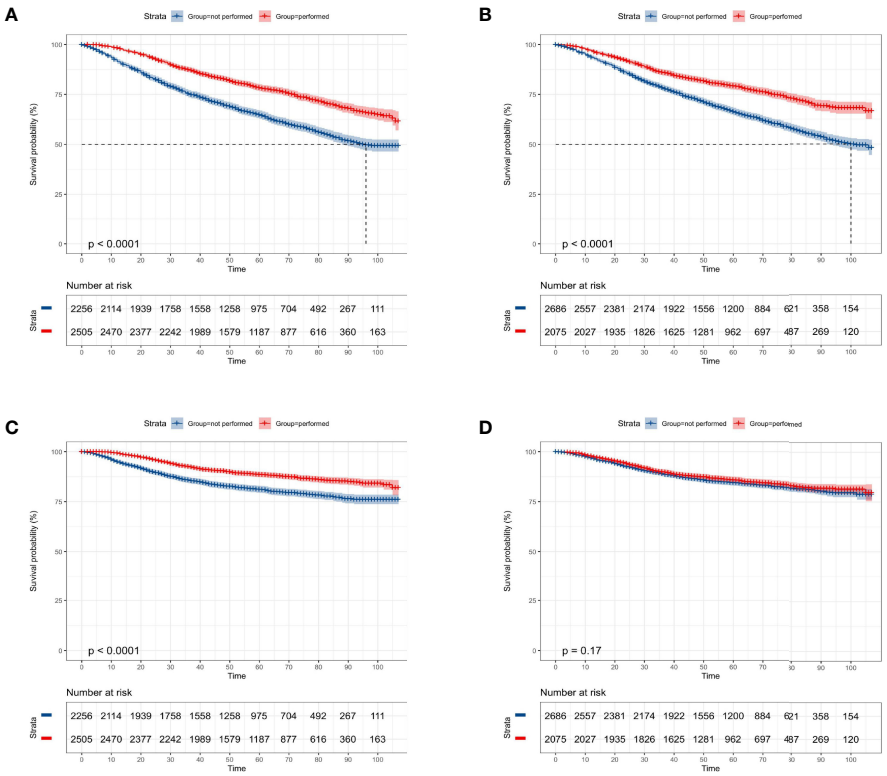

FIGURE 2 | Kaplan–Meier overall survival and cancer-specific survival analyses of 4,761 women aged 70 years or older with primary operable, triple-negative breast cancer regarding the adjuvant treatment. **(A)** Radiotherapy for OS; **(B)** chemotherapy for OS; **(C)** radiotherapy for CSS; **(D)** chemotherapy for CSS. OS, overall survival; CSS, cancer-specific survival. Tick marks indicate censored data.

p = 0.001), larger tumor size (T_{1c}: HR = 2.95, 95% CI: 1.37–6.34; T_2: HR = 5.61, 95% CI: 2.62–12.01, T_3: HR = 10.61, 95% CI: 4.86–24.09, p < 0.001), positive N stage (N_{1mi}: HR = 2.03, 95% CI: 1.49–2.87; N_1: HR = 2.06, 95% CI: 1.68–2.53, p < 0.001), receiving chemotherapy (HR = 0.79, 95% CI: 0.64–0.98, p = 0.035), and radiation therapy (HR = 0.63, 95% CI: 0.50–0.79, p < 0.001) were the independent prognostic factors for CSS (**Table 3**).

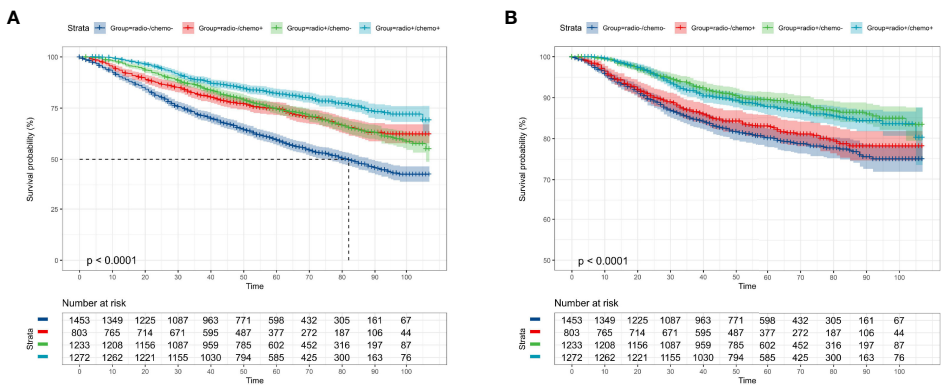

FIGURE 3 | Kaplan–Meier overall survival and cancer-specific survival analyses of 4,761 women aged 70 years or older with primary operable, triple-negative breast cancer who received both chemotherapy and radiotherapy (radio+/chemo+), received only radiotherapy (radio+/chemo-), received only chemotherapy (radio-/chemo+), or did not receive radiotherapy and chemotherapy (radio-/chemo-). **(A)** adjuvant treatment for OS; **(B)** adjuvant treatment for CSS. OS, overall survival; CSS, cancer-specific survival. Tick marks indicate censored data.

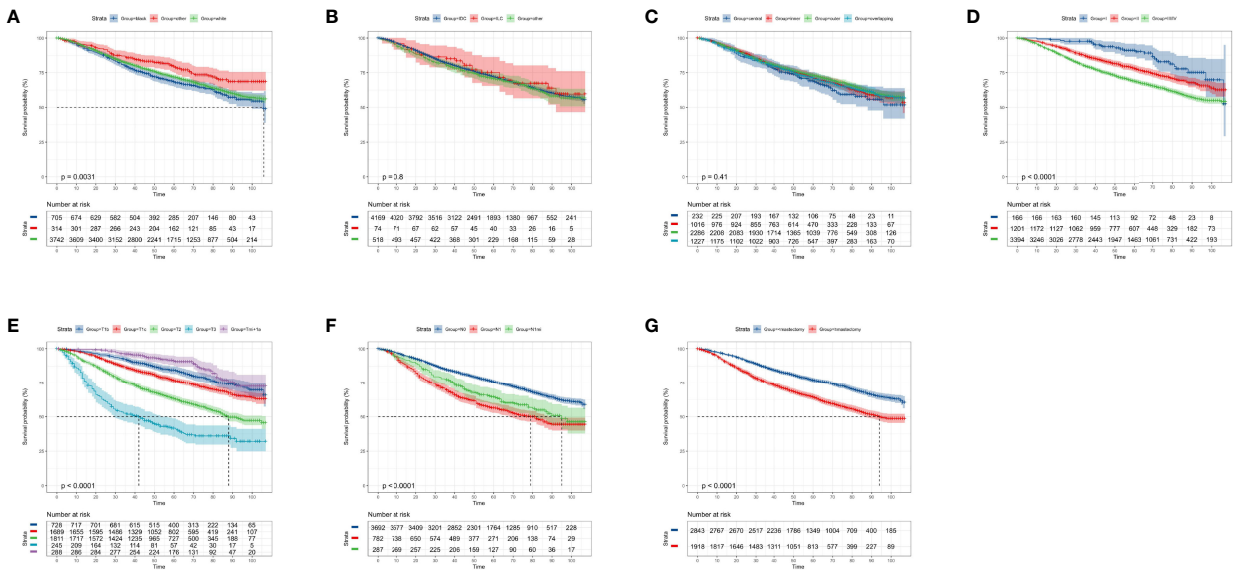

FIGURE 4 | Kaplan–Meier overall survival analysis of 4,761 women aged 70 years or older with primary operable, triple-negative breast cancer regarding the clinicopathological characteristics. **(A)** Race. **(B)** Histology. **(C)** Tumor location. **(D)** Differentiation grade. **(E)** T stage. **(F)** N stage. **(G)** Surgical extension. other: defined as the Asian/Pacific Islander and American Indian/Alaska Native; Grade: I: well-differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated; central: central portion of breast combined with nipple; other: other types of breast cancer. TNBC, triple-negative breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma. Tick marks indicate censored data.

Development and Validation of Nomograms for Predicting the OS and CSS

Based on the multivariate Cox regression analysis above, any variable with a significant correlation was included in developing clinical nomogram models (**Figure 6A**). Each factor was given a score on the point scale and the total point could be calculated by adding up all the specific values from an individualized patient (**Tables S1**, **S2**).

For predicting the 3-, 5-, and 7- year OS probability, the C-index of the nomogram was 0.716 (95% CI: 0.687–0.751), and the

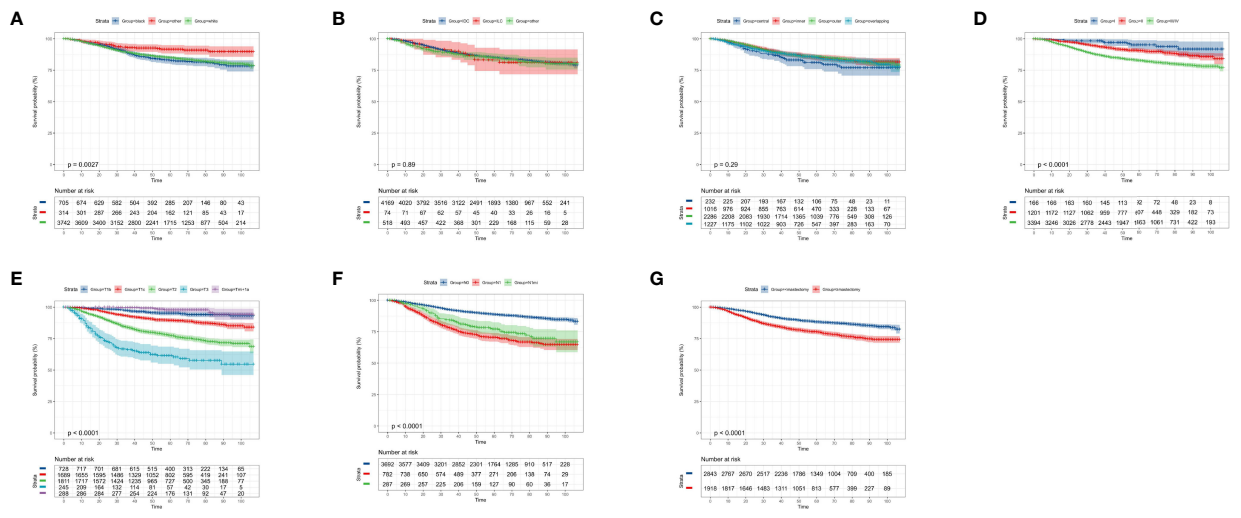

FIGURE 5 | Kaplan–Meier cancer-specific survival analysis of 4,761 women aged 70 years or older with primary operable, triple-negative breast cancer regarding the clinicopathological characteristics. **(A)** Race. **(B)** Histology. **(C)** Tumor location. **(D)** Differentiation grade. **(E)** T stage. **(F)** N stage. **(G)** surgical extension. other: defined as the Asian/Pacific Islander and American Indian/Alaska Native; Grade: I: well-differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated; central: central portion of breast combined with nipple; other: other types of breast cancer. TNBC, triple-negative breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma. Tick marks indicate censored data.

TABLE 2 | Univariate and multivariate Cox regression analyses of predictive variables correlated with OS in elderly primary operable TNBC patients (≥70 years old).

Variables	Subgroup	Univariable		Multivariable	
		Hazard ratio	*p*	Hazard ratio	*p*
Age (year)	Per year	1.08 (1.07–1.09)	**<0.001**	1.05 (1.04–1.06)	**<0.001**
Race	White	Reference	**0.025**	Reference	**0.029**
	Black	1.16 (0.98–1.38)		1.19 (0.93–1.31)	
	Other[a]	0.76 (0.58–1.01)		0.73 (0.55–0.96)	
Location	Central[b]	Reference	0.657	/	
	Inner	0.84 (0.63–1.13)			
	Outer	0.84 (0.64–1.10)			
	Overlap	0.87 (0.65–1.15)			
Grade	I	Reference	**<0.001**	Reference	**<0.001**
	II	2.31 (1.34–3.97)		2.01 (1.16–3.45)	
	III/IV	3.37 (1.98–5.72)		2.67 (1.57–4.55)	
Histology	IDC	Reference	0.836	/	
	ILC	0.94 (0.56–1.56)			
	Other	1.05 (0.87–1.27)			
T stage	T_{mi+1a}	Reference	**<0.001**	Reference	**<0.001**
	T_{1b}	1.29 (0.86–1.92)		1.28 (0.85–1.91)	
	T_{1c}	1.87 (1.30–2.70)		1.83 (1.26–2.64)	
	T_2	3.30 (2.31–4.73)		2.78 (1.93–4.04)	
	T_3	6.81 (4.58–10.12)		4.93 (3.26–7.48)	
N stage	N_0	Reference	**<0.001**	Reference	
	N_{1mi}	1.65 (1.31–2.07)		1.60 (1.27–2.01)	**<0.001**
	N_1	1.91 (1.65–2.21)		1.54 (1.32–1.79)	
Surgical extension	<Mastectomy	Reference	**<0.001**	Reference	0.406
	≥Mastectomy	1.76 (1.55–1.98)		0.93 (0.80–1.09)	
Radiation	Not	Reference	**<0.001**	Reference	**<0.001**
	performed	0.51 (0.45–0.58)		0.66 (0.56–0.77)	
Chemotherapy	Not	Reference	**<0.001**	Reference	**<0.001**
	performed	0.56 (0.49–0.64)		0.61 (0.52–0.71)	

[a]*Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native.*

[b]*Grade: I: well differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated; central: central portion of breast combined with nipple; other: other types of breast cancer.*

TNBC, triple-negative breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

Bold values indicate statistical significance ($p < 0.05$).

AUC of the 3-, 5-, and 7- year time-dependent ROC reached 0.720, 0.740, and 0.750, respectively (**Figure 6B**). Moreover, to validate the accuracy of our nomogram, an internal validation cohort with 1,429 cases was adopted. The results in the validating cohort presented good discrimination with an AUC of 0.680 in predicting 3-year OS, 0.710 in predicting 5-year OS, and 0.740 in predicting 7-year OS (**Figure 6C**). To evaluate the utility of the nomogram, three calibration curves of the nomogram were displayed. The curves (apparent, ideal, and bias-corrected lines) indicated a high agreement in predicting the 3-, 5-, and 7-year OS (**Figures 7A–C**). The decision curve analysis (DCA) curves presented that the score derived from the nomogram would be more effective than a treat-none or treat-all strategy when the threshold probability reached 75% in three cohorts (**Figures 7D–F**).

For predicting the 3-, 5-, and 7-year CSS probability, a novel nomogram (**Figure 8A**) was established with a C-index of 0.746 (95% CI: 0.713–0.803) and the AUC of the 3-, 5-, and 7-year time-dependent ROC reached 0.750, 0.750, and 0.780, respectively (**Figure 8B**). Moreover, the AUC of the 3-, 5-, and 7-year time-dependent ROC in the validating cohort was 0.710, 0.730, and 0.770, respectively (**Figure 8C**). Similarly, the calibration curves (**Figures 9A–C**) and DCA (**Figures 9D–F**) suggested the feasibility of the nomogram in applying for clinical use.

DISCUSSION

With the aging process, an increasing number of older women would be diagnosed with breast cancer and many are diagnosed at stages requiring more aggressive treatment, which needs efforts to increase rates of earlier stage diagnosis and the development of less toxic treatments that could help improve postoperative survival while preserving the quality of life (6, 21, 26, 28, 30). Currently, many clinical trials have demonstrated that elderly women with TNBC had the worst outcome when compared with other subtypes of breast cancer. Regarding the unique molecular subtype of TNBC patients, adjuvant chemotherapy modality, therefore, plays a crucially important role in deescalating the tumor progression and reducing the risk of recurrence as well as cancer-specific death. Unfortunately, the value of adjuvant chemotherapy in old patients with early breast cancer remains controversial (14, 19, 26, 30–32). There is a

TABLE 3 | Univariate and multivariate Cox regression analyses of predictive variables correlated with CSS in elderly primary operable TNBC patients (≥70 years old).

Variables	Subgroup	Univariable		Multivariable	
		Hazard ratio	*p*	Hazard ratio	*p*
Age (year)	Per year	1.06 (1.04–1.08)	**<0.001**	1.03 (1.01–1.05)	**<0.001**
Race	White	Reference	**0.004**	Reference	**0.004**
	Black	1.19 (0.93–1.51)		1.08 (0.84–1.37)	
	Other[a]	0.48 (0.29–0.79)		0.44 (0.26–0.73)	
Location	Central[b]	Reference	0.509	/	
	Inner	0.78 (0.52–1.17)			
	Outer	0.74 (0.50–1.09)			
	Overlap	0.76 (0.51–1.14)			
Grade	I	Reference	**<0.001**	Reference	**0.001**
	II	2.50 (1.01–6.16)		1.93 (0.78–4.78)	
	III/IV	4.61 (1.91–11.14)		2.83 (1.17–6.88)	
Histology	IDC	Reference	0.484	/	
	ILC	1.25 (0.65–2.43)			
	Other[c]	1.15 (0.87–1.51)			
T stage	T_{mi+1a}	Reference	**<0.001**	Reference	**<0.001**
	T_{1b}	1.51 (0.65–3.50)		1.43 (0.61–3.31)	
	T_{1c}	3.39 (1.58–7.26)		2.95 (1.37–6.34)	
	T_2	7.89 (3.72–16.71)		5.61 (2.62–12.01)	
	T_3	17.24 (7.89–37.69)		10.61 (4.86–24.09)	
N stage	N_0	Reference	**<0.001**	Reference	
	N_{1mi}	2.25 (1.65–3.07)		2.03 (1.49–2.78)	**<0.001**
	N_1	2.86 (2.35–3.48)		2.06 (1.68–2.53)	
Surgical extension	<Mastectomy	Reference	**<0.001**	Reference	0.542
	≥Mastectomy	1.90 (1.59–2.27)		0.93 (0.74–1.16)	
Radiation	Not	Reference	**<0.001**	Reference	**<0.001**
	performed	0.51 (0.43–0.62)		0.63 (0.50–0.79)	
Chemotherapy	Not	Reference	0.161	Reference	**0.035**
	performed	0.87 (0.73–1.05)		0.79 (0.64–0.98)	

[a]*Other: defined as the Asian/Pacific Islander and American Indian/Alaska Native.*

[b]*Grade: I: well differentiated, II: moderately differentiated, III/IV: poorly differentiated and undifferentiated; central: central portion of breast combined with nipple.*

[c]*Other: other types of breast cancer.*

TNBC ,triple-negative breast cancer; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

Bold values indicate statistical significance ($p < 0.05$).

paucity of data about the benefits of chemotherapy in elderly women with breast cancer (11). Few prospective data exist for chemotherapy in older breast cancer patients (≥70 years old) concerning efficacy or toxicity, but previous studies did suggest that the whole TNBC population could benefit from the adjuvant chemotherapy treatment (26, 33). As for elderly primary operable TNBC patients, whether active or omitting adjuvant treatment could further improve survival rates after local therapy still lacks robust evidence.

Among 4,761 elderly primary operable TNBC patients in our cohort, only 52.6% of them received radiation therapy and less than half of the patients received chemotherapy (43.6%). Notably, the KM curves showed that OS but not CSS benefits from the addition of adjuvant chemotherapy to surgery. Based on literature review (11, 34) and clinical experience, chemotherapy appears to influence the prognosis of TNBC patients. We hereby add this factor for further multivariate analysis, regardless of the p-value derived from the univariate analysis. Remarkably, after adjusting other confounders, the results demonstrated that patients who received chemotherapy presented longer OS and CSS probability. Similarly, in an earlier study derived from the SEER database (30), Elkin et al. determined that adjuvant chemotherapy was associated with a significant reduction in mortality among older women with negative hormone receptor status but lymph node-positive breast cancer. However, some confounders, like Her-2 status, were unavailable at that time. In the present study, we reanalyzed the cases from the latest version of the SEER database (between 2010 and 2015 years) with the target population. We addressed this limitation and further validated and highlighted the beneficial role of adjuvant treatment in reducing the long-term mortality of elderly primary operable TNBC. Most recently, Morita et al. conducted a retrospective multicenter study in Japan (27). However, they did not find any significant difference in OS among older patients who received adjuvant chemotherapy or not ($p = 0.333$). Alternatively, patients who received adjuvant chemotherapy had significantly prolonged disease-free survival ($p = 0.037$). The different results of our study and theirs might be contributed to the varied study population (operable TNBC vs. whole breast cancer population) and chemotherapy rate (43.6% vs. 14%).

On the other hand, based on existing evidence, the value of adjuvant radiotherapy in elderly TNBC patients also remains in conflict. One earlier meta-analysis (twelve studies were included within 5,507 TNBC cases) showed that adjuvant radiotherapy was not likely to benefit the OS of the elderly population but women with late-stage disease and younger patients (15). Moreover, in another Asian multicenter comparative study,

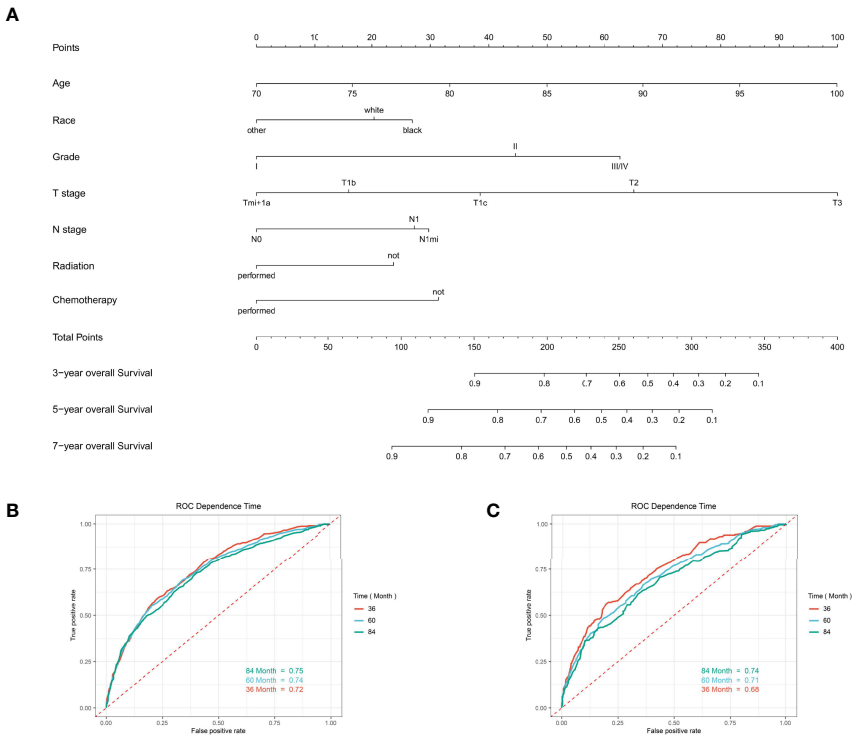

FIGURE 6 | The predictive model for predicting the long-term overall survival probability in women aged 70 years or older with primary operable, triple-negative breast cancer in the training cohort. **(A)** Nomogram for predicting the 3-, 5-, and 7- year OS for elderly primary operable TNBC patients. **(B)** The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC) in the training cohort. **(C)** The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC) validating cohort.

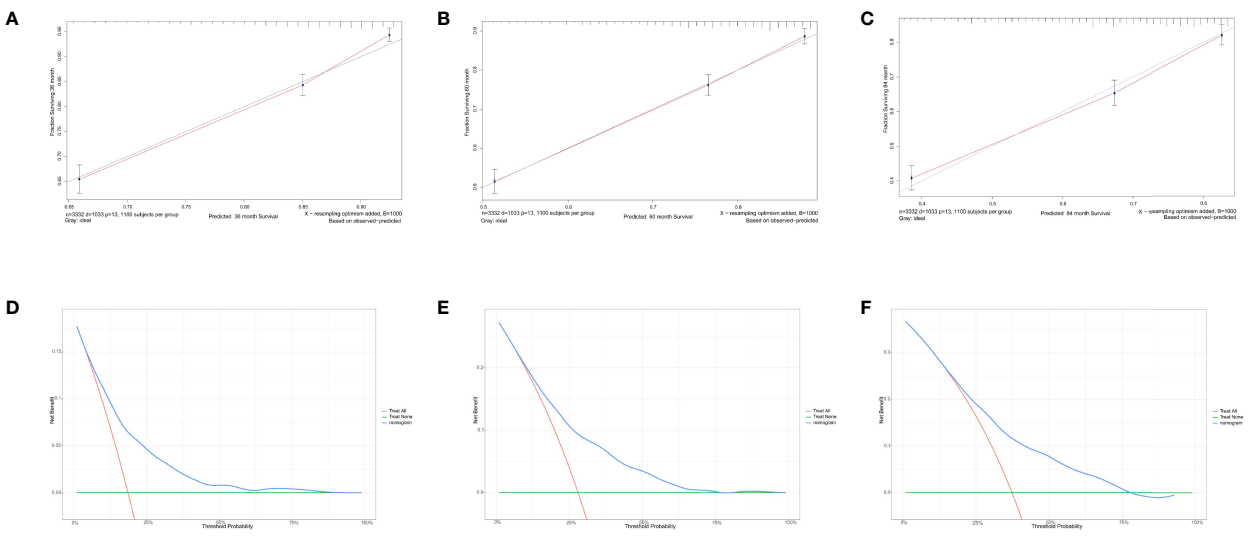

FIGURE 7 | Calibration curves and decision curve analysis for evaluating the accuracy of the nomogram in predicting the overall survival. The solid red line represented the performance of the nomogram, of which the closer fit to the gray line represents the better prediction of the nomogram we constructed. **(A)** 3-year OS in elderly primary operable TNBC patients, **(B)** 5-year OS in elderly primary operable TNBC patients; **(C)** 7-year OS in elderly primary operable TNBC patients; **(D)** DCA for 3-year OS in elderly primary operable TNBC patients in the training cohort; **(E)** DCA for 5-year OS in elderly primary operable TNBC patients in the training cohort; **(F)** DCA for 7-year OS in elderly primary operable TNBC patients in the training cohort. OS, overall survival; DCA, decision curve analysis; TNBC, triple-negative breast cancer.

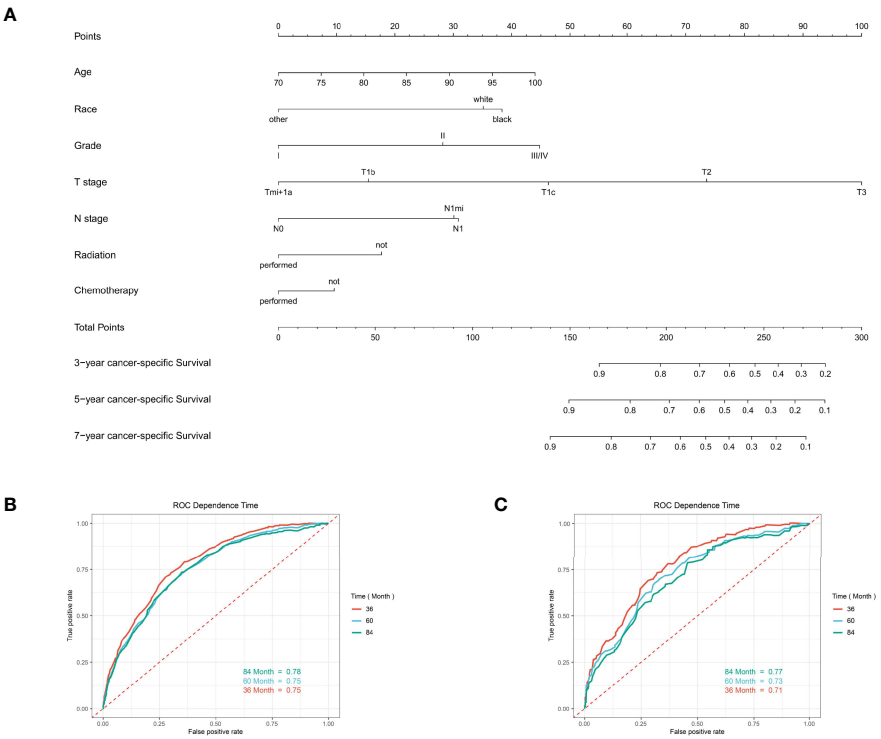

FIGURE 8 | The predictive model for predicting the long-term cancer-specific survival probability in women aged 70 years or older with primary operable, triple-negative breast cancer in the training cohort. **(A)** Nomogram for predicting the 3-, 5-, and 7- year CSS for elderly primary operable TNBC patients. **(B)** The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC) in the training cohort. **(C)** The receiver operating characteristics (ROC) curve and area under the ROC curve (AUC) validating cohort.

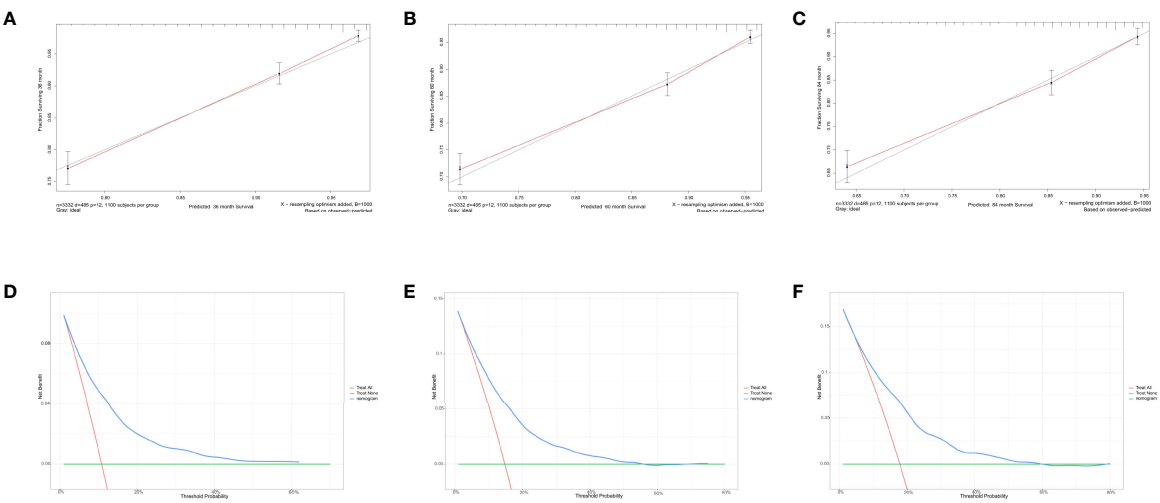

FIGURE 9 | Calibration curves and decision curve analysis for evaluating the accuracy of the nomogram in predicting cancer-specific survival. The solid red line represents the performance of the nomogram, of which the closer fit to the gray line represents the better prediction of the nomogram we constructed. **(A)** 3-year CSS in elderly primary operable TNBC patients; **(B)** 5-year CSS in elderly primary operable TNBC patients; **(C)** 7-year CSS in elderly primary operable TNBC patients; **(D)** DCA for 3-year CSS in elderly primary operable TNBC patients in the training cohort; **(E)** DCA for 5-year CSS in elderly primary operable TNBC patients in the training cohort; **(F)** DCA for 7-year CSS in elderly primary operable TNBC patients in the training cohort. CSS, cancer-specific survival; DCA, decision curve analysis; TNBC, triple-negative breast cancer.

Bhoo-Pathy et al. also showed that adjuvant radiotherapy was only associated with better survival in locally advanced or very young TNBC patients (16). However, in one study from the SEER database (median follow-up was 45 months), Zhai et al. determined that adjuvant radiotherapy after breast-conserving surgery (BCS) was associated with better OS and CSS in patients aged ≥70 years (35). Collectively, most published studies on the role of adjuvant radiotherapy in improving the OS for elderly patients are either retrospective observational or comparative studies (16, 18, 35). In our study, the effect of radiotherapy was significant for OS as well as CSS of operable TNBC patients, which could reduce the risk of mortality by about half (OS: HR = 0.66; CSS: HR = 0.63). The subgroup analysis indicated that patients receiving both radiotherapy and chemotherapy showed the highest survival probability, whereas patients omitting chemoradiotherapy had the worst OS and CSS. Therefore, prospective randomized controlled studies focused on adjuvant treatment in older breast cancer should be carried out in the future to improve the care quality for this population and the level of evidence-based medicine (11, 17).

In addition, some clinicopathological parameters including tumor differentiation grade, tumor size, and regional lymph node status which were well known associated with the prognosis of TNBC survival were again confirmed in the present study. Interestingly, the primary surgical extension was observed to be a significant predictor for OS and CSS during the univariate analysis. By contrast, the significance of this relationship with survival probability was eliminated in the stepwise multivariate analysis. Besides, while the lymph node stage was a pivotal indicator for the prognosis of elderly primary operable TNBC patients, there was only a small difference between N_{mi} and N_{1macro} (HR = 1.60 vs. HR = 1.54 in OS; HR = 2.03 vs. HR = 2.06 in CSS, respectively). It is suggested that N_{mi} was equally essential to assigning patients for more active treatment modalities. Moreover, recent studies have demonstrated that age at diagnosis and heterogeneous health backgrounds were significantly associated with the clinical decision-making for this population (8, 9).

Regarding race/ethnicity, it was recently determined to be associated with the prognosis of breast cancer (36–39). Especially, young black women with breast cancer had more adverse pathological factors and worse prognosis, when compared with white or Asian women. The potential intrinsic biological differences and socioeconomic status factors might be the contributors to these disparities. However, among elderly TNBC patients in our study, only Asian or Pacific Islander and American Indian/Alaska Native subgroups showed a survival advantage in OS and CSS, while there was no significant survival difference among black and white race patients. There were some possible explanations for our diverging findings. For instance, a study from San Miguel et al. suggested that insurance status played a pivotal role in breast cancer mortality, namely, uninsured women had the highest risk for breast cancer death, regardless of age (40). For this reason, insurance could be a pivotal factor but missed in our research which might influence the results we determined.

Based on the prognostic factors we determined, we further established an individualized predicting model for quantitatively analyzing the long-term OS and CSS probability for elderly primary operable TNBC patients. For example, one 75-year-old black TNBC ($T_2N_1M_0$, moderate differentiation) patient after radiation without chemotherapy was met in the outpatient room. The physicians could calculate the 3-, 5-, and 7-year OS (78%, 66%, and 53%, respectively) and CSS (85%, 75%, and 73%, respectively) probability. The C-index derived from the training and validating cohorts supported that the two nomograms we developed had promising predicting value in clinical use. Moreover, the calibration curves and DCA graphically highlighted the accuracy and clinical utility of the model. The calculation outcome will help oncologists to choose adjuvant treatment regimens.

Reviewing recently published literature, our study partially confirmed their results and took it a step further (15, 16, 18, 26). To the best of our knowledge, this is the first population-based study to investigate the clinicopathological characteristics associated with the prognosis of elderly primary operable TNBC patients. The primary strength of our study is the large population-based sample size within 4,761 cases, which was significantly larger than previous studies on this topic (16, 33). Thus, the results, especially in terms of the favorable role in chemotherapy and radiotherapy for this population, provided further evidence-based suggestions for clinical practice guideline improvement. Moreover, the developed nomogram model included individuals of different races and ethnicities present in the US, which was different from other retrospective single-center designed studies.

Nevertheless, this observational study has some limitations which need to be mentioned. First, this is a retrospective study in which selection bias inevitably exists. Second, while ten pivotal variables were enrolled for analysis, some information regarding important confounders including but not limited to Ki-67 index (41) and 21-Gene Recurrence Score (21-GRS) (42) as well as medical comorbidities and functional status, which tend to correlate with age and the prognosis of breast cancer, is now unavailable from the SEER database. Third, the adjuvant chemotherapy regimens and cycles as well as the scope and dose of the radiotherapy were not given in the present study. Thus, whether chemotherapy and radiotherapy could benefit the elderly primary operable TNBC patients should be discussed cautiously and the determined results need to be interpreted carefully. Lastly, another limitation of this study is the lack of external independent cohorts which prohibits further enforcing the reliability and clinical application of the nomograms. Herein, a prospective, multicenter cohort study with more detailed indicators is urgently needed to further evaluate the independent prognostic factors we determined and get a higher level of evidence for clinical guideline updates.

CONCLUSION

In conclusion, our results highlight that receiving adjuvant chemotherapy and radiotherapy could be favorable prognostic factors for elderly primary operable TNBC patients after local surgery. Besides, age, race, differentiation grade, T stage, and N

stage were identified as the independent prognostic indicators for predicting the long-term survival of this population. The two novel nomograms could help physicians to evaluate the survival probability and make tailored clinical decisions in elderly TNBC patients. Nevertheless, these findings need to be further validated and explored in future studies.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. ZT and YJ organized the database. YM, ZT, XZ, WX, and LZ performed the statistical analysis. All authors wrote the first draft of the manuscript. All authors wrote sections of the manuscript. All authors contributed to the manuscript revision and 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/fendo.2022.856268/full#supplementary-material

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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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CASE REPORT
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Partial Response After Toripalimab Plus Anlotinib for Advanced Metaplastic Breast Carcinoma: A Case Report

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Metaplastic breast carcinoma (MBC) is an aggressive subtype of breast cancer, accounting for <1%. The clinical outcome is unknown due to the lack of treatment options. Here, we present the case of a 58-year-old woman with advanced MBC, in which standard adjuvant chemotherapy was unsuccessful. In the second-line therapy, she received anti-angiogenic(anlotinib) therapy plus chemotherapy. Finally, she was subsequently treated with immunotherapy (toripalimab) combined anlotinib and achieved partial response (PR); thus, immunotherapy plus anti-angiogenic therapy might be a novel option for advanced MBC patients.

Keywords: metaplastic breast carcinoma, immunotherapy, anti-angiogenic, outcome, PR

INTRODUCTION

Metaplastic breast carcinoma (MBC) is a rare and aggressive subtype of breast cancer (about 1%) (1). MBC occurs commonly in women over the age of 60 years, typically presenting as a larger tumor size (2, 3). According to the WHO breast tumor classification in 2019, MBC was divided into five subtypes including adenosquamous carcinoma, pure squamous cell carcinomas, pure spindle cell carcinoma, metaplastic carcinoma with mesenchymal differentiation, and mixed metaplastic carcinoma (1). More than 80% of MBC did not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 receptor (HER2) (4). Advanced MBC has a poor prognosis compared to non-MBC triple-negative breast cancer (TNBC) due to rapid tumor growth and insensitivity to standard chemotherapy (5, 6). Immune checkpoint inhibitors (ICIs) block the PD-L1/PD-1 and CTLA-4/B7 signaling pathways, thereby preventing effector T cells from being inactivated and maintaining/keeping them to be able to kill tumor cells. In the past decade, immunotherapy improved survival benefits for patients with TNBC; whether immunotherapy is effective for MBC is still unknown (7–12). Here, we report an advanced MBC patient who failed with standard chemotherapy in the first-line therapy and anlotinib plus chemotherapy in the second-line therapy. She was subsequently treated with toripalimab plus anlotinib and achieved partial response (PR). Thus, immunotherapy combined with anti-angiogenic therapy might be a novel option for advanced MBC patients in later-line treatment.

CASE REPORT

A 58-year-old female came to our hospital with a chief complaint of finding a left breast mass. Breast ultrasound showed a left BI-RADs 4c breast mass and enlarged left axillary lymph nodes (**Figure 1**). The tumor biomarkers such as carcinoembryonic antigen (CEA) (1.2 ng/ml) and cancer antigen 153 (CA 153) (8.63 U/ml) were in the normal range. She underwent modified radical mastectomy, axillary lymph node dissection, and breast reconstruction. MBC (squamous cell carcinoma and sarcomatoid components) was established by pathological examination and confirmed by immunohistochemistry (IHC) staining, which demonstrated ER (−), PR (−), Her2 (0), Ki67(+, 20%), GATA3 (+), PCK (+), P63 (+), CK5/6 (+), SMA (+), P53 (−), Desmin (−), Myogenin (−), and STAB2 (−). CD31 stain was negative in the tumor cell and positive in the tumor vasculature. PD-L1 expression in the tumor cell and tumor vasculature was assessed using antibody 22C3 (Agilent Technologies, USA) and a combined positive score of <1% (**Figure 2**). All of the three excised lymph nodes were free of tumor cells (T3N0M0, stage IIB). Adjuvant chemotherapy was prescribed after surgery, and chemotherapy regimen consisted of anthracycline plus cyclophosphamide followed by paclitaxel plus carboplatin (AC-TCb). After four cycles of AC and one cycle of TCb, multiple pulmonary metastases (>5, **Supplementary Figure S1** in the **Supplementary Appendix**) in the lung were shown in the following chest CT scan. The efficacy was evaluated as progression disease (PD). Anlotinib (10 mg, qd, days 1–14) combined with gemcitabine (1,400 mg, every 3 weeks) was prescribed for the second-line therapy. After two cycles of combined therapy, the metastases in the lung achieved PR, while after four cycles, we rechecked the enhanced CT images, and the efficacy evaluation was PD (**Supplementary Figure S2**). After multi-disciplinary treatment, we changed the original scheme, and toripalimab (an anti-PD1 antibody, Junshi Inc., China, Shanghai) at a dose of 160 mg(3 mg/kg) combined with anlotinib (10 mg, qd, days 1–14) was given every 3 weeks. After two cycles of combined therapy, the size of pulmonary metastases became smaller with no treatment-related adverse events. Up to now, the patient sustained remission more than 8 months without further complaints and side effects (sustained PR) and continue to receive anlotinib plus toripalimab regularly.

DISCUSSION

MBC is a rare and aggressive subtype of breast cancer. Reddy et al. showed that MBC has a lower OS than non-MBC (64.4 vs. 159.2 m, $p < 0.001$) (13). No standard of care for this disease is established, while the current therapy often contains chemotherapy. Our patients received immunotherapy plus anti-angiogenic therapy, which has been shown to improve the prognosis of MBC.

Previous studies showed that MBC had a unique tumor environment. Several studies observed a high level of PD-L1 expression and high density of CD8+ tumor infiltrating lymphocytes (TILs) in this tumor. Upasana et al. showed that MBC has the highest PD-L1 expression and CD8+ TILs than the other subtypes of breast cancer (14). Lien et al. observed that the positivity for TILs, combined positive score (CPS), and tumor proportion score (TPS) were 34.1%, 47.6%, and 17.1%, respectively. In addition, squamous cell carcinoma components in MBC had the highest positivity rates of TILs and CPS (15). Kalaw et al. enrolled 146 MBC patients, and 73% of them had PD-L1 expression in tumor ≥5% (16). In addition, Joneja et al. tested the expression of PD-L1 in 72 MBC patients, and the results showed positive PD-L1 of tumor and immune cells at 46% and 43%, respectively (14). High PD-L1 expression and TILs are generally associated with good response to ICIs.

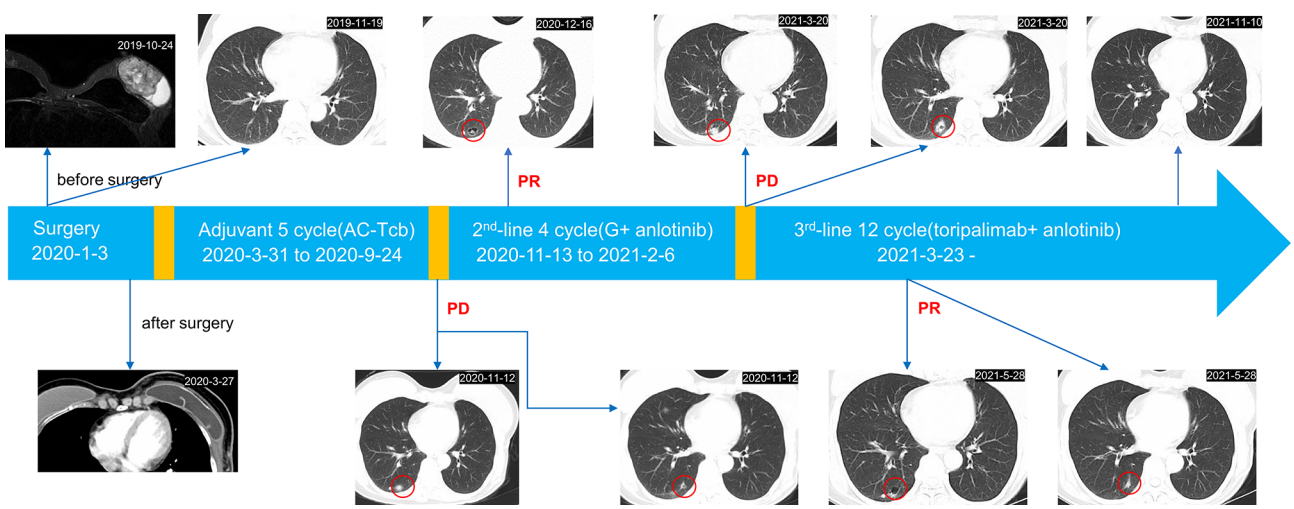

FIGURE 1 | Complete treatment process of the patient since diagnosis.

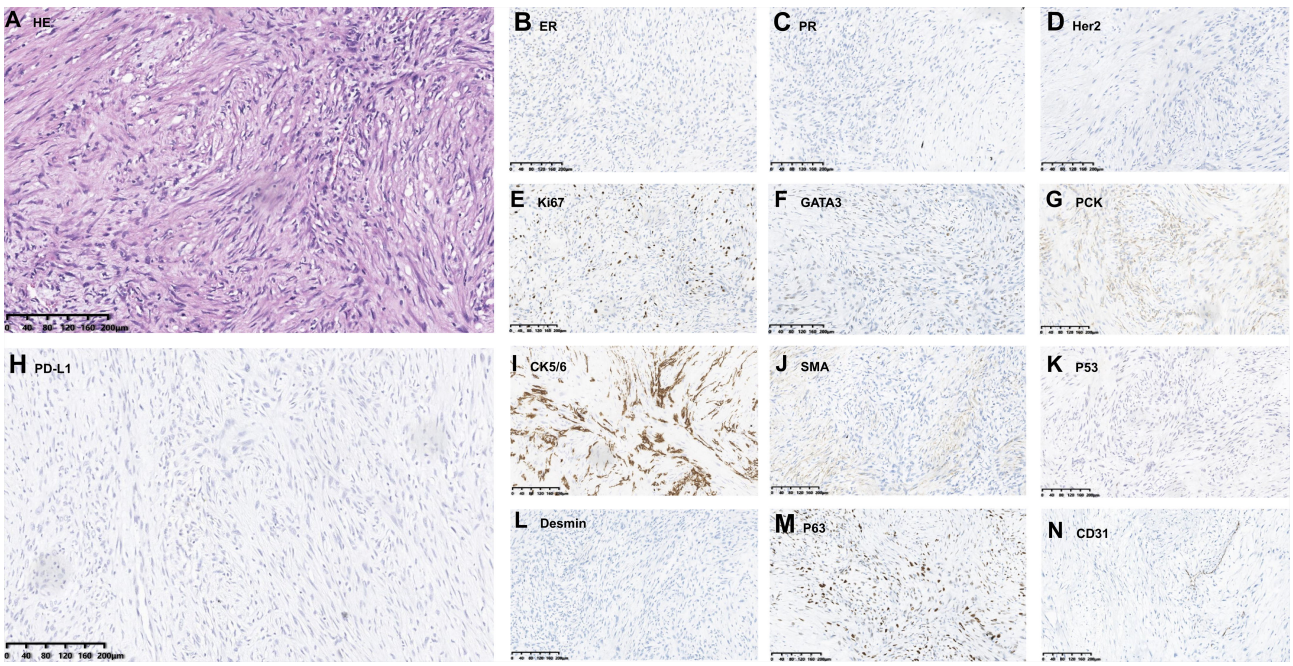

FIGURE 2 | Pathological examination showed spindle cell morphology (**A**, H&E). **(B–N)** Immunohistochemistry data: ER (–), PR (–), Her2(–), Ki-67 (+, positive proportion about 20%), GATA3 (+), PCK (+), PD-L1(–, positive proportion about <1%), CD5/6 (+), SMA (+), P53(–), Desmin (–), P63(+), and CD31 (–) supported the diagnosis. Original magnification: **(A–N)**, 200×.

ICIs have changed the treatment landscape of advanced breast cancer. Impassion 130 was a randomized phase III study that tested the efficiency of atezolizumab combined with nab-paclitaxel in the first-line treatment. The chemo-immunotherapy showed benefit in both median progression-free survival (PFS) (7.5 m vs. 5.3 m, p<0.001) and OS (21.0 m vs. 18.7 m, p = 0.078) compared with placebo plus nab-paclitaxel (8). A similar good outcome was also achieved by pembrolizumab. In the phase III KEYNOTE-355 study, the combination of pembrolizumab and chemotherapy achieved an mPFS of 9.7m (CPS > 10) (7). In a single-arm, phase 2 trial (DART trial), 17 MBC patients received the nivolumab and ipilimumab combination therapy. The ORR, median PFS, and median OS were 18%, 2 months, and 12 months, respectively (17). Previously, four case reports observed the efficacy of ICIs in advanced MBC (**Table 1**). Six of eight patients showed a good prognosis after immunotherapy (18–21).

Anlotinib is a Chinese multitarget tyrosine kinase inhibitor (TKI), which can inhibit VEGFR1, VEGFR2, VEGFR3, c-Kit, and PDGFR, and is approved by the Chinese National Medical

TABLE 1 | Summary of case reports observing the efficacy of ICI in MBC.

Source	Age (y)	Histology	ER	PR	HER2	PD-L1	Line	Therapy	PFS (m)	Response	OS (m)	Death
Adams (18)	53	Spindle cell carcinoma	–	–	–	100%	3rd	Pembrolizumab+ nab-paclitaxel	6	PR	>6	No
Sayed et al. (19)	49	Squamous cell carcinomas	–	–	+	20%	4th	Durvalumab+ paclitaxel	>24	PR	>24	No
Gorshein et al. (20)	72	Mixed metaplastic carcinoma	–	–	–	positive	1st	Pembrolizumab	24	PR	32	No
Kim et al. (21)	63	Metaplastic squamous carcinoma	–	–	–	0%	1st	Pembrolizumab+ capecitabine	6	PR	>6	No
	58	Metaplastic carcinoma mesenchymal	–	–	–	0%	1st	Pembrolizumab+ capecitabine	6	PR	NR	No
	82	Mixed metaplastic carcinoma	+	+	–	30%	3rd	Nivolumab+ bicalutamide	8	CR	NR	NR
	60	Metaplastic squamous carcinoma	–	–	–	10%	1st	Pembrolizumab+ capecitabine	3	PD	NR	NR
	62	Metaplastic carcinoma mesenchymal	–	–	–	0%	1st	Pembrolizumab+ paclitaxel	NR	PD	NR	Yes

NR, not reported; CR, complete response; PR, partial response; PD, progressive disease; m, months; y, years.

Products Administration for the treatment of advanced non-small-cell lung carcinoma (NSCLC), SCLC, soft-tissue sarcoma, and medullary thyroid carcinoma in the later line (22–25). Hu et al. investigate anlotinib for HER2-negative breast cancer in later-line therapy. The mPFS was 5.22 m, and the disease control rate (DCR) was 80.8%. Meanwhile, severe adverse events (AEs, ≥G3) were hypertension (26.92%) and hand–foot syndrome (3.85%). These results showed that anlotinib had good efficacy and limited toxicity with HER2-negative breast cancer (26). Only one case of advanced MBC treated with anlotinib has been reported. This MBC patient underwent anlotinib (12 mg/day, 2 weeks on, 1 week off), and achieved a durable PR for more than 25 months (27).

Several studies demonstrated that anti-angiogenic agents have synergistic effects with ICIs. Antiangiogenic therapy can make abnormal tumor vessels normalization, which increases the infiltration of immune effector cells in TME (28). In three phase 3 trials (IMBrave150, KEYNOTE-426, and IMpower150), atezolizumab combined with bevacizumab, pembrolizumab combined with axitinib, and atezolizumab combined with bevacizumab+ chemotherapy were shown to bring survival benefit to advanced hepatocellular carcinoma, advanced renal cell carcinoma, and advanced NSCLC, respectively (29–32). No case report described the efficacy of these combination therapy in advanced MBC. However, there were no clinical studies that reported the anlotinib plus ICI in earlier line treatment for MBC. We look forward to observing prospective clinical trials to explore the efficacy of the combined scheme on MBC in the future. In addition, it is necessary to explore the relationship of PD-L1 expression and vascularization for the efficacy of anlotinib and toripalimab.

In conclusion, we described a case of advanced MBC treated with toripalimab plus anlotinib after failure of standard chemotherapy and chemotherapy plus anti-angiogenic therapy. Immuno-combined anti-angiogenic therapy might be a useful candidate for advanced MBC.

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. The full original source data can access in https://www.jianguoyun.com/p/DcmPFKQQ7oeDChiYyJsE.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee on Biomedical Research, West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YJ and JL contributed to conception of the study. YF drafted the manuscript. YJ reviewed the manuscript. JL edited 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/fendo.2022.810747/full#supplementary-material

Supplementary Figure 1 | The CT showed that mutiple metastases of lung after adjuvant chemotherapy.

Supplementary Figure 2 | The CT showed that multiple pulmonary metastases after anlotinib plus gemcitabine.

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Epidemiology, Treatment and Prognosis Analysis of Small Cell Breast Carcinoma: A Population-Based Study

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Background: Primary small cell breast carcinoma (SCBC) is an uncommon malignancy with highly invasive behavior. The aim of this study was to find out more about the incidence, clinicopathologic characteristics and identify potential prognostic factors of SCBC.

Methods: Data of patients with primary diagnosis of SCBC between 1975 and 2018 were extracted from the Surveillance, Epidemiology, and End Results (SEER) database. The incidence after adjustment for age and percentage change per year in incidence were calculated. Disease-specific survival (DSS) and overall survival (OS) were analyzed among these SCBC patients identified from the SEER database. The whole cohorts were randomized into training and validation cohorts as ratio of 7: 3. Cox regression analysis was performed to determine predictors of survival with the training cohorts. Predictive models were constructed with training cohorts, and nomogram validation was performed using receiver operating characteristic curves, concordance indices and calibration curves in both training and validation cohorts.

Results: 323 SCBC patients were enrolled finally during the research period. The overall incidence after adjustment for age between 1990 and 2018 was 0.14 per million per year, and the prevalence of the incidence has plateaued. Most of these tumors were poorly differentiated or undifferentiated. The most prevalent presenting stage was Stage II. Patients identified in this study were randomly divided into training (n = 226) and testing (n = 97) cohorts. Multivariate Cox proportional hazards model showed that chemotherapy, surgery and stage were important predictors of DSS and OS.

Conclusion: SCBC is considered an infrequent breast neoplasm with aggressive characteristics. Tumor stage is associated with poor prognosis. Combination of surgery and chemotherapy is the main treatment for SCBC.

Keywords: breast, small cell carcinoma, epidemiology, prognosis, disease-specific survival, overall survival

INTRODUCTION

Primary small cell breast carcinoma (SCBC) is an uncommon neoplasm that makes up less than 1% of all invasive breast cancer cases and approximately 7% of all extrapulmonary small cell carcinomas (1, 2). SCBC, a subtype of neuroendocrine neoplasm, was first described by Wade et al. in 1983, and diagnostic criteria were first proposed by Sapino et al. in 2000 (3, 4). Because of the rarity of instances, an agreement on the nomenclature and diagnostic criteria of SCBC could not be reached for a long period. Recently, a new classification system arisen by the World Health Organization expert panel defined SCBC as a neuroendocrine carcinoma with poor differentiation (1). A study published in 2021 demonstrated that disease-specific survival (DSS) and overall survival (OS) of neuroendocrine neoplasm was significantly worse than invasive ductal carcinoma of no special type (All $P < 0.001$). However, further analyse of SCBC was not conducted in this study (5).

In terms of treatment, the standardized therapy protocol for SCBC is largely undefined. Given the similar histologic and morphologic features with small cell lung cancer (SCLC), the current clinical management of SCBC is mostly extrapolated from the therapeutic strategies of SCLC, mainly combining surgery, chemotherapy and radiotherapy. The main chemotherapy schedules used include etoposide and platinum agents, even anthracycline and taxane (6). Morever, A case of SCBC patient treated with regimen of doxorubicin and cyclophosphamide then followed by carboplatin and etoposide achieved favourable therapeutic effect (7)[Append 21]. The administration of adjuvant radiotherapy is given based on the size of tumor and status of lymph node (8). Moreover, endocrine treatment is added when SCBC expresses the relative hormone receptors (9, 10). About 75% of SCBC patients were detected with TP53 mutations and 33% cases detected with PIK3CA mutations, which means TP53 and PIK3CA could serve as the potential therapeutic targets (11). Because of the limited number of prospective studies and large sample investigations for consolidating the medical evidence of SCBC, the standard for a definitive and preferable management strategy varies among different medical institutions or clinicians.

In our study, we gathered a sizable sample of SCBC patients and extracted clinical information from the Surveillance, Epidemiology and End Results (SEER) database for analysis. We investigated the incidence, tumor characteristics and outcomes of SCBC and explored optimal treatments and potential prognostic factors for SCBC.

MATERIALS AND METHODS

Patients Selection

The SEER 18 Registries data set was utilized to identify individuals who were first diagnosed with SCBC from 1975 to 2018 (12). Patients with SCBC were identified in the SEER database using topographical and histology codes from the International Classification of Diseases (ICD-O-3). C50.0 to C50.6, C50.8, and C50.9 were the topographical codes utilized in this investigation. Cases of small cell carcinoma was identified using the ICD-O-3 histological codes 8041, 8042, 8043, 8044, and 8045. Clinical, pathological and survival information was collected by using SEER*Stat version 8.3.6. Patients who met following criteria were recruited in to our study: 1) female patient with age more than 18 years; 2) The diagnosis of SCBC was confirmed by pathology; 3) SCBC was the primary cancer and no other primary cancers; 4) complete survival duration and cause

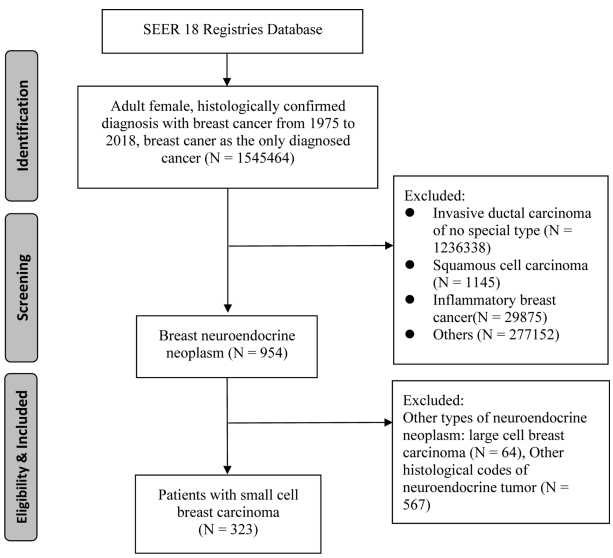

FIGURE 1 | Flow diagram of patients enrollment in this study.

of death. Finally, we identified a total of 323 patients for this study cohort (**Figure 1**).

Primary cohort with 323 patients met enrollment criteria was further divided into training cohort (n = 226) and validation cohort (n = 97) randomly as ratio of 7:3. Training cohort with 226 patients were used to construct the nomograms and validation cohort with 97 cases were used for nomograms validation.

Endpoint Definition

Patients with a first primary diagnosis of SCBC who had complete staging and survival information were chosen for survival analysis. The endpoints of this research were (DSS, which was the interval between the primary diagnosis of SCBC to the death related with SCBC, and OS, which was the interval from the primary diagnosis of SCBC to death or the last visit.

Epidemiological Analysis

The incidence rates were calculated, and age was adjusted to the 2000 US population, as the number of new occurrences per 1,000,000 person-years. The yearly percent change was calculated using the weighted least squares technique. For incidence trend analysis, the percentage change was assessed by comparison to zero.

Clinical Characteristic Analysis

In the current study, Grade I or highly differentiated was defined as G1, grade II or moderately differentiated as G2, and grade III or badly differentiated and grade IV or undifferentiated as G3. For all recruited patients, Kaplan–Meier curves were used to predict survival rates, and the log-rank test was used to examine differences in survival distributions across groups. The multivariate Cox model was used to determine independent prognostic variables with training cohort. Then, A nomogram based on the regression coefficients of each element in the multivariate study was used to visualize the prediction model with training cohort. Validation cohort was used for external validation. Survival prediction value of the nomograms was calculated by performing the area under the receiver operating characteristic (ROC) curve (AUC), calculating concordance index (C-index) and conducting calibration curves. Specificity and sensitivity were derived from the areas under the ROC curves (AUCs). The AUC was used to assess the signature's prediction abilities. The C-index was used to assess the prediction accuracy and discriminating capabilities of each component and the nomogram. To test the nomogram's calibration, calibration curves (500 bootstrap resamples) were produced.

Immunohistochemistry

Pathology and immunohistochemistry were conducted with SCBC tissue. CD56 and Syn localization and Ki-67 expression were evaluated with immunohistochemistry. The tumor tissues of one SCBC patient from Jiangnan University's Affiliated Hospital were sliced in order, then dewaxed and rehydrated in graded alcohols. The slides were stained with immunohistochemistry according to the manufacturer's directions. Antibodies for the identification of CD56, Syn and Ki-67 protein expression were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An Aperio pathology workstation (Aperio) was used for quantitative evaluation. The percentage of cells that stained positively was automatically calculated. The immunohistochemistry data and use of tumor tissue for this investigation were approved by the patient and the institutional review board of the Affiliated Hospital of Jiangnan University.

Statistical Analysis

R software version 3.6.0 and SPSS version 24.0.0 (SPSS, Chicago, IL) were used for statistical analysis. The primary cohort was randomized into training cohort and testing cohort using R software. All tests were two-sided. A P value < 0.1 was defined as the criterion for eliminating variables in the multivariate Cox model, and a P value < 0.05 was considered to be significant for further testing.

RESULTS

Incidence

Due to the limitation of population data acquisition, only the incidences of SCBC between 1990 and 2018 were calculated. The overall age-adjusted incidence of SCBC was 0.14 per 1,000,000 per year during this period. The incidence of SCBC reached a peak in 2003 (0.19 per 1,000,000), but the overall prevalence of the incidence has plateaued during the last 28 years (**Figure 2A**). The incidence was the highest in the 65–69 age group (**Figure 2B**).

Clinical Characteristics and Survival

A total of 323 patients initially diagnosed with SCBC were recruited during the study period. At the time of diagnosis, the median age was 65 years (range: 28–97 years). The most frequent stage among these individuals was stage II and the median tumor size was 3.5 cm. The tumors were mostly poorly differentiated or undifferentiated (133/155). The majority of the patients (94/152) tested negative for estrogen receptor (ER) and progesterone receptor (PR) expression. Of note, human epidermal growth factor receptor 2 (HER-2) status was only reported in patients after 2010 in SEER database, and only one patient (1/67) had positive HER-2 status. Approximately half of these patients (37/67) had triple-negative breast cancer (TNBC). In terms of treatment, most SCBC patients underwent surgery (238/313). Of these 238 patients, 82 received postoperative adjuvant radiotherapy, and 4 received neoadjuvant radiotherapy before surgery. The summary of clinical characteristics is shown in **Table 1**.

The 3- and 5-year DSS rates were 64.9% and 61.6%, respectively (**Figure 3A**). The 3- and 5-year OS rates were 53.1% and 47.7%, respectively, and the median OS was 50 months (95% confidential interval (CI), 0.432–0.589) (**Figure 3B**). Because some patients had incomplete clinical information, 159 patients with overall stage disease were

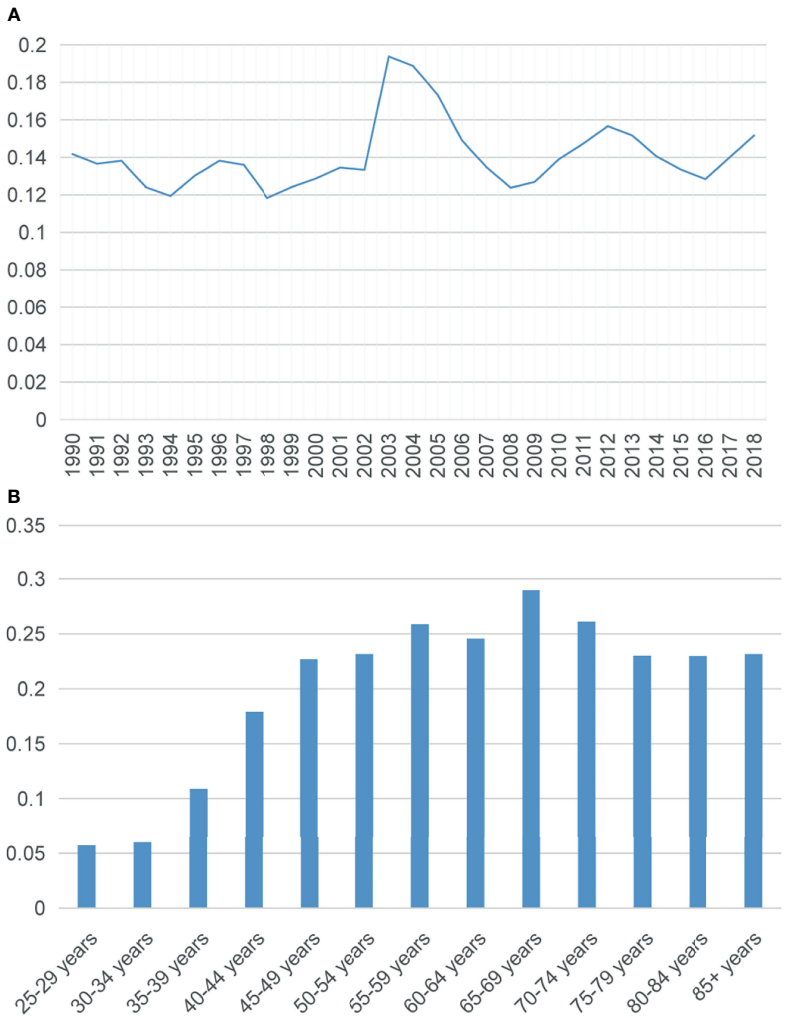

FIGURE 2 | **(A)** Trend in the incidence of small cell carcinoma of the breast from 1990 to 2018. **(B)** Age-wise incidence. All rates were per 1,000,000, and age was adjusted to the 2000 US standard population.

recruited for further survival analysis. The 3- and 5-year DSS rates for stage I disease were 94.3% and 94.3%, respectively, while those for stage IV disease were 26.6% and 8.8%. The 3- and 5-year OS rates for stage I disease were 88.6% and 85.7%, respectively, while those for stage IV disease were 13.9% and 4.6%, respectively. Significant differences were observed among the different stages in both DSS (P<0.0001) and OS (P<0.0001) (**Figures 3C, D**). Patients who treated with both surgery and chemotherapy had greater DSS and OS rates than those who received just surgery or chemotherapy, or none of the two therapies. (P<0.0001) (**Figures 3E, F**).

Differentiation, tumor location, stage, surgery, radiation therapy and chemotherapy were significantly correlated with poor DSS in the univariate Cox analysis (P<0.10). Age at diagnosis, tumor differentiation, tumor location, stage, surgery, and radiation therapy were related to poor OS (P<0.10). Given the actual clinic practice the risk factor of chemotherapy and these significant variables were enrolled into multivariate analysis. Stage (P = 0.000) (stage II vs. stage I, HR:7.632; 95% CI, 1.105-16.439; P = 0.014) (stage III vs. stage I, HR:26.314; 95% CI, 2.476-68.421; P = 0.000) (stage IV vs. stage I, HR:55.968; 95% CI, 6.678-126.573; P = 0.000), surgery (HR:2.108; 95% CI, 1.063-3.585; P = 0.046), and chemotherapy (HR:3.543; 95% CI, 1.564-6.593; P = 0.003) were the independent variables in DSS, according to multivariate analysis. Stage (P = 0.000) (stage II vs. stage I, HR:2.896; 95% CI, 1.061-6.386; P = 0.019) (stage III vs. stage I, HR:7.472; 95% CI, 2.659-14.351; P = 0.000) (stage IV vs. stage I, HR:21.876; 95% CI, 5.109-48.215; P = 0.000), surgery (HR:1.493; 95% CI, 1.113-3.879; P = 0.036), and chemotherapy (HR:2.469; 95% CI, 2.279-7.361; P = 0.000) were also independent factors for OS in multivariate analysis. **Table 2** summarizes the outcomes of the univariate and multivariate analysis for DSS and OS. All of the independent factors were included in the predictive models for DSS and OS and visually presented as nomograms (**Figures 4A, B**). The calibration curves showed good consistency in the 3- and 5-year DSS and OS probabilities between the actual observations and the nomogram predictions in the

TABLE 1 | Patient, tumor and treatment characteristics.

Parameter	All patients (Percent)	Training Cohort (Percent)	Validation Cohort (Percent)	*P*
Total number of cases (1975-2018)	**323 (100)**	**226 (100)**	**97 (100)**	
Age at diagnosis				0.453
Median (years)	65	63	66	
Range	28-97	28-97	30-87	
Tumor size				0.685
Median (cm)	3.5	3.3	3.6	
Range	0.3-15.7	0.3-15.5	0.5-15.7	
Race				0.355
White	275 (85.1)	191 (84.5)	84 (86.6)	
Black	38 (11.8)	29 (12.8)	9 (9.3)	
Other	9 (2.8)	6 (2.7)	3 (3.1)	
Unknown	1 (0.3)	0 (0.0)	1 (1.0)	
Laterality				0.794
Right	155 (48.0)	106 (46.9)	49 (50.5)	
Left	160 (49.5)	114 (50.4)	46 (47.4)	
Bilateral	1 (0.3)	1 (0.4)	0 (0.0)	
	7 (2.2)	5 (2.2)	2 (2.1)	
Unknown				
Tumor location				0.291
Medial/central	62 (19.2)	45 (19.9)	17 (17.5)	
Outer	125 (38.7)	83 (36.7)	42 (43.3)	
Other	50 (15.5)	32 (14.2)	18 (18.6)	
Unknown	86 (26.6)	66 (29.2)	20 (20.6)	
Hormone receptor status				0.016
ER-/PR-	94 (29.1)	63 (27.9)	31 (32.0)	
ER+/PR-	11 (3.4)	5 (2.2)	6 (6.2)	
ER-/PR+	9 (2.8)	5 (2.2)	4 (4.1)	
ER+/PR+	38 (11.8)	21 (9.3)	17 (17.5)	
Unknown	171 (52.9)	132 (58.4)	39 (40.2)	
HER2				0.476
Positive	1 (0.3)	1 (0.4)	0 (0.0)	
Negative	66 (20.4)	49 (21.7)	17 (17.5)	
Unknown	256 (79.3)	176 (77.9)	80 (82.5)	
Differentiation				0.325
Grade 1	6 (1.9)	5 (2.2)	1 (1.0)	
Grade 2	16 (5.0)	10 (4.4)	6 (6.2)	
Grade 3	133 (41.1)	87 (38.5)	46 (47.4)	
Unknown	168 (52.0)	124 (54.9)	44 (45.4)	
T-stage				0.092
1-2	89 (27.6)	56 (24.8)	33 (34.0)	
3-4	30 (9.3)	25 (11.1)	5 (5.2)	
Unknown	204 (63.1)	145 (64.2)	59 (60.8)	
N-stage				0.173
N0	81 (25.1)	50 (22.1)	31 (32.0)	
N+	68 (21.0)	49 (21.7)	19 (19.6)	
Unknown	174 (53.9)	127 (56.2)	47 (48.5)	
Overall staging (AJCC)				0.107

(Continued)

TABLE 1 | Continued

Parameter	All patients (Percent)	Training Cohort (Percent)	Validation Cohort (Percent)	*P*
Total number of cases (1975-2018)	**323 (100)**	**226 (100)**	**97 (100)**	
I	35 (10.8)	24 (10.6)	11 (11.3)	
II	56 (17.3)	33 (14.6)	23 (23.7)	
III	32 (9.9)	27 (11.9)	5 (5.2)	
IV	36 (11.1)	23 (10.2)	13 (13.4)	
Unknown	164 (50.9)	119 (52.7)	45 (46.4)	
Surgery				0.253
Yes	238 (73.7)	146 (64.6)	92 (94.8)	
No	75 (23.2)	52 (23.0)	23 (23.7)	
Unknown	10 (3.1)	8 (3.5)	2 (2.1)	
Radiation therapy				0.088
Yes	89 (27.6)	56 (24.8)	33 (34.0)	
No	234 (72.4)	170 (75.2)	64 (66.0)	
Chemotherapy				0.264
Yes	135 (41.8)	99 (43.8)	36 (37.1)	
No/Unknown	188 (58.2)	127 (56.2)	61 (62.9)	
Marital status at diagnosis				0.052
Single	27 (8.4)	14 (6.2)	13 (13.4)	
Married or ever married	282 (87.3)	200 (88.5)	82 (84.5)	
Unknown	14 (4.3)	12 (5.3)	2 (2.1)	

HER2, human epidermal growth factor receptor 2.

training cohort (**Figures 4C, D**) and in the valiation cohort (**Figures 4E, F**). The C-index of the two nomograms for DSS and OS in the training cohort were 0.834 (95% CI, 0.773–0.894) and 0.829 (95% CI, 0.775–0.883), reflecting the good discrimination ability of the models. In the validation cohort, the C-index for the constructed nomogram to predict DSS and OS were 0.780 (95% CI, 0.670–0.891) and 0.769 (95% CI, 0.707–0.831).

The AUCs for 3- and 5-year DSS in the whole cohort were 0.823 (95% CI, 0.753–0.879) and 0.848 (95% CI, 0.807–0.916), respectively (**Figure 5A**). The AUCs for 3- and 5-year OS in the whole cohort were 0.775 (95% CI, 0.711–0.839) and 0.801 (95% CI, 0.753–0.851), respectively (**Figure 5B**). In the training cohort, the AUCs for 3- and 5-year DSS were 0.864 (95% CI, 0.782–0.917) and 0.877 (95% CI, 0.821–0.938), respectively (**Figure 5C**). The AUCs for 3- and 5-year OS in the training cohort were 0.798 (95% CI, 0.723–0.872) and 0.815 (95% CI, 0.756–0.892), respectively (**Figure 5D**). In the validation cohort, the AUCs for 3- and 5-year DSS were 0.780 (95% CI, 0.637–0.878) and 0.802 (95% CI, 0.707–0.912), respectively (**Figure 5E**). The AUCs for 3- and 5-year OS in the validation cohort were 0.720 (95% CI, 0.597–0.844) and 0.769 (95% CI, 0.659–0.881), respectively (**Figure 5F**).

Immunohistochemistry

Histopathological examination of the stained specimen under a high-power field (10x, 20x) showed neoplastic cells arranged in solid sheets and short fusiform with light nuclei, fine chromatin, unclear nucleoli and a high mitotic rate, which is compatible with a SCBC diagnosis (**Figures 6A, B**). Immunohistochemical analysis showed that Syn (**Figure 6C**) and CD56 (**Figure 6D**) were positive and Ki67 was 65% (**Figure 6E**).

DISCUSSION

Primary SCBC is a rare malignancy, and the available information is limited to individual case reports or small case series in the literature. The clinical characteristics of 56 SCBC patients were analyzed in the study of Boutrid et al., but further survival analysis was not conducted (13). Similar outcomes were reported by Shin et al. (9) and Kanat et al. (14), who managed 9 and 7 patients, respectively. In this study, 323 SCBS patients between 1975 and 2018 were recruited through the SEER program. Our study is the largest scale report on SCBC with long-term follow-up and comprehensively analyze the incidence, survival, and prognostic factors of this malignancy.

SCBC is a rare malignant tumor, according to the outcome of our study, with a total incidence of 0.14 instances per million women each year between 1990 and 2018. The incidence reached the peak in 2003, and then a slight decline in SCBC was observed. However, the incidence of SCBC remained stable during the study period. Improved histological classification may serve as an influencing factor accounting for this phenomenon. Patients in the 65–69 age group had the highest incidence of SCBC, and the disease was more common in older women aged > 60 years,

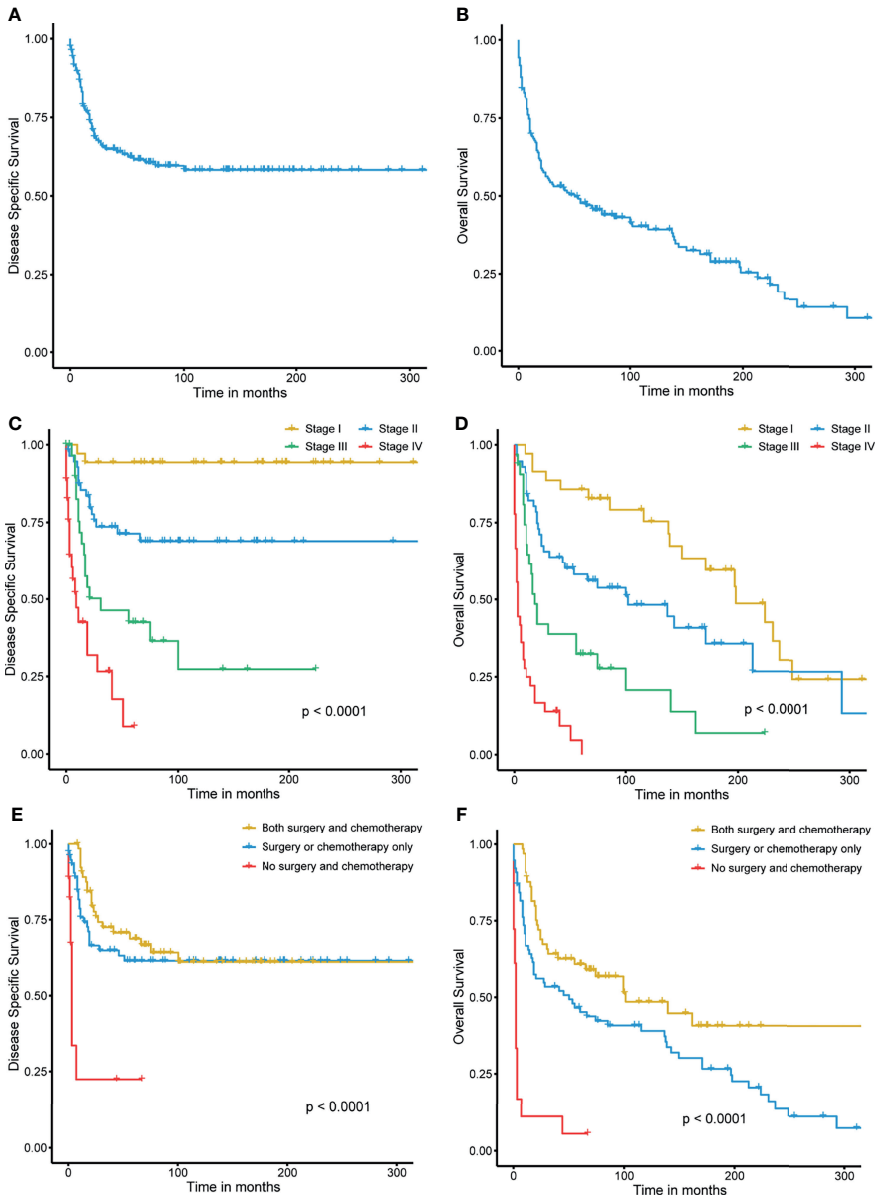

FIGURE 3 | **(A, B)** Disease-specific survival and overall survival of small cell carcinoma of the breast. **(C, D)** Stage-wise disease-specific survival and overall survival of small cell carcinoma of the breast. **(E, F)** Treatment-wise disease-specific survival and overall survival of small cell carcinoma of the breast.

which is in line with a previous report (15). In terms of the prognosis of SCBC, the 5-year DSS and OS were 61.6% and 53.1%, respectively, and patients with SCBC had poorer survival than patients with any no uncommon type of invasive ductal carcinoma, who had 5-year DSS and OS rates of 89.2% and 83.2%, respectively. Patients with neuroendocrine neoplasms of the breast (containing 28.3% SCBC patients) had slightly higher 5-year DSS (63.4%) and OS (55.7%) rates (5). Stage-stratified prognostic analysis showed that patients with an initial diagnosis of stage IV disease had the worst DSS and OS. A high risk of recurrence and metastasis may contribute to poor survival.

Primary SCBC has similar biological and clinical characteristics to small SCLC. Most of patents with these two diseases have positive chromogranin A and synaptophysin in terms of Immunohistochemistry, but it is not necessary for diagnosis. While ductal carcinoma *in situ* promotes the diagnosis of breast carcinoma (16). More than half of the two malignancy patients have positive expression of TTF-1, which needs imaging methods to differentiate at diagnosis (13). In the early stages of the two diseases, SCBC has a more favorable outcomes compared with SCLC. And SCBC even has the best prognosis among the extrapulmonary small cell carcinomas,

TABLE 2 | Univariate and multivariate analysis for disease specific survival and overall survival with training cohort.

Variables	UVA (DSS)			MVA (DSS)			UVA (OS)			MVA (OS)		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Year of diagnosis	0.528	(0.364-1.115)	0.156				0.985	(0.897-1.043)	0.574			
Age at diagnosis	1.013	(0.975-1.011)	0.673				1.083	(1.114-1.538)	0.001	1.106	(0.847-1.178)	0.125
Race			0.640						0.328			
White	Ref						Ref					
Black	1.163	(0.651-2.486)	0.518				0.832	(0.548-1.379)	0.388			
Other	1.879	(0.214-3.981)	0.968				1.015	(0.164-2.642)	0.591			
Differentiation			0.081			0.325			0.026			0.207
Grade 1	Ref			Ref			Ref			Ref		
Grade 2	2.127	(1.003-4.921)	0.049	1.927	(0.683-4.538)	0.219	2.717	(0.183-5.467)	0.793	1.015	(0.245-2.739)	0.889
Grade 3	4.645	(1.682-10.682)	0.037	2.645	(0.908-5.682)	0.059	2.645	(0.704-5.468)	0.252	2.210	(0.579-6.427)	0.428
Unknown	3.495	(0.452-7.718)	0.421	2.016	(0.561-4.438)	0.382	4.329	(0.658-9.356)	0.443	3.170	(0.473-8.591)	0.657
ER/PR status			0.301						0.312			
ER-/PR-	Ref						Ref					
ER+/PR-	1.128	(0.613-2.233)	0.687				1.645	(0.438-4.414)	0.425			
ER-/PR+	0.818	(0.121-2.272)	0.385				2.190	(0.631-4.952)	0.532			
ER+/PR+	1.433	(0.298-3.512)	0.951				0.636	(0.246-1.479)	0.105			
Unknown ER or PR status	0.459	(0.181-1.136)	0.101				1.242	(0.609-2.072)	0.482			
Laterality			0.131						0.106			
Left	Ref						Ref					
Right	1.158	(0.665-1.643)	0.788				1.109	(0.873-1.732)	0.432			
Bilateral	5.288	(1.019-10.768)	0.063				5.267	(1.443-11.365)	0.003			
Tumor Location			0.000			0.078			0.001			0.318
Medial or Central	Ref			Ref			Ref			Ref		
Outer	1.358	(0.561-3.889)	0.282	1.649	(0.467-4.583)	0.453	1.485	(0.796-3.328)	0.172	1.698	(0.739-3.191)	0.326
Other	3.126	(1.009-8.793)	0.046	1.749	(0.577-5.756)	0.310	1.892	(0.980-4.478)	0.056	1.602	(0.669-3.896)	0.293
Unknown	6.388	(2.313-13.528)	0.021	3.679	(1.001-9.773)	0.050	3.365	(1.968-8.341)	0.001	1.769	(0.863-5.347)	0.134
Stage			0.000			0.000			0.000			0.000
I	Ref			Ref			Ref			Ref		
II	5.768	(1.412-11.826)	0.029	7.632	(1.105-16.439)	0.014	1.687	(1.056-3.653)	0.038	2.896	(1.061-6.386)	0.019
III	18.394	(3.675-45.852)	0.000	26.314	(2.476-68.421)	0.000	3.416	(1.786-8.816)	0.003	7.472	(2.659-14.351)	0.000
IV	35.235	(8.287-109.257)	0.000	55.968	(6.678-126.573)	0.000	11.615	(6.296-24.663)	0.000	21.876	(5.109-48.215)	0.000
Surgery												
Yes	Ref			Ref			Ref			Ref		
No	4.732	(1.879-10.856)	0.000	2.108	(1.063-3.585)	0.046	4.318	(3.013-6.463)	0.000	1.493	(1.113-3.879)	0.036
Radiation therapy												
Yes	Ref			Ref			Ref			Ref		
No	2.496	(1.103-3.467)	0.038	0.687	(0.365-2.014)	0.558	1.921	(1.421-2.926)	0.003	1.493	(0.687-2.396)	0.732
Chemotherapy												
Yes	Ref			Ref			Ref			Ref		
No/Unknown	0.694	(0.511-1.384)	0.259	3.543	(1.564-6.593)	0.003	1.102	(0.867-1.782)	0.203	2.469	(2.279-7.361)	0.000

CI, confidence interval; DSS, disease specific survival; HR, hazard ratio; MVA, multivariate analysis; OS, overall survival; UVA, univariate analysis.

which attributed to the early detection and diagnosis of SCBC (2, 17).

Given the rarity of primary SCBC and limited reports in the previous literature, the optimum therapeutic modalities for this malignancy are still unknown. Surgery remains the major treatment for SCBC, including modified radical mastectomy and lumpectomy, especially for patients with disease in early stages of the condition. In this current study, 73.7% (238/323) of SCBC patients and 90.1% (9/91) SCBC patients with stage I or II disease underwent surgery. However, large cases and long follow-up studies comparing the outcomes of different surgical treatments are lacking. In addition to surgery, chemotherapy has a significant impact on the survival of SCBC, as shown in our study. Although a previous study showed that neuroendocrine neoplasms of the breast, gastrointestinal and pulmonary neuroendocrine system were not sensitive to chemotherapy, several case review series observed the prognosis benefit of adjuvant chemotherapy in the treatment of SCBC, especially in those with a high risk of recurrence (9, 13, 18). The mainstay chemotherapy regimens include anthracycline- and taxane-based chemotherapy regimens are commonly utilized for invasive breast cancers, whereas platinum-based chemotherapy is uregularly employed for small cell lung cancer (10, 14, 16, 19). Yildirim et al. proposed that platinum compounds and etoposide should be recommended for SCBC with Ki67 >15%; otherwise, an adriamycin-based regimen is preferred (20). The limited number of SCBC patients who received neoadjuvant chemotherapy with various chemotherapy regimens did not show satisfactory outcomes (9, 21, 22). With the advent of the era of immunotherapy, chemotherapy combined with immune checkpoint inhibitors (ICIs) could improve the survival of

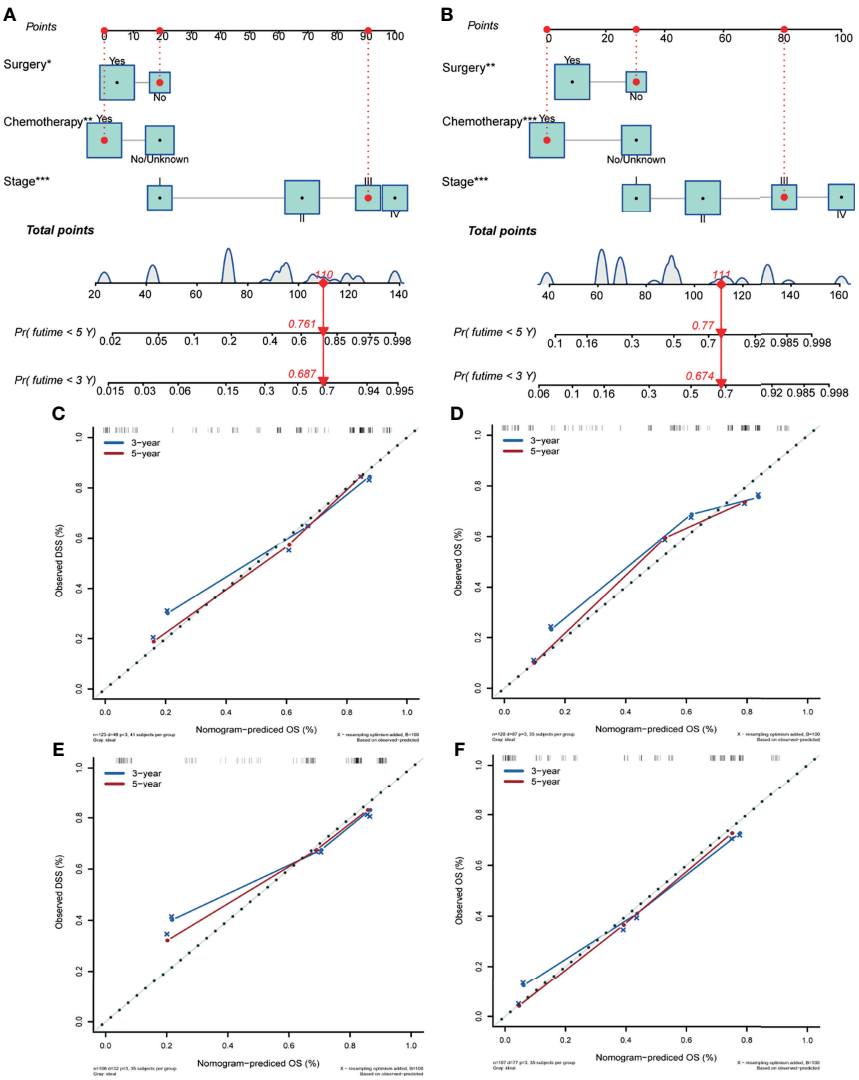

FIGURE 4 | Nomogram predicting the disease-specific survival **(A)** and overall survival **(B)** of patients with initially diagnosed small cell carcinoma of the breast. Calibration plots in the training **(C, D)** and validation **(E, F)** cohorts for 3- and 5-year disease-specific survival and overall survival. Significant differences are defined by *p < 0.05, **p < 0.01, and ***p < 0.001.

extensive-stage SCLC and advanced triple negative breast cancer patients based on the outcomes of phase III study such as IMpower 133 and IMpassion 130 (23, 24). Given the similar histologic and morphologic between SCBC and SCLC and triple negative breast cancer accounting for more than half SCBC, we consider that SCBC patients may benefit from ICIs treatment.

The use of radiotherapy in the management of SCBC patients remains controversial. Grossman et al. discovered that radiation had a survival benefit of radiotherapy among patients with extrapulmonary small cell carcinomas, including SCBC (2). Hare et al. analyzed the survival value of adjuvant radiotherapy in patients with localized and regional SCBC (25). However, no significant improvement in OS was observed in the adjuvant radiation group in their investigation, which is similar to the outcomes of our survival analysis and those of another study by Abbasi et al. (26). Molecular subtype may affect the treatment response. We observed that most SCBCs are TNBC. A recent BEATRICE trial-based retrospective study demonstrated that postmastectomy radiation therapy did not improve survival in TNBC with N0 or N1 status (27). Moreover, postoperative radiation is always recommended for patients with high-risk pathological characteristics, and the real benefit of radiotherapy is underestimated. Therefore, patients may potentially benefit from postoperative radiotherapy, and a larger sample size study is warranted.

Currently, immunohistochemical staining remains the most commonly used tool at the molecular biology level for SCBC. A

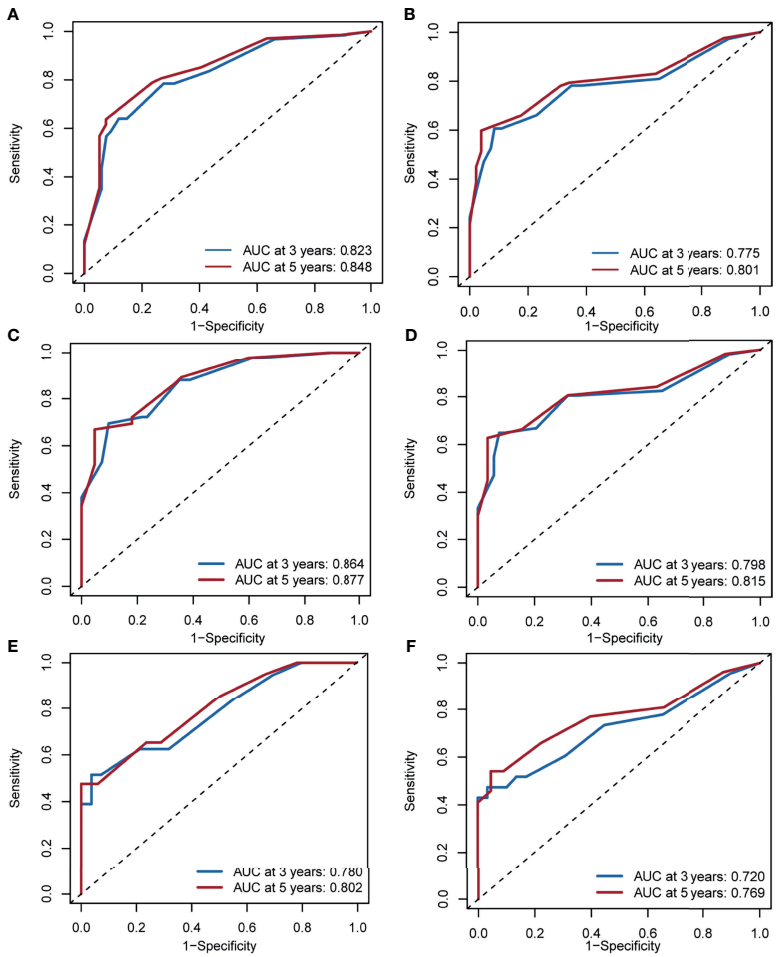

FIGURE 5 | Area under the receiver operating characteristic curve for 3- and 5-year disease-specific survival and overall survival in the whole cohorts **(A, B)**, training cohorts **(C, D)** and validation cohorts **(E, F)**.

wide variety of ER/PR statuses were observed among previous case series reports, while negative HER-2 status was commonly reported (9, 13, 28, 29). A high proportion of TNBC in SCBC was observed in both our study and others' (10). To explore potential treatment targets, the genomic landscape of SCBC and small cell lung cancer was compared by McCullar et al. with next-generation sequencing (11). PIK3CA mutations only occurred in SCBC, with a 33% mutation rate, and a high level of TOP2A expression (77%) in SCBC was observed in their study. Therefore, PIK3CA and TOP2A were considered possible targets for treatment. PIK3CA mutations that activate the phosphatidylinositol-3-kinase (PI3K) pathway have association with poor prognosis in breast cancer patients (30). The SOLAR-1 trial found that adding alpelisib, a PI3Kα-specific inhibitor, provided a progression-free survival benefit and OS improvement in PIK3CA-mutated, hormone receptor-positive, HER2-negative advanced breast cancer patients (31). Alteration of TOP2A was reported to be related with restricted responsiveness to anthracycline-based chemotherapy in breast cancer (32). Thus, PIK3CA-targeted immunochemotherapy may also serve as an optional treatment option for SCBC, and the detection of TOP2A status before chemotherapy is necessary.

Several flaws exist in this study. First, the SEER database was used in this study. Although our study has the largest sample size of SCBC patients, the number of cases is still small compared to that of other common histologies. Subgroup analysis and external validation of the predictive model could not be conducted. Second, information on several variables in the SEER database is incomplete, such as molecular subtype, staging, regimen of chemotherapy and radiotherapy dose, which may affect the accuracy of the predictive model. Third, due to the uniform diagnostic criteria of SCBC, inconsistent recognition and diagnosis may exist during the study period. Last, inherent biases were inevitable in this retrospective analysis.

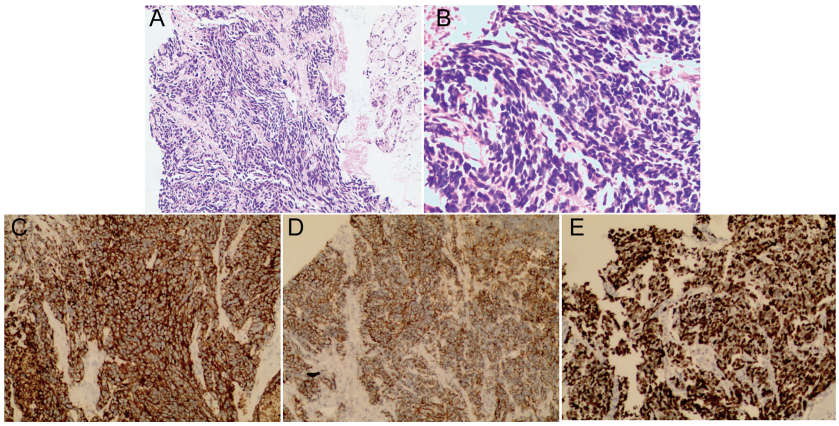

FIGURE 6 | Histopathological examination of the stained specimen under a high-power field showed neoplastic cells arranged in solid sheets and short fusiform with light nuclei, fine chromatin, unclear nucleoli and a high mitotic rate consistent with the diagnosis of SCBC. (**A** 10x, **B** 20x). Immunohistochemical analysis showed that Syn **(C)** and CD56 **(D)** were positive and Ki67 was 65% **(E)**.

CONCLUSION

SCBC is a rare, aggressive tumor that needs uniform multimodality therapies. The incidence of this malignancy is stable. Surgery and chemotherapy still play important roles in the treatment of SCBC and serve as independent factors, in addition to staging. A nomogram for predicting the DSS and OS of patients with initially diagnosed SCBC was established using the three above mentioned factors. Future external validation is needed, and prospective clinical trials are warranted to explore better treatment strategies.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JZ drafted the primary manuscript. KG and SJ conceived and supervised this research and revised the manuscript. SJ and GW contributed to the design of this research. JZ, YZ, BY, QC, and JJ collected and analyzed data. YM contributed to statistical analysis. All authors contributed to the article and approved the submitted version.

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The Effect of HER2 Status on Metaplastic Breast Cancer A Propensity Score-Matched Analysis

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Background: The role of human epidermal growth factor receptor 2 (HER2) in metaplastic breast cancer (MBC) patients remains unclear. The present study aimed to evaluate the effect of HER2 status on MBC patients by propensity-score matching (PSM).

Methods: The SEER data from 2010 to 2016 were extracted. The breast cancer-specific survival (BCSS) of MBC patients, diagnosed from 2001 to 2016, was compared using Kaplan–Meier analysis. The multivariate Cox proportional model between groups was performed. PSM was used to make 1:1 case-control matching.

Results: We included 1887 patients with a median follow-up time of 28 months (range 1-83 months). 1749 (92.7%) and 138 (7.3%) patients presented in the HER2-negative group and HER2-positive group. 833 (44.1%) patients received post-mastectomy radiotherapy (PMRT). The HER2-positive group had younger patients, lower tumor grades, and more advanced tumor stages. The prognoses were related to age of diagnosis, race/ethnicity, TNM stage, and PMRT in multivariate Cox analysis. ER status and HER2 status had no impact on BCSS. In the Kaplan-Meier analysis, PMRT was associated with a better prognosis. Importantly, patients with HER2-negative status can benefit from PMRT, but not those with HER2-positive status. After PSM, on multivariate Cox analysis, the prognosis was related to HER2 status and PMRT. In the Kaplan-Meier analysis, PMRT was related to a better prognosis for HER2-negative patients.

Conclusions: Our findings supported that PMRT and HER2-positive status were associated with a better prognosis after PSM. However, HER2-negative, but not HER2-positive patients could benefit from PMRT.

Keywords: metaplastic breast cancer, post-mastectomy radiotherapy, human epidermal growth factor receptor 2, prognosis, propensity score-matched

BACKGROUND

Metaplastic breast cancer (MBC) was rare and the World Health Organization identified it as a unique pathological type in 2000 (1). MBC is a rare histologic subtype, accounting for about 2-5% of breast cancer (2). It was classified into 5 subtypes: squamous cell carcinoma, spindle cell carcinoma, matrix-producing carcinoma, carcinosarcoma, and metaplastic carcinoma with osteoclastic giant cells (3–7). With the improvement of pathologists' awareness of MBC, the incidence also increases (8). However, due to its rarity, the role of human epidermal growth factor receptor 2 (HER2) status in the treatments and prognoses of MBC is unclear.

Of note, although treatments of MBC are parallel to that of infiltrating ductal carcinoma (IDC) (9), the prognosis of MBC patients was worse than that of IDC even after receiving comprehensive treatment (10, 11). However, there is no consensus on post-mastectomy radiotherapy (PMRT) in the management of MBC. On the one hand, some researchers reported that PMRT of patients showed a better prognosis than that non-PMRT (9, 12–17). On the other hand, others debated that no connection was presented between PMTR and outcomes (18–21). The management strategy and sample sizes of the study populations may result in this conflict.

HER2-positive (HER2 +) status in traditional breast cancers is an aggressive disease related to drugs resistance, regional recurrence, metastases, and outcomes (22). It had been proved that HER2+ patients that underwent radiotherapy and anti-HER2 therapy had better survival outcomes (23). However, our published report showed that HER2 + patients diagnosed with MBC receiving RT had not a superior breast cancer-specific survival (BCSS) than that not RT (24). This discrepancy may be due to several reasons. Firstly, the characteristics of MBC are different from traditional breast cancer. Secondly, there is no study to explore the role of HER2 status in MBC patients underwent PMRT.

Therefore, to improve the comprehensive treatment of MBC, it is urgent to explore PMRT. Based on the above factors, the information of MBC patients was extracted from the Surveillance, Epidemiology, and End Results (SEER) registry to explore the effect of PMRT on MBC patients under different HER2 statuses.

MATERIALS AND METHODS

Patients

Data from 2010 to 2016 were obtained from the SEER database. The demographic and clinicopathological information was obtained from the database. The international classification of diseases for oncology Version 3 (ICD-O-3) codes identified the metaplastic histology, including 8560, 8562, 8570–8572, 8575, and 8980–8982 (24–26). Finally, 1887 patients were included. **Figure 1** showed the inclusion criteria.

Demographic and Clinicopathologic Variables

Although it is rare, we still include more comprehensive study variables. Demographic variables, including age at diagnosis, race/ethnicity recorded in the SEER database (White, Black, other), and insurance status, were enrolled. The clinical and pathologic variables included grade, histology, tumor size (T1, T2, T3, T4), regional node status (N0, N1, N2, N3), PMRT, post-mastectomy chemotherapy (PMCT), and biomarker parameters (ER, HER2). HER2 status, according to the SEER database, was stratified as HER2-negative and HER2-positive groups.

The BCSS, defined as the date of diagnosis to the date of death from MBC, was considered the primary clinical outcome in our study.

Detection the Status of ER and HER2

In the SEER database, 1) If ER was reported on multiple tumor specimens, the highest value was recorded; 2) In case sample had any positive, that record is positive; 3) If ER status of all tested invasive specimens was negative, the status of ER was negative whatever ER status was *in situ* specimen; 4) The criterion of ER-positive status was that ≥ 1% cells stained positive; 5) HER2 negative status was defined as staining with a score of 0/1+ by IHC; 6) HER2 positive status was defined as staining with a score of 3+ by IHC; 7) The score of 2+ was interpreted as equivocal. The test of fluorescence *in situ* hybridization or silver *in situ* hybridization order to be performed. Only when the ratio of HER2 to CEP17 was >2.2, the results of HER2 amplification was interpreted as positivity.

Ethics Statement

Since the patient information in the SEER database has been de-identified, the study was exempted from the approval process of the institutional review committee. In addition, consent papers are not applicable.

Statistical Analysis

The differences between groups were analyzed by the $\chi 2$ test. The univariate Cox proportional hazards model was implemented to evaluate the risk factors of BCSS, and then the variables with P-value < 0.1 and with clinically valuable were included in the multivariate Cox proportional hazards model. The Kaplan–Meier method plotted Survival curves, and the difference between the two group was tested by log-rank. Hazard ratios were showed with 95% confidence intervals (CIs). All statistical analyses were conducted using SPSS (version 24.0; IBM, Armonk, New York, USA). $P < 0.05$ was statistically significant.

Because of the retrospective design, there was a selection bias when patients were divided into HER2-negative and HER2-

Abbreviations: MBC, Metaplastic breast cancer; HER2, Human epidermal growth factor receptor 2; IDC, Invasive ductal carcinoma; PMRT, Post-mastectomy radiotherapy; SEER, Surveillance, Epidemiology, and End Results; ICD-0-3, International Classification of Diseases for Oncology Version 3; PMCT, Post-mastectomy chemotherapy; BCSS, Breast cancer-specific survival; ER, Estrogen receptor; HRs, Hazard ratios; CI, Confidence interval.

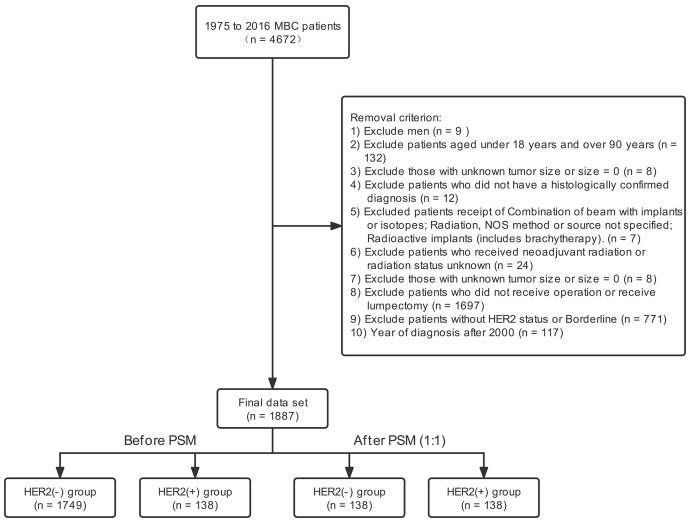

FIGURE 1 | Stepwise inclusion and exclusion counts. MBC, metaplastic breast cancer; PSM, propensity score-matching; Her2, Human epidermal growth factor receptor 2.

positive groups. We compared the clinical and pathologic parameters between the groups and found that those parameters were different, including age of diagnosis, tumor grade, TNM, and PMCT. To reduce the confounding factors and treatment selection bias, propensity score matching (PSM) was conducted (27).

RESULT

Demographic and Clinical Characteristics

The SEER registry recorded 2240 patients diagnosis of MBC from 2010 to 2016. The final sample comprised 1887 cases. In this study, 1749 (92.7%) patients had HER2-negative tumors, and 138 (7.3%) had HER2-positive tumors. The median age of the whole cohort was 63 years (range, 20-89 years). There are more white women (n=1558, 82.6%) and more poorly differentiated patients (n=1341, 71.1%). In addition, 48.6% were stage T2. 1478 (78.3%) and 405 (21.5%) patients had ER-negative and ER-positive status. In terms of treatment, 833 (44.1%) patients underwent PMRT, and 1212 underwent PMCT. Also, 1356 (71.9), 241 (12.8%), 68 (3.6%), and 222 (11.8%) patients diagnosed in N0, N1, N2, and N3 stage. Meanwhile, 427 cases (22.6%) died, including 310 cases (16.4%) related to breast cancer.

The characteristics of clinical and pathological between the two subgroups were showed in **Table 1**. Compared with HER2-negative tumors, HER2-positive tumors were not different concerning race/ethnicity, tumor histology, tumor size, regional node involvement, and PMRT, but HER2-positive patients received more PMCT ($P < 0.001$). HER2-positive tumors had younger patients (HER2-negative 42.4% vs. HER2-positive 53.6%, $P = 0.007$) and had higher tumor grade ($P < 0.001$) than HER2-negative tumors. After PSM, no difference existed between the two groups (**Table 1**).

Prognostic Factors Associated with BCSS

Univariate analysis showed that those parameters were associated with BCSS, including the age of diagnosis, race/ethnicity, insurance, tumor histology, tumor size, and regional node involvement. HER2 status was not related to better BCSS. Interestingly, patients could benefit from PMRT but not PMCT (**Table 2**). After PSM, PMRT also benefits for MBC patients. Tumor size, regional node involvement were associated with a better BCSS.

the multivariate Cox proportional hazards model was conducted to explore the independent prognostic factors related to BCSS. the results showed that HER2 status was not associated with better BCSS (hazard ratio [HR]: 0.740; 95%CI: 0.453–1.209; $P = 0.230$), as well as ER status (HR:0.756, 95%CI: 0.562-1.017). Older patients had a worse prognosis (HR: 1.620; 95%CI:1.248–2.102; $P < 0.001$). In addition, patients could benefit from PMRT (HR: 0.626; 95%CI: 0.489–0.802; $P < 0.001$) but not PMCT (HR: 0.853; 95%CI: 0.656–1.110; $P = 0.237$). Independent prognostic factors associated with BCSS including tumor size (T1 as reference; T2, HR:1.132, 95%CI: 0.541-2.369, $P = 0.743$; T3, HR: 3.202, 95%CI: 1.527-6.713; $P = 0.002$; T3, HR: 2.815, 95%CI: 1.288-6.154; $P = 0.010$) and regional node involvement (N0 as reference; N1, HR: 1.366, 95%CI: 0.987-1.889; $P = 0.060$; N2, HR: 1.294, 95%CI:0.811-2.065; $P = 0.279$; N3, HR: 1.025, 95%CI: 0.718-1.464; $P = 0.890$). (**Table 3**) After PSM, patients undergoing PMRT (HR: 0.200; 95%CI: 0.089–0.451; $P < 0.001$) had a better BCSS than patients not undergoing PMRT. Of note, HER2-positive MBC was associated with better prognoses than HER2-negative MBC.

TABLE 1 | Characteristics in MBC patients.

Variables	Before PSM			After PSM		
	HER2(-)	HER2(+)	*p*	HER2(-)	HER2(+)	*p*
Age group			0.007			0.47
≤ 60 years	741 (42.4)	74 (53.6)		67 (48.6)	74 (53.6)	
> 60 years	1008 (57.6)	64 (46.4)		71 (51.4)	64 (46.4)	
Race/ethnicity (n, %)			0.694			0.328
White	1446 (82.7)	111 (80.5)		119 (86.2)	112 (81.2)	
Black	296 (16.9)	26 (18.8)		19 (13.8)	26 (18.8)	
Other	7 (0.4)	1 (0.7)		–	–	
Insurance (n, %)			0.395			0.634
No	281 (16.1)	26 (18.8)		22 (15.9)	26 (18.8)	
Yes	1468 (83.9)	112 (81.2)		116 (84.1)	112 (81.2)	
Grade (n, %)			< 0.001*			0.102*
Well differentiated	81 (4.6)	0 (0)		5 (3.6)	0	
Moderately differentiated	223 (12.8)	8 (5.8)		13 (9.4)	8 (5.8)	
Poorly differentiated	1219 (69.7)	122 (88.4)		112 (81.2)	122 (88.4)	
Undifferentiated	46 (2.6)	2 (1.4)		4 (2.9)	2 (1.4)	
Unknown	180 (10.3)	6 (4.3)				
Histology (n, %)			0.096			0.480*
Metaplastic carcinoma	1534 (87.7)	116 (84.1)		122 (88.4)	116 (84.1)	
Adenosquamous carcinoma	91 (5.2)	14 (10.1)		10 (7.2)	14 (10.1)	
Carcinosarcoma	82 (4.7)	6 (4.3)		6 (4.3)	6 (4.3)	
Others	42 (2.4)	2 (1.4)		0	2 (1.4)	
Tumor size (n, %)			0.240			0.119*
T1	481 (27.5)	41 (29.7)		34 (24.6)	41 (29.7)	
T2	860 (49.2)	57 (41.3)		78 (56.5)	57 (41.3)	
T3	271 (15.5)	25 (18.1)		16 (11.6)	25 (18.1)	
T4	129 (7.4)	13 (9.4)		9 (6.5)	13 (9.4)	
Unknown	8 (0.5)	2 (1.4)		1 (0.7)	2 (1.4)	
Lymph node state			0.083*			0.349
N0	1267 (72.4)	89 (64.5)		91 (65.9)	89 (64.5)	
N1	218 (12.5)	23 (16.7)		24 (17.4)	23 (16.7)	
N2	59 (3.4)	9 (6.5)		3 (2.2)	9 (6.5)	
N3	205 (11.7)	17 (12.3)		20 (14.5)	17 (12.3)	
TNM stage (n, %)			0.022*			0.083*
I	437 (25.0)	37 (26.8)		31 (22.5)	37 (26.8)	
II	1016 (58.1)	64 (46.4)		88 (63.8)	64 (46.4)	
III	230 (13.2)	29 (21.0)		11 (8.0)	29 (21.0)	
IV	50 (2.9)	7 (5.1)		7 (5.1)	7 (5.1)	
Unknown	16 (0.9)	1 (0.7)		1 (0.7)	1 (0.7)	
ER status			0.171			0.087
positive	367 (21.0)	38 (27.5)		26 (18.8)	38 (27.5)	
negative	1382 (79.0)	100 (72.5)		112 (81.2)	26 (18.8)	
PMRT			0.16			0.276
No	983 (56.2)	71 (51.4)		80 (58.0)	71 (51.4)	
Yes	766 (43.8)	67 (48.6)		58 (42.0)	67 (48.6)	
PMCT			< 0.001			0.755
No	649 (37.1)	26 (18.8)		24 (17.4)	26 (18.8)	
Yes	1100 (62.9)	112 (81.2)		114 (82.6)	112 (81.2)	

*MBC, metaplastic breast cancer; PSM, propensity score-matching; HER2, Human epidermal growth factor receptor 2; ER, estrogen receptor; PMRT, post-mastectomy radiotherapy; PMCT, post-mastectomy chemotherapy. *Fisher test.*

Kaplan-Meier Analysis for Patients Undergoing PMRT

The median follow-up time in the HER2 negative group was 28 months (range 1-82 months), and the median follow-up time in the HER2 positive group was 29 months (range 1-78 months). 5-year survival rate in patients receiving PMRT was 78.0% and 74.2% in patients not receiving PMRT (P = 0.001, **Figure 2A**). After PSM, the median follow-up time in the HER2-negative group was 28 months (range, 1–82 months) and the median follow-up time in the HER2-positive group was 29 months (range, 1–78 months). 5-year survival rate was 83.4% in patients receiving PMRT and 64.1% in patients not receiving PMRT (P < 0.001, **Figure 2B**)

Subgroup Analysis for the Role of HER2 Status in PMRT

To explore the effect of PMRT on MBC patients under different HER2 statuses, this study conducted a subgroup analysis.

TABLE 2 | Univariate analysis for BCSS in MBC patients.

Variables	Befor PSM			After PSM		
	HRs	95% CI	P	HRs	95% CI	P
Age group						
≤ 60 years	1	[Reference]		1	[Reference]	
> 60 years	1.318	1.048-1.657	0.018	1.142	0.651-2.006	0.643
Race/ethnicity (n, %)						
White	1	[Reference]		1	[Reference]	
Black	1.395	1.063-1.831	0.017	1.072	0.520-2.210	0.850
Other	0.921	0.129-6.567	0.935	–	–	
Insurance (n, %)						
No	1	[Reference]		1	[Reference]	
Yes	0.684	0.496-0.847	0.001	0.483	0.263-0.887	0.019
Grade (n, %)						
Undifferentiated	1	[Reference]		1	[Reference]	
Poorly differentiated	0.774	0.433-1.382	0.387	1.427	0.196-10.361	0.725
Moderately differentiated	0.386	0.190-0.784	0.008	0.674	0.061-7.436	0.748
Well differentiated	0.284	0.107-0.758	0.012	0.876	0.055-14.013	0.925
Unknown	0.692	0.358-1.341	0.276	0.616	0.039-9.851	0.732
Histology (n, %)						
Metaplastic carcinoma	1	[Reference]		1	[Reference]	
Adenosquamous carcinoma	0.211	0.079-0.567	0.002	0.567	0.175-1.832	0.343
Carcinosarcoma	1.637	1.079-2.484	0.021	2.440	0.964-6.177	0.060
Others	0.765	0.341-1.718	0.517	–	–	–
Tumor size (n, %)						
T1	1	[Reference]		1	[Reference]	
T2	2.273	1.519-3.401	<0.001	1.375	0.560-3.372	0.487
T3	7.795	5.177-11.736	<0.001	4.300	1.713-10.790	0.002
T4	13.221	8.591-20.346	<0.001	17.252	6.688-44.503	< 0.001
Unknown	2.219	0.303-16.274	0.433	3.794	0.466-30.863	0.212
Lymph node state						
N0	1	[Reference]		1	[Reference]	
N1	1.846	1.340-2.544	<0.001	2.110	1.023-4.355	0.043
N2	1.993	1.204-3.299	0.007	3.978	1.506-10.507	0.005
N3	1.576	1.090-2.279	0.016	3.288	1.592-6.792	0.001
TNM stage (n, %)						
I	1	[Reference]		1	[Reference]	
II	3.089	1.990-4.795	<0.001	1.326	0.519-3.390	0.555
III	10.396	6.600-16.376	<0.001	6.260	2.447-16.015	<0.001
IV	34.276	20.439-57.479	<0.001	23.360	8.138-67.058	<0.001
Unknown	1.616	0.218-11.969	0.638	4.069	0.489-33.840	0.194
ER status						
negative	1	[Reference]		1	[Reference]	
positive	0.787	0.588-1.053	0.107	0.420	0.179-0.987	0.047
HER2 status						
negative	1	[Reference]		1	[Reference]	
positive	0.784	0.487-1.262	0.316	0.584	0.327-1.044	0.069
PMRT						
No	1	[Reference]		1	[Reference]	
Yes	0.727	0.579-0.913	0.006	0.370	0.196-0.697	0.002
PMCT						
No	1	[Reference]		1	[Reference]	
Yes	0.904	0.718-1.139	0.393	0.754	0.376-1.511	0.426

MBC, metaplastic breast cancer; PSM, propensity score-matching; ER, estrogen receptor; HER2, Human epidermal growth factor receptor 2; PMRT, post-mastectomy radiotherapy; PMCT, post-mastectomy chemotherapy; BCSS, Breast cancer-specific survival; HRs, Hazard ratios; CI, Confidence interval.

Patients receiving PMRT had a higher survival rate when HER2 was negative than patients not receiving PMRT in the Kaplan-Meier analysis (P = 0.017, **Figure 2C**) even though after PSM (P = 0.006, **Figure 2D**). When HER2 status was positive, patients receiving PMRT had no better survival than those without PMRT (P = 0.298, **Figure 2E**) After PSM, HER2-negative patients could benefit from PMRT. However, HER2-positive patients undergoing PMRT were not associated with better prognoses. (P = 0.084, **Figure 2F**).

DISCUSSION

Our study explored the role of PMRT in the prognosis of MBC patients and verified the effectivity of HER2 status in prognosis. After propensity score matching, our results showed that PMRT and HER2-positive status were associated with a better prognosis. However, only HER2-negative patients could benefit from PMRT.

The effectiveness of radiotherapy (RT) on MBC is still controversial. Jung et al. (28) reported that RT was not associated with a better prognosis. Those patients' information was extracted from the Center for Breast Cancer Database and they diagnosed from 2001 to 2008. However, only 35 patients were diagnosed with MBC in those studies. Cecilia et al. (15) included stage I–III MBC patients diagnosed from 2010 to 2014. They illustrated that RT was associated with improved survival. The reasons for this effect could be the fact that, firstly, the sample size of the study varies greatly. Secondly, different eras might exist different results. As pathologists' understanding and

TABLE 3 | Multivariate analysis forBCSS in MBC patients.

Variables	Befor PSM			After PSM		
	HRs	95% CI	P	HRs	95% CI	P
Age group						
≤ 60 years	1	[Reference]		1	[Reference]	
> 60 years	1.620	1.248-2.102	<0.001	0.798	0.379-1.677	0.551
Race/ethnicity (n, %)						
White	1	[Reference]		1	[Reference]	
Black	1.440	1.086-1.911	0.011	0.540	0.249-1.174	0.120
Other	0.950	0.116-7.754	0.962	–	–	
Insurance (n, %)						
No	1	[Reference]		1	[Reference]	
Yes	0.825	0.623-1.093	0.180	0.823	0.360-1.878	0.643
Grade (n, %)						
Undifferentiated	1	[Reference]		1	[Reference]	
Poorly differentiated	0.834	0.461-1.509	0.548	1.532	0.170-13.813	0.704
Moderately differentiated	0.630	0.304-1.308	0.215	1.666	0.118-23.598	0.706
Well differentiated	0.936	0.331-2.652	0.901	0.021	0.000-3.194E+14	0.839
Unknown	0.644	0.328-1.266	0.202	0.503	0.026-9.694	0.649
Histology (n, %)						
Metaplastic carcinoma	1	[Reference]		1	[Reference]	
Adenosquamous carcinoma	0.275	0.101-0.754	0.012	0.390	0.091-1.679	0.206
Carcinosarcoma	1.138	0.737-1.757	0.559	1.291	0.399-4.176	0.670
Others	0.989	0.438-2.234	0.978	–	–	
Tumor size (n, %)						
T1	1	[Reference]		1	[Reference]	
T2	1.132	0.541-2.369	0.743	0.715	0.088-5.789	0.754
T3	3.202	1.527-6.713	0.002	3.076	0.356-26.589	0.307
T4	2.815	1.288-6.154	0.010	2.610	0.268-25.421	0.409
Unknown	0.597	0.066-5.400	0.646	0.001	0.000-2.183E+257	0.983
Lymph node state						
N0	1	[Reference]		1	[Reference]	
N1	1.366	0.987-1.889	0.060	1.308	0.559-3.062	0.536
N2	1.294	0.811-2.065	0.279	1.221	0.349-4.276	0.755
N3	1.025	0.718-1.464	0.890	1.931	0.755-4.944	0.170
ER status						
negative	1	[Reference]		1	[Reference]	
positive	0.756	0.562-1.017	0.065	0.283	0.099-0.736	0.051
HER2 status						
negative	1	[Reference]		1	[Reference]	
positive	0.740	0.453-1.209	0.230	0.379	0.192-0.746	0.005
PMRT						
No	1	[Reference]		1	[Reference]	
Yes	0.626	0.489-0.802	<0.001	0.200	0.089-0.451	<0.001
PMCT						
No	1	[Reference]		1	[Reference]	
Yes	0.853	0.656-1.110	0.237	0.414	0.163-1.050	0.063

MBC, metaplastic breast cancer; PSM, propensity score-matching; ER, estrogen receptor; HER2, Human epidermal growth factor receptor 2; PMRT, post-mastectomy radiotherapy; PMCT, post-mastectomy chemotherapy; BCSS, Breast cancer-specific survival; HRs, Hazard ratios; CI, Confidence interval.

surgeons' recognition of MBC has been improved, the prognosis might have also been improved.

As we all know, to minimize local recurrence after patients undergoing lumpectomy, post-surgery radiotherapy is considered as a standard component of lumpectomy to treat patients with IDC. Dave et al. (29) and Yu et al. (10) found that patients receiving lumpectomy but not total mastectomy can benefit from radiotherapy. The National Comprehensive Cancer Network breast cancer guidelines recommended that the T1-2N1 stage patients should receive PMRT, while those with stage N2 might undergo PMRT (30). In addition, 5-year survival rates of the MBC patients ranged from 49 to 83%, which suggested that the effect of PMRT in those tumors is not clear. In the present study, PMRT was associated with a better prognosis for MBC patients.

According to previously published studies, the rate of MBC underwent CT ranged from 33 to 86% (31–33). The reasons might be that, firstly, the widely gapped rates might suggested that the effectivity of patients underwent CT remained unclear, but some studies with small sample size showed that patients receiving CT had a superior prognosis (34–36). Secondly, the high rate may be that the triple-negative phenotype was the common molecular subtype of MBC, which is characterized by more aggressive cancer (37). The next but not the last reason is

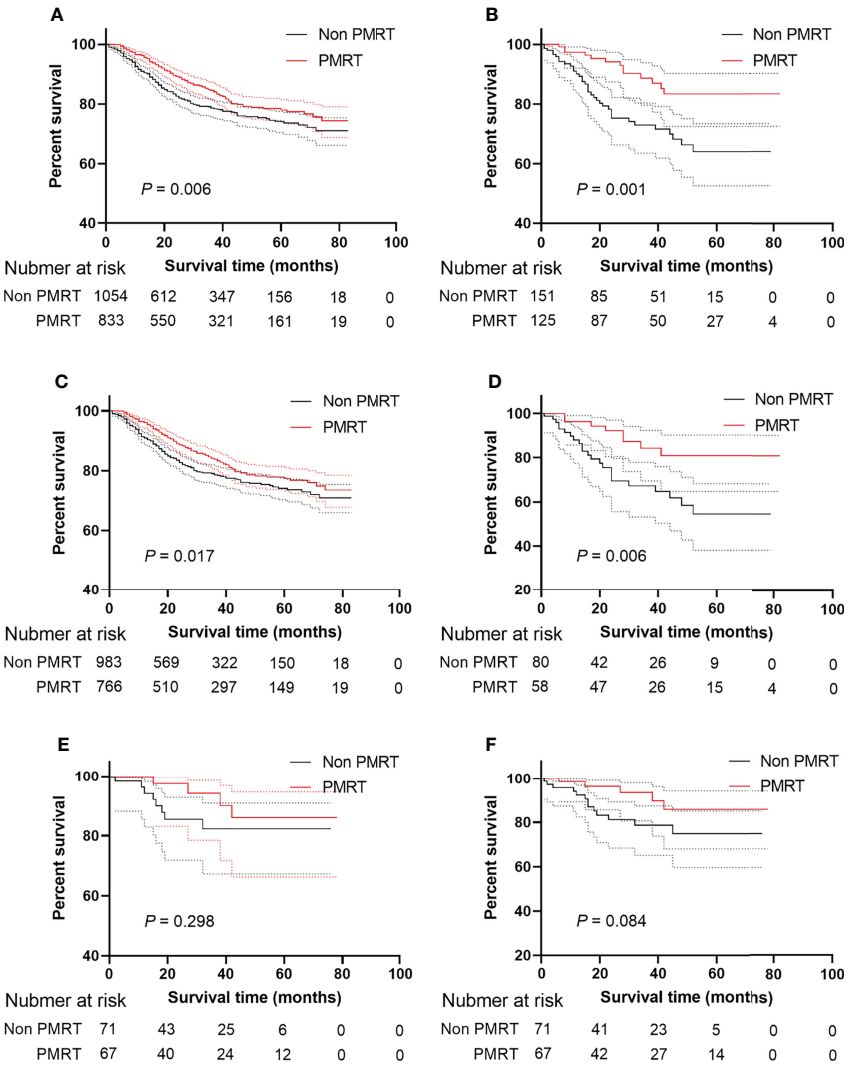

FIGURE 2 | BCSS and OS of MBC patients displayed as Kaplan-Meier curve stratified according to PMRT. **(A)** BCSS curve of Non PMRT group versus PMRT group; **(B)** BCSS curves of Non PMRT group versus PMRT group after PSM; **(C)** BCSS curve of Non PMRT group versus PMRT group patients with Her2 negative status; **(D)** BCSS curves of Non PMRT group versus PMRT group with Her2 negative status after PSM; **(E)** BCSS curve of Non PMRT group versus PMRT group patients with Her2 positive status; **(F)** BCSS curves of Non PMRT group versus PMRT group with Her2 positive status after PSM. MBC, metaplastic breast cancer; BCSS, breast cancer-special survival; PSM, propensity score-matching; PMRT, post mastectomy radiotherapy; Her2, Human epidermal growth factor receptor 2.

that in the NCCN guideline, its treatment was paralleled to that of IDC (38). Nevertheless, CT can not affect the prognosis of MBC patients, which is supported by most researchers (28, 39–41). 64.2% of patients received CT but they had no better outcomes than that not receiving CT, in the present study, which was consistent with the previous study (15, 42). The presence of more than one metaplastic component may be one of reasons for chemotherapy-resistant.

Although the triple-negative phenotype was the common molecular subtype in MBC, HR-positive and HER2 over-expression tumors do exist (43).

A published study reported that HER2 status was associated with a better prognosis for MBC patients (37). This is in contrast to invasive ductal and lobular carcinoma of the breast (22). Interestingly, some small sample reports suggest that anti-estrogen therapy does not improve the disease-free and overall survival of HR-positive MBC (8, 32, 44). In our study, HER2-positive status was associated with better outcomes, this conclusion is consistent with a recent study by Schroeder et al. that was published. Additionally, little is known about the presentations and prognoses of HER2 positive MBC, due to lack of reports of tumor HER2 receptor status. There was a particularly significant gap when consider the availability and use of HER2-directed therapy. In addition, by investigating the response of MBC to HER2 targeted therapy, we can understand the vulnerability to antibodies (37).

Owing to the rarity of HER2 over-expression tumors, clinicopathologic features need to be fully determined. The incidence of MBC is unknown, so the association of these therapeutic factors with MBC is unknown. Her2-positive breast cancer is an invasive disease, and until recently the overall survival rate for this subtype of breast cancer had been the worst (45, 46). Overall survival in this subtype had been greatly improved due to the use of HER2-targeted therapies by antibody-based approaches (e.g., trastuzumab, pertuzumab) and small-molecule inhibitors (lapatinib, neratinib) (47, 48). However, the involvement of HER2 over-expression in MBC prognoses is unknown. Previous studies have found the rate of HER2 over-expression ranging from 0% to 25% (49, 50). In our study, 7.3% of MBC patients had HER2 over-expression, which is consistent with previous studies. According to the current consensus guidelines, the degree of HER2 overexpression or amplification was thought to be intermediate between typical breast cancer and MBC, as reported in previous studies (51).

Our study has several key strengths. The role of HER2 status and PMRT in the prognosis of MBC is unclear. From our results, the prognosis was improved in MBC patients receiving PMRT. In addition, HER2 status can redefine the role of PMRT in the prognosis of MBC.

Our study has several limitations. First, due to its retrospective study, it is characterized by the nature of observation and the possibility of selection bias. Second, the SEER database lacks information on hormone therapy, anti-Her-2 therapy, and baseline characteristics including working status, comorbidity, and socio-economic environmental parameters. Third, the SEER database can not provide detailed chemotherapy and radiotherapy information, so it is impossible to conduct further case-control studies. However, our results will help researchers understand the role of HER2 in the prognosis of MBC.

CONCLUSIONS

Our findings supported that, after PSM, PMRT and HER2-positive status were associated with a better prognosis. However, only HER2-negative patients could benefit from PMRT.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found online at https://doi.org/10.6084/m9.figshare.19800526.

AUTHOR CONTRIBUTIONS

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Identification of the lymph node metastasis-related automated breast volume scanning features for predicting axillary lymph node tumor burden of invasive breast cancer *via* a clinical prediction model

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Breast cancer has become the malignant tumor with the highest incidence in women. Axillary lymph node dissection (ALND) is an effective method of maintaining regional control; however, it is associated with a significant risk of complications. Meanwhile, whether the patients need ALND or not is according to sentinel lymph node biopsy (SLNB). However, the false-negative results of SLNB had been reported. Automated breast volume scanning (ABVS) is a routine examination in breast cancer. A real-world cohort consisting of 245 breast cancer patients who underwent ABVS examination were enrolled, including 251 tumor lesions. The ABVS manifestations were analyzed with the SLNB results, and the ALND results for selecting the lymph node metastasis were related to ABVS features. Finally, a nomogram was used to construct a breast cancer axillary lymph node tumor burden prediction model. Breast cancer patients with a molecular subtype of luminal B type, a maximum lesion diameter of ≥5 cm, tumor invasion of the Cooper's ligament, and tumor invasion of the nipple had heavy lymph node tumor burden. Molecular classification, tumor size, and Cooper's ligament status were used to construct a clinical prediction model of axillary lymph node tumor burden. The consistency indexes (or AUC) of the training cohort and the validation cohort were 0.743 and 0.711, respectively, which was close to SLNB (0.768). The best cutoff value of the ABVS nomogram was 81.146 points. After combination with ABVS features and SLNB, the AUC of the prediction model was 0.889, and the best cutoff value was 178.965 points. The calibration curve showed that the constructed nomogram clinical prediction model and the real results were highly consistent. The clinical prediction model constructed using molecular classification, tumor size, and Cooper's ligament status can effectively predict

the probability of heavy axillary lymph node tumor burden, which can be the significant supplement to the SLNB. Therefore, this model may be used for individual decision-making in the diagnosis and treatments of breast cancer.

KEYWORDS

ABVS, ultrasound, breast cancer, ALND, SLNB

Introduction

At present, breast cancer has become the malignant tumor with the highest incidence in women (1), and the onset of breast cancer has been occurring at younger and younger ages. Axillary lymph node dissection (ALND) is an effective method of maintaining regional control; however, it is associated with a significant risk of complications such as lymphedema, numbness, axillary web syndrome, and decreased upper-extremity range of motion (2, 3). The Z0011 trial conducted by the American College of Surgeons Oncology Group (ACOSOG) showed that if the postoperative treatments are standardized, patients with one or two positive lymph nodes in sentinel lymph node biopsy (SLNB) do not need an ALND (2, 4). Only breast cancer patients with three or more metastatic axillary lymph nodes are required to undergo surgical dissection. Therefore, Li et al. (5)proposed the concept of lymph node tumor burden, which defines fewer than three axillary lymph node metastases as a mild lymph node tumor burden, and three or more as a heavy lymph node tumor burden. The results of the ACOSOG Z0011 trial have changed the treatment of breast cancer. Studies have shown that the overall proportion of patients who met the Z0011 standard for parallel surgery has dropped from 34.0% to 22.7%, and there is a declining trend year by year (4, 6). Currently, lymphatic metastasis is mainly determined by SLNB; however, false-negative results (9.8%) had been reported (7).

Automated breast volume scanning (ABVS) is an emerging technology of breast ultrasound examination that can obtain images of multiple planes, including cross section, sagittal plane, and coronal plane. In addition, it can observe the lesions in real time, dynamically, continuously, and multi-sectionally, providing more information on the imaging manifestations of the lesions and the surrounding tissues of the lesions (8). Ultrasound is a common method for screening breast diseases, and the ultrasound manifestations of different molecular subtypes of breast cancer are slightly different, especially in ABVS technology (9). In addition, studies have shown that different molecular subtypes of breast cancer have different biological behaviors (10), different prognosis (11), and different distant metastasis statuses, i.e., the axillary lymph node metastasis status is different (12, 13). Therefore, if the relationship between the ABVS manifestations and the molecular subtype of the primary breast cancer lesion with the status of axillary lymph node metastasis can be ascertained, more imaging evidence for assessing the status of the lymph node metastasis can be provided.

Therefore, we initiated a real-world analysis. First, we performed some analyses of the clinical features, ABVS features, and lymph node tumor burden. Then, the features related to the lymph node tumor burden were selected. Finally, a clinical prediction model of lymph node tumor burden was developed. Our work indicated there are strong links between ABVS features and lymph node tumor burden, and the clinical prediction model can be the significant supplement to the SLNB, and this model may be used for individual decision-making.

Materials and methods

Xiangya real-world cohort patients

The patients who underwent ABVS examination in the Department of Ultrasound of Third Xiangya Hospital of Central South University and were confirmed to have breast cancer by postoperative pathological examination from June 2017 to June 2019 were included. There was a total of 245 patients and 251 tumor lesions. The patients were screened according to the inclusion criteria and exclusion criteria. This study was approved by the ethics committee of Third Xiangya Hospital of Central South University.

The inclusion criteria were (1) preoperative ABVS examination in our hospital and postoperative pathological confirmation of breast cancer and (2) complete clinical and pathological data.

The exclusion criteria were as follows (1): not newly diagnosed with breast cancer (2); the patients without the ABVS results before neoadjuvant chemotherapy (chemotherapy before surgery); and (3) the clinical data and pathological results were incomplete (4); the patients with poor-quality ABVS images: the scanning operation is not standardized— the breast gland scanning is incomplete, the scanning depth is too large or too small, and the gain is too large or too small, the gray-scale setting is based on fat tissue, and the fat lobules are medium gray, not black—and artifacts: the probe does not fit well with the patient's

skin, causing artifacts, and the glands are not flattened; the posterior echo attenuation of the image generated by the wrinkles in the nipple and areola area, and coupling agent solidification, small bubbles.

The patients with the relative contraindication for ABVS examination were described as the following: there is no absolute contraindication, but it is recommended to use it with caution or check it after full communication with the patient in the following cases—in the middle and late trimesters of pregnancy, lactation, acute mastitis, great pain of breast, breast prosthesis, and breast skin ulceration.

ABVS examination

A Siemens ACUSON S2000 ABVS acquisition system was used for image acquisition for all of the selected subjects; the probe model was 14L5BV, the frequency was 5.0–12.0 MHz, and the maximum scan volume was 154 mm × 168 mm × 60 mm. The patient was in a supine position with both hands raised over the head to fully expose the breasts on both sides. The mechanical arm was adjusted so that the probe could exert proper pressure to contact the breast without causing patient discomfort. The settings of the instrument were preset according to the size of the patient's breasts. Then the machine scanned the median, lateral, and medial positions of the breast sequentially and, when necessary, scanned other planes. After the scan was completed, the position of the nipple was marked, and the images were uploaded to the image processing workstation for image reconstruction. If a mass was identified, the image features of the mass on the ABVS images were extracted, including tumor size (≤2 cm/2–5 cm/≥5 cm). Clinically, the TNM staging method is used for clinical staging of breast cancer, where T represents the size of the tumor, N represents lymph node invasion, and M represents distant metastasis. T1 indicates that the maximum diameter of the lesion is ≤2 cm, T2 indicates that the maximum diameter of the lesion is 2–5 cm, and T3 indicates that the maximum diameter of the lesion is ≥5 cm, which is the current T staging standard and also the size grouping method used in this study. The use of tumor size to determine the degree of breast cancer malignancy and the range of invasiveness has been recognized. Shape (regular/irregular), margin (circumscribed/angular/microlobulated/spiculated), orientation (parallel/non-parallel), echo pattern (hypoechoic/mixed solid echo), posterior acoustic pattern (enhanced/shadow/no change), retraction phenomenon (present/absent), acoustic halo (present/absent), microcalcification (present/absent: microcalcifications were observed as echogenic dots within the mass or as a dilated duct on the ABVS images (14)), invasion of Cooper's ligament (present/absent: Cooper's ligaments were considered shortened, thickened, pulled, and straightened, when there were hyperechogenic lines near the mass, radiating toward the skin and thus differing from other parts of normal breast tissue. Cooper's ligaments were considered normal if this feature was absent (15)), and BI-RADS (breast imaging reporting and data system) classification (class 3/4a/4b/4c/5) were determined. Two doctors independently evaluated all of the acoustic image characteristics with intermediate or higher titles. Disagreements were resolved by a third doctor with a senior title. According to the study of Eda et al., for mass lesions, malignant features include irregular margin, irregular shape, non-parallel growth, peripheral hyperechoic halo, posterior acoustic pattern attenuation, and microcalcification. In addition, one malignant sign is categorized as class 4a, two malignant signs as class 4b, three as class 4c, and more than three as class 5 (16).

Determination of surrogate molecular subtypes

According to the St. Gallen consensus and ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) guidelines, if the stained cells exceed 1% of the total number of cells, the patient is considered PR- (progesterone receptor) and ER- (estrogen receptor) positive; if the number of stained cells is less than 1% of the total number of cells, the patient is considered PR- and ER-negative (17, 18). The Ki-67 proliferation index is determined by the percentage of the number of stained cells in the total number of tumor cells, with 20% as the cutoff value (<20% is considered low proliferation, and ≥20% is considered high proliferation (19, 20)). The *HER2* (human epidermal growth factor receptor 2) gene was detected using immunohistochemistry (IHC), and the IHC results were scored as 0, 1+, 2+, and 3+ according to the standards. The 2+ specimens were further examined using fluorescence *in situ* hybridization (FISH), and the result was used as a basis for further judgment of the amplification of the *HER2* gene. *HER2*-positive cases included IHC 3+ and FISH-positive individuals of IHC 2+ cases, and *HER2*-negative cases included IHC 0, 1+, and FISH-negative individuals of IHC 2+ cases (21). Surrogate molecular subtypes were as follows (1): luminal A: ER or PR positive, and Ki-67 of less than 20% (2); luminal B: with ER or PR positive, and Ki-67 of 20% or greater (3); HER2: ER negative, PR negative, and HER2 positive; and (4) triple negative: ER negative, PR negative, and HER2 negative.

Grouping criteria for axillary lymph node tumor burden

According to the ACOSOG Z0011 trial results, if follow-up tumor surgery and postoperative comprehensive treatment are standardized, patients with two or fewer positive SLNB do not need to undergo ALND (22). Therefore, sentinel/axillary lymph node metastasis ≥3 is defined as heavy lymph node tumor

burden, and sentinel/axillary lymph node metastasis <3 is defined as mild lymph node tumor burden (5).

Statistics

Using R software (RStudio 1.2) and SPSS 25.0 software, the relationship between the ABVS manifestations of different molecular subtypes of breast cancer and lymph node tumor burden was explored through logistic univariate and multivariate regression risk factor analyses. Using SPSS 25.0 software, the chi-square test or Fisher's exact test was performed to analyze the difference between the mild axillary lymph node tumor burden group and the heavy axillary lymph node tumor burden group. (1. For all theoretical numbers T ≥5 and total sample size n ≥ 40, the Pearson chi-square test was used; 2. If theoretical number T < 5 but ≥ 1, and n ≥ 40, a continuity correction chi-square test was performed; 3. If the theoretical number T <1 or n <40, a Fisher's test was used.) Finally, based on the logistic regression analysis results, R software was used to construct a nomogram model for breast cancer axillary lymph node tumor burden prediction. MedCalc software (18.2) was used to graph the ROC curve of axillary lymph node tumor burden detected using SLNB and calculate the area under the curve.

Results

Clinical characteristics and pathological data of the study subjects

According to the inclusion and exclusion criteria, a total of 245 eligible patients were screened, and a total of 251 lesions were included in the statistical analysis (Table S1).

Relationship between ABVS manifestations of different molecular subtypes of breast lesions and different levels of axillary lymph node tumor burden

Difference of lymph node tumor burden in the ABVS manifestations and molecular subtypes of different breast lesions

A total of 251 breast lesions were included. Among the 144 lesions that underwent SLNB, the size, orientation, echo pattern, shape, margin, posterior acoustic pattern, presence of acoustic halo, presence of microcalcification, presence of retraction phenomenon, lesion type, and invasion of the Cooper's ligament were not significantly different between the mild lymph node tumor burden group and the heavy lymph node tumor burden group (P > 0.05).

In the 178 cases of lesions that performed ALND, the shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, and microcalcification were not significantly different between the mild lymph node tumor burden group and the heavy lymph node tumor burden group (P> 0.05). However, the tumor size in the heavy lymph node burden group was larger than in the mild lymph node tumor burden group with statistical significance (χ^2 = 7.594, P = 0.022). The incidence of acoustic halo in the heavy lymph node tumor burden group was higher than in the mild lymph node tumor burden group with statistical significance (χ^2 = 5.753, P = 0.016). The Cooper's ligament invasion proportion in the heavy lymph node tumor burden group was higher than in the mild lymph node tumor burden group with statistical significance (χ^2 = 11.992, P = 0.001).

We did not detect a significant difference in the sentinel lymph node tumor burden in the analysis of different molecular subtypes. However, in the analysis of axillary lymph node tumor burden, we found that in the luminal A type, *HER-2* overexpression type, and triple-negative breast cancer, the proportion of patients with a mild lymph node tumor burden was significantly higher than that of patients with a heavy lymph node tumor burden. In contrast, in luminal B breast cancer, the proportion of patients with heavy lymph node tumor burden was significantly higher than that of patients with mild lymph node tumor burden (72.549% vs. 57.480%, χ^2 = 8.050, P = 0.046). Our results suggest that molecular classification is an important factor affecting the axillary lymph node tumor burden. Therefore, we further analyzed the subgroups of different molecular subtypes (Table 1).

Differences of lymph node tumor burden of luminal A-type breast cancer regarding different ABVS manifestations

In 44 cases of luminal A-type lesions, the lymph node tumor burden was not significantly different with respect to tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, microcalcification, invasion of the Cooper's ligament, and BI-RADS classification (P > 0.05). The incidence of acoustic halo in the heavy lymph node tumor burden group was higher than in the mild lymph node tumor burden group with statistical significance (χ^2 = 8.734, P = 0.003) (Table 2).

Differences of lymph node tumor burden of luminal B-type breast cancer regarding different ABVS manifestations

A total of 138 cases of luminal B-type breast lesions were included. Among the 73 lesions that underwent SLNB, the lymph node tumor burden was not significantly different with respect to tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, invasion of the Cooper's ligament, and BI-RADS classification (P > 0.05). In the 113 cases of lesions that underwent ALND, the lymph node tumor burden was not significantly different concerning tumor size, shape, margin,

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TABLE 1 Difference of lymph node tumor burden in the ABVS features and molecular subtypes of different breast lesions.

ABVS features and molecular subtype	Sentinel lymph node tumor burden			Axillary lymph node tumor burden		
	Mild	Heavy	P	Mild	Heavy	P
Tumor size						
≥5 cm	8 (5.970%)	2 (20.000%)	0.155	6 (4.724%)	8 (15.686%)	0.022
2–5 cm	74 (55.224%)	6 (60.000%)		71 (55.906%)	30 (58.824%)	
≤2 cm	52 (38.806%)	2 (20.000%)		50 (39.370%)	13 (25.490%)	
Orientation						
Not parallel	45 (33.582%)	2 (20.000%)	0.499	48 (37.795%)	17 (33.333%)	0.699
Parallel	89 (66.418%)	8 (80.000%)		79 (62.205%)	34 (66.667%)	
Shape						
Regular	17 (12.687%)	0 (0.000%)	0.609	12 (9.449%)	3 (5.882%)	0.560
Irregular	117 (87.313%)	10 (100.000%)		115 (90.551%)	48 (94.118%)	
Echo pattern						
Hypoechoic	119 (88.806%)	10 (100.000%)	0.600	118 (92.913%)	49 (96.078%)	0.731
Mixed solid echo	15 (11.194%)	0 (0.000%)		9 (7.087%)	2 (3.922%)	
Margin						
Circumscribed	10 (7.462%)	0 (0.000%)	0.116	3 (2.362%)	0 (0.000%)	0.085
Angular	51 (38.060%)	4 (40.000%)		46 (36.220%)	15 (29.412%)	
Microlobulated	18 (13.433%)	0 (0.000%)		20 (15.748%)	3 (%5.882)	
Spiculated	55 (41.045%)	6 (60.000%)		58 (45.669%)	33 (64.706%)	
Posterior acoustic pattern						
Enhancement	22 (16.418%)	1 (10.000%)	1.000	21 (16.535%)	5 (9.804%)	0.103
Shadow	22 (16.418%)	2 (20.000%)		22 (17.323%)	16 (31.373%)	
No change	90 (67.164%)	7 (70.000%)		84 (66.142%)	30 (58.824%)	
Microcalcifications						
Present	52 (38.806%)	3 (30.000%)	0.742	59 (46.457%)	18 (35.294%)	0.233
Absent	82 (61.194%)	7 (70.000%)		68 (53.543%)	33 (64.706%)	
Acoustic halo						
Present	28 (20.896%)	2 (20.000%)	1.000	22 (17.323%)	18 (35.294%)	0.016
Absent	106 (79.104%)	8 (80.000%)		105 (82.677%)	33 (64.706%)	
Retraction phenomenon						
Present	17 (12.687%)	1 (10.000%)	1.000	25 (19.685%)	10 (19.608%)	1.000
Absent	117 (87.313%)	9 (90.000%)		102 (80.315%)	41 (80.392%)	
Invasion of Cooper's ligament						
Yes	29 (21.642%)	3 (30.000%)	0.693	37 (29.134%)	29 (56.863%)	0.001
No	105 (78.358%)	7 (70.000%)		90 (70.866%)	22 (43.137%)	
BI-RADS						
3	11 (8.209%)	0 (0.000%)	0.882	0 (0.000%)	0 (0.000%)	0.068
4a	25 (18.657%)	2 (20.000%)		14 (11.024%)	5 (9.804%)	
4b	44 (32.836%)	3 (30.000%)		48 (37.795%)	10 (19.608%)	
4c	18 (13.433%)	2 (20.000%)		24 (18.898%)	10 (19.608%)	
5	36 (26.865%)	3 (30.000%)		41 (32.283%)	26 (50.980%)	
Molecular subtype						
Luminal A	41 (30.597%)	1 (10.000%)	0.226	26 (14.173%)	4 (7.843%)	0.046
Luminal B	60 (44.776%)	8 (80.000%)		65 (57.480%)	37 (72.549%)	
HER-2	17 (12.687%)	1 (10.000%)		20 (15.748%)	4 (7.843%)	
Triple negative	16 (11.940%)	0 (0.000%)		16 (12.598%)	6 (11.765%)	

orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, invasion of the Cooper's ligament, and BI-RADS classification. The proportion of Cooper's ligament invasion in the heavy lymph node tumor burden group was higher than in the mild lymph node tumor burden group with statistical significance (χ^2 = 7.749, P= 0.005) (Table 3).

Differences of lymph node tumor burden of HER-2 overexpression type breast cancer regarding different ABVS manifestations

A total of 35 cases of *HER-2* overexpression breast lesions were included. Among the 18 lesions that underwent SLNB, the lymph node tumor burden was not significantly different with respect to tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, invasion of the cooper's ligament, and BI-RADS classification (P > 0.05). In the 24 cases of lesions that underwent ALND, the lymph node tumor burden was not significantly different with respect to tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, invasion of the Cooper's ligament, and BI-RADS classification (P > 0.05). The proportion of posterior acoustic pattern in the heavy lymph node tumor burden group was higher than in the mild lymph node tumor burden group with statistical significance (χ^2 = 6.900, P = 0.032) (Table 4).

Differences of lymph node tumor burden of triple-negative type breast cancer regarding different ABVS manifestations

A total of 34 cases of triple-negative breast lesions were enrolled, of which 16 cases underwent SLNB and 22 cases underwent ALND. According to the results of SLNB, no patients were with heavy lymph node tumor burden. In the 22 cases of lesions that underwent ALND, the lymph node tumor burden was not significantly different concerning tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, Cooper's ligament invasion, and BI-RADS classification (P > 0.05). The BI-RADS classification of the heavy lymph node tumor burden group was higher than that of the mild lymphoid tumor burden group with statistical significance (χ^2 = 13.387, P = 0.004) (Table 5).

Relationship between ABVS manifestations and different levels of axillary lymph node tumor burden

Relationship between breast cancer ABVS manifestations and clinical features with sentinel lymph node tumor burden

To better explore the relationship between the clinical features of breast cancer and sentinel lymph node tumor burden, which can rule out the influence of other factors, including age, molecular subtype, Ki-67, neoadjuvant chemotherapy, menopause, and tumor site, a univariate logistic regression analysis was performed. The results showed that none of the above factors was statistically significant (P > 0.05) (Table S2).

ABVS manifestations, tumor size, shape, margin, orientation, echo Pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, Cooper's ligament invasion, and BI-RADS classification were included in an univariate logistic regression analysis. The results showed that the maximum lesion diameter of ≥5 cm significantly influenced the aggravation of sentinel lymph node tumor burden. At the same time, the difference was not statistically significant (OR = 6.500, 95% CI 0.701–60.974, P= 0.080; Table S3). Therefore, indicators with a P-value <0.05 can be included in the multivariate logistic regression analysis. However, because none of the univariate logistic regression analysis results of this study was statistically significant, no indicator could be included in multivariate logistic regression analysis in this study.

Relationship between breast cancer ABVS manifestations and clinical features with axillary lymph node tumor burden

To better explore the relationship between the clinical features of breast cancer and axillary lymph node tumor burden, which can rule out the influence of other factors, including age, molecular subtype, Ki-67, neoadjuvant chemotherapy, menopause, and tumor site, an univariate logistic regression analysis was performed. Neoadjuvant chemotherapy was a risk factor of heavy axillary lymph node tumor burden (OR = 4.181, 95% CI 1.509–12.202, P = 0.006), and nipple invasion significantly increased the risk of heavy axillary lymph node tumor burden (OR = 6.793, 95% CI Is 1.411–48.598, P = 0.025) (Table S4).

Studies have shown that different molecular subtypes of breast cancer have different prognoses (11). Neoadjuvant chemotherapy can downgrade the clinical stage of breast cancer patients and has different responses in different molecular subtypes (23), suggesting that it may affect postoperative lymph node tumor burden. Therefore, we included indicators with a P-value <0.05 and clinical significance in the multivariate logistic regression analysis. Molecular classification and Ki-67 were included in the multivariate logistic regression analysis together with neoadjuvant chemotherapy and nipple invasion. The results showed that the molecular subtype of the luminal B type (OR = 7.766, 95% CI 2.022–43.649, P = 0.008) was an independent risk factor of heavy axillary lymph node tumor burden; in addition, neoadjuvant chemotherapy (OR = 6.657, 95% CI 2.017–24.579, P = 0.003) was also one of the risk factors. We conducted a literature review and data analysis and found a

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TABLE 2 Differences of lymph node tumor burden of luminal A-type breast cancer regarding different ABVS feathers.

ABVS feathers	Sentinel lymph node tumor burden			Axillary lymph node tumor burden		
	Mild	Heavy	P	Mild	Heavy	P
Tumor size						
≥5 cm	4 (9.756%)	1 (100.000%)	0.119	1 (3.846%)	1 (25.000%)	0.328
2–5 cm	19 (46.341%)	0 (0.000%)		12 (46.154%)	1 (25.000%)	
≤2 cm	18 (43.902%)	0 (0.000%)		13 (50.000%)	2 (50.000%)	
Orientation						
~	13 (31.707%)	0 (0.000%)	1.000	8 (30.769%)	0 (0.000%)	0.550
Parallel	28 (68.293%)	1 (100.000%)		18 (69.231%)	4 (100.000%)	
Shape						
Regular	4 (9.756%)	0 (0.000%)	1.000	0 (0.000%)	0 (0.000%)	1.000
Irregular	37 (90.244%)	1 (100.000%)		26 (100.000%)	4 (100.000%)	
Echo pattern						
Hypoechoic	35 (85.366%)	1 (100.000%)	1.000	24 (92.308%)	3 (75.000%)	0.360
Mixed solid echo	6 (14.634%)	0 (0.000%)		2 (7.692%)	1 (25.000%)	
Margin						
Circumscribed	5 (12.195%)	0 (0.000%)	1.000	1 (3.846%)	0 (0.000%)	0.154
Angular	16 (39.024%)	1 (100.000%)		7 (26.923%)	2 (50.000%)	
Microlobulated	3 (7.317%)	0 (0.000%)		1 (3.846%)	1 (25.000%)	
Spiculated	17 (41.463%)	0 (0.000%)		17 (65.385%)	1 (25.000%)	
Posterior acoustic pattern						
Enhancement	6 (14.634%)	0 (0.000%)	1.000	2 (7.692%)	1 (25.000%)	0.452
Shadow	7 (17.073%)	0 (0.000%)		5 (19.231%)	0 (0.000%)	
No change	28 (68.293%)	1 (100.000%)		19 (73.077%)	3 (75.000%)	
Microcalcifications						
Present	11 (26.829%)	0 (0.000%)	1.000	12 (46.154%)	0 (0.000%)	0.130
Absent	30 (70.171%)	1 (100.000%)		14 (53.846%)	4 (100.000%)	
Acoustic halo						
Present	6 (14.634%)	1 (100.000%)	0.167	4 (15.385%)	4 (100.000%)	0.003
Absent	35 (85.366%)	0 (0.000%)		22 (84.615%)	0 (0.000%)	
Retraction phenomenon						
Present	4 (9.756%)	0 (0.000%)	1.000	7 (26.923%)	1 (25.000%)	1.000
Absent	37 (90.244%)	1 (100.000%)		19 (73.077%)	3 (75.000%)	
Invasion of Cooper's ligament						
Yes	9 (21.951%)	0 (0.000%)	1.000	10 (38.462%)	2 (50.000%)	1.000
No	32 (78.049%)	1 (100.000%)		16 (61.538%)	2 (50.000%)	
BI-RADS						
3	7 (17.073%)	0 (0.000%)	1.000	0 (0.000%)	0 (0.000%)	0.220
4a	8 (19.512%)	0 (0.000%)		0 (0.000%)	1 (25.000%)	
4b	12 (29.268%)	1 (100.000%)		14 (53.846%)	2 (50.000%)	
4c	4 (9.756%)	0 (0.000%)		4 (15.385%)	0 (0.000%)	
5	10 (24.390%)	0 (0.000%)		8 (30.769%)	1 (25.000%)	

false-positive result, which will be discussed in the discussion section (Table 6).

Tumor size, shape, margin, orientation, echo pattern, posterior acoustic pattern, retraction phenomenon, acoustic halo, microcalcification, Cooper's ligament invasion, and BI-RADS classification were included in univariate logistic regression analysis. The results showed that the maximum lesion diameter of ≥5 cm significantly increased the risk of heavy axillary lymph node tumor burden (OR = 5.128, 95% CI 1.530–18.232, P = 0.009), as well as a lesion with acoustic halo

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TABLE 3 Differences of lymph node tumor burden of luminal B-type breast cancer regarding different ABVS features.

ABVS features	Sentinel lymph node tumor burden			Axillary lymph node tumor burden		
	Mild	Heavy	P	Mild	Heavy	P
Tumor size						
≥5 cm	3 (5.000%)	1 (12.500%)	0.173	3 (4.615%)	5 (13.514%)	0.150
2–5 cm	34 (56.667%)	6 (75.000%)		33 (50.769%)	21 (56.757%)	
≤2 cm	23 (38.333%)	1 (12.500%)		29 (44.615%)	11 (29.730%)	
Orientation						
Not parallel	22 (36.667%)	1 (12.500%)	0.250	29 (44.615%)	12 (32.432%)	0.295
Parallel	38 (63.333%)	7 (87.500%)		36 (55.385%)	25 (67.568%)	
Shape						
Regular	7 (11.667%)	0 (0.000%)	0.587	3 (4.615%)	3 (8.108%)	0.665
Irregular	53 (88.333%)	8 (100.000%)		62 (95.385%)	34 (91.892%)	
Echo pattern						
Hypoechoic	57 (95.000%)	8 (100.000%)	1.000	63 (96.923%)	36 (97.297%)	1.000
Mixed solid echo	3 (5.000%)	0 (0.000%)		2 (3.077%)	1 (2.703%)	
Margin						
Circumscribed	4 (6.667%)	0 (0.000%)	0.301	1 (1.538%)	0 (0.000%)	0.133
Angular	24 (40.000%)	2 (25.000%)		24 (36.923%)	10 (27.027%)	
Microlobulated	10 (16.667%)	0 (0.000%)		10 (15.385%)	2 (5.405%)	
Spiculated	22 (36.667%)	6 (75.000%)		30 (46.154%)	25 (67.568%)	
Posterior acoustic pattern						
Enhancement	8 (13.333%)	0 (0.000%)	0.723	8 (0.123%)	3 (8.108%)	0.501
Shadow	12 (20.000%)	2 (25.000%)		14 (21.538%)	12 (32.432%)	
No change	40 (66.667%)	6 (75.000%)		43 (66.154%)	22 (59.459%)	
Microcalcifications						
Present	33 (55.000%)	3 (37.500%)	0.461	33 (50.769%)	18 (48.649%)	1.000
Absent	27 (45.000%)	5 (62.5%)		32 (49.231%)	19 (51.351%)	
Acoustic halo						
Present	17 (28.333%)	1 (12.500%)	0.671	13 (20.000%)	13 (35.135%)	0.104
Absent	43 (71.667%)	7 (87.500%)		52 (80.000%)	24 (64.865%)	
Retraction phenomenon						
Present	12 (20.000%)	1 (12.500%)	1.000	15 (23.076%)	9 (24.324%)	1.000
Absent	48 (80.000%)	7 (87.500%)		50 (76.923%)	28 (75.676%)	
Invasion of Cooper's ligament						
Yes	18 (30.000%)	3 (37.500%)	0.695	19 (29.231%)	22 (59.459%)	0.005
No	42 (70.000%)	5 (62.500%)		46 (70.769%)	15 (40.541%)	
BI-RADS						
3	2 (3.333%)	0 (0.000%)	0.803	0 (0.000%)	0 (0.000%)	0.126
4a	9 (15.000%)	2 (25.000%)		9 (13.846%)	2 (5.405%)	
4b	17 (28.333%)	1 (12.500%)		18 (27.692%)	5 (13.514%)	
4c	13 (21.667%)	2 (25.000%)		14 (21.538%)	9 (24.324%)	
5	19 (31.667%)	3 (37.500%)		24 (36.923%)	21 (56.757%)	

(OR = 2.603, 95% CI 1.242–5.446, P = 0.011) and invasion of the Cooper's ligament (OR = 3.206, 95% CI 1.645–6.353, P = 0.001) (Table S5).

The multivariate logistic regression analysis included indicators with a P-value <0.05. To exclude the influence of other factors, the significant factors in the multivariate logistic regression analysis of the relationship between clinical features and axillary lymph node tumor burden were also included in the multivariate logistic regression analysis. The included factors were molecular subtype, neoadjuvant chemotherapy, lesion size, acoustic halo, posterior acoustic pattern, and Cooper's ligament invasion.

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TABLE 4 Differences of lymph node tumor burden of HER-2 overexpression type breast cancer regarding different ABVS features.

ABVS features	Sentinel lymph node tumor burden			Axillary lymph node tumor burden		
	Mild	Heavy	P	Mild	Heavy	P
Tumor size						
≥5 cm	1 (5.882%)	0 (0.000%)	0.389	1 (5.000%)	1 (25.000%)	0.405
2–5 cm	11 (64.706%)	0 (0.000%)		15 (75.000%)	3 (75.000%)	
≤2 cm	5 (29.412%)	1 (100.000%)		4 (20.000%)	0 (0.000%)	
Orientation						
Not parallel	3 (17.647%)	0 (0.000%)	1.000	5 (25.000%)	3 (75.000%)	0.091
Parallel	14 (82.353%)	1 (100.000%)		15 (75.000%)	1 (25.000%)	
Shape						
Regular	1 (5.882%)	0 (0.000%)	1.000	3 (15.000%)	0 (0.000%)	1.000
Irregular	16 (94.118%)	1 (100.000%)		17 (85.000%)	4 (100.000%)	
Echo pattern						
Hypoechoic	15 (88.235%)	1 (100.000%)	1.000	17 (85.000%)	4 (100.000%)	1.000
Mixed solid echo	2 (11.765%)	0 (0.000%)		3 (15.000%)	0 (0.000%)	
Margin						
Circumscribed	1 (5.882%)	0 (0.000%)	0.389	0 (0.000%)	0 (0.000%)	0.135
Angular	4 (23.529%)	1 (100.000%)		8 (40.000%)	0 (0.000%)	
Microlobulated	1 (5.882%)	0 (0.000%)		4 (20.000%)	0 (0.000%)	
Spiculated	11 (64.706%)	0 (0.000%)		8 (40.000%)	4 (100.000%)	
Posterior acoustic pattern						
Enhancement	2 (11.765%)	0 (0.000%)	0.278	7 (35.000%)	1 (25.000%)	0.032
Shadow	2 (11.765%)	1 (100.000%)		3 (15.000%)	3 (75.000%)	
No change	13 (76.471%)	0 (0.000%)		10 (50.000%)	0 (0.000%)	
Microcalcifications						
Present	6 (35.294%)	0 (0.000%)	1.000	10 (50.000%)	0 (0.000%)	0.114
Absent	11(64.706%)	1 (100.000%)		10 (50.000%)	4 (100.000%)	
Acoustic halo						
Present	2 (11.765%)	0 (0.000%)	1.000	1 (5.000%)	0 (0.000%)	1.000
Absent	15 (88.235%)	1 (100.000%)		19 (95.000%)	4 (100.000%)	
Retraction phenomenon						
Present	0 (0.000%)	0 (0.000%)	1.000	1 (5.000%)	0 (0.000%)	1.000
Absent	17 (100.00%)	1 (100.000%)		19 (95.000%)	4 (100.000%)	
Invasion of Cooper's ligament						
Yes	2 (11.765%)	0 (0.000%)	1.000	4 (20.000%)	1 (25.000%)	1.000
No	15 (88.235%)	1 (100.000%)		16 (80.000%)	3 (75.000%)	
BI-RADS						
3	1 (5.882%)	0 (0.000%)	0.645	0 (0.000%)	0 (0.000%)	0.223
4a	4 (23.529%)	0 (0.000%)		0 (0.000%)	0 (0.000%)	
4b	6 (35.294%)	1 (100.000%)		9 (45.000%)	0 (0.000%)	
4c	0 (0.000%)	0 (0.000%)		2 (10.000%)	1 (25.000%)	
5	6 (35.294%)	0 (0.000%)		9 (45.000%)	3 (75.000%)	

The results showed that the molecular subtype of luminal B type (OR = 4.405, 95% CI was 1.194–20.368, P = 0.037), maximum lesion diameter of ≥5 cm (OR = 8.734, 95% CI was 2.156–38.796, P = 0.003), and tumor invasion of Cooper's ligament (OR = 3.295, 95% CI 1.529–7.303, P = 0.004) were independent influence factors of heavy axillary lymph node tumor burden. Moreover, similar to the above analysis, neoadjuvant chemotherapy (OR = 6.951, 95% CI 2.133–25.144, P = 0.002 was also one of the risk factors. We conducted a literature review and data analysis and found that this is a false-positive result, which will be discussed in the discussion section (Table 7).

TABLE 5 Differences of lymph node tumor burden of triple-negative type breast cancer regarding different ABVS features.

ABVS features	Sentinel lymph node tumor burden			Axillary lymph node tumor burden		
	Mild	Heavy	P	Mild	Heavy	P
Tumor size						
≥5 cm	0 (0.000%)	0 (0.000%)	/	1 (6.250%)	1 (16.667%)	0.424
2–5 cm	10 (62.500)	0 (0.000%)		11 (68.750%)	5 (83.333%)	
≤2 cm	6 (37.500%)	0 (0.000%)		4 (25.000%)	0 (0.000%)	
Orientation						
Not parallel	7 (43.750%)	0 (0.000%)	/	6 (37.500%)	2 (33.333%)	1.000
Parallel	9 (56.250%)	0 (0.000%)		10 (62.500%)	4 (66.667%)	
Shape						
Regular	5 (31.250%)	0 (0.000%)	/	6 (37.500%)	0 (0.000%)	0.133
Irregular	11 (68.750%)	0 (0.000%)		10 (62.500%)	6 (100.000%)	
Margin						
Circumscribed	0 (0.000%)	0 (0.000%)	/	1 (6.250%)	0 (0.000%)	0.340
Angular	7 (43.750%)	0 (0.000%)		7 (43.750%)	3 (50.000%)	
Microlobulated	4 (25.000%)	0 (0.000%)		5 (31.250%)	0 (0.000%)	
Spiculated	5 (31.250%)	0 (0.000%)		3 (18.750%)	3 (50.000%)	
Echo pattern						
Hypoechoic	12 (75.000%)	0 (0.000%)	/	14 (87.500%)	6 (100.000%)	1.000
Mixed solid echo	4 (25.000%)	0 (0.000%)		2 (12.500%)	0 (0.000%)	
Posterior acoustic pattern						
Enhancement	6 (37.500%)	0 (0.000%)	/	4 (25.000%)	0 (0.000%)	0.183
Shadow	1 (6.250%)	0 (0.000%)		0 (0.000%)	1 (16.667%)	
No change	9 (56.250%)	0 (0.000%)		12 (75.000%)	5 (83.333%)	
Microcalcifications						
Present	2 (12.500%)	0 (0.000%)	/	4 (25.000%)	0 (0.000%)	0.541
Absent	14 (87.500%)	0 (0.000%)		12 (75.000)	6 (100.000%)	
Acoustic halo						
Present	3 (18.750%)	0 (0.000%)	/	4 (25.000%)	1 (16.667%)	1.000
Absent	13 (81.250%)	0 (0.000%)		12 (75.000%)	5 (83.333%)	
Retraction phenomenon						
Present	1 (6.250%)	0 (0.000%)	/	2 (12.500%)	0 (0.000%)	1.000
Absent	15 (93.750%)	0 (0.000%)		14 (87.500%)	6 (100.000%)	
Invasion of Cooper's ligament						
Yes	0 (0.000%)	0 (0.000%)	/	4 (25.000%)	4 (66.667%)	0.137
No	16 (100.000%)	0 (0.000%)		12 (75.000%)	2 (33.333%)	
BI-RADS						
3	1 (6.250%)	0 (0.000%)	/	0 (0.000%)	0 (0.000%)	0.004
4a	4 (25.000%)	0 (0.000%)		5 (31.250%)	1 (16.667%)	
4b	9 (55.250%)	0 (0.000%)		7 (43.750%)	1 (16.667%)	
4c	1 (6.250%)	0 (0.000%)		4 (25.000%)	0 (0.000%)	
5	1 (6.250%)	0 (0.000%)		0 (0.000%)	4 (66.667%)	

The accuracy of SLNB in the determination of axillary lymph node tumor burden

A total of 251 cases of breast lesions were included. One hundred forty-four cases underwent SLNB, 178 cases underwent ALND, and 71 cases underwent both operations. The 71 patients who underwent both SLNB and ALND were grouped according to the results of SLNB: 10 cases (14.085%) with heavy lymph node tumor burden and 61 cases (85.915%) with mild lymph node tumor burden. The results of SLNB were compared with the results of ALND, and the comparison showed a sensitivity of

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TABLE 6 Multivariate-logistic regression analysis of the clinical features and axillary lymph node tumor burden.

Variable	OR (95% CI)	P
Molecular subtype		
Luminal A	1.666 (0.292-10.933)	0.570
Luminal B	7.766 (2.022-43.649)	0.008
Triple negative	3.288 (0.645-20.811)	0.169
HER-2	1.000	
Ki-67		
≥20%	1.705 (0.427-9.279)	0.483
<20%	1.000	
Neoadjuvant chemotherapy		
Yes	6.657 (2.017-24.57)	0.003
No	1.000	
Nipple invasion		
Present	14.147 (2.186-133.948)	0.009
Absent	1.000	

57.143%, a specificity of 96.491%, and an accuracy of 88.732%. The graphed ROC curve is shown in Figure 1E, and the area under the ROC curve (AUC) is 0.768.

Nomogram for predicting the probability of heavy lymph node tumor burden

All of the patients who underwent ALND were included in the cohort. The cohort was divided into a training set and a validation cohort at a 1:1 ratio in chronological order. A total of 178 cases of lesions were included, with 89 cases in each of the training sets and the validation cohort. The breast cancer preoperative examination indicators with statistical significance in the multivariate logistic analysis were included as predictors to establish a nomogram scoring system. The predictors included tumor size, molecular classification, and Cooper's ligament invasion. Among them, the molecular subtype of luminal B type was assigned a score of 25 points, and other molecular subtypes were assigned 0 points; the maximum lesion diameter of ≤2 cm was assigned 0 points, the maximum lesion diameter of 2–5 cm was assigned 50 points, and the maximum lesion diameter of ≥5 cm was assigned 100 points; the presence of Cooper's ligament invasion was assigned 42.5 points, and its absence was assigned 0 points. The statistical model automatically generated all of the assigned scores (Figure 1A). The concordance index (C-index) of the nomogram scoring system for predicting the probability of heavy lymph node tumor burden on the training set is 0.743, the average absolute error is 0.05 (Figure 1B), and the area under the curve is 0.743 (Figure 1D). The validation cohort was used to calibrate the nomogram scoring system for predicting the probability of heavy lymph node tumor burden. The calibration curve is shown in Figure 1C with a consistency index of 0.711 and an average absolute error of 0.054. The results of the validation set and the training set are consistent. The best cutoff value of the ABVS nomogram is 81.146 points according to the ROC curve.

To confirm whether the ABVS nomogram can be a supplement to SLNB, we developed a new model based on ABVS features and SLNB. The results showed that the AUC and C-index are 0.889, and the average absolute error is 0.029. Meanwhile, the best cutoff value is 178.965 points according to the ROC curve (Figure 2).

Discussion

In this study, we showed the landscape of ABVS features in breast cancer, including the analyses in different clinical subgroups and molecular subtypes. Then, we successfully identified tumor size and invasion of Cooper's ligament as the lymph node tumor burden-related ABVS features, combined with the molecular subtype; we developed a nomogram prediction model, which has a convincible AUC (0.743), while the AUC of SLNB is 0.768. Furthermore, when in combination with ABVS and SLNB, the AUC can increase to 0.889. Therefore, this model may be used for individual decision-making.

In breast cancer, the expression status of ER, PR, and HER-2 has important predictive values for prognosis. The recurrence rate of ER- or PR-positive breast cancer changes with time (24–26). In this study, compared with other molecular subtypes of breast cancer, the luminal B type was more closely associated with heavy axillary lymph node tumor burden. Previously, it has been reported that poorly differentiated breast tumors are mainly of the luminal B type (27), and breast tumors with positive axillary lymph nodes are often of the luminal B type (27, 28). Luminal B-type breast cancer is more likely to have a heavier axillary lymph node tumor burden. This result may be due to the interaction of several steroid receptors. The plasminogen activator inhibitor is one of the predictors of axillary lymph node metastasis, but it only functions in PR-positive tumors (29). The expression of vimentin and Ki-67 may indicate that the long-term prognosis of ER-positive tumors is poor (27), and studies have shown that vimentin is positively correlated with the expression of ER in breast cancer (30, 31). Although whether the expression of ER and PR can be used as a predictor of axillary lymph node status is still controversial (32), there are studies suggesting the correlation between the expression status of ER and lymph node involvement (33). The expression level of Ki-67 can be used to measure the level of cell proliferation. Ki-67 <14% is considered a low proliferation state, and ≥14% is considered a high proliferation state (34). At present, the cutoff level of Ki-67 is still controversial (35, 36). Some studies suggested that using

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TABLE 7 Multivariate-logistic regression analysis of the ABVS features and axillary lymph node tumor burden.

Variable	OR (95% CI)	P
Molecular subtype		
Luminal A	0.939 (0.153-5.851)	0.945
Luminal B	4.405 (1.194-20.368)	0.037
Triple negative	2.028 (0.381-11.803)	0.412
HER-2	1.000	
Neoadjuvant chemotherapy		
Yes	6.951 (2.133-25.144)	0.002
No	1.000	
Tumor size		
≥5 cm	8.734 (2.156-38.796)	0.003
2–5 cm	1.491 (0.629-3.648)	0.370
≤2 cm	1.000	
Acoustic halo		
Present	2.205 (0.910-5.358)	0.078
Absent	1.000	
Invasion of Cooper's ligament		
Yes	3.295 (1.529-7.303)	0.004
No	1.000	
Posterior acoustic pattern		
Enhancement	1.596 (0.440-5.237)	0.319
Shadow	1.584 (0.634-3.910)	0.451
No change	1.000	

20% as the cutoff value for Ki-67 could better reflect the proliferation status of tumor cells (37). Therefore, in the logistic regression analysis of this study, 20% was used as the cutoff value of Ki-67. The difference between luminal A-type and luminal B-type breast cancer lies in the different expression levels of Ki-67. The luminal B-type breast cancer has a higher expression level of Ki-67 than the luminal A type, and then the proliferation of its tumor cells is more active.

A study has shown that tumor size is one of the predictors of axillary lymph node metastasis (38). Some scholars have identified a linear relationship between tumor size and axillary lymph node metastasis (39). There were 20 cases with a maximum lesion diameter of ≥5 cm in this study. In this group, the risk of heavy axillary lymph node tumor burden was eight times the risk in other groups, which is basically consistent with the results of a previous study (5). The Cooper's ligament is a fiber bundle between the breast's lobules that connects the deep and top layer of thesuperficial fascia and supports and secures the breast. When the lesion invades the Cooper's ligament, the ultrasound manifests traction and thickening of the Cooper's ligament. In this study, according to whether the Cooper's ligament was invaded, all of the patients were divided into two groups. The results showed that the risk of heavy axillary lymph node tumor burden when the Cooper's ligament was invaded was three times higher than that of the non-invaded group, which is consistent with previous studies (40, 41). Neoadjuvant chemotherapy has a positive effect on prolonging the survival time of breast cancer patients; however, some studies have also shown that neoadjuvant chemotherapy cannot achieve the expected effect for all breast cancer patients (42). Neoadjuvant chemotherapy is not effective on lymph nodes, the efficacy of complete remission is only about 40%, and different molecular subtypes respond differently to neoadjuvant chemotherapy (23). Therefore, we included it in our multivariate analysis. The results showed that patients who received neoadjuvant chemotherapy had a higher lymph node tumor burden. The reason is that patients with late-stage cancer were included in neoadjuvant chemotherapy. At the same time, the effective rate of the treatment was low, and the response of lymph nodes was even lower, which led to false-positive results. Therefore, this result's essential cause is that these patients were in an advanced stage and not because neoadjuvant chemotherapy aggravated lymph node metastasis.

Studies have suggested that pathological classification is one of the prognostic factors of breast cancer (43), but no significant statistical difference was found in this study. The possible reason may be that there is no linear correlation between the pathological classification and the malignant degree of breast cancer. The evaluation index of this study was lymph node tumor burden, i.e., classifying the degree of lymphatic metastasis instead of analyzing whether there is axillary lymph node metastasis in breast cancer, which may have caused indistinguishable pathological classification.

In the malignant and benign breast lesion differentiation, ABVS diagnostic performance is similar to that of handheld ultrasound (HHUS), based on the evidence available in the previous studies (8, 44, 45). However, a great advantage of ABVS in breast lesion characterization in comparison to HHUS is its capability of obtaining details on the reconstructed coronal plane's morphological features (8). Therefore, it can be sensibly concluded that in terms of differential findings assisted by coronal reconstruction, ABVS might be better when compared to HHUS (8). In our analysis, the Cooper's ligament has been confirmed to have a relation with lymph node tumor burden, owing to the sensibly and completely ability of ABVS. On the other hand, in thedifferentiation of breast lesions that are malignant and benign, the ABVS coronal plane retraction phenomenon is perceived as having high probability as a diagnostic feature. However, we have not found any reports exploring the relationship between retraction phenomenon and lymphatic metastasis of breast cancer. Our findings suggest that the retraction phenomenon may not be closely related to the lymphatic metastasis of breast cancer, and further verification is needed.

This study has limitations. This research is a retrospective study. All of the acoustic features of breast lesions were extracted from saved images. Although the saved images can be reconstructed by the workstation and viewed repeatedly, there are still possible information omissions or misjudgments. Some breast cancer lesions would not be identified well by sonography;

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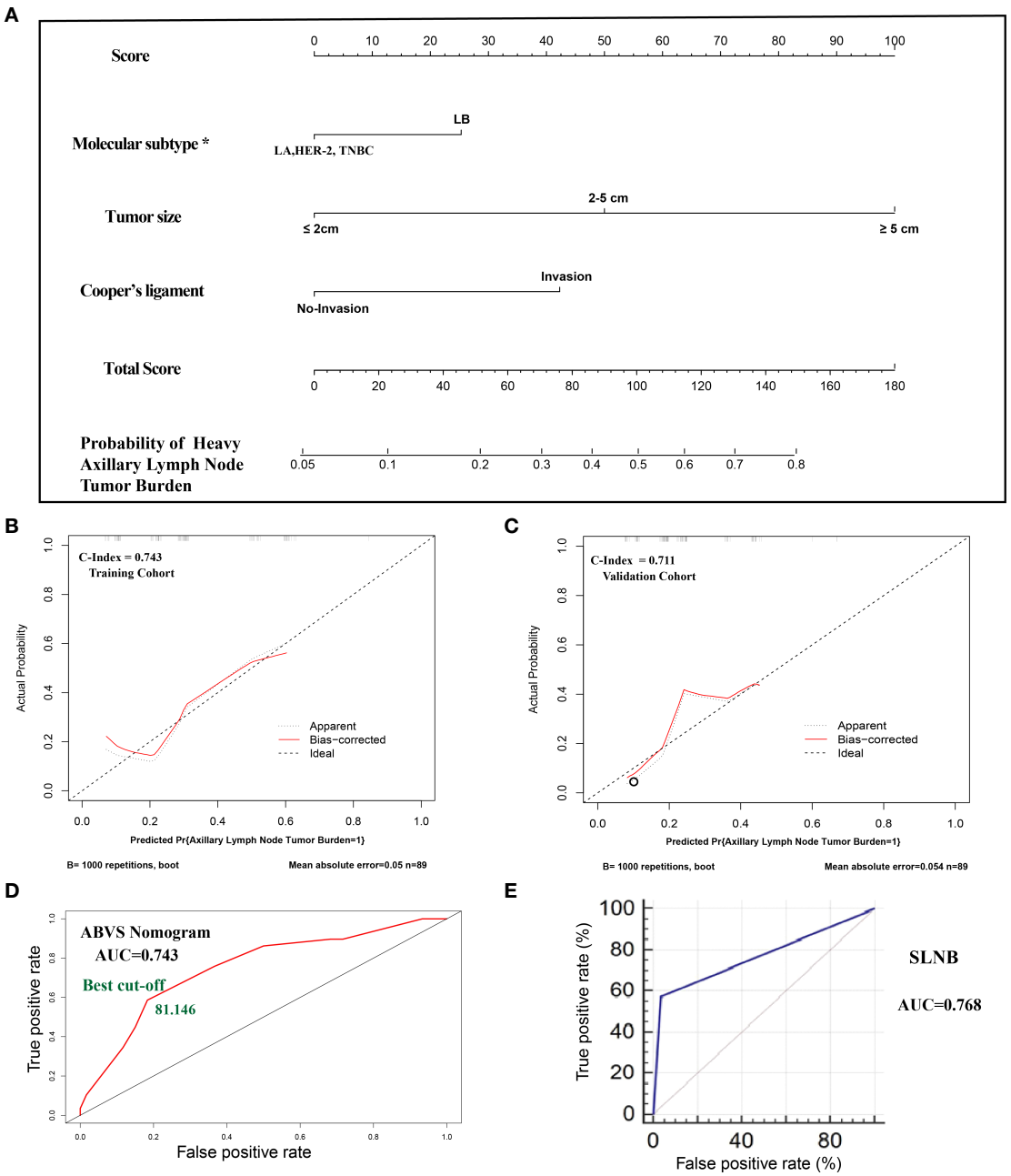

FIGURE 1
(A). The nomogram clinical model. The predictors included tumor size, molecular classification, and Cooper's ligament invasion. Among them, the molecular subtype of luminal B type was assigned a score of 25 points, and other molecular subtypes were assigned 0 points; the maximum lesion diameter of ≤2 cm was assigned 0 points, the maximum lesion diameter of 2–5 cm was assigned 50 points, and the maximum lesion diameter of ≥5 cm was assigned 100 points; the presence of the Cooper's ligament invasion was assigned 42.5 points, and its absence was assigned 0 points. The probability of axillary lymph node tumor burden can be calculated after generating all of the assigned scores. **(B)**. The calibration of the training cohort. **(C)**. The calibration of the validation cohort. **(D)**. The ROC curve and best cutoff value of the nomogram clinical model. **(E)**. The AUC of sentinel lymph node biopsy. *LA: luminal A, LB: luminal B, TNBC: triple-negative breast cancer.

therefore, the ABVS model may not be suitable for all the breast cancer patients, and more studies focusing on these patients are needed. However, the ABVS and SLNB model may be the solution for these patients; further studies are needed.

In conclusion, by integrating the real-world data, we showed the landscape of ABVS features in the breast cancer, including the analyses in different clinical subgroups and molecular subtypes. Then, we successfully identified the

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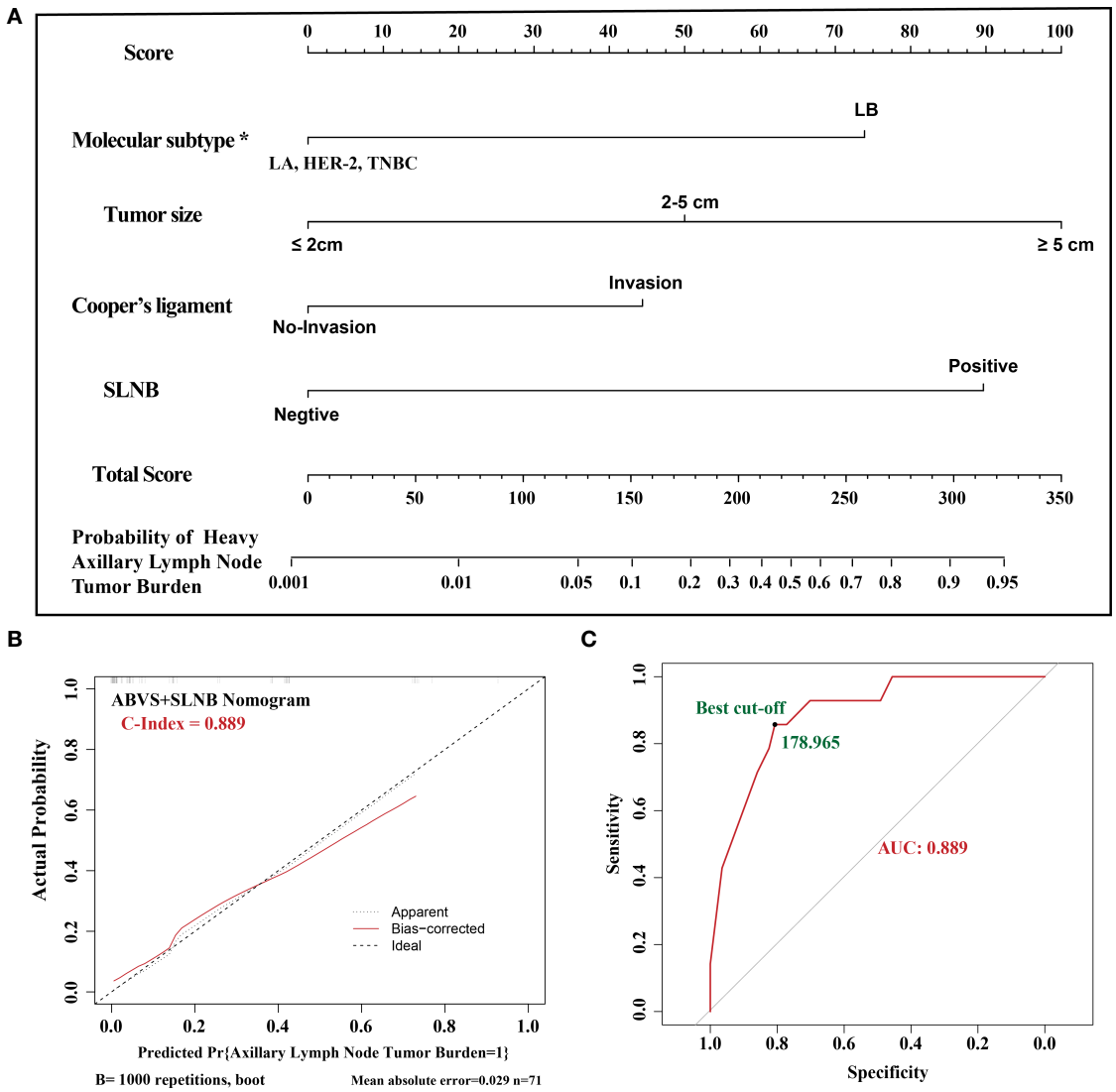

FIGURE 2
(A). The ABVS and SLNB nomogram clinical model. The predictors included tumor size, molecular classification, and Cooper's ligament invasion, SLNB. The probability of axillary lymph node tumor burden can be calculated after generating all of the assigned scores. **(B)**. The calibration. **(C)**. The ROC curve and best cutoff value. *LA: luminal A, LB: luminal B, TNBC: triple-negative breast cancer.

lymph node tumor burden-related ABVS features, combined with the molecular subtype, and we developed a nomogram prediction model, which may be used for individual decision-making in the diagnosis and treatment of breast cancer.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Third Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FZ, CC, and JX designed the study. CC, FZ, and ML collected the data and performed the major analysis. JX supervised the study. CC and FZ analyzed and interpreted the data. CC and FZ

did the statistical analysis. CC and FZ drafted the manuscript. All authors read and approved the final 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.881761/full#supplementary-material

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