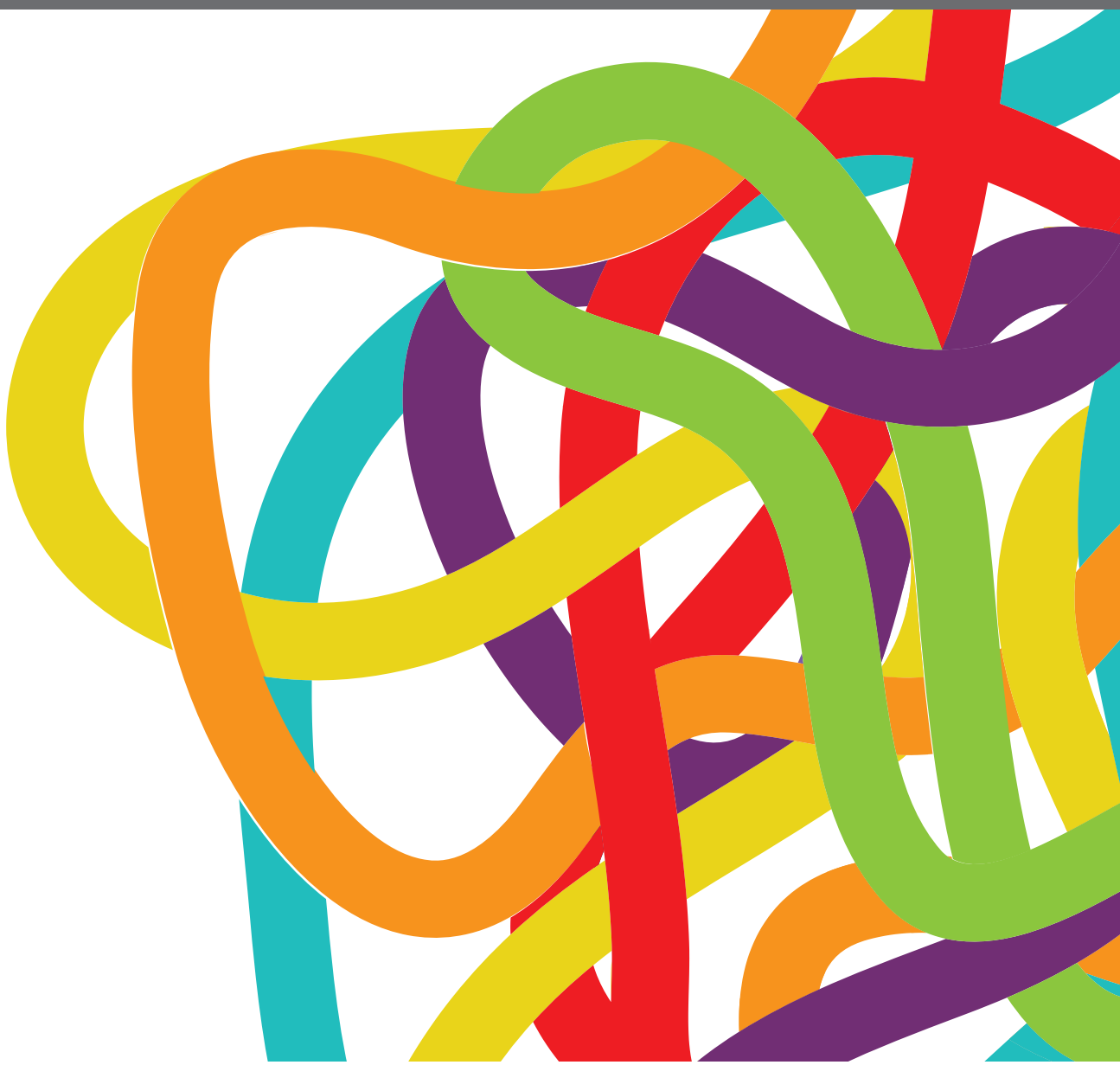
METABOLIC ABNORMALITIES AND BREAST CANCER: CHALLENGES FROM BENCH TO BEDSIDE

EDITED BY: Xiaosong Chen, Mark Daniel Pegram, Pu Li and Zheng Wang
PUBLISHED IN: Frontiers in Oncology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-187-6
DOI 10.3389/978-2-88976-187-6

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METABOLIC ABNORMALITIES AND BREAST CANCER: CHALLENGES FROM BENCH TO BEDSIDE

Topic Editors:
Xiaosong Chen, Shanghai Jiao Tong University, China
Mark Daniel Pegram, Stanford University, United States
Pu Li, Shanghai Jiao Tong University, China
Zheng Wang, Shanghai Jiao Tong University, China

Citation: Chen, X., Pegram, M. D., Li, P., Wang, Z., eds. (2022). Metabolic Abnormalities and Breast Cancer: Challenges From Bench to Bedside. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-187-6

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EDITORIAL
published: 27 April 2022
doi: 10.3389/fonc.2022.890810

Editorial: Metabolic Abnormalities and Breast Cancer: Challenges From Bench to Bedside

Zheng Wang[1*], *Pu Li*[2*], *Mark Daniel Pegram*[3*] *and Xiaosong Chen*[1*]

[1] Department of General Surgery, Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3] Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, United States

Keywords: breast cancer, metabolism abnormalities, metabolic reprogramming, tumor microenvironment, obesity

Editorial on the Research Topic

Metabolic Abnormalities and Breast Cancer: Challenges from Bench to Bedside

OPEN ACCESS

Edited and reviewed by:
San-Gang Wu,
First Affiliated Hospital of Xiamen University, China

***Correspondence:**
Xiaosong Chen
chenxiaosong0156@hotmail.com
Mark Daniel Pegram
mpegram@stanford.edu
Pu Li
leerockygood@yahoo.com
Zheng Wang
wilsonwangzheng@163.com

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *06 March 2022*
Accepted: *04 April 2022*
Published: *27 April 2022*

Citation:
Wang Z, Li P, Pegram MD and Chen X (2022) Editorial: Metabolic Abnormalities and Breast Cancer: Challenges From Bench to Bedside. Front. Oncol. 12:890810. doi: 10.3389/fonc.2022.890810

Breast cancer (BC) ranks as the first malignant disease and the second leading cause of death by cancer in women (1). Despite the development and progress in multi-disciplinary treatments, metastasis and drug resistance pose a huge impediment to further improving the clinical outcome of BC patients. Cancerous cells own the capacity to get nutrition in a tough environment in order to sustain the transformed state, build biomass, and promote proliferation and invasion (2). Thus, metabolic characteristics play an important role in the formation, invasion, and metastasis of breast cancer as well as the development of therapeutic resistance.

Literature reported that the tumor progression, treatment response, and clinical outcome would be affected by host metabolic abnormalities, including diabetes, obesity, and metabolic syndrome (3). Meanwhile, metabolic reprogramming is termed as another emerging hallmark of breast cancer. The distinct metabolic phenotypes of tumor cells, such as glycolysis and altered metabolism of carbohydrates, fat, and protein, could facilitate the tumorigenesis and evolution of breast cancer, which opens up a new scenario for overcoming disease progression (4). In addition, the metabolic pathway of BC cells alters with the tumor microenvironment, including fibroblasts, immune cells, and adipocytes, which ultimately lead to changes in cellular behavior (5). Thus, elucidating the molecular mechanisms, clinical implications, and potential targets involved in the above metabolism abnormalities can further reveal the biology of breast cancer and improve the prognosis of patients. Under this circumstance, the current Research Topic collected 17 scientific studies (11 original research articles, and six reviews) focused on metabolic abnormalities and breast cancer. Those researches narrate the latest progress and reviewed the recent advances in this field, from the basic, translational and clinical aspects.

In the review by Dong et al., metabolic syndrome could affect prevalence, treatment response, progression and survival of breast cancer. As for the initiation of breast cancer, Yan et al. studied the association between mammary tumorigenesis and metabolome in a novel mouse model and showed that MCP-1 derived from adipose could contribute to breast tumorigenesis. By performing a nationwide population-based cohort study, Seol et al. found that elevated GGT level could be a risk factor for breast cancer, especially in the obese post-menopausal group.

In terms of progression and metastasis, Wang et al. illustrated that Lactate Dehydrogenase-A (LDHA) mediated a loop between breast cancer stem cell plasticity and tumor-associated

macrophage infiltration, which would a potential target for combating metastasis. Since both intrinsic and extrinsic factors contributed to metabolic reprogramming phenotypes, Wang et al. comprehensively reviewed the metabolic mechanisms underlying BC metastasis.

Several authors focused on the therapeutic response and resistance. Lu et al. found that the UCHL1, a deubiquitinating enzyme, could lead to chemoresistance by modulating free fatty acid synthesis. Wang et al. found that pyrotinib and adriamycin had synergistic effects on HER2-positive BC. Qiu et al. reviewed the newly published studies in the correlation between hyperglycemia and chemoresistance, as well as the hyperglycemic microenvironment and glucose metabolism. Li and Li. further summarized the recent studies about mitochondrial metabolism and therapeutic resistance in breast cancer. And Wang et al. made a review of the mechanisms by which salinomycin protected against breast cancer and discussed its future clinical applications. In additional, He et al. studied the lipid changes during endocrine therapy and found that tamoxifen would improve total cholesterol and low-density lipoprotein levels in premenopausal patients, and that aromatase inhibitors had no adverse effects on lipid profiles.

Furthermore, Qin et al. studied the relationship between hormone receptor status and prognosis between medullary breast carcinoma and atypical medullary carcinoma of the breast. And Tong et al. analyzed the correlation between 21-gene recurrence score (RS) and obesity, indicating RS varied among different obesity status.

Several articles studied the molecular mechanism beyond metabolic changes in breast cancer, including non-coding RNAs, RNA modification, and hypoxia. Du et al. and Ni et al. indicated that long non-coding RNAs MIR210HG and ADAMTS9-AS2 could modulate the metabolic reprogramming and progression of triple-negative breast cancer (TNBC). Sheng et al. further illustrated the latest progress in DNA N6-Methyladenine modification and drug resistance in TNBC. And Zhang et al. reviewed the role of hypoxia in breast cancer, and discussed the relationship between hypoxia and therapeutic response, as well as the clinical values of hypoxia biomarkers.

In conclusion, all these publications in the present Research Topic provide new insights into the role of metabolic abnormalities in the disease development, treatment response, and prognosis of breast cancer. We hope that the finding of the articles in this Research Topic would provide novel treatment strategies to improve survival of BC patients.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this Editorial. All authors contributed to the article and approved the submitted version.

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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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 17 December 2020
doi: 10.3389/fonc.2020.580176

Long Noncoding RNA MIR210HG Promotes the Warburg Effect and Tumor Growth by Enhancing HIF-1α Translation in Triple-Negative Breast Cancer

Ye Du[1], *Na Wei*[1], *Ruolin Ma*[1], *Shu-Heng Jiang*[2] *and Dong Song*[1]*

[1] Departments of Breast Surgery, The First Hospital of Jilin University, Changchun, China, [2] State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

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Edited by:
Zheng Wang,
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Reviewed by:
Xinyuan Ding,
Suzhou Municipal Hospital, China
Yan Du,
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University of Naples Federico II, Italy
Zuoren Yu,
Tongji University, China

***Correspondence:**
Dong Song
songdong@jlu.edu.cn

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *04 July 2020*
Accepted: *20 November 2020*
Published: *17 December 2020*

Citation:
Du Y, Wei N, Ma R, Jiang S-H and Song D (2020) Long Noncoding RNA MIR210HG Promotes the Warburg Effect and Tumor Growth by Enhancing HIF-1α Translation in Triple-Negative Breast Cancer. Front. Oncol. 10:580176. doi: 10.3389/fonc.2020.580176

Background: Hypoxia is an important environmental factor and has been correlated with tumor progression, treatment resistance and poor prognosis in many solid tumors, including triple-negative breast cancer (TNBC). Emerging evidence suggests that long noncoding RNA (lncRNA) functions as a critical regulator in tumor biology. However, little is known about the link between hypoxia and lncRNAs in TNBC.

Methods: TNBC molecular profiles from The Cancer Genome Atlas (TCGA) were leveraged to identify hypoxia-related molecular alterations. Loss-of-function studies were performed to determine the regulatory role of MIR210HG in tumor glycolysis. The potential functions and mechanisms of hypoxia-MIR210HG axis were explored using qPCR, Western blotting, luciferase reporter assay, and polysome profiling.

Results: We found that MIR210HG is a hypoxia-induced lncRNA in TNBC. Loss-of-function studies revealed that MIR210HG promoted the Warburg effect as demonstrated by glucose uptake, lactate production and expression of glycolytic components. Mechanistically, MIR210HG potentiated the metabolic transcription factor hypoxia-inducible factor 1α (HIF-1α) translation via directly binding to the 5'-UTR of HIF-1α mRNA, leading to increased HIF-1a protein level, thereby upregulating expression of glycolytic enzymes. MIR210HG knockdown in TNBC cells reduced their glycolytic metabolism and abolished their tumorigenic potential, indicating the glycolysis-dependent oncogenic activity of MIR210HG in TNBC. Moreover, MIR210HG was highly expressed in breast cancer and predicted poor clinical outcome.

Conclusion: Our results decipher a positive feedback loop between hypoxia and MIR210HG that drive the Warburg effect and suggest that MIR210HG may be a good prognostic marker and therapeutic target for TNBC patients.

Keywords: long noncoding RNA, triple-negative breast cancer, Warburg effect, MIR210HG, HIF-1α

BACKGROUND

Triple-negative breast cancer (TNBC) a specific subtype of breast cancer that does not express progesterone receptor, estrogen receptor, and human epidermal growth factor receptor 2 (HER2, overexpression and/or amplification). TNBC constitutes ~15% of all breast cancer subtypes and exhibits high invasiveness, increased metastatic potential, high risk of recurrences, and poor outcomes (1). Due to the limited targeted therapy available for this deadly disease, the clinical treatment of TNBC is still hindered by metastasis or recurrence (2, 3). Therefore, a more comprehensive understanding of the molecular mechanisms that implicated in TNBC progression will likely aid in clinical developmental therapeutics.

A common feature of many solid tumors is hypoxia due to insufficient blood supply (4). Hypoxia-inducible factor 1 alpha (HIF-1α), functioning as first responder under hypoxic conditions, is highly expressed in TNBC. Hypoxia leads to HIF-1α stabilization and rapid protein accumulation and increased transcriptional activity (5). Through transcription of several hundred of target genes, HIF-1α is profoundly implicated in many malignant phenotypes, such as angiogenesis, metabolic reprogramming, stemness maintenance, cell survival and proliferation, tumor motility and invasion, immune evasion, and resistance to chemoresistance (6–9). Recent preclinical studies showed that the combination of cytotoxic chemotherapy with drugs that targeting hypoxia-inducible factors may improve the clinical outcome for TNBC patients (10–12).

Long non-coding RNAs (lncRNAs) are a class of nonprotein-coding RNAs with a length of more than 200 nucleotides (13). Accumulating evidence has shown that lncRNAs are participated in many fundamental cellular functions, such as histone modification, alternative splicing, chromatin structure modification, and gene expression regulation (14). Recently, lncRNAs have emerged as crucial regulators of cell differentiation, organogenesis, and tumorigenesis (15, 16). In TNBC, dysregulation of many lncRNAs has been reported to promote cell survival, tumor metastasis, immune evasion, and chemoresistance (17–20). For example, lncRNA LINK-A expression in TNBC cells contributes to downregulation of antigenicity and facilitates intrinsic tumor suppression (21). Moreover, lncRNA NRAD1 regulates expression of genes involved in differentiation and catabolic processes, which are essential for TNBC development (22).

Reprogrammed energy metabolism is an emerging hallmark of human cancers (23). Highly proliferative cancer cells preferentially metabolize glucose by aerobic glycolysis rather than through the more energetically efficient oxidative phosphorylation, even in the presence of sufficient oxygen, a phenomenon known as the Warburg effect (24, 25). Accumulating evidence suggests that the Warburg effect is closely associated with a poor clinical outcome and exerts critical implications on tumor progression (26, 27). In this study, we first performed integrated analysis to characterize hypoxia-related lncRNAs in TNBC through leveraging large-scale TNBC molecular profiles from The Cancer Genome Atlas (TCGA). Subsequently, functional verification showed that MIR210HG is a hypoxia-induced lncRNA and acts as a key glycolytic regulator in TNBC. Importantly, MIR210HG regulates glycolytic gene expression through increased HIF-1α mRNA translation. Therefore, our data revealed a novel feedback loop between HIF-1α and MIR210HG that facilitates the Warburg effect in TNBC, suggesting that targeting HIF-1α/MIR210HG axis could be a potential therapeutic target.

Abbreviations: TNBC, triple-negative breast cancer; lncRNA, long noncoding RNA; HER2, human epidermal growth factor receptor 2; HIF-1α, hypoxia-inducible factor 1 alpha; TCGA, The Cancer Genome Atlas; IHC, Immunohistochemistry; co-IP, chromatin immunoprecipitation; GTEx, Genotype-Tissue Expression; HUVECs, human umbilical vein endothelial cells; PTECs, human proximal tubular epithelial cells; ceRNA, competing endogenous RNA.

MATERIALS AND METHODS

Bioinformatics Analysis

The RNA-sequencing data of TNBC and corresponding non-tumor tissues were downloaded from The Cancer Genome Atlas (TCGA, https://gdc.cancer.gov/) database. A well-documented 15-gene expression signature (ACOT7, ADM, ALDOA, CDKN3, ENO1, LDHA, MIF, MRPS17, NDRG1, P4HA1, PGAM1, SLC2A1, TPI1, TUBB6, and VEGFA) was used to classify hypoxia status (11). These genes are mainly targets of HIF1A. As reported previously, this gene signature was derived by selecting genes that were consistently co-expressed with the hypoxia seeds in multiple cancers and defined based on gene function and analysis of *in vivo* co-expression patterns (28). Differentially expressed lncRNAs related to hypoxia status were analyzed by estimating an exact test P-value.

Cell Lines

Human breast cancer cell lines (MCF7, T47D, ZR-75-1, Hs578T, MDA-MB-231, and HCC1937) and the non-malignant human mammary epithelial cell lines MCF10A were all obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (Hyclone, USA) or Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, USA). All cell lines were cultured in a humidified incubator of 5% CO_2 at 37°C.

Small Interfering RNA and Generation of Stably Expressing Cell Lines

For siRNA transfection, Hs578T, MDA-MB-231, and HCC1937 cells were seeded in six-well plates and allowed to grow to 50–70% confluence. Then, the cells were transfected with HIF-1α siRNA or negative control (GenePharma Inc., Shanghai, China) at a concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. To generate stable MIR210HG-depleted TNBC cells, the pCDH-CMV-MCS-EF1-copRFP lentiviral vector was used. The shRNA

sequences were shown as follows: sh-MIR210HG-1, 5′-GCATTAGTACAGGCACCAGCCTA-3′; sh-MIR210HG-2, 5′-UUUAGACCCAUUCUCGUAUGGAGGU-3′. A non-silencing shRNA (sh-Ctrl) oligonucleotide was used as a negative control.

Real-Time Quantitative PCR

Total RNAs from breast cancer cells or tumor tissues were extracted by the RNAiso Plus kit (Takara Bio Inc., Japan). Twenty-two TNBC specimens were also obtained from Departments of Breast Surgery, The First Hospital of Jilin University. All the patients were provided with written informed consent before enrollment, and the study was approved by the Research Ethics Committee of The First Hospital of Jilin University. The RNA concentration and quality were determined by spectrophotometry using NanoDrop™ 2000 (Thermo Scientific, USA). Then, RNA was reverse transcribed to complementary DNA (cDNA) by using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) according the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green using the ViiA7 System (AB Applied Biosystems, USA). ACTB mRNA was used for normalization. The primers used in this study were shown in **Supplementary Table 1**.

Isolation of Cytoplasmic and Nuclear RNA

Nuclear and cytoplasmic fractions were isolated by the PARIS Kit (Life Technologies, USA) according to the manufacturer's instruction. GAPDH and U1 were used as cytoplasmic and nuclear controls, respectively. Then, isolated RNA was subjected to reverse transcription reaction and real-time qPCR. The primers used were shown as follows: GAPDH, forward: 5'-CTGGGCTACACTGAGCACC-3', reverse: 5'-AAGTGG TCGTTGAGGGCAATG-3'; U1, forward: 5'-GTGGT TTTTCCAGAGCAAGG-3', reverse: 5'-CAGGGGAAA ACACAGACACA-3'.

Western Blotting

Cells were lysed with lysis buffer containing 0.1% Triton X-100, 20 mM Tris-Cl, 125 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail. Protein concentration was detected by Pierce BCA Protein assay kit (Thermo Fisher Scientific, USA). Lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membrane was then blocked with 5% non-fat milk and hybridized overnight with primary antibodies against HIF-1α (#36169, Cell Signaling Technology), GLUT1 (21829-1-AP, ProteinTech), PKM2 (15822-1-AP, ProteinTech), LDHA (19987-1-AP, ProteinTech), and β-actin (ab8227, Abcam). The next day, blots were detected with horseradish peroxidase-conjugated anti-IgG for 1 h at room temperature and visualized with an ECL kit (Millipore, USA).

Measurement of Glucose and Lactate Level

Glucose utilization and lactate release by cancer cells were used to detect cellular glycolytic activity as reported previously (29). Briefly, 1×10^6 indicated cells were seeded in 60-mm plates and supplemented with FBS-free medium. After incubation for 24 h, culture medium was collected and subjected for glucose and lactate level analysis using a commercial glucose assay kit (Sigma-Aldrich, MAK263, Shanghai, China) and a Lactate Assay Kit (BioVision, K607-100, USA) according to the manufacturer's instruction. Total cell protein was used for normalization.

Polysome Profiling

Polysome profiling is a method widely used to monitor the translation activity of mRNAs. Once each polysome fractions are collected, the translation activity of each mRNA can be analyzed using various molecular biology techniques such as Northern blotting and RT-PCR. Polysome profiling was performed as reported elsewhere (30). Briefly, cell lysates were collected with polysome lysis buffer and then loaded onto 10 to 50% sucrose density gradients prepared in polysome buffer. After centrifugation for 3 h at 35,000 rpm at 4°C, gradients were recovered in 12 fractions using gradient fractionators and RNA was isolated from each fraction. The expression of HIF-1α mRNA was detected by quantitative reverse transcription PCR.

Immunofluorescence

RNA-FISH was performed with MIR210HG specific probe designed and synthesized by ServiceBio Company (Wuhan, China). In brief, MDA-MB-231 and HCC1937 cells were fixed with 4% paraformaldehyde for 10 min. Then, the cells were permeabilized by 0.5% TritonX-100 for 5 min at 4°C and washed with PBS for three times. Hybridization was performed with MIR210HG probe in a moist chamber at 37°C overnight, flowing by co-staining with DAPI for 10 min. After that, the cells were washed and photographed with a fluorescence confocal microscope.

Immunohistochemistry (IHC)

IHC was performed on formalin-fixed paraffin-embedded sections as reported previously (31). After deparaffinization and citrate-based antigen retrieval, endogenous peroxidase was blocked by 3% H_2O_2. Then, the sections were washed and incubated with primary antibodies against Ki67 (#9027, Cell Signaling Technology) or HIF-1α (#36169, Cell Signaling Technology) at 4°C overnight. The next day, slides were incubated with a second antibody labeled by HRP at room temperature for 1 h. Finally, slides were developed with the HRP substrate diaminobenzidine and counterstained with hematoxylin. Scoring was mainly conducted based on the percentage of positive-staining cells: 0–5% scored 0, 6–30% scored 1, 30–70% scored 2, and more than 70% scored 3. The final score was designated as low or high expression as follows: low expression (score 0–1), high expression (score 2–3). These scores were determined independently by two senior pathologists in a blinded manner.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) experiment was performed using the ChIP assay kit (Pierce Agarose ChIP Kit). In brief, HS578T, MDA-MB-231, and HCC1937 cells were

crosslinked and sonicated, and DNA was immunoprecipitated with HIF-1α (#36169, Cell Signaling Technology) or isotype-matched control IgG (Cell Signaling Technology) from the sonicated cell lysates and quantified using Premix Taq™ PCR analysis (Takara, Japan). The primers used in the assay were shown as follows. Site 1: forward, 5'-CCCGGGCAGACGTGC-3'; reverse, 5'-CCTGGTCCCTCAGCCAATG-3'; Site 2: forward, 5'-GTCACGGCCCGGGATAC-3'; reverse, 5'-GGAGCTG CCCCTCTTCCC-3'. The known target of HIF-1α vascular endothelial growth factor A (VEGFA) was used as a positive control.

uciferase Reporter Assay

Two hundred ninety-two base pairs of human HIF-1α, 5'-untranslated region (UTR) upstream of the start codon (identified by UCSC Genome Browser, http://www.genome.ucsc.edu/), were amplified and cloned into bicistronic reporter plasmid. MDA-MB-231 and HCC1937 cells were seeded in 6-well plates at a density of 2×10^5 cells per well. After incubation overnight, the reporter was transfected into stable sh-MIR210HG or sh-Ctrl MDA-MB-231 and HCC1937 cells for 48 h. Finally, the cells were harvested and luciferase activity was analyzed using Dual-Glo Luciferase Assay (Promega). Renilla activity was used to an internal control. Moreover, mRNA expression of firefly-luciferase was measured as an additional control.

Measurement of HIF-1α Transcription Activity

Briefly, a specific double stranded DNA sequence containing the HIF-1α response element (5'-ACGTG-3') is immobilized to the wells of a 96-well plate. The nuclear extract lysates from sh-Ctrl and sh-MIR210HG MDA-MB-231 and HCC1937 cells were harvested using the Nuclear Extraction kit (ab113474; Abcam, Cambridge, UK). Then, HIF-1α activity was detected by addition of a specific primary antibody directed against HIF-1α according to the manufacturer's instruction (ab133104; Abcam, Cambridge, UK). A secondary antibody conjugated to HRP is added to provide a sensitive colorimetric readout at 450 nm.

CCK-8 Assay

For CCK-8 experiment, MDA-MB-231 and HCC1937 cells were seeded in 96-well plates at 3,000 cells per well. At indicated time points (day 1, 3, 5), cell viability was measured by treatment with 10% (v/v) CCK-8 solution (Dojindo, Kumamoto, Japan) for 1 h at 37°C. Then, the absorbance value at 450 nm in each well was detected by a microplate reader.

Animal Experiment

BALB/c nude male mice aged 6 weeks were obtained from Shanghai Jiesijie Laboratory Animal Co., Ltd. Mice were manipulated and housed according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences. Mice were kept on a 12-hour day/night cycle with free access to food and water. Mice were divided into two groups at random. The investigator was blinded to the group allocation during the experiment. For generation of subcutaneous xenograft model, mice were subcutaneously injected with 1×10^6 sh-control or sh-MIR210HG-depleted MDA-MB-231 cells under the right lower limbs, followed by growth under specific pathogen-free condition for 5 weeks. At the end of the experiment, all mice were euthanized and the tumors were resected, weighed and collected for IHC and real-time qPCR analysis. This study was approved by the Research Ethics Committee of The First Hospital of Jilin University.

Statistical Analysis

Data were presented as the means ± SD. The statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The two-sided Student's t test or one-way ANOVA followed by Student-Newman-Keuls (SNK) test was used to compare data between groups. Survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Correlation analysis was evaluated by Spearman's rank correlation. For all tests, a p-value of less than 0.05 was considered statistically significant.

RESULTS

Integrated Analysis of Hypoxia-Related lncRNAs in TNBC

RNA sequencing data of TNBC patient samples was acquired from The Cancer Genome Atlas (TCGA). To classify the hypoxia status of TNBC samples, we focused on a 15-gene expression signature (ACOT7, ADM, ALDOA, CDKN3, ENO1, LDHA, MIF, MRPS17, NDRG1, P4HA1, PGAM1, SLC2A1, TPI1, TUBB6, and VEGFA) that was well documented in a recent article assessing hypoxia-associated molecular features (11). Then, TNBC samples were stratified into hypoxia score-low (n = 57) or score-high (n = 58) groups (**Supplementary Table 2**). By comparing the differentially expressed lncRNAs, 10 lncRNAs (XIST, LINC01152, AL365361.1, C1orf132, FAM30A, AC009041.2, LINC00861, AP001453.2, WFDC21P and MIR210HG) had at least two-fold change (**Figure 1A**). Moreover, we revealed that 160 lncRNAs were differentially expressed in TNBC tissues compared with normal breast tissues (**Supplementary Figure 1**). Of note, 2 hypoxia-related lncRNAs (MIR210HG and XIST) were dysregulated (**Figure 1B**); MIR210HG expression was upregulated approximately 5 times in glycolysis-high TNBC tissues compared with glycolysis-low TNBC tissues (**Figure 1C**).

MIR210HG Is Induced by Hypoxia in TNBC Cells

By real-time qPCR analysis, we found that MIR210HG expression was highly expressed in TNBC cells (Hs578T, MDA-MB-231 and HCC1937) compared to non-TNBC cells (MCF7, T47D, and ZR-75-1) and the non-malignant breast epithelial MCF-10A cells (**Figure 2A**). To test whether hypoxia is responsible for MIR210HG expression, we cultured MCF7, T47D and ZR-75-1 cells under normoxia or hypoxia condition

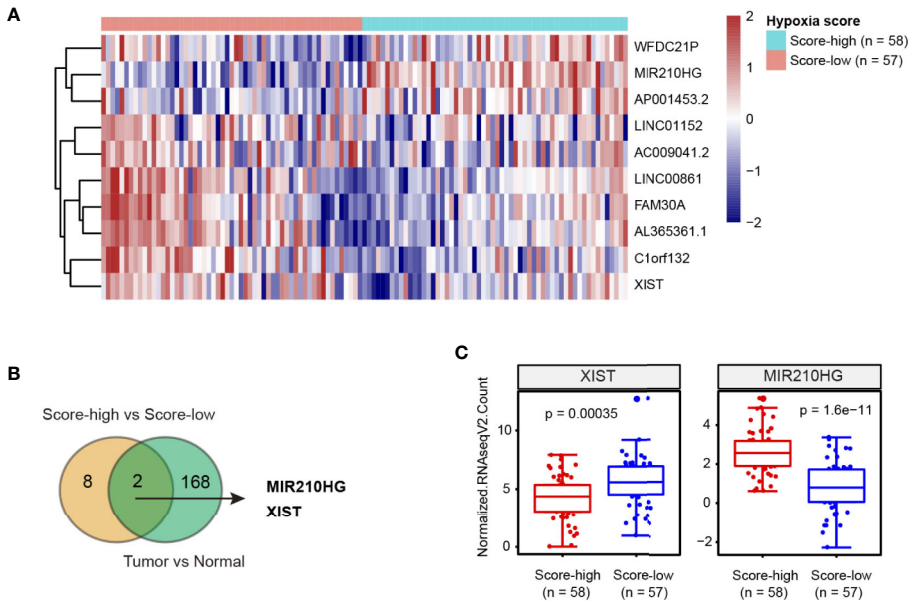

FIGURE 1 | Integrated analysis of hypoxia-related lncRNAs in TNBC. **(A)** Heatmap of 10 lncRNAs related to hypoxia in TNBC; hypoxia score-low: n = 57 and hypoxia score-high: n = 58. **(B)** Venn diagram showed that 2 hypoxia-related lncRNAs were dysregulated in TNBC tissues. **(C)** Expression level of XIST and MIR210HG in hypoxia score-high (n = 58) and score-low (n = 57) groups.

for 24 h. The result showed that MIR210HG expression was differentially increased by hypoxia in these cell lines (**Figure 2B**). To mimic hypoxia, we treated MCF7, T47D and ZR-75-1 cells with 100 μM $CoCl_2$, a known chemical inducer of HIF-1α. Expectedly, $CoCl_2$ also significantly promoted MIR210HG expression (**Figure 2C**). Next, we genetically silenced HIF-1α in three TNBC cell lines. Two specific siRNAs against HIF-1α led to significant reduction in HIF-1α protein level (**Figure 2D**). As a result, HIF-1α knockdown remarkably suppressed MIR210HG expression in Hs578T, MDA-MB-231 and HCC1937 cells (**Figure 2E**). Moreover, chromatin immunoprecipitation experiment demonstrated that HIF-1α interacted directly with MIR210HG promoters (**Figures 2F, G**). Taken together, these findings suggest that MIR210HG is a hypoxia-induced lncRNA in TNBC.

MIR210HG Facilitates Aerobic Glycolysis in TNBC Cells

To elucidate the cellular functions of MIR210HG in TNBC, we compared the gene expression profiles between MIR210HG-low group and MIR210HG-high group by using the RNA-seq data in the TCGA cohort. Interestingly, gene set enrichment analysis (GSEA) showed that glycolysis gene signature was significantly enriched in samples with high MIR210HG expression (**Figure 3A**). To further confirm this observation, we generated stably sh-MIR210HG expressing cell lines (**Figure 3B**). MIR210HG knockdown led to marked reduction in glucose consumption and lactate production in Hs578T, MDA-MB-231 and HCC1937 cells (**Figures 3C, D**). Western blotting analysis showed that MIR210HG knockdown attenuated the protein expression of three key glycolytic components (GLUT1, PKM2 and LDHA) (**Figure 3E**). Moreover, real-time qPCR analysis revealed that MIR210HG expression was closely associated with GLUT1, PKM2 and LDHA mRNA expression in 22 TNBC tissues (**Figure 3F**). Collectively, MIR210HG may act as a positive glycolysis modulator in TNBC.

MIR210HG Promotes HIF1α Translation in TNBC

In light of the critical role of HIF-1α in regulating the Warburg effect (32), we tested whether MIR210HG facilitates aerobic glycolysis via HIF-1α. Real-time qPCR analysis showed that no significant change in HIF-1α mRNA levels in response to MIR210HG knockdown (**Figure 4A**). In contrast with this observation, western blotting result showed that MIR210HG knockdown led to marked reduction in HIF-1α protein expression (**Figure 4B**), suggesting that the regulation of HIF-1α by MIR210HG is post-transcriptional. Consistently, HIF-1α transcriptional activity was also downregulated by MIR210HG knockdown (**Figure 4C**). To uncover the mechanism by which MIR210HG promotes HIF-1α mRNA translation, we detected the subcellular localization of MIR210HG in MDA-MB-231 and HCC1937 cells. The result showed that MIR210HG was predominantly located in the cytoplasm, which was consistent with its potential role in post-transcriptional regulation of HIF-1α (**Figure 4D**). Fluorescence in situ hybridization of MIR210HG in MDA-MB-231 and HCC1937 cells also confirmed the cytoplasm localization (**Figure 4E**). Next, we characterized polysome-associated HIF-1α mRNA in MIR210HG knockdown cells. As a result, MIR210HG knockdown increased association of HIF-1α to free and light ribosome fractions and decreased association of HIF-1α mRNA to heavy ribosome fractions (**Figure 4F**), indicating that

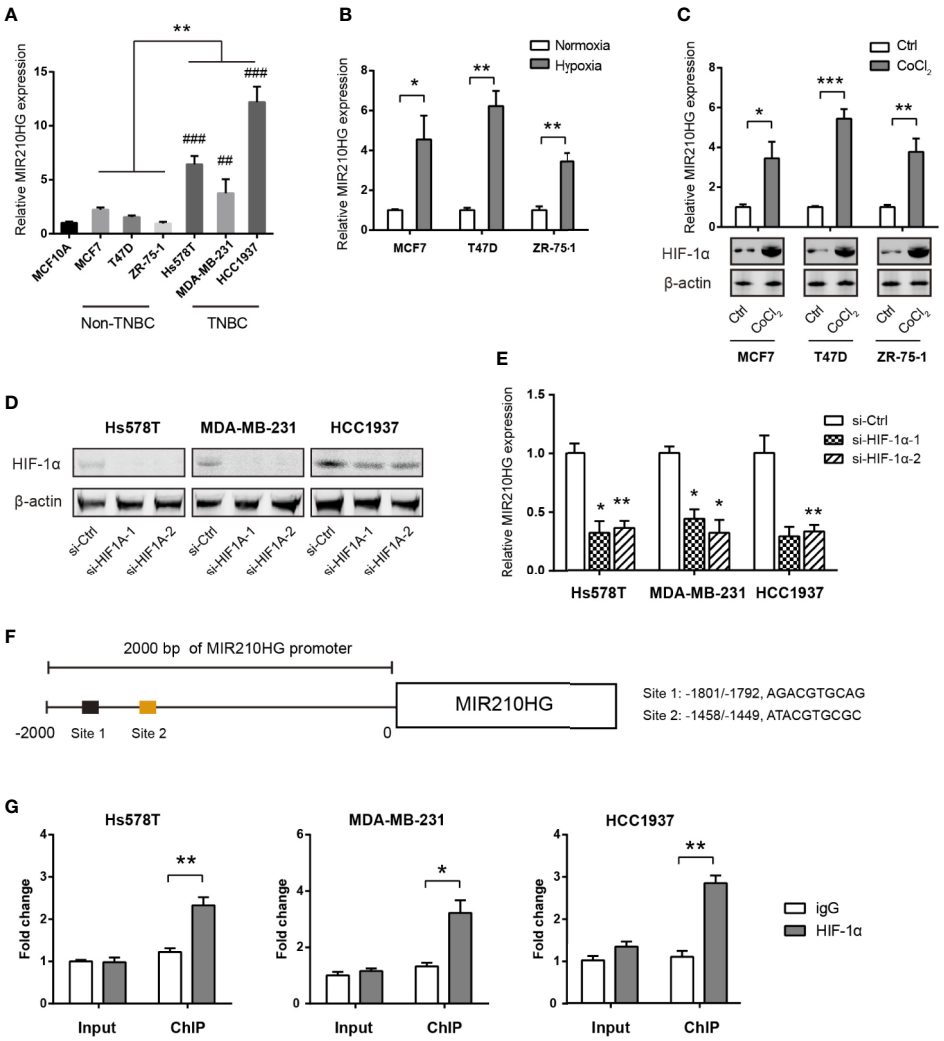

FIGURE 2 | MIR210HG is induced by hypoxia in TNBC cells. **(A)** Real-time qPCR analysis of MIR210HG expression in breast cancer cells and the non-malignant MCF10A cells; indicates statistical significance of comparison to normal MCF10A cells; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. **(B)** Real-time qPCR analysis of MIR210HG expression in three breast cancer cell lines (MCF7, T47D, and ZR-75-1) under hypoxia (1% O_2) or normoxia (20% O_2) condition. **(C)** Real-time qPCR analysis of MIR210HG expression in three breast cancer cell lines (MCF7, T47D, and ZR-75-1) after treatment with $CoCl_2$ (50 μM) for 24 h. **(D)** Western blotting analysis of HIF-1α knockdown efficiency in Hs578T, MDA-MB-231, and HCC1937 cells. **(E)** Real-time qPCR analysis of MIR210HG expression in three breast cancer cell lines (MCF7, T47D, and ZR-75-1) after HIF-1α knockdown. **(F)** A schematic diagram showed the HIF-1α locus in the MIR210HG promoter. **(G)** HIF-1α occupation on MIR210HG promoter was evaluated by ChIP-qPCR, IgG was used as negative control (n= 3). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

MIR210HG regulates HIF-1α translation. To determine whether MIR210HG regulates HIF-1α translation through binding its 5'-UTR, we generated a bicistronic reporter containing HIF-1α 5'-UTR and performed luciferase reporter assay. Indeed, the luciferase reporter activity was significantly reduced in sh-MIR210HG MDA-MB-231 and HCC1937 cells compared with control cells (**Figure 4G**) whereas the mRNA levels of luciferase were not changed (**Figure 4H**).

MIR210HG Knockdown Suppresses Tumor Growth

By CCK-8 experiment, we showed that MIR210HG knockdown significantly inhibited cell proliferation of MDA-MB-231 and HCC1937 cells (**Figure 5A**). Moreover, reduced cell viability induced by MIR210HG knockdown can be largely restored by ectopic expression of HIF-1α (**Figure 5A**). To test the *in vivo* oncogenic role of MIR210HG in TNBC, we established subcutaneous xenograft tumor model by injection of sh-Ctrl or sh-MIR210HG MDA-MB-231 cells into nude mice (n = 5 per group). Tumor volume was monitored for 5 consecutive weeks. As shown in **Figure 5B**, MIR210HG knockdown significantly retarded tumor growth. Five weeks later, mice were sacrificed and tumors were weighted. MIR210HG knockdown drastically reduced tumor weight (**Figure 5C**). IHC analysis showed that Ki67 and HIF-1α positive staining were also decreased in sh-MIR210HG tumor tissues compared with that in sh-Ctrl group

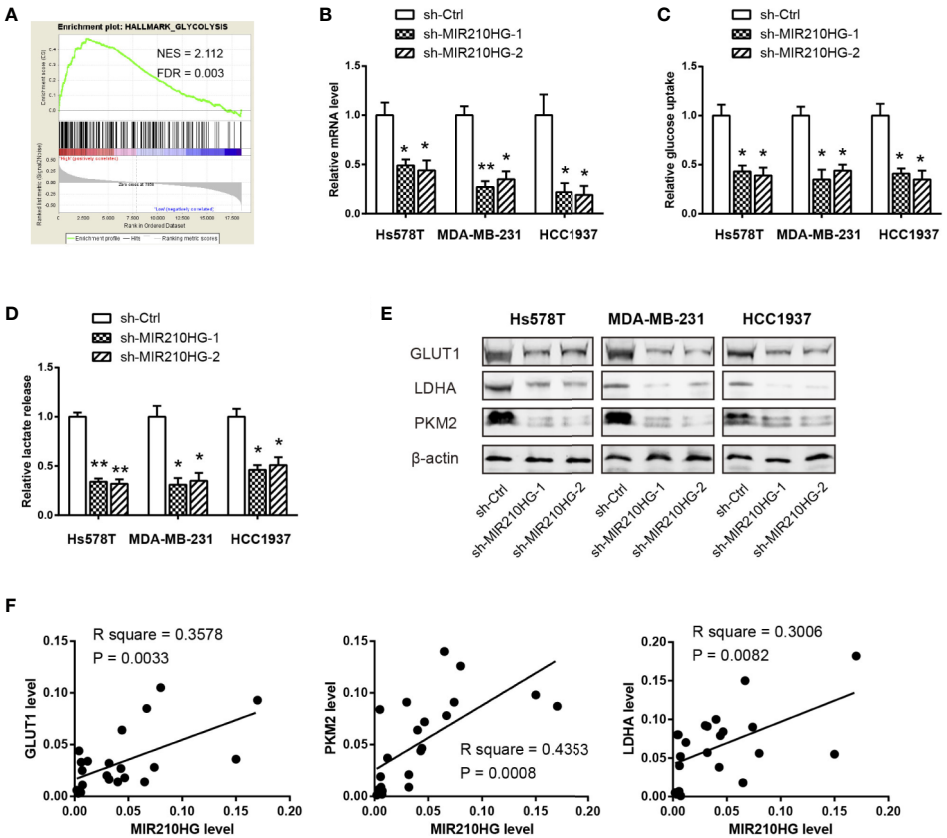

FIGURE 3 | MIR210HG facilitates aerobic glycolysis in TNBC cells. **(A)** Gene set enrichment analysis (GSEA) showed that MIR210HG is closely related to glycolysis gene signature in TNBC. **(B)** Real-time qPCR analysis of MIR210HG knockdown efficiency in Hs578T, MDA-MB-231, and HCC1937 cells. **(C, D)** Quantification of glucose consumption and lactate production in Hs578T, MDA-MB-231, and HCC1937 cells after MIR210HG knockdown. **(E)** Western blotting analysis of the effect of MIR210HG knockdown on GLUT1, LDHA, and PKM2 expression in Hs578T, MDA-MB-231, and HCC1937 cells. **(F)** Correlation analysis of MIR210HG and three glycolytic components in 22 TNBC tissues. *p < 0.05; **p < 0.01.

(**Figure 5D**). Consistently, real-time qPCR analysis showed that MIR210HG knockdown did not affect HIF-1α mRNA but reduced the expression of GLUT1, PKM2 and LDHA (**Figure 5E**). Taken together, these findings suggest that a positive feedback loop between MIR210HG and HIF-1α may enhance Warburg effect, which ultimately promotes tumor growth in TNBC (**Figure 5F**).

MIR210HG Is Highly Expressed in Breast Cancer and Predicts a Poor Prognosis

Using the transcriptomic profiles from TCGA and the Genotype-Tissue Expression (GTEx) portal, we analyzed MIR210HG in breast cancer tissues and normal breast tissues. The result showed that MIR210HG expression was significantly upregulated in breast tumor tissues compared with normal controls (**Figure 6A**) and there was an increase in stage IV tumors (**Figure 6B**). By Kaplan-Meier plotter analysis (https://kmplot.com/analysis/) (33), we found that MIR210HG expression predicted a poor prognosis in relapse-free survival (**Figure 6C**) and overall survival (**Figure 6D**) in breast cancer patients. Importantly, higher MIR210HG expression showed a more significant hazard ratio for relapse-free survival in TNBC patients (**Figure 6E**).

DISCUSSION

Recently, lncRNAs are emerged as important regulators of gene expression at chromatin, transcriptional and posttranscriptional levels with diverse functions in many physiological and pathological processes, especially cancer. Hypoxia is a typical feature of tumor microenvironment and is essential for aggressive cancer phenotypes. Under hypoxia, HIF-1α stimulates expression of multiple hypoxia responsive genes via binding to the hypoxia response elements (HREs), eliciting a wide spectrum of cellular adaptations, such as angiogenesis, proliferation, and metabolic reprogramming. Not surprisingly, lncRNAs are also downstream targets of HIF-1α and act as effectors in response to hypoxia. In this study, we identified two dysregulated hypoxia-related lncRNAs (XIST and MIR210HG) in TNBC. Many studies have documented the tumor suppressor function of XIST in breast cancer and TNBC, which is consistent

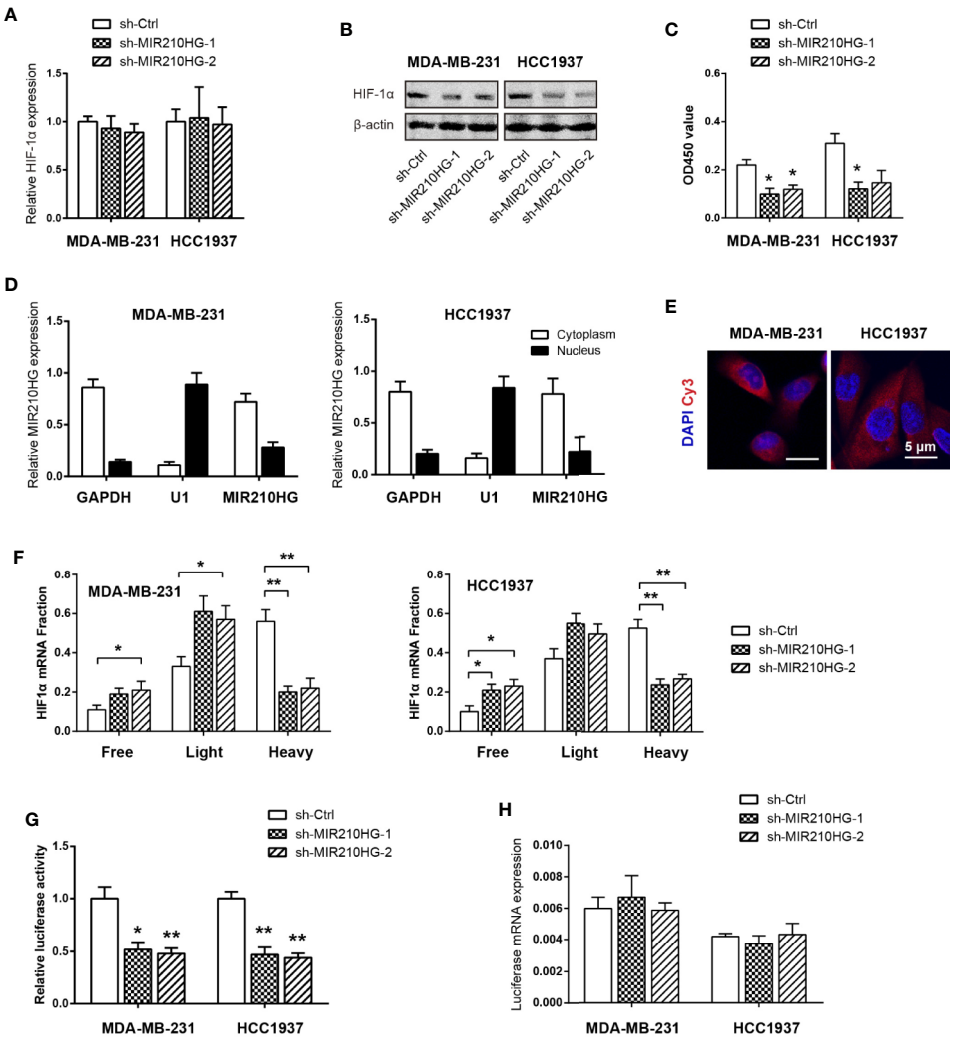

FIGURE 4 | MIR210HG upregulates HIF1α translation in TNBC. **(A)** Real-time qPCR analysis of HIF-1α mRNA expression in MDA-MB-231 and HCC1937 cells stably expressing sh-MIR210HG or sh-Ctrl. **(B)** Western blotting analysis of HIF-1α protein expression in MDA-MB-231, and HCC1937 cells stably expressing sh-MIR210HG or sh-Ctrl. **(C)** Effects of MIR210HG knockdown on the HIF-1α transcriptional activity. **(D)** Real-time qPCR analysis of HIF-1α location in MDA-MB-231 and HCC1937 cells. GAPDH and U1 were used as internal cytoplasmic and nuclear control, respectively. **(E)** FISH analysis of HIF-1α location in MDA-MB-231 and HCC1937 cells. Scale bar: 5 μm. **(F)** Relative distribution of HIF-1α mRNA across the polysome fractions in cells stably expressing sh-MIR210HG or sh-Ctrl. **(G)** Effect of MIR210HG knockdown on the translation activity of 5'-UTR of HIF-1α mRNA in MDA-MB-231 and HCC1937 cells was detected by luciferase reporter assay. **(H)** Real-time qPCR analysis of the luciferase transcript expression in MDA-MB-231 and HCC1937 cells. *$p < 0.05$; **$p < 0.01$.

with its expression pattern as revealed in this study (34–36). From the therapeutic point of view, we focused on the study of the role and mechanism of MIR210HG in TNBC.

MIR210HG is the host gene of miR-210 and is well-known hypoxia lncRNA induced by HIF-1α.

In varicocele-related male infertility, MIR210HG was identified as a hypoxia-related long noncoding RNAs (37). Similarly, MIR210HG expression was also induced in hypoxic human umbilical vein endothelial cells (HUVECs) (38) and human proximal tubular epithelial cells (PTECs) (39). Consistent with these reports, we for the first time revealed that MIR210HG was transcriptionally activated by in HIF-1α under hypoxic conditions. Previously, MIR210HG has been demonstrated to be overexpressed in hepatocellular carcinoma (40), non-small cell lung cancer (NSCLC) (41), osteosarcoma (42), glioma (43) and chemoresistant pancreatic cancer (44). In invasive breast cancer patients, MIR210HG is highly expressed and confers a poor prognosis (45). Here, we confirmed this finding and showed that MIR210HG was closely associated with a relapse-free survival in TNBC. The high expression of MIR210HG in TNBC is associated with poor prognosis, suggesting that MIR210HG may be used as a potential prognostic predictor. Moreover, we revealed that MIR210HG was highly expressed in TNBC cells in comparison to the non-TNBC cells and the nonmalignant MCF10A cells, suggesting a specific role of MIR210HG in TNBC development. However, the

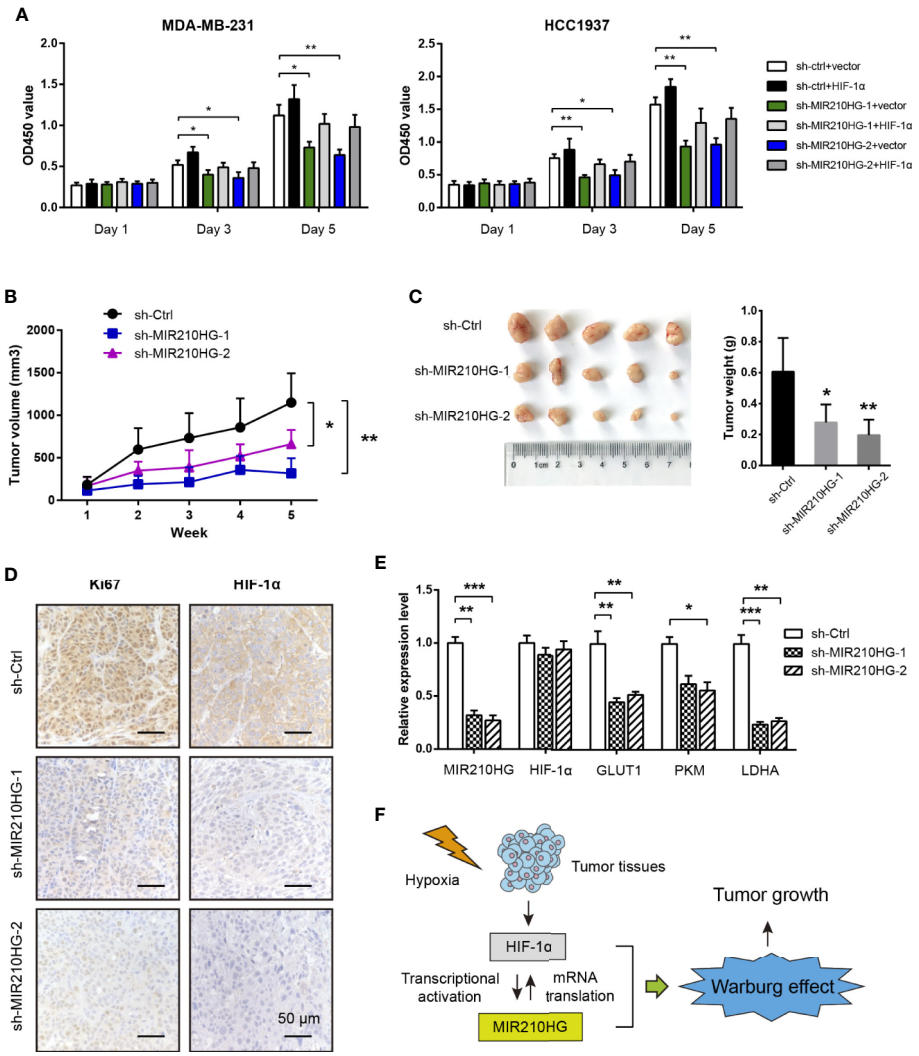

FIGURE 5 | MIR210HG knockdown suppresses tumor growth. **(A)** CCK-8 analysis of the effect of MIR210HG knockdown in the cell proliferation of MDA-MB-231 and HCC1937 cells with or without HIF-1α expression. **(B)** The curve of tumor volume in the indicated three groups (sh-Ctrl, sh-MIR210HG-1, and sh-MIR210HG-1). **(C)** The tumor weights in the indicated three groups (sh-Ctrl, sh-MIR210HG-1, and sh-MIR210HG-1). **(D)** IHC analysis of Ki67 and HIF-1α protein in xenograft tumor tissues. Scale bar: 50 μm. **(E)** Real-time qPCR analysis of MIR210HG, HIF-1α, GLUT1, PKM2, and LDHA in xenograft tumor tissues. **(F)** Proposed model illustrating the mechanism by which HIF-1α-MIR210HG feedback loop promotes the Warburg effect and facilitate tumor growth in TNBC. *p < 0.05; **p < 0.01; ***p < 0.001.

reason for differentially expressed MIR210HG in TNBC warrants further investigations. Given its expression pattern and prognostic value in TNBC, MIR210HG may represent a novel therapeutic target for TNBC treatment.

Several molecular mechanisms underlying the oncogenic roles of MIR210HG have been reported. In cervical cancer, MIR210HG might act as a competing endogenous RNA (ceRNA) of miR-503-5p to relieve the suppressive effect of miR-503-5p on TRAF4 expression, resulting in increased cell proliferation and invasive capacity (46). In NSCLC, MIR210HG is able to promote cell proliferation and invasion through targeting miR-874/STAT3 axis and regulating methylation of CACNA2D2 promoter via binding to DNMT1 (41, 47). In this study, we identified a novel function of MIR210HG in regulating the Warburg effect. In line with our result, RUAN et al. showed that higher MIR210HG expression was associated with shorter overall survival in colon cancer and MIR210HG may play a role in the modulation of energy metabolism, especially glucose metabolism (48). Polysome profiling of HIF-1α mRNA showed reduced translation of HIF-1α in MIR210HG knockdown cells, suggesting the regulatory role if MIR210HG in HIF-1a translation process. Thus, we provided a previous unprecedented mechanism by which MIR210HG promotes tumor progression. Our study advances the knowledge of the regulation of HIF-1α, and underlines the essential relevance of lncRNA in gene regulation. Although our data indicated that MIR210HG exerts a regulatory role in HIF-1α, the underlying mechanisms of MIR210HG in interacting with HIF-1α remained to be determined in following studies. Additionally, MIR210HG can

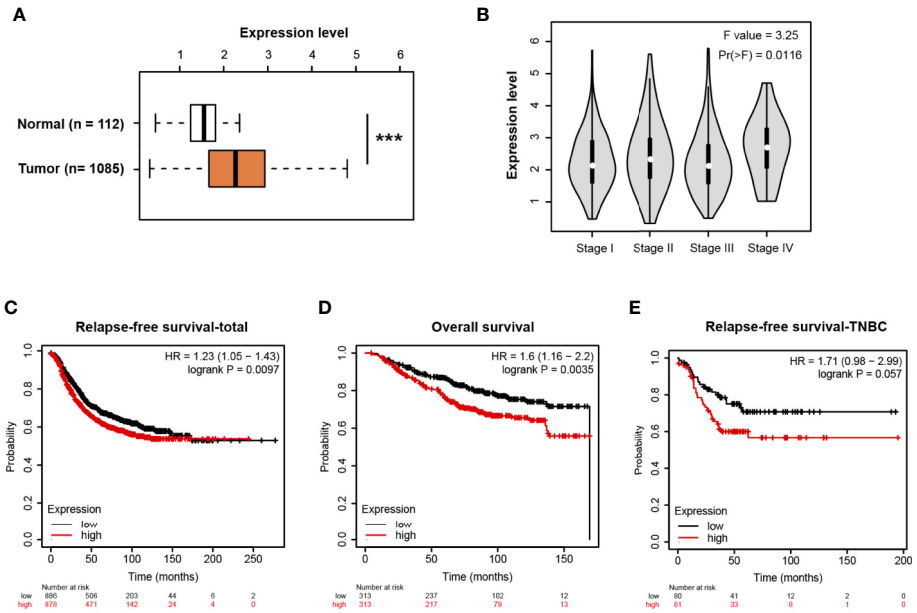

FIGURE 6 | MIR210HG is highly expressed in breast cancer and predicts a poor prognosis. **(A)** MIR210HG expression in breast cancer and normal control tissues; data was acquired from TCGA + GTEx cohort. **(B)** MIR210HG expression in different stages of breast cancer tissues. **(C)** Kaplan-Meier survival curves showing an association between MIR210HG expression and relapse-free survival in patients with breast cancer. **(D)** Kaplan-Meier survival curves showing an association between MIR210HG expression and overall survival in patients with breast cancer. **(E)** Kaplan-Meier survival curves showing an association between MIR210HG expression and relapse-free survival in patients with TNBC. ***: $p < 0.001$.

act as a ceRNA of miR-1226-3p to regulate mucin-1c expression resulting in increased breast cancer metastasis (45). Therefore, we cannot exclude other alternative targets and potential cellular mechanisms of MIR210HG in regulating the glycolytic phenotype of TNBC.

Conclusion

To the best of our knowledge, the present study provides the first evidence that MIR210HG acts as a metabolic regulator to promote TNBC cell proliferation and tumor growth. Our study revealed that hypoxia-induced MIR210HG might act as a tumor promoter by enhancing the Warburg effect. Molecular mechanism showed that MIR210HG regulates the expression of HIF-1α at the translational level. Our findings shed lights on the HIF-1α/MIR210HG feedback loop in TNBC glucose metabolism and suggest that targeting HIF-1α/MIR210HG axis might serve as new strategies for TNBC prevention and therapy.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Hospital of Jilin University. The patients/ participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The First Hospital of Jilin University.

AUTHOR CONTRIBUTIONS

DS and S-HJ designed and performed the research. YD, NW, and RM performed the experiments. DS, YD, and NW analyzed and interpreted data, and wrote the draft manuscript. All authors contributed to the writing and reviewing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from National Natural Science Foundation of China (81773171) and Science and Technology Department of Jilin Province (20170311005YY, 20200404197YY, and 20200201349JC).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.580176/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.

frontiers in Oncology

REVIEW
published: 07 January 2021
doi: 10.3389/fonc.2020.602416

The Metabolic Mechanisms of Breast Cancer Metastasis

Lingling Wang[1,2], *Shizhen Zhang*[3*] and *Xiaochen Wang*[1*]

[1] Department of Breast Surgery, Zhejiang Provincial People's Hospital, Hangzhou, China, [2] Department of Surgical Oncology and Cancer Institute, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [3] Institute of Translational Medicine, Zhejiang University School of Medicine, Hangzhou, China

Breast cancer is one of the most common malignancy among women worldwide. Metastasis is mainly responsible for treatment failure and is the cause of most breast cancer deaths. The role of metabolism in the progression and metastasis of breast cancer is gradually being emphasized. However, the regulatory mechanisms that conduce to cancer metastasis by metabolic reprogramming in breast cancer have not been expounded. Breast cancer cells exhibit different metabolic phenotypes depending on their molecular subtypes and metastatic sites. Both intrinsic factors, such as *MYC* amplification, *PIK3CA*, and *TP53* mutations, and extrinsic factors, such as hypoxia, oxidative stress, and acidosis, contribute to different metabolic reprogramming phenotypes in metastatic breast cancers. Understanding the metabolic mechanisms underlying breast cancer metastasis will provide important clues to develop novel therapeutic approaches for treatment of metastatic breast cancer.

Keywords: breast cancer, metabolism, metastasis, molecular mechanisms, metabolic phenotypes, glycolysis, hypoxia

OPEN ACCESS

Edited by:
Xiaosong Chen,
Shanghai Jiao Tong University, China

Reviewed by:
Yingying Xu,
The First Affiliated Hospital of China Medical University, China
Kunwei Shen,
Shanghai Jiao Tong University, China

***Correspondence:**
Shizhen Zhang
zhangshizhen@zju.edu.cn
Xiaochen Wang
wangxiaochen@zju.edu.cn

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *03 September 2020*
Accepted: *23 November 2020*
Published: *07 January 2021*

Citation:
Wang L, Zhang S and Wang X (2021) The Metabolic Mechanisms of Breast Cancer Metastasis. Front. Oncol. 10:602416. doi: 10.3389/fonc.2020.602416

INTRODUCTION

Breast cancer is the most common malignant tumor and the second capital reason for cancer death among women worldwide (1, 2). Metastatic breast cancer, not the primary tumor, is responsible for more than 90% cancer-related deaths (3). A SEER based study showed that for metastatic breast cancer patients: 30–60% have metastases in the bone, 21–32% in the lung, 15–32% in the liver and 4–10% in the brain. Moreover, the preferred metastatic sites appear to depend on the specific pathological subtypes of primary breast cancers (4).

Recently, increasing evidence point out that cancer is not only a genetic disease but also a metabolic disease, in which oncogenic signaling pathways participate in energy regulation and anabolism to support rapidly spreading tumors (5). In this sense, metabolic reprogramming is considered a hallmark of cancer (6, 7). Notably, metabolic reprogramming and its complex regulatory networks also affect the tumorigenesis and progression of breast cancer (8). Considered as a high heterogeneous disease, breast cancer includes four main intrinsic molecular subtypes: Luminal A, luminal B, HER2-positive, and triple-negative breast cancer (TNBC). Each subtype has different proliferation and metastasis capabilities, as well as metabolic genotypes and phenotypes (9–16) (**Table 1**). Specifically, TNBC cells possess particular metabolic traits characterized by high glycolysis and low mitochondrial respiration (22). HER2-positive tumors display higher glutamine metabolic activity and higher lipid metabolism than other subtypes

TABLE 1 | Metabolic differences in different breast cancer subtypes.

	Expression level	Luminal A subtype	Luminal B subtype	HER2+ subtype	Basal-like/TNBC
Glucose metabolism					
	G6PD and 6PGL (17)	lower		Higher	
	6PGDH (17)				only
	HIF-1α, IGF-1, and MIF (18)			Notedly increased	
	GLUT-1 and CAIX (18)				Notedly Increased
Amino acid metabolism					
	Stromal GLS1 (19)	Lowest		Highest	
	Stromal GDH (19)	Lowest		Highest	
	Tumoral GDH (19)			Highest	lowest
	Tumoral ASCT2 (19)	Lowest		Highest	
	Stromal PSPH and SHMT1 (14).	Lowest		Highest	
	Stromal and tumoral GLDC (14)			Highest	lowest
Lipid metabolism					
	Tumoral PLIN1, CPT-1A, and FASN (20)			Highest	lowest
	Tumoral FABP4, and ACOX-1 (20)			Highest	
		ER+ tumor		ER- tumor	
		Inhibition of 27-hydroxycholesterol synthesis decreases cell proliferation in ER+ cancers but not in ER- cancers (12).		Higher ACAT activity, higher caveolin-1 protein levels, greater LDL uptake, and lower *de novo* cholesterol synthesis (10); Products of *de novo* fatty acid synthesis, such as palmitate-containing phosphatidylcholine, were high (11).	
Genes related with metabolism		Luminal B tumors displayed higher glutamine metabolic activity driven by *MYC* than Luminal A tumors (15).		Highest glutamine metabolic activity and higher *MYC* amplification (15).	Loss of *p53* collaborates with MYC_{high}/$TXNIP_{low}$-driven metabolic dysregulation to drive the aggressive clinical behavior in TNBC but not in other subclasses of breast cancer (21).

ER, estrogen receptor; TNBC, triple-negative breast cancer; G6PD, glucose-6-phosphate dehydrogenase; 6PGL, 6-phosphogluconolactonase; GLS1, glutaminase 1; GDH, glutamate dehydrogenase; ASCT2, alanine-serine-cysteine transporter2; HIF-1α, hypoxia-inducible factor 1α; SHMT1, serine hydroxymethyltransferase 1; IGF-1,insulin-like growth factor-1; MIF, macrophage migration inhibitory factor; GLDC, glycine decarboxylase; PLIN1, perilipin-1; FASN, fatty acid synthase; CPT-1A, carnitine palmitoyltransferase-1; FABP4, fatty acid binding protein 4; ACOX-1, acyl-CoA oxidase 1; GLUT-1 glucose transporter protein-1; CAIX, carbonic Anhydrase IX; ACAT, acetyl-CoA acetryltransferase; sPLA2, secreted phospholipase A2; TXNIP, thioredoxin-interacting protein.

(15, 20). Nevertheless, metabolic changes may not only be varied in different breast cancer subtypes, but also diverged relying on the interplay of cancer cells with the complex microenvironment (16, 23).

This review addresses the current knowledge on the crosstalk between metabolic reprogramming and metastatic process in breast cancer. A better understanding of the metabolic mechanisms driving breast cancer metastasis may provide clues for discovering new anticancer therapeutics.

OVERVIEW OF METABOLIC PROGRAMMING IN BREAST CANCER

Glucose Metabolism

In response to external growth signals, normal cells in a rapidly proliferating state activate assorted signaling pathways to suppress oxidative phosphorylation (OXPHOS), and advance glycolysis and anabolic metabolism for cell growth. Cancer cells is able to hijack this mechanism to meet developmental needs even if there are no external signals (24) (**Figure 1**). Different from normal cells where glycolysis and OXPHOS are always negatively correlated, cancer cells possess these two modes coexisting to disparate degrees (25). Moreover, unlike normal cells, which mainly produce adenosine triphosphate (ATP) from glucose-derived pyruvate by OXPHOS through the TCA cycle, most cancer cells depend on glycolysis to generate energy even under aerobic conditions (26). It was found that tumors displayed dual metabolic natures that tumor cells could switch from the aerobic glycolysis back to OXPHOS phenotype upon lactic acidosis (27). Furthermore, some tumors exhibit two-compartment tumor metabolism, called the reverse Warburg effect or metabolic coupling, which indicates that glycolytic metabolism in the cancer-related stroma sustains the adjacent cancer cells. Such metabolic phenotype will contribute to chemotherapy resistance, and also explain the contradictory phenomenon of high mitochondrial respiration and low glycolysis rate in some tumor cells (28–30). Moreover, a large sample data study showed luminal subtype correlated with

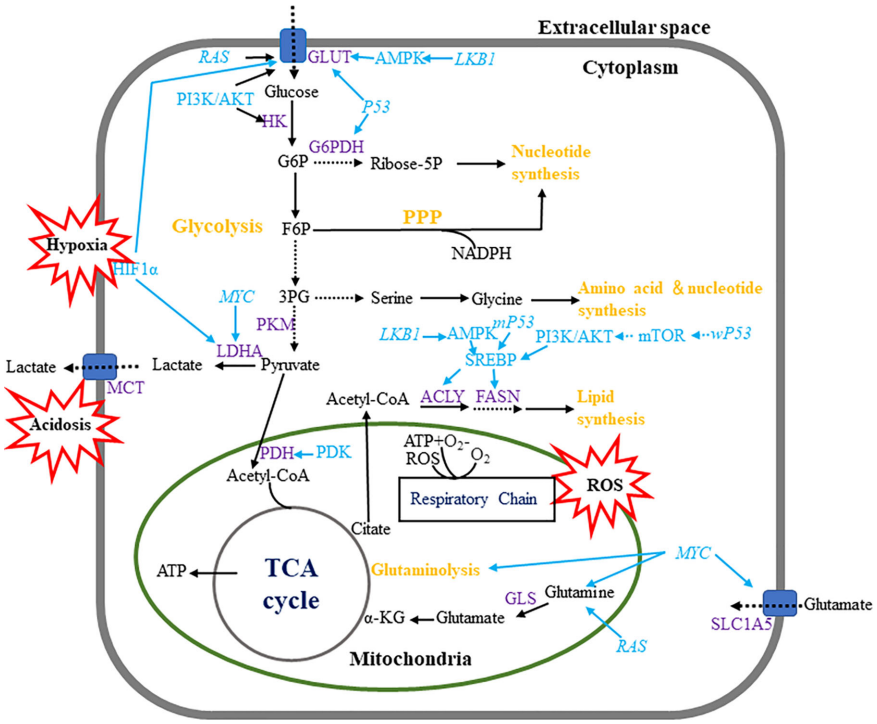

FIGURE 1 | Metabolic pathways in breast cancer cells. Breast cancer cells enhance metabolism of glucose, amino acid lipid by regulating multiple metabolic pathways. Breast tumor cells mainly use aerobic glycolysis to produce ATP and utilize the pentose phosphate pathway to produce macromolecules such as NADPH. Wild-type and mutant *P53* have contrary effects in monitoring fatty acid metabolism. Hypoxia, acidosis and ROS are regarded as important events which influence multiple metabolic pathways.

reverse-Warburg/null phenotypes that are metabolically inactive, while TNBC correlated with Warburg/mixed phenotypes that are metabolically active (31). Additionally, hypoxic environment in breast tumors brings about increased production of reactive oxygen species (ROS) (32), at the same time, induced hypoxia-inducible factor 1 (HIF-1) is able to boost glucose metabolism to maintain the redox homeostasis (33).

Glucose transport cross cell membrane through the glucose transporter proteins (GLUTs) and different GLUTs expression in breast cancers are related to dissimilar pathological grades and prognosis. GLUT1-5 and GLUT12 are functionally in breast cancer cells (34–37), and GLUT1 appears to play the most important role (38). Interestingly, TNBC had the highest expression of GLUT1 when contrasted with other subtypes, suggesting the highly active metabolic status in TNBC (18). Moreover, some critical glycolysis-related enzymes, such as hexokinase (HK) and lactate dehydrogenase-A (LDHA), are highly activated in breast cancer and related to cancer growth and progression (39, 40).

The pentose phosphate pathway (PPP) is another way of oxidative decomposition of glucose besides glycolysis and TCA cycle, which produces nicotinamide adenine dinucleotide phosphate (NADPH), ribose phosphate, fructose-6-phosphate (F6P) to make cancer cells satisfy their anabolic needs and respond to oxidative stress (41). Proteins involved in PPP are distinctively expressed in different molecular subtypes of breast cancers. For example, the expression of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconolactonase (6PGL) were elevated, implying a more activated PPP in HER2 subtype than other subtypes of breast cancer (17). It has been suggested that the expression of G6PD and transketolase (TKT) are positively correlated to the decreased overall and relapse-free survival in breast cancer (42).

Amino Acid Metabolism

Glutamine and its metabolic intermediates such as antioxidants nicotinamide adenine dinucleotide (NADH), and glutathione (GSH), participate in energy supply, supplement glucose metabolism and help cells resist oxidative stress to uphold proliferation and progression of tumor cells (43, 44). Some cancer cells exhibit "glutamine addiction" that cannot survive in the absence of exogenous glutamine (45). More importantly, certain oncogenic transcription factors, such as *c-MYC* and *RAS*, can increase the cancer cell glutamine metabolic activity by upregulating glutamine transporters such as alanine-serine-cysteine transporter 2 (ASCT2) and enzymes participating in the conversion of glutamine-to-glutamate, such as glutaminase (GLS)-1 (46). For example, *c-MYC* activates the expression of ASCT2 and GLS-1 under the induction of lactic acid, leading to elevated glutamine uptake and catabolism in cancer cells (47). Notably, a metabolomic analysis indicted that breast tumor tissues had a higher glutamate-to-glutamine ratio (GGR) than

normal tissues, especially in estrogen receptor (ER) negative tumors and the GGR levels dramatically correlated with ER status and tumor grade (48). The glutamine metabolism-related proteins, such as GLS-1, glutamate dehydrogenase (GDH), and ASCT2 were found to be highly expressed in HER2-positive breast cancer than other subtypes, which indicated that HER2-positive breast cancer had the highest glutamine metabolism activity (19).

One-carbon metabolism, also known as network of folate utilization reactions, participates in multiple metabolic pathways such as amino acid biosynthesis and degradation, *de novo* nucleotide biosynthesis, and methylation and reductive metabolism (49). It has been widely accepted that one-carbon metabolism acts a pivotal part in supporting the high proliferative rate of tumor cells (50). Folate (vitamin B9), a carrier of one-carbon units, and other B vitamins, such as B6 and B12, take part widely in one-carbon metabolism, which is requisite for DNA biosynthesis and methylation (51). Although the relationship between folic acid intake and the risk of breast cancer is still controversial, a recent meta-analysis, analyzing 23 prospective studies, found that an increment of folate intake decreased the risk of ER-, ER-/PR-, premenopausal breast cancer and had the preventive effects against breast cancer in individuals with alcohol consumption (52).

In addition to glutamine, upregulation of serine/glycine metabolism closely connected with folate metabolism is relevant to high proliferation of tumor cells and poor prognosis of patients (50). Tryptophan and arginine are involved in the manipulation of immunity and tolerance, which are generally deregulated in cancers (53). The activity of arginase, the key enzyme catalyzing L-arginine, in breast tumor environments is strengthened, which generates an unfavorable milieu for T cell adaptability (54).

Lipid Metabolism

The fatty acids (FAs) and lipid metabolic programming also play significant parts in promoting breast cancer growth and progression (55). Cancer cells maintain a highly proliferative state by activating the uptake of exogenous lipids and lipoproteins, or by reinforcing *de novo* lipid and cholesterol biosynthesis, showing active lipid and cholesterol metabolisms (56). Moreover, tumor cells mostly rely on *de novo* fatty acid synthesis (FAS) to satisfy the augmented demand for membrane metabolism in favor of rapid growth and proliferation. The expression of fatty acid synthase (FASN), a key enzyme essential for the FAS, is elevated in breast cancer (57), and its upregulation appears to be connected with cancer development, recurrence and poor prognosis (58), suggesting the augmented FAS activity is important for breast cancer progression. Notably, FASN was found to be expressed highest in HER2-positive breast cancer and lowest in TNBC at both cell and tissue levels (20, 59). It has been assumed that a two-way regulatory system between FASN and HER2, the "HER2-FASN axis", may enhance breast cancer proliferation, metastasis and chemoresistance (60). Sterol regulatory element-binding protein (SREBP)-1, a lipogenic transcription factor, can regulate FASN expression by binding with the FASN promoter site (61, 62). And phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signal transduction pathways are also likely to regulate FASN expression (63, 64). Under hypoxic conditions, *FASN* gene is upregulated due to the arousal of AKT and SREBP-1 in breast tumor cells (65). Inhibition of MAPK pathway and mTOR inhibitor rapamycin both can decrease FASN expression in breast cancer cells (66, 67).

OVERVIEW OF METASTASIS IN BREAST CANCER

Tumor metastasis is a sequential multi-step process, which includes local invasion, intravasation, migration through the lymphatics or blood vessels, extravasation and colonization giving rise to the formation of metastases in distant organs (68). Particularly, organ-specific colonization hinge on the dynamic and mutual interrelation between tumor cells and tumor microenvironment (TME), comprised by varieties of non-cancerous cells such as immune cells, endothelial cells, fibroblasts, adipocytes, together with extracellular matrix (ECM) and soluble factors (69). In addition to the linear metastasis model, breast cancers prefer to the parallel metastasis model, which means that breast cancer cells begin to spread in the early stages of tumor development (70), and the spread of cancer cells may be independent of the progression of the primary tumors (71). Studies have shown that the genetic changes of the bone marrow disseminated breast cancer cells are usually not identical to their corresponding primary tumors (72). Different breast cancer subtypes have been found to show different metastatic sites preference governed by different molecular mechanisms (73). The molecular characteristics of breast cancers and target tissues appear to confirm the organotropism of metastasis (74). All the breast cancer subtypes are apt to develop bone metastasis, luminal A subtype is regarded as a risk factor for recurrence in the bone (75), and luminal B subtype is more likely to have bone as a first relapse site when compared to other subtypes (76). Moreover, the incidence of luminal subtype tumors to have bone metastasis is much higher (80.5%) than HER2-positive tumors (55.6%) and basal-like tumors (41.7%) (77). While luminal B and basal-like subtypes present higher levels of lung-specific metastasis (78). Compared with the HER2-negative subtype, the HER2-positive subtype is more often observed with liver metastases (4). Another study showed that basal-like tumors had a higher rate of metastasis to the brain, lungs and distant lymph nodes, while the rate of liver and bone metastasis is much lower (79).

The Process of Metastasis

The step one of the metastasis is that tumor cells break away from the tumor bed and migrate from the stroma into the bloodstream (80). In order to leave the primary tumor and invade surrounding tissues, these tumor cells need to reduce

their tight cell adhesion through undergoing epithelial-mesenchymal transitions (EMT) (81, 82). EMT is typified by loss of epithelial traits (including cell polarity and cell-cell junctions) and acquisition of mesenchymal traits (including fibroblastic spindle-shaped morphology) to increase the mobility of tumor cells. EMT also links to cancer metastasis with stem cell properties (83, 84). Moreover, the integrin-mediated adhesion and debonding interactions with matrix components is critical for regional migration. And the intratumoral blood vessels characterized by increased permeability allow cancer cells to enter the systemic circulation readily (85).

After escaping from the original tumor site to blood circulation, breast tumor cells begin to migrate to remote organs. The first obstacle encountered by circulating tumor cells is the blood vessel wall, especially endothelial cells. In some organs, such as bone marrow and liver, microvessels are composed of sinuses with strong permeability, which make cancer cells easier to break through (86). Whereas in most other organs, including brain, endothelial cells form a continuous barrier that prevents cancer cells from penetrating freely. Platelets and white blood cells can help tumor cells pass through the vasculature by forming complexes with tumor cells through L- or P-selectin (87, 88). As such, increased expression of selectin ligands by tumor cells is well connected with metastatic progression and bad prognosis (89). The induction of angiopoietin-like 4 (ANGPTL4) by transforming growth factor-beta (TGFβ)/small mother against decapentaplegic (SMAD) signaling pathway in cancer cells is reported to enhance their subsequent retention in the lungs and empower breast cancer cells to destroy lung capillary wall and form pulmonary metastases (90). Chemokines in target cell tissues can also induce directed cell migration, initiate signal pathways, and monitor cytoskeletal rearrangment and adhesion (91).

Adjusting to new environment is another hurdle for circulating tumor cells (CTCs) to form metastasis. Disseminated cancer cells will spring up in targeted tissues and organs through a way that is significantly different from their origins. Cancer cells must acquire new capabilities, especially the ability to interact with cells in the ECM and new microenvironment. Tumor cells form a two-way connection with circumferent stroma in the early stage of invasion and after that, tumor-stroma interaction helps the tumor to develop toward metastasis (6).

Pre-Metastatic Niche

An appropriate microenvironment, namely, pre-metastatic niche, can be established in secondary tissues and organs before metastases occurring through a complicated mechanism by interaction between the primary tumors and organs stromal components (92). Kaplan et al. emphasized the role of tumor-mobilized bone marrow-derived cells (BMDCs) in developing a satisfactory microenvironment for lung metastatic colonization. The factors, such as vascular endothelial growth factor (VEGF), and placental growth factor (PlGF), released by the primary tumor act on the bone marrow mesenchymal stem cells to induce the BMDCs to reach the expected metastasis site before the disseminated tumor cells arrive (93). Hiratsuka et al. demonstrated that matrix metalloproteinase (MMP)-9 is particularly motivated in premetastatic lung endothelial cells and macrophages mediated by primary tumors *via* the VEGFR-1/Flt-1 pathway, which is important for lung metastasis (94). The integrin β1/α5/JNK/c-JUN signaling pathway in cancer cells is able to upregulate the higher matrix stiffness-induced lysyl oxidase like (LOXL)-2, then subsequently promote production of fibronectin, expression of MMP-9 and C-X-C motif chemokine ligand (CXCL)-12 and recruitment of BMDCs to encourage pre-metastatic niche establishment (95). Chemokines binding to specific receptors on the target cell membrane help to recruit immune cells into the tumor microenvironment, thereby managing immune surveillance, angiogenesis, invasion and metastasis (96). The CXCL-12/C-X-C motif chemokine receptor (CXCR)-4 axis provides a fit microenvironment before breast cancer bone metastasis formation (97). Carmen et al. suggested that HIF-1 is a crucial regulatory factor inducing breast cancer metastatic niche forming through activation of several elements of the lysyl oxidase (LOX) family, which catalyze collagen cross-linking in the lungs before BMDC recruitment (98). Dickkopf (DKK)-1 suppresses prostaglandin endoperoxide synthase (PTGS)-2-induced macrophage and neutrophil recruitment to lung metastases by antagonizing cancer cell non-classical WNT/Planar cell polarity (PCP)-RAC1-JNK signaling, whereas it encourages breast-to-bone metastasis by modulating classical WNT signaling of osteoblasts (99).

Organotropism

The site-specific metastasis of breast cancer is related to subtypes and divergent gene signatures of metastatic cancer cells. Functional studies have identified many key genes that mediate breast cancer organ-specific metastasis, and the expression of these genes in the primary tumor is likely to forecast the patient's organ-specific metastasis (100, 101).

Bone is the most frequent site of breast cancer metastasis (73). Bone metastasis is usually connected with osteolytic-type lesions as a result of the overactive bone resorption mediated by osteoclasts (102). Integrin complexes, such as integrin αvβ3, α4β1 and α5, play important roles in the attraction and adhesion of breast tumor cells to the bone (103–105). Some clinical, genetic, and functional evidence suggest that the SMAD tumor suppressor pathway may diverted into potent pro-metastatic factor in breast cancer, and signaling through the SMAD pathway can facilitate breast cancer bone metastasis (106). Moreover, both hypoxia (via HIF-1α) and TGFβ signaling can independently stimulate the VEGF and CXCR4 expression to drive breast cancer bone metastases (107). In basal-like TN breast cancer, CCL20 promotes bone metastasis by raising the secretion of MMP-2/9 and increasing the receptor activator of nuclear factors-kappa B (NF-κB) ligand/osteoprotegerin ratio in breast cancer and osteoblastic cells (108).

The second most common metastatic site of breast cancer is the brain (73). Brain metastasis of breast cancer can be located in the parenchymal brain (around four-fifths) or in leptomeningeal region (109). CTCs need to break through the blood-brain barrier (BBB), interplay with the local microenvironment to

survive, and then set up brain metastatic colonies. CD44, VEGF and CXCR4 can impair endothelial integrity to raise the transendothelial migration of tumor cells (110). Angiopoietin-2 (Ang-2) expression is elevated in brain microvascular endothelial cells (BMECs) and secreted Ang-2 can increase BBB permeability by disrupting tight junction protein structures between ZO-1 and Claudin-5 in TNBC models of brain metastasis (111). Cyclooxygenase (COX)-2, heparin-binding epidermal growth factor-like growth factor (HBEGF), and ST6GALNAC5 are all able to help tumor cell pass through the BBB (112). Additionally, astrocytes and microglia are related with brain metastases. Astrocytes-derived factors, such as MMP-2 and MMP-9, are able to enhance the migration and invasion of breast tumor cells, thus leading to brain metastasis (113). Similarly, microglia can also be stimulated by culturing with cancer cells, so that it boosts cancer cell colonization in a WNT-dependent manner (114).

Compared to other metastatic lesions, lung metastasis generally show phenotypes of aggressive growth and invasiveness (101). EGFR, COX2, MMP-1, and MMP-2 expressed in breast cancers jointly facilitate lung metastasis by promoting the angiogenesis, emancipating cancer cells into the circulation and breaking through lung capillaries (115). Studies have determined that compared with primary breast cancer, the degree of pyruvate carboxylase (PC)-dependent anaplerosis in lung metastasis of breast cancer is higher, as a result of responding to the lung microenvironment (116). Bone morphogenetic proteins (BMPs) secreted by lung resident cells can restrict cancer development by turning cancer cells into a dormant state, while Coco and GALNTs derived from lung metastatic breast tumor cells are able to inhibit the effect of BMPs and reactivate dormant tumor cells to seed in the lung, thereby leading to metastasis (117).

Breast cancer cells preferred to liver-specific homing display unique transcriptional profiling (118). The status of ER, progesterone receptor (PR) and HER2 between the primary and liver metastatic tumors of breast cancer can be changed after treatment (119). Development of breast cancer liver metastasis is reported to be associated with the activation of β-catenin-independent WNT signaling (120). A model for breast cancer liver metastasis was established involving diverse factors from breast tumor cells and the liver microenvironment such as integrin complexes, HIFs and LOX (121).

Breast Cancer Stem Cells and Dormant Cells

Breast cancer stem cells (BCSCs), a small number of cells with self-renewal and unlimited replication capabilities, have been shown in numerous cancer models to be involved in tumor development and metastatic dissemination. Moreover, the occurrence of BCSCs with the properties of stemness, EMT and drug resistibility, is the main cause for cancer recurrence and treatment failure (122). Multiple researches revealed that several signaling pathways, such as WNT/β-catenin and Notch, contribute significantly to the development of BCSCs (123). Devon A et al. showed that early metastatic breast cancer cells had unique stem-like gene expression characteristics and prefer to proliferate and differentiate to produce advanced metastatic disease at the single-cell level (124). Additionally, BCSCs isolated from primary human breast cancers possess the advanced metastasis potential and the CD70+ subpopulations appear to preferentially mediate lung-specific metastasis by enhancing self-renewal potential (125).

After colonizing the distant metastatic site, BCSC can enter into a metastatic dormant state, showing the halted proliferation and activated cellular stress response, while maintaining metabolic activity (126–128). The dormant phenotype is able to be reversed by manipulating of intrinsic and/or extrinsic factors and then the proliferative program restarts *in vivo* (129, 130). However, the biological mechanisms of cell dormancy and re-awakening are still elusive (131). The dormant state is regarded as a high risk of cancer recurrence and is supposedly limit the efficiency of chemotherapy. Targeting the metastatic dormancy, therefore, could be an promising treatment strategy to improve long-term control of cancer progression (132).

METABOLIC REPROGRAMMING AND ORGAN-SPECIFIC METASTASIS

Metabolic plasticity is one of the important characteristics that distinguishes the tumor cells with high metastatic potentiality from non-metastatic tumor cells. Metastatic cancer cells always operate multiple metabolic pathways concurrently, thus they can adjust the application of diverse pathways according to their adaptive requirements (133, 134). Cancer cells are challenged by diverse environmental and cellular stresses during metastatic progression (135). Strikingly, cancer cells are capable of manipulating one or more metabolic pathways according to their stage in the metastatic cascade and the site they metastasize (133, 136–139). For instance, extracellular acidification by the release of CO_2, lactic acid and other organic acids from metabolically vigorous tumor cells promotes intravasation of cells from the primary tumor. Once tumor cells enter the circulatory system, they produce NADPH and GSH through the PPP pathway to protect themselves from oxidative stress. The coordination of the metabolism between cancer cells and adjacent microenvironment is critical for successful colonization of distant sites and survival during dormancy. Most importantly, anabolic metabolism is reactivated in cancer cells to facilitate the growth of macro-metastatic tumors (140). Tumor metabolism reprogram also occurs when tumors progress in order to adapt to lack of sufficient blood supply. While during adaptation to environmental stress, such as cyclic hypoxia, tumor metabolism reprogram contributes to selection of drug-resistant and metastatic clones (141, 142).

Primary breast tumor cells exhibit metabolic heterogeneity and participate in different metabolic reprogramming according to metastatic sites (**Figure 2**). Liver-metastatic breast cancer cells display a distinct metabolic reprogramming characterized by accumulation of glucose-derived lactate and reduction in the TCA cycle and OXPHOS (138). In brain metastatic breast cancer, the significant metabolic changes are mainly the

enhanced glycolysis, mitochondrial respiration and the PPP. Intriguingly, breast cancer cells metastasized to the brain are less sensitive to glucose deficiency (136), which may attribute to upregulation of glutamine and branched chain amino acid oxidation (143).

Preference for metastatic sites is determined by many factors, including proximity to the primary tumor site and breast cancer subtype. Not only pro-metastatic genes in these subtypes, but also related metabolic mechanisms are closely related to the propensity of metastatic organs. Monica et al. utilized Raman spectroscopy (RS) and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) analysis to study biochemical differences between metastasis tropisms in two TNBC cell lines and showed that bone metastasis tropism was characterized by the increase of amino acids and the decrease of mitochondrial signal, while high lipid and mitochondrial (cytochrome C and RNA) levels for lung metastasis (144). NETosis is an important neutrophil function that can promote liver metastasis of breast cancer and different pro-metastatic neutrophil populations are highly metabolically adaptable, which facilitates the formation of liver metastases (145). The products of pathologically deposited lipids can promote metastasis and nonalcoholic fatty liver disease (NAFLD) activates tumor-induced triglyceride lipolysis in juxtaposed hepatocytes, thereby promoting breast-to-liver metastasis (146). In addition to genetic tendency, metastatic cells that inhabit the brain are adaptive to crosstalk with many different brain residential cells (112, 147). The important role of Notch signaling in breast cancer brain metastasis has been recognized, and it has recently been considered to regulate metabolism (148, 149). Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signals in the brain and astrocyte-derived cytokines contribute to the metastatic brain specificity of breast cancer cells (150, 151). In addition to the similarity of certain metabolic signaling pathways such as the Wnt/β-catenin pathway, Heregulin-HER3-HER2 signaling and the EGFR/PI3K/Akt pathway, brain metastatic cancer cells also share certain metabolic characteristics with neuronal cells (152). Metastatic cells with neuron-like properties thrive in the brain microenvironment. For example, neurons typically catabolize gamma-aminobutyric acid (GABA) to create NADH to support biosynthetic processes and breast tumor cells with a GABAergic phenotype have a strong growth advantage in the brain by converting GABA to succinate to boost the citric acid cycle (153). Enzymes involved in lipid metabolism may also be the appropriate target to prevent the formation of brain metastases, because oncogenic lipid signaling can promote

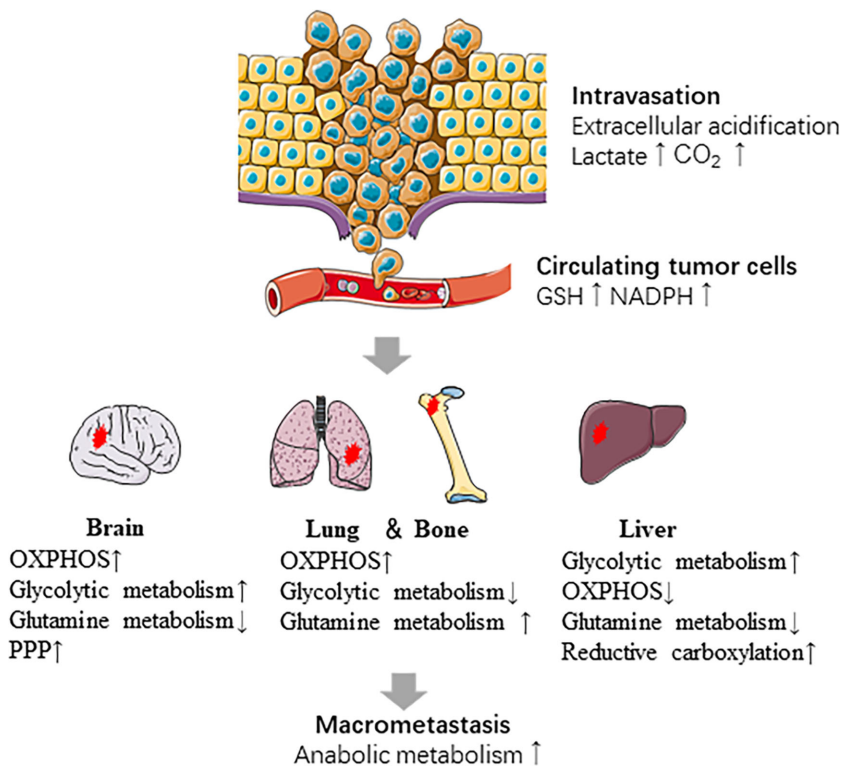

FIGURE 2 | Metabolic reprogramming in the metastatic cascade. Metabolic reprogramming occurs at several steps of metastasis. The intravasation of cells from the primary tumor is promoted by extracellular acidification. CTCs survive in oxidative stress by producing NADPH and GSH. Cancer cells show different metabolic characteristics based on the sites which they metastasize. Last, anabolic metabolism is reactivated during macro-metastatic tumor proliferation.

the metastasis of breast tumor cells to the brain by supporting cell survival, migration, and invasion (154).

CROSSTALK BETWEEN METABOLIC REGULATORS AND METASTATIC PATHWAYS

Intrinsic Factors: Tumor-related Genes that Regulate Metabolic Pathways in Breast Cancer Metastatic Cascade

TP53

TP53 mutations are very common in breast cancers, especially in triple-negative and HER2-positive subtypes (155). TP53 is recognized to mediate its tumor-suppressive functions by adjusting the expression of genes that promote cell cycle arrest, apoptosis, and senescence (156). Moreover, TP53 is able to suppress tumorigenesis by regulation of metabolism and reactive oxygen species (ROS) production (157). There are several mechanisms involved in TP53-mediated metabolic changes (158, 159). For example, wild-type *TP53* is able to inhibit glycolysis by suppressing the expression of GLUT1, GLUT3, and GLUT4 (160, 161), and regulating the expression of enzymes involved in the glycolytic pathway, such as HK2 (162), phosphofructokinase 1 (PFK1) (163), phosphoglycerate mutase (PGM) (163), pyruvate dehydrogenase (PDH), parkin 2 (PARK2) (164), and pyruvate dehydrogenase kinase (PDK2) (165). *TP53* also regulates mitochondrial respiration in cancer progression. *TP53* loss results in downregulation of mitochondrial respiration and oxidative metabolism, which contribute to the Warburg effect in tumor cells, thus linking to tumor progression (166). Besides, by upregulating the cytochrome c oxidase 2 (SCO2) (167), *TP53* initiates several transcriptional programs to promote the expression of genes related to mitochondrial biogenesis (168), such as apoptosis-inducing factor (AIF) (169, 170) and ferredoxin reductase (FDXR) (171). The expression of GLS-2 is also positively regulated by wild-type *TP53*, as such the conversion of glutamine-to-glutamate increases, which is requisite for supplement of NADPH and GSH (172, 173). In contrast, mutant *TP53* has been proved to drive the glycolysis by activating the RhoA/ROCK/GLUT1 signaling cascade (164), repress the catabolic activities, such as fatty acid oxidation (FAO), by inhibiting 5′-AMP-activated protein kinase (AMPK) pathways, and enhance the anabolic processes, such as enhanced fatty acid synthesis (174).

Wild-type and mutant *TP53* have contrary effects in managing the fatty acid metabolism. Wild-type *TP53* hampers the shunt of the glucose carbon to anabolic pathways by binding to and inhibiting the G6PD, whereas mutant *TP53* is unable to affect the G6PD activity (175). Moreover, wild-type *TP53* appears to negatively control the mTOR pathway and the PPP, therefore governing fatty acid synthesis (175, 176). However, mutant *TP53* enhances lipid synthesis through interacting with SREBPs (177). In particular, *TP53* mutation connects with raised expression of genes involved in mevalonate pathway in human breast cancer, and most importantly, mutant *TP53* upregulates these genes and activates the mevalonate pathway, which is indispensable to keep the malignant status of breast cancer (178).

c-MYC

Amplification of *c-MYC* and activation of its downstream effectors are related with high metastatic ability, endocrine resistance and poor disease outcome in breast tumors (179). The c-MYC pathway is well known to enhance the cancer cell growth and proliferation. Its role in the orchestration of metabolic pathways, which provides nutrients and other essential factors to motivate DNA replication and cell division, was recently identified. Specifically, *MYC* amplification mediates the glutamine-related metabolic rewiring in breast cancers, that promotes the excessive uptake of glutamine by inducing the expression of glutamine transporters and glutamine-metabolizing enzymes (180). Such *MYC* amplification-mediated molecular mechanism is specifically upregulated in the luminal B, HER2-positive, and TN breast cancers (15). Moreover, c-MYC activation links to TCA cycle overactivation in HER2-positive and TN breast tumors by increasing the uptake of serine, glycine, and tryptophan and the synthesis of one-carbon units (181).

Beside, c-MYC and other transcription factors, such as mTOR and HIF-1, can act synergistically to improve glycolysis and promote cancer proliferation (182, 183). c-*MYC* is also a direct target and a coregulator of ERα (184), it can act synergistically with ERα to induce breast cancer cell proliferation (185). Furthermore, ER regulates the glutamine metabolism by crosstalking with HER2 signaling in a way dependent on c-MYC in aromatase inhibitor-resistant breast cancer cells (186). Other studies have reported that c-MYC drives glucose metabolism in TNBC by inhibiting thioredoxin-interacting protein (TXNIP)—an inhibitor of glycolysis (21).

PI3K/AKT/mTOR Pathway

PI3K/AKT/mTOR pathway is an intracellular signaling pathway significant for cell cycle and metabolism involved in cancer progression (187). The activation of PI3K/Akt/mTOR pathway is able to enhance expression of genes related to glucose uptake and glycolysis through normoxic upregulation of HIF-1α (188–191). Activation of mTORC1 is also likely to be a latent mechanism driving the Warburg effect by upregulation of *c-MYC* (182, 183). Moreover, the PI3K/AKT/mTOR pathway can facilitate the expression of lipogenic genes in an SREBP-dependent manner (192), and mTORC1 has been regarded as a vital effector in advancing the trafficking or processing of SREBP to stimulate *de novo* lipogenesis (193). Activation of mTORC1 is adequate to provoke the expression of genes encoding the enzymes of both the oxidative and non-oxidative branches of the PPP, thus activating specific bioenergetic and anabolic cellular processes (194). The PI3K/AKT/mTOR pathway was recently showed to reduce oxidative stress and promote cell survival of breast epithelial cells segregated from the ECM by strengthening flux through the oxidative PPP (195)

PIK3CA mutation, which leads to increased PI3K activity, is the most common somatic mutation in breast cancer, and 36% of patients with HR+/HER2- breast cancer are *PIK3CA* mutated (196). It was suggested that crosstalk between the ER and PI3K/

AKT/mTOR signaling pathway exists during breast cancer development (197). Estrogens stimulate PI3K/AKT/mTOR pathway to conduct the migratory and invasive features of ER tumors (198, 199). Reciprocally, mTOR signaling monitors the expression and activity of ERα (200). A recent study reported that PI3K pathway repression triggered the activity of the histone-lysine N-methyltransferase 2D (KMTD2), which leads to the activation of ER in breast cancer cells (201). Interestingly, reactivation of AKT/mTOR signaling by using small molecule PI3K antagonists activates the transport of energy-active mitochondria to the cortical cytoskeleton of cancer cells, therefore heightening tumor cell invasion (202). Although PI3K pathway inhibitors reduce cancer growth, they could accidentally increase tumor invasion by inducing reprograming of mitochondrial trafficking, OXPHOS, and promoting cell motility (203). Moreover, suppression of the mTOR-p70S6K axis is able to induce possessing of unique metabolic features, distinguished by high glucose uptake, incremental lactate production, and low mitochondrial respiration, in TNBC cells (22).

Estrogen Receptor

More than two-thirds of the breast cancer cases present as ERα-positive, and cancers with ERα-positive without HER2-positive is termed as luminal breast cancer (204). Luminal breast cancer appears to have a metabolic phenotype that balances the glycolysis and OXPHOS, while TNBC is more relying on OXPHOS (22). ER-positive tumors have lower levels of glycine, lactate, and glutamate (high glutamine) and lower GGR with lower levels of glutaminolysis, which suggest that ER is implicated in regulation of tumor metabolism (205). ER plays a central role in metabolic regulation through crosstalk with multiple pivotal regulators and pathways, such as TP53, c-MYC, HIF, Ras/Raf/MAPK and PI3K/AKT/mTOR pathway, enabling tumors to reprogram their metabolism to fit various kinds of environment (16). 17β-estradiol (E2) is capable of increasing the expression of insulin receptors and decreasing the lipogenic activity of lipoprotein lipase in adipose tissue by activating ERα (206). Moreover, E2 and ERα can regulate the metabolism reprogram based on glucose availability. In high glucose conditions, E2 enhances glycolysis *via* enhanced AKT kinase activity and suppresses TCA cycle activity, while in low extracellular glucose conditions, E2 stimulates the TCA cycle *via* the upregulation of PDH activity and suppresses glycolysis to satisfy the energy requirements of the tumor cell (207). Besides, a study employing the nuclear magnetic resonance spectroscopy illustrated that E2 appeared to induce glycolysis, whereas tamoxifen reduced it (208–210). Mechanically, E2 is able to transcriptionally upregulate GLUT1, thus promote glycolysis (210).

Contrary to ERα, ERβ is expressed in more than 50% of normal mammary epithelial cells, but less than 10% of tumor cells in invasive ductal carcinoma (211). In general, expression of ERβ is downregulated or lost in high grade breast tumors, but its relation to clinical outcome does not reach an agreed conclusion (212). In glucose metabolism, ERβ, similar to ERα, seems to enhance glycolysis while repress OXPHOS (213). Most importantly, ERβ is suggested to play a key role in regulating the metabolism of BCSCs, given several glycolysis-related pathways are upregulated in ERβ-activated mammospheres (214).

HER2

HER2-positive breast tumors generally exhibit a glycolytic phenotype (215, 216) and display the increased uptake levels of glutamine, glycine, creatinine, and succinate while a reduction in alanine levels (205). Moreover, the expression of FASN, carnitine perilipin-1 (PLIN1), and palmitoyltransferase-1A (CPT1A) are elevated in HER2-positive breast cancers (20). HER2 is involved in multiple signaling pathways that promote glucose utilization (216), regulate LDHA (40) and 6-phosphofructo-2-kinase (PFKFB3) expression levels (217), and induce lactate accumulation in tumors (218). Additionally, HER2 can be translocated to the mitochondria by the intercourse with mitochondrial heat shock protein-70 (mtHSP70), which negatively controls oxygen consumption and thus enhancing glycolysis (219). Inhibition of HER2 pathways by a dual novel EGFR/HER2 inhibitor, KU004, significantly inhibits the Warburg effect by downregulating HK2, thus decreasing cancer cell proliferation (220). Overactivated HER2 signaling results in increased HIF-1α and VEGF expression, which in turn activate the downstream kinase FKBP-rapamycin-associated protein (FRAP), therefore contributing to tumor progression by mediating angiogenesis and metabolic adaptation (221).

Breast Cancer Type 1 Susceptibility

BRCA1-mutated breast tumors are usually phenotyped as aggressive, high-grade, aneuploidy tumors (222, 223), and with a worse prognosis (224). Loss of BRCA1 function caused by *BRCA1* mutation results in the production of hydrogen peroxide in both epithelial breast tumor cells and adjacent stromal fibroblasts, which is able to promote the onset of a reactive glycolytic stroma, suggesting the metabolic phenotype of stromal cells in the TME may also be affected by *BRCA1* mutation in tumor cells (225). Moreover, the BRCA1 loss mutation, like oncogene activation (RAS, NF-κB, TGF-β), in cancer cells will drive the initiation of metabolic symbiosis phenotype between tumor cells and fibroblasts in both primary and metastatic cancers (226).

PGC-1α

PGC-1α is a transcriptional co-activator that actively participates in gene regulation of energy metabolism. Elevated expression of PGC-1α in breast cancer is well associated with the formation of distant metastases. Notably, breast cancer cells with higher levels of PGC-1α may preferentially metastasize to some specific tissues, such as lung and bone (139). Silencing of PGC-1α appears to suspend cancer cell invasive potential and attenuate metastasis (137). The invasive cancer cells particularly do favor mitochondrial respiration with augmented production of ATP. As such, the circulating and metastatic cancer cells upregulate the PGC-1α to facilitate oxygen consumption rate oxidative phosphorylation, and mitochondrial biogenesis to uphold metastasis (137).

RB1

RB1 is a tumor suppressor that is commonly disrupted in many human tumors, including breast cancer (227). *RB1* deficiency is connected with cancer invasion and metastasis (228, 229). It is

evaluated that *RB1* and *TP53* are lost together in 28–40% of human TNBCs, and *RB/P53*-double mutant mouse breast tumor cells exhibit more mesenchymal phenotypes than only *P53*-deficient cells (230, 231). *RB1* loss links to increased mitochondrial OXPHOS, which links to enhanced anabolic metabolism and augmented cancer cell stemness and metastatic spread (232). Additionally, *RB1* deficiency is able to enhance tumor metastasis by increasing OXPHOS to generate more ATP fueling for tumor invasion and cooperating oncogenic alterations to uphold EMT and metastasis (232).

LKB1-AMPK Signaling

AMPK is a universally expressed metabolic sensor, which can be phosphorylated and activated under some stress conditions, such as energy deprivation. Phosphorylated AMPK activates multiple downstream elements to regulate adaptive changes and maintain metabolic homeostasis, including glucose, lipid or protein metabolism. Recently, the latent roles of AMPK signaling in tumorigenesis and progression have been gradually revealed (233). Activated AMPK signaling regulates protein and lipid synthesis by inhibiting mTORC1 through activation of tuberous sclerosis complex 2 (TSC2) and phosphorylation of raptor (234–237). The chief activator of AMPK is the serine-threonine tumor suppressor kinase LKB1, which contributes to phosphorylation of AMPK to activate energy sensors (235, 238). As long as LKB1-AMPK signaling is activated, the regulation of the metabolic branch of mTOR signaling cannot be impaired in spite of the abnormal of PI3K/AKT or receptor tyrosine kinase signaling (237). LKB1 inactivation has recently been reported to drive tumor progression by cooperating with certain activating oncogene mutations in various models of cancer (239–242). Lysine demethylase 5B (also known as KDM5B) is upregulated in breast tumors and play an important role in lipid metabolic reprogramming (243). A recent study clearly demonstrated the knockdown of KDM5B reversed the EMT process to inhibit breast tumor cell migration by activating AMPK signaling-mediated lipid metabolism (244).

Extrinsic Factors: Interaction Between Metabolic Pathways/Fluxes and Breast Cancer Metastasis Induced by Hypoxia, Oxidative Stress, Acidosis, and Tumor Microenvironment

Hypoxia

Hypoxia represents an important characteristic in the TME arising as a mismatch between cellular oxygen consumption and supply (245). About 25%–40% of invasive breast tumors display hypoxic situations (246). Hypoxia is able to regulate glycolysis, glycogen synthesis, lipid metabolism and oxidative phosphorylation, thus playing a vital role in tumor cell survival and growth during all stages of metastasis (247).

Hypoxia-inducible factors, including HIF-1α and HIF-2α, are main regulators in adaptation to hypoxia and nutrient deprivation during tumor progression (141). The activated HIFs is able to induce the expression of various gene products, such as glycolysis- and EMT program-associated molecules (CXCR4, SNAIL and TWIST), the induced pluripotency-associated transcription factors (OCT-3/4, NANOG, and SOX2), angiogenic factors (VEGF) and microRNAs, which are vital to self-renewal, survival, invasion, metastasis, angiogenesis, metabolic reprogram, and treatment resistance of cancer cells. Furthermore, elevated HIF-1α level is a predictive marker of early relapse and metastasis, and correlated with bad clinical outcome in human breast cancer (248–250). Inhibition of HIF-1 activity has a significant inhibitory effect on primary tumor proliferation and metastasis to lymph nodes and lungs in mice by orthotopic transplantation of TNBC (251, 252). Notably, HIF-1 mediates adaptive metabolic responses to hypoxia by enhancing glycolytic pathway, serine synthesis and one-carbon metabolism to promote mitochondrial antioxidant production (NADPH and GSH), and inhibiting the TCA cycle so as to diminish mitochondrial ROS production (247). HIF-1α has recently been shown to increase the expression levels of pro-collagen prolyl (P4HA1 and P4HA2) and lysyl (PLOD1 and PLOD2) hydroxylases in both tumor and stromal cells, thereby enhancing cancer cell alignment along collagen fibers, thereby promoting invasion and metastasis to lymph nodes and lungs (253–255). Hypoxia raises the proportion of BCSCs in a HIF-1α–dependent manner (256, 257), which will contribute to cancer metastasis. A recent study demonstrated that HIF-1α appeared to dynamically regulate glucose metabolism based on oxygen availability to prevent the risk of continuous incremental ROS production to keep redox homeostasis. This HIF-1α-induced effect is vital for induction of the BCSC phenotype in breast cancer when in response to hypoxia or cytotoxic chemotherapy (33). PDK1, a HIF-1α target that antagonizes the function of PDH, a main rate-limiting enzyme for pyruvate converting to acetyl-coA and entering the TCA cycle, has been reported to be a critical regulator of breast cancer metabolism and metastasis (138). Liver metastatic breast cancer cells are recognized to depend on the HIF-1/PDK1 axis for their metabolic reprogramming to accelerate their efficient colonization and proliferation in the liver (138). Some metabolic enzymes, such as succinate dehydrogenase (SDH), fumarate hydratase (FH), IDH and pyruvate kinase 2 (PKM2) are likely to activate HIF-1 pathway by stabilizing HIF-1α, therefore enhancing cancer metastasis (258).

Reactive Oxygen Species and Antioxidants

Tumor cells can only survive within a narrow window of ROS levels. Inhibition of ROS clearance is a therapeutic approach (259), and on the contrary, prohibition of ROS enhances tumor metastasis (260). Among the detachment from ECM during the procedure of metastasis, cancer cells can undergo alterations in metabolic pathways harmful to survival, such as moderated glucose uptake, PPP flux, and ATP levels while promoting the producing of ROS (261). Antioxidant enzymes support survival of breast tumor cells deprived of ECM, implying that eliminating antioxidant enzyme activity in ECM-detached tumor cells may be an efficacious strategy to stop metastatic spreading (262). Additionally, the untransformed breast epithelial cells upregulate PDK4 to inhibit PDH and attenuate the flux of glycolytic carbon

into mitochondrial oxidation, consequently suppressing anoikis (the absence of the home environment) upon detachment from ECM (261). By stimulating PDH in cancer cells to normalize glucose metabolism, it can restore their sensitivity to anoikis and weaken their metastatic potential, suggesting that PDKs are potential targets for anti-metastasis therapy (261). Another way to counter increased ROS production in breast cancer cells is to induce the expression of catalases, such as manganese superoxide dismutase (MnSOD). The expression of MnSOD is elevated in metastatic breast cancer, and its overexpression is correlated with histologic tumor grades (263). Isaac et al. has suggested that combined inhibition of endogenous antioxidant GSH and thioredoxin antioxidant pathways can produce a synergistic anti-cancer effect both *in vitro* and *in vivo* (264).

Extracellular Acidification

Lactate, the final product of glycolysis, is released from cells together with H^+ ions by means of monocarboxylate transporters and hydrogen ion pumps, and the excess carbon dioxide produced in the process of mitochondrial metabolism diffuses into the extracellular space and is then converted into H + and HCO3- by carbonic anhydrase (265). In situations of metabolic stress, such as nutrient deprivation and hypoxia, these reactions are strengthened, leading to extracellular acidification and enhancing the proteolytic activity of MMPs. Afterwards, the ECM is remodeled, which facilitates tumor invasion (265, 266). It has been reported that extracellular lactate also increase tumor invasion and metastasis by facilitating the fibroblast expression of hyaluronan and CD44 (267). Besides, increased extracellular lactate induces tumor-associated stromal cells to secrete VEGF, thus reinforcing angiogenesis (268). The augment in extracellular lactate has also been reported to provide an immune-conducive environment for tumor cells by reducing the activation and function of dendrites and T cells (269, 270).

Cancer-Associated Fibroblasts

Cancer-asscociated fibroblasts (CAFs), the paramount stromal cells in breast tumor microenvironment, contribute to tumor progression through many mechanisms, such as releasing of assorted secretory proteins (e.g. TGF-β, IGF, and IL6), direct interplaying with tumor cells, regulating immune-response, ECM remodeling, and inducing cancer metabolic reprogramming (271). Breast cancer cells MCF-7 exhibited increased aerobic glycolysis when co-cultured with adjacent fibroblasts. Mechanically, the lactate produced by the CAFs can be used by cancer cells, thus enhancing aerobic glycolysis, which is called the "reverse Warburg effect" (28, 272). Similarly, metabolomic analysis showed that CAFs also produce glutamine and other metabolites that can be utilized by tumor cells (273). Subsequent researches demonstrated that co-culture with MCF-7 and CAFs resulted in promoted glutamine catabolism and inhibited glutamine synthesis in cancer cells, thereby promoting cancer cell growth and progression (274).

Cancer-Associated Adipocytes

The "cancer-associated adipocytes (CAAs)" are generated by the transformation of tumor adjacent adipocytes (275). It has been reported that tumor-surrounding adipocytes exhibit an distinct phenotype comparing to normal adipocytes, which is characterized by upregulated beige/brown adipose markers and increased catabolism and the release of metabolites, including lactate, free fatty acids, pyruvate, and ketone bodies. Importantly, the tumor-adipocyte interaction can reprogram energy metabolism and foster tumor progression (276).

Accumulation of lipids is found in breast tumor cells when co-cultured with adipocytes (277). Tumor cells can switch from glycolysis to lipid-dependent energy production and also store excess lipids, which provides energy to support their expansion and metastasis (278). The ketone bodies produced and released by glycolytic fat cells are the ideal fuel for ATP production and they can be burned more efficiently than other mitochondrial substrates, even during hypoxia, potentially allowing the tumor grow when without adequate blood supply (279). It is worth noting that the co-existence of adipocytes and cancer cells enhances both ketogenesis in adipocytes and ketolytic activity in breast cancer cells (276). In addition, β-hydroxybutyrate secreted from adipocytes is able to induce several tumor-promoting genes in breast cells, and facilitate breast tumor cells malignant growth *in vitro* (280). Moreover, elevated ketone-specific gene expression is related with worse outcomes in breast cancer patients (281).

Immune cells in Tumor Microenvironment

The local immune surveillance environment is increasingly recognized as a significant factor inhibiting tumor metastasis. Apart from fundamental competition for nutrients required by cancer cells and immune cells in TME, metabolic pathways change in tumor cells may influence tumor-infiltrating immune cells, and different immune cell subgroups in TME have specific metabolic characteristics (282). The limitation of glucose and amino acids within the TME can significantly affect the T cell response and the determinants of metabolic dysfunction and associated T cell exhaustion within the TME are also being explored. Studies have shown that cancer itself can cause effector T (Teff) cell metabolism disorders, and there is a negative correlation between the degree of glycolytic activity of cancer cells and the antitumor function of infiltrating T cells (282). A study has confirmed that the expression of glycolysis-related genes in tumor samples from patients with melanoma and non-small-cell lung cancer is negatively correlated with T cell infiltration, and that tumor glycolysis is related to the efficacy of adoptive T cell therapy (ACT), suggesting that the glycolytic pathway may be a candidate target for combined therapeutic intervention (283). Inhibition of cholesterol esterification in T cells by genetic ablation or pharmacological inhibition of ACAT1 (a key cholesterol esterase) can lead to potentiated effector function and enhanced proliferation of CD8 (+) T cells by increasing plasma membrane cholesterol levels, which causes enhanced T-cell receptor clustering and signaling as well as more efficient formation of the immunological synapse, thereby controlling the growth and metastasis of mouse melanoma (284). However, such studies are still lacking in breast cancer. Tumor-derived myeloid-derived suppressor cells (MDSCs) are critical tumor immunosuppression components. Glycolysis restriction limited the development of MDSCs by inhibiting tumor expression of granulocyte colony-stimulating factor (G-CSF) and

granulocyte macrophage colony-stimulating factor (GM-CSF), therefore enhanced T cell immunity, reduced tumor growth and metastasis, and prolonged survival in two TNBC mouse models (285). Interestingly, hypoxia through HIF-1α significantly changes the function of MDSC in TME and shifts its differentiation direction to tumor-associated macrophages (TAMs) (286). TAMs are well-known parts of breast cancer microenvironment and most TAMs within TME are closely related to the M2-like phenotype, which participate in almost all metastatic processes, including local invasion, blood vessel intravasation, extravasation at distant sites and metastatic cell growth (287, 288).The hypoxic areas in tumors are related to the accumulation of macrophages, which assist tumor progression by producing angiogenic factors, mitogenic factors and cytokines related to tumor metastasis (289–291). In addition, hypoxia can promote the differentiation and functional capabilities of immunosuppressive macrophages (292). Blockade of Eotaxin/Oncostatin M not only prevented hypoxic breast tumor cells from recruiting and polarizing macrophages towards the M2-like phenotype and hindered cancer progression in 4T1 breast cancer model but also improved the efficacy of antiangiogenic Bevacizumab, suggesting these two cytokines as novel targets for devising effective anticancer therapy (293). Lactic acid production by tumor cells, as a byproduct of aerobic or anaerobic glycolysis, has also been shown to play a vital role in the M2-like polarization of TAMs, which is mediated by HIF- 1α (294).

The metabolites produced by cancer cells may hinder the antitumor immune response by affecting different tumor infiltrating immune cells (295). Cholesterol metabolites, oxysterols, which act as endogenous regulators of lipid metabolism through the interaction with the nuclear Liver X Receptors-(LXR)α and LXRβ, aid tumor progression by inhibiting antitumor immune responses, and by recruiting proangiogenic and immunosuppressive neutrophils. A recent study showed that in the 4T1 breast cancer model the enzymatic depletion of oxysterols in primary tumors decreases the formation of lung metastases by regulating the levels of immune cells infiltrating the metastatic TME, and tumor-associated neutrophils are the main driving force of local immunosuppression (296). Another work also proved that by recruiting immunosuppressive neutrophils in the metastatic niche, oxysterol 27-HC played a role in promoting metastasis in breast cancer models (297).

DRUGS TARGETING METABOLISM IN METASTATIC BREAST CANCER

Breast cancer patients who have not yet found metastasis are at high risk of metastasis, and those metastatic breast cancers are not curable due to lack of effective treatments. Early intervention in the early stage of distant metastasis, during the period of colonization and growth will be more beneficial to the survival of the patient. There are many promising drugs targeting altered metabolism pathways undergoing disparate stages of preclinical studies and clinical trials (**Table 2**). However, there is currently no clear conclusion of the clinical benefit of metabolic interfering drugs in the treatment of breast cancer. A Phase II clinical trial involving 164 patients was recently showed that in patients with HER2-metastatic breast cancer, addition of indoximod, the Indoleamine 2,3-dioxygenase 1 (IDO1) pathway inhibitor, to taxane did not improve PFS compared with taxane alone (300). The use of glucose metabolic inhibitors such as 2-deoxy-D-glucose (2-DG) and metformin in combination with chemotherapy has shown encouraging results in combating chemotherapy resistance (301). One patient with medullary breast cancer metastatic to lung and lymph nodes underwent extensive pretreatment (8 previous systemic treatment options) was reported to have a confirmed partial response (PR) with a duration of 65 days when treated with 45 mg/kg 2-DG every other week (298). Dichloroacetate (DCA) can enhance metformin-induced oxidative damage with simultaneous reduce of metformin promoted lactate production through PDK1 inhibition, suggesting the innovative combinations, such as metformin and DCA, will be promising in expanding breast cancer therapies (302). Nevertheless, due to the limited sample and the lack of evidence for benefit, further researches are needed.

Metabolic inhibitors combined with checkpoint inhibitors holds promise to enhance the efficacy of immunotherapy and the relationship of tumor-intrinsic metabolism and successful immunotherapy is being explored. Tumor-imposed metabolic restrictions can mediate T cell hyporesponsiveness during cancer. Checkpoint blockade antibodies against CTLA-4, PD-1, and PD-L1, can restore glucose in tumor microenvironment, permitting T cell glycolysis and IFN-γ production, and blocking PD-L1 directly on tumors dampens glycolysis by inhibiting mTOR activity and decreasing expression of glycolysis enzymes (270). Because breast cancer immunotherapy is in the ascendant, understanding the metabolic dependence between infiltrating immune cells and cancer is an important direction for future research.

For ER-positive breast cancer patients, endocrine therapy is very beneficial, but some patients will develop endocrine therapy resistance. Whether endocrine therapy combined with metabolic therapy will achieve better results still needs a lot of preclinical studies and clinical trials to verify. It has been reported that trastuzumab resistant cells exhibit enhanced glycolysis phenotype, and glycolytic restraint is able to sensitize trastuzumab resistant HER2+ breast cancers to trastuzumab treatment (303). The TN/basal-like breast cancer lacks the therapeutic targets, and chemotherapy is currently the main treatment strategies. Based on the TNBC unique metabolic phenotype, there are many existing researches focus on the metabolic interference in chemotherapy resistance models and spontaneously metastatic preclinical models (304, 305). What is more, the metabolic characteristics of tumor cells and their microenvironment in different metastatic sites are different, therefore, the corresponding targeting treatment plans can also be considered in the future (306).

Although anti-cancer therapy targeting metabolism has achieved some gratifying results, it is still currently believed that this field has the following shortcomings for possible future breakthroughs: 1) The side effects of such drugs limit their clinical effects as the optimal dose window is hard to be determined; 2) Due to the extremely complex signaling pathways in the regulation of normal cellular biology, inhibition of a specific signaling pathway will definitely have feedback activation or upregulation of other

TABLE 2 | Current metabolic interventions in metastatic breast cancer.

Targeting	Drugs	Phase	Populations	Clinical Trials	Study Date	Results
Glucose metabolism						
Hexokinase	2-deoxy-D-glucose (2DG) (alone and combined with docetaxel)	Phase I	Locally advanced or metastatic solid tumors including breast cancer	NCT00096707	2004.02–2008.07	Feasible but need further evidence (298)
Pyruvate dehydrogenase kinase(PDK)	Dichloroacetate (DCA)	Phase II	Previously Treated metastatic breast cancer or non-small-cell lung cancer.	NCT01029925	2009.12–2011.11	Suspended
Complex I	Metformin	Phase I/II/III	All breast cancer	Multiple clinical trials		Feasible but need further evidence (299)
Lipid metabolism						
FASN	TVB-2640 (combined with paclitaxel and trastuzumab)	Phase II	HER2+ metastatic breast cancer resistant to trastuzumab and taxane-based therapy	NCT03179904	2017.08-	Unpublished
Amino acid metabolism						
Glutaminase	CB-839	Phase I	Advanced solid tumors including triple-negative breast cancer	NCT02071862	2014.02–2019.03	Unpublished
	CB-839 (combined with paclitaxel)	Phase II	Locally-advanced or metastatic triple-negative breast cancer	NCT03057600	2017.05–2019.11	Unpublished
Indoleamine 2,3 dioxygenase (IDO1)	Indoximod (combined standard of care therapy (docetaxel or paclitaxel))	Phase II	HER2- metastatic breast cancer	NCT01792050	2013.02–2017.07	Cannot improve PFS (300)
	Indoximod	Phase II	metastatic invasive breast cancer that is positive for p53 staining by IHC (>= 5%)	NCT01042535	2009.12–2018.02	Unpublished
Arginine deiminase (ADI)	ADI-PEG20	Phase I	HER2- metastatic breast cancer or advanced solid tumor	NCT01948843	2014.04–2016.04	Unpublished

alternative signaling pathways, therefore causing ultimately treatment failure; 3) To specifically target related mutations involved in metabolic pathways is challenging. 4) Accurate screening of the beneficiaries is the key to improve the drug effect, and is an urgent problem to be solved in the future.

CONCLUSIONS

Metabolic programming supports several steps of successful metastasis in breast cancer. Breast cancer cells exhibit different metabolic phenotypes in different metastasis sites. Both intrinsic factors, properties arising in the malignant cells, such as *MYC* amplification, *PIK3CA*, and *TP53* mutations, and extrinsic factors, metabolic stresses imposed by the microenvironment, such as hypoxia, oxidative stress, acidosis, contribute to different metabolic programming phenotypes in metastatic breast cancer. More importantly, interfering with tumor metabolism to control tumor progression is a very promising approach in cancer treatment, although it is full of challenges. More researches are required to further discover the related genes and molecular mechanisms involved in metabolism reprograming during cancer progression, so that they can be used for targeting therapy in clinical practice in the future. We also look forward to further advances in approaches to judge and quantify metabolic phenotypes in human breast cancers *in vivo*, including metabolomics, metabolic imaging and isotope tracing studies, so that clinical oncologists will develop treatment strategies by matching the treatment to the patient-specific tumor metabolic characteristics.

AUTHOR CONTRIBUTIONS

LW wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Zhejiang Provincial Natural Science Foundation of China (grant no. Y19H160283).

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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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 03 February 2021
doi: 10.3389/fonc.2020.616098

DNA N6-Methyladenine (6mA) Modification Regulates Drug Resistance in Triple Negative Breast Cancer

Xianneng Sheng, Jinqiu Wang, Yu Guo, Jiabo Zhang and Jin Luo*

Department of Breast and Thyroid Surgery, Ningbo First Hospital, Ningbo, China

Triple negative breast cancer (TNBC) is a subtype of breast cancer with strong aggressiveness and poor clinical treatment effect, accounting for about 10–20% of breast cancer cases. N(6)-methyldeoxyadenosine (6mA) is the most conservative DNA modification in prokaryotes and eukaryotes. It is widely found in bacteria and has such functions as DNA mismatch repair, chromosome separation and virulence regulation. We determined that 6mA was modified in TNBC cell line MDA-MB-231 and the TNBC tissue. Meanwhile, compared with normal tissues, the expression level of 6mA and its methylase N6AMT1 was significantly decreased in TNBC tissue. MDA-MB-231cells were cultured with 8μM Olaparib for 2 months to construct drug-resistant cell line 231-RO. It was found that the level of 6mA also increased significantly, and the expression of N6AMT1 or ALKBH1 could effectively influence the drug resistance. Subsequently, we found that LINP1 was highly expressed in 231-RO, which was involved in DNA repair, and the expression of LINP1 could be positively regulated by 6mA modification. LINP1 expression level is directly related to TNBC drug resistance. The above results indicate that 6mA may be a new biological marker of TNBC. Meanwhile, 6mA modification may be involved in the regulation of Olaparib resistance.

Keywords: triple negative breast cancer (TNBC), 6mA, MDA-MB-231, Olaparib resistance, LINP1

OPEN ACCESS

Edited by:
Xiaosong Chen,
Shanghai Jiao Tong University, China

Reviewed by:
Yunhui Hu,
GeneNet Pharmaceuticals Co. Ltd., China
Hai Hu,
Sun Yat-Sen Memorial Hospital, China

****Correspondence:***
Yu Guo
guoyu308@163.com

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *11 October 2020*
Accepted: *14 December 2020*
Published: *03 February 2021*

Citation:
Sheng X, Wang J, Guo Y, Zhang J and Luo J (2021) DNA N6-Methyladenine (6mA) Modification Regulates Drug Resistance in Triple Negative Breast Cancer. Front. Oncol. 10:616098. doi: 10.3389/fonc.2020.616098

INTRODUCTION

Breast cancer, worldwide, is one of the most common types of cancer in women, and it also leads to the highest number of deaths in women aged 20–59 (1). TNBC accounted for 10.0~20.0% of all the pathological types of breast cancer, with a poor prognosis compared with other types of cancer and special biological behavior and clinicopathological characteristics (2, 3). Immunohistochemistry of TNBC showed that estrogen receptor (ER), progesterone receptor (PR) and proto-oncogene HER-2 were negative (2–5). Chemotherapy is the main way to treat TNBC, however, resistance to cytotoxic drugs often leads to treatment failure and death. Therefore, the understanding of the mechanism of drug resistance and effective new treatment strategies become urgent clinical needs.

Poly ADP-Ribose polymerase (PARP) is a DNA repair enzyme. It plays an important role in DNA damage repair and apoptosis (6). To date, several inhibitors of PARP (PARPi) have been applied in clinical cancer treatment with promising results. Olaparib is a highly effective PARPi

approved by FDA for clinical application (7), which can treat a variety of cancers including breast cancer (8–10), especially for cancers containing BRCA1/2 mutation with better therapeutic effect (11, 12). However, the emergence of drug resistance also makes the application of these drugs in a difficult situation.

The 6mA modification on DNA is widely present in the genomes of prokaryotes and regulates the functions of bacterial DNA replication, repair, transcription, and bacterial resistance (13). Recent studies have found that 6mA also exists in eukaryotes and plays a regulatory role in DNA transcription and other functions (14–16). Data showed that 6mA was involved in the occurrence and development of tumors and had an impact on tumor progression (16, 17). Based on the above conclusions, we concluded that 6mA May play an important role in TNBC resistance.

In this study, we first collected and detected the modification level of 6mA in TNBC, and compared it with normal tissue to determine the change trend of 6mA. By constructing the Olaparib resistance model, we explored the relationship between 6mA modification and drug resistance. We then preliminarily explained the mechanism of 6mA regulating TNBC resistance generation by regulating corresponding proteins.

MATERIALS

Cell Culture

TNBC cell line MDA-MB-231 was purchased from ATCC. MDA-MB-231 was cultured in RPMI1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco). When resistant cell lines were constructed, the screening medium (Basal medium supplemented with 8 μM Olaparib) was changed every 48 h and the culture was continued for 2 months for detection.

Cell Proliferation Assay

The cells to be tested were seeded in 24-well plates at a density of 25,000 cells per well. After 24 h of culture reached logarithmic growth stage, the culture medium was replaced with experimental medium, and continuously processed for another 48 h. Cell count is measured by manual counting with Cell Counting instrument or by Cell Counting Kit-8 (Beyotime, C0038). Three biological replicates were performed for each set of experiments.

DNA Extraction and Dot Blot

Tissue samples or cells were homogenized in a lysis buffer with protease K (Sigma-Aldrich) and digested overnight in a 56°C metal bath. On the second day, the samples were treated with RNase A for 12 h, the DNA was purified and dissolved with 10 mM Tris-Hcl (pH 8.0).

After denaturation, purified DNA samples were dripped onto membranes (Amersham Biosciences) according to the experimental design, heated in a hybrid furnace at 80°C for 30 min and sealed with 5% skimmed milk for 1 h, followed by incubation with an 6mA primary antibody (Active Motif, 61755) at 4°C overnight. On the second day, the secondary antibody was incubated at room temperature for 30 min, and then the 6mA level was detected and quantified. Then we soaked the membrane in methylene blue solution and stained the DNA. To compare the total amount of DNA and use it as a positive control.

Immunofluorescence

We digested the cells with trypsin to make cell suspension. Drops containing a small number of cells were added to the frozen slide at high altitudes to expose the chromosomes. Then chromosomes fixed with 4%PFA and sealed with 5% BSA at room temperature for 1 h. Primary Antibody of 6mA (Active Motif, 61755) was incubated overnight at 4°C, followed by fluorescent secondary antibody (Invitrogen, A32723), with DAPI (Invitrogen, P36931) marking of chromosomes.

Q-PCR

Total RNA was extracted from tissue samples or cells using TRIzol (Invitrogen), and 500 mg of total RNA was reverse-transcript using a reverse-transcription kit (Vazyme). The SYBR qPCR kit (Vazyme) was then used to detect the expression level of the target gene. $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expression. The following primers were used:

GAPDH FW TCGGAGTCAACGGATTTGGT
GAPDH RV TGAAGGGGTCATTGATGGCA
LINP1 FW CCCGAAATTCAAGCCACACA
LINP1 RV TCCCCATACCCTCTCCTACC
ALKBH1 FW CCTGGTGCCCAAAAGGTGAT
ALKBH1 RV TGAGTCCATAGGCTTGCCAC
N6AMT1 FW GCAGCAGCTTGTACCCTAGA
N6AMT1 RV GGTAGCAAGCCTTTGACCAAATC

Western Blot

Proteins were extracted from tissue or cell samples using RIPA (Abcam). The 20 mg protein was separated by SDS-PAGE and then transferred to the nitrocellulose membrane. A buffer containing 5% skimmed milk powder was used to seal at room temperature for 1 h, followed by incubation with primary antibody overnight. On the second day, secondary antibody was incubated and color and quantitative analysis were performed. GAPDH (Abcam, 1:10,000, ab8245), N6AMT1 (Invitrogen, 1:1,000, PA5-42782), ALKBH1 (Abcam, 1:1,000, ab126596), P53 (Abcam, 1:1,000, ab26), γ-H2AX (Abcam, 1:1,000, ab124781).

γ-H2AX Resolution Assay

The cells were seeded in a six-well plate and irradiated with 5 Gy when the growth rate reached 80%. The irradiated cells were then collected and used for protein extraction. Western detection for γ-H2AX level.

The Statistics

All quantitative data was counted and analyzed using Graphpad 5. The data were shown as the percentage of controlled mean ± standard deviation, and were evaluated by the two-tailed, double-sample, and equal variance student T test. P value ≤ 0.05 was considered to have a significant difference.

RESULTS

6mA Is the Biological Marker of Triple Negative Breast Cancer

6mA plays an important role in the life cycle of prokaryotes and is involved in bacterial replication and drug resistance generation. We speculated that 6mA played a specific role in the occurrence of TNBC and had impacted on the regulation of drug resistance. In order to explore the above inference, we collected and extracted DNA from TNBC tissue and TNBC cell line MDA-MB-231. Then we detected the modification level of 6mA in TNBC tissues and TNBC cell lines. The results of Dot blot showed that the 6mA modification on DNA was present in TNBC and TNBC cell line (**Figures 1A**, **S1A**). To confirm this result, we performed immunofluorescence staining on the chromosomes. The result showed that there was a 6mA positive stain on the chromosome (**Figure S1B**). It is worth noting that 6mA levels in TNBC tissues were significantly lower than those in normal tissues (**Figures 1B, C**, **S1C**, $p < 0.01$). Meanwhile, we found that in the 15 TNBC tissues detected, compared with the control tissues, the expression level of 6mA demethylase ALKBH1 showed no significant trend of change, while that of methylase N6AMT1 showed a significant trend of decrease (**Figures 1D, E**, $p < 0.001$).

N6AMT1 Is the Stress Protein From Olaparib That Regulates 6mA Levels

In order to explore the relationship between 6mA and TNBC resistance, we constructed the Olaparib resistance cell model. The MDA-MB-231 cell line with Olaparib resistance was cultured for 2 months in a medium containing 8 μM Olaparib, and the MDA-MB-231-RO (231-RO) cell line was obtained. CCK-8 results showed that the survival rate of 231-RO was significantly higher than that of Ctrl (**Figure 2A**, $P < 0.001$), after 48 h treatment with different Olaparib concentrations. Meanwhile, IC50 results showed that the drug resistance of 231-RO was about six times that of the Ctrl group (**Figure 2B**, $P < 0.001$).

Then we compared the modifications of the two cell lines, and the results showed that the level of 6mA of 231-RO was higher than that of Ctrl (**Figures 2C, D**, $P < 0.01$), and methylene blue staining showed no difference in the amount of DNA samples between the two lines (data not shown). Interestingly, the statistics of 6mA modifications in different time periods showed that the trend was first significant rise and then slow decline (**Figure S2A**, $n = 3$). In line with this, the mRNA level of N6AMT1 also increased significantly (**Figure 2E**, $n = 3$, $P < 0.001$), but the expression of ALKBH1 showed no difference (**Figure S2B**, $n = 3$). In addition to MDA-MB-231 cell lines, we also explored another TNBC cell line (MDA-MB-468), and one ER-positive line (MCF7) as the models for drug resistance experiments. Similar to MDA-MB-231, 6mA of MDA-MB-468 and MCF7 also increased after long-term cultivation with Olaparib, and it was also found that the expression of methylase N6AMT1 also increased. However, both of the above trends were weaker than MDA-MB-231, which may be caused by cell heterogeneity, but their changing trends still showed a high degree of consistency (**Figures S2C, D**).

6mA Regulates the Resistance to Olaparib of MDA-MB-231

In order to determine the effect of 6mA modification on Olaparib drug resistance, we increased ALKBH1 or decreased the expression of N6AMT1 in 231-RO cell lines (**Figures 3A, B**). IC50 results showed that the reverse regulation of both proteins

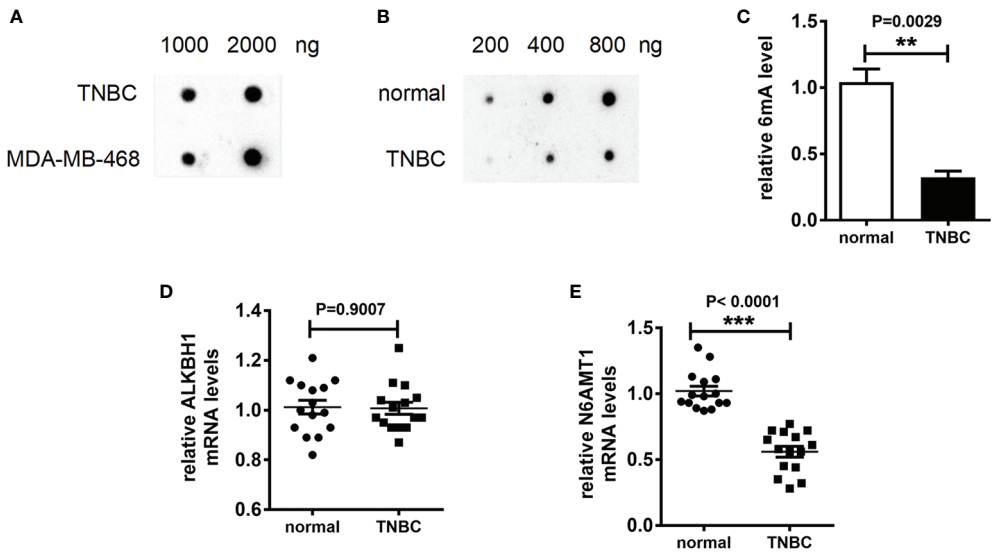

FIGURE 1 | 6mA is the biological marker of TNBC. **(A–C)** Dot blot detection of 6mA level; Q-PCR was used to detect mRNA expression levels **(D)** kbh1, **(E)** N6AMt1. **p < 0.01, ***p < 0.001, n = 15.

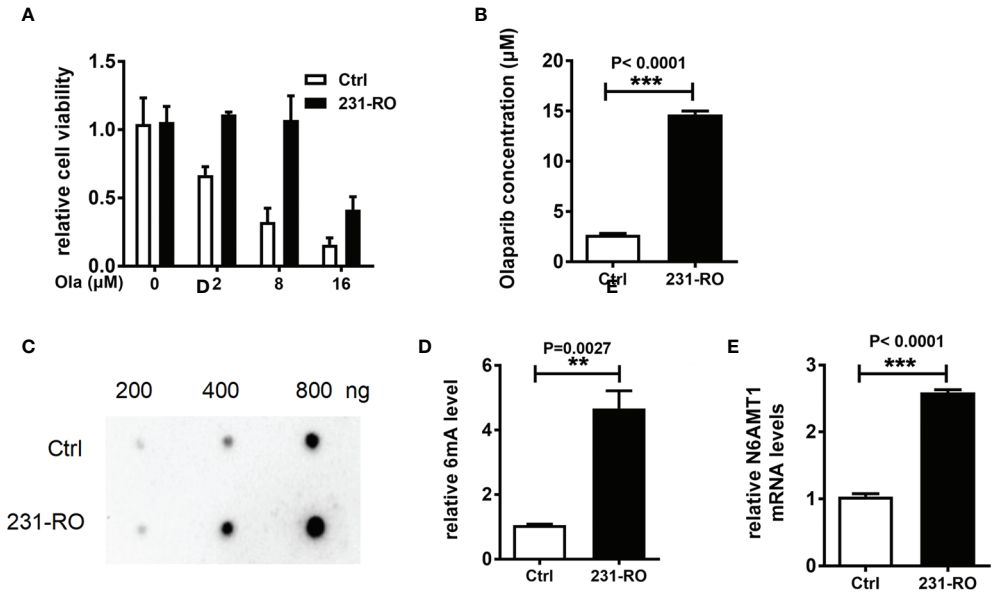

FIGURE 2 | N6AMT1 is the stress protein from Olaparib that regulates 6mA levels. **(A)** Cell survival rate was detected by CCK-8; **(B)** IC50 analysis of drug resistance of Ctrl and 231-RO; **(C, D)** Dot blot detection of 6mA level in each group; **(E)** Q-PCR was used to detect the expression level of N6AMT1. **p < 0.01, ***p < 0.001, n = 3.

could reduce Olaparib drug resistance (**Figures 3C, D**) and significantly reduce the level of 6mA (**Figure S3A**). Similarly, increasing the expression of N6AMT1 in MDA-MB-231 cell lines can also improve the drug resistance of Olaparib (**Figure S3B**). Since Olaparib is a PARP inhibitor which targets at DNA repair, we speculate that 6mA plays an important role on DNA repair. Then we examined the effects of regulation of 6mA level on DNA damage repair. The expression level of γ-H2AX, a damage marker, after regulating 6mA levels. The results showed that γ-H2AX expression in N6AMT1 overexpressed cells was significantly lower than that in the control group (**Figure S3C**) under the same irradiation condition.

6mA Affects Triple Negative Breast Cancer Resistance by Regulating LINP1

Previous studies have shown that 6mA regulates gene expression and shear in eukaryotes (15), especially the transcription of non-coding RNA (18). LINP1 was highly expressed in TNBC (19), and its main function was to enhance DNA damage repair (20). In order to explore the potential mechanism of 6mA affecting the resistance of MDA-MB-231 to Olaparib, we detected the expression level of LINP1 in 231-RO. Compared with MDA-MB-231, the expression level of LINP1 in 231-RO was dramatically increased (**Figure 4A**), while the regulation of 6mA-related proteins positively regulated the expression of LINP1 (**Figures S4A, B**). Subsequently, the expression level of LINP1 was down-regulated by overexpression of p53 protein (20) (**Figures 4B, C**), and changes in 231-RO drug resistance were detected. IC50 results showed that p53 could effectively reduce the resistance of 231-RO to Olaparib (**Figure 4D**).

DISCUSSION

TNBC has high treatment difficulty and poor prognosis due to its special biological characteristics. However, the emergence of drug resistance also leads to the gradual weakening of the therapeutic effect of TNBC. In the process of exploring the correlation between 6mA and TNBC, we found that the level of 6mA in TNBC samples was lower than that of normal tissues. These results are in contravention of the higher levels of 6mA found by Xie Q et al. in primary Glioblastoma (18), but as the same as Xiao et al. found in primary gastric and liver cancer tissues (17). The opposite trend may be due to tissue heterogeneity, or the different stages of tumor development. Subsequently, our detection results of the expression levels of N6AMT1 and ALKBH1 also showed that 6mA methylase generally had higher expression levels in TNBC tissues. Therefore, the high 6mA should be one of the biological markers of TNBC.

6mA modification is very common in the genomes of prokaryotes, and it is involved in various life activities of bacteria (13). In eukaryotes, 6mA also has a variety of biological functions (21, 22). The TNBC cell lines cultured with 8 μM Olaparib and the cell lines with strong Olaparib resistance were selected. And then, we found that 6mA had a high level in the cell line. We observed that 6mA showed a trend of fluctuation during the screening process. These results indicate that 6mA has a high stress on Olaparib, and the stress also regulates the life characteristics of cells. Q-PCR results suggested that the trend of 6mA was more controlled by N6AMT1, indicating that N6AMT1 might be the stress protein of Olaparib and thus affect the modification level of 6mA. In the subsequent regulation of

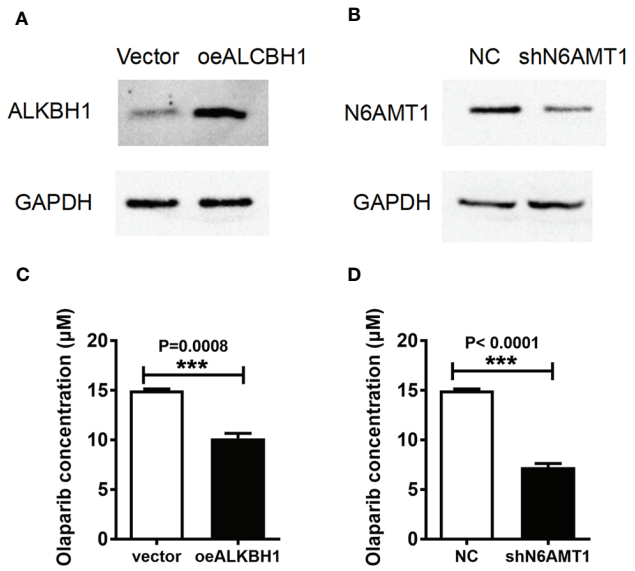

FIGURE 3 | 6mA regulates the resistance to Olaparib of MDA-MB-231. The protein expressions of **(A)** ALKBH1 and **(B)** N6AMT1 were detected by Western blot; **(C, D)** IC50 analyzed the changes of drug resistance in each group. ***p < 0.001, n = 3.

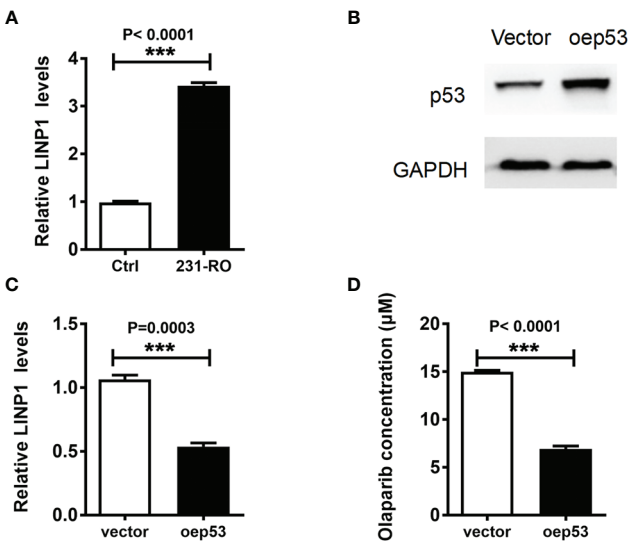

FIGURE 4 | 6mA affects TNBC resistance by regulating LINP1. **(A, C)** LINP1 levels was detected by Q-PCR; **(B)** p53 expression level was measured by western blot; **(D)** IC50 analysis the level of Olaparib resistance. ***p < 0.001, n = 3.

N6AMT1 and ALKBH1, the variation trend of 6mA modification level consistent with drug resistance was observed. In other words, increasing N6AMT1 or decreasing ALKBH1, respectively, can lead to increased cell resistance to Olaparib, and *vice versa*. This result indicates that changing the 6mA level by regulating different proteins can have expected effects on drug resistance, that is, 6mA can positively regulate the resistance of MDA-MB-231 to Olaparib. Further, it can be inferred that since each protein has multiple functions, the effect of N6AMT1 and ALKBH1 on drug resistance is through the function of regulating 6mA level, rather than other potential protein functions.

LINP1 is an LncRNA highly expressed in TNBC (19), and its main function is to promote DNA repair (20). During the regulation of 6mA, we observed that it had a positive regulatory effect on LINP1 expression level. This phenomenon seems to be contrary to the phenomenon of high expression in TNBC, but in fact it can be inferred that LINP1 is co-regulated by multiple mechanisms. Subsequently, in the regulation of p53 protein, we also found that reducing LINP1 level could restore the sensitivity of 231-RO to Olaparib. This data suggests that LINP1 is indeed a potential mechanism for 6mA to regulate TNBC resistance. It was also shown that LINP1 was regulated by both P53 and 6mA.

In conclusion, we found that 6mA is one of the biomarkers of TNBC. It is also preliminarily proved that N6AMT1 is Olaparib stress protein and can regulate the modification of 6mA. Subsequently, 6mA further regulates the sensitivity of TNBC to Olaparib by regulating the expression level of LINP1. However, there are still many unanswered questions, such as the mechanism by which 6mA regulates the expression of LINP1, and the mechanism by which multiple DNA repair mechanisms work together to make cells develop drug resistance, which still needs further study.

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 Ningbo First Hospital Ethics Committee (approval no.2019-R046). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XS, JW, and YG contributed to the concept design, planning of the study, revision, and final approval of the present article. XS, JZ, and JL are responsible for doing the experiments, writing, analysis, interpretation, revision, and final approval of the present article. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the clinical pharmacy project of the Association of Integrated Chinese and Western Medicine of Zhejiang (grant no. 2019LY009).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.616098/full#supplementary-material

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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 24 February 2021
doi: 10.3389/fonc.2021.629640

The Deubiquitinating Enzyme UCHL1 Induces Resistance to Doxorubicin in HER2+ Breast Cancer by Promoting Free Fatty Acid Synthesis

Guangxian Lu[1†], Jianhua Li[2,3†], Leyun Ding[1], Chenping Wang[4], Lian Tang[1], Xin Liu[1], Jinhui Xu[1], Qin Zhou[1], Jiantong Sun[1], Wenjuan Wang[5*] and Xinyuan Ding[1*]*

[1] Department of Pharmacy, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou, China, [2] Department of General Surgery, Huashan Hospital, Fudan University, Shanghai, China, [3] Institute of Organ Transplantation, Fudan University, Shanghai, China, [4] Department of Pharmacy, Nantong Third Hospital Affiliated to Nantong University, Nantong, China, [5] Department of Pharmacy, Children's Hospital of Soochow University, Soochow University, Suzhou, China

OPEN ACCESS

Edited by:
Zheng Wang,
Shanghai Jiao Tong University, China

Reviewed by:
Pei Zhang,
China Pharmaceutical University, China
Zhichao Wang,
Shanghai Jiao Tong University, China

***Correspondence:**
Xinyuan Ding
aladdine@163.com
Wenjuan Wang
wangwenjuan1110@163.com
Jiantong Sun
18915591290@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: 15 November 2020
Accepted: 04 January 2021
Published: 24 February 2021

Citation:
Lu G, Li J, Ding L, Wang C, Tang L, Liu X, Xu J, Zhou Q, Sun J, Wang W and Ding X (2021) The Deubiquitinating Enzyme UCHL1 Induces Resistance to Doxorubicin in HER2+ Breast Cancer by Promoting Free Fatty Acid Synthesis. Front. Oncol. 11:629640. doi: 10.3389/fonc.2021.629640

Ubiquitin C-terminal hydrolase L1 (UCHL1), which is a deubiquitinating enzyme, is known to play a role in chemoresistance in cancers. However, its potential roles and mechanisms in the chemoresistance of breast cancer (BC) remain unclear. In this study, we examined its expression in patients with BC and employed Kaplan–Meier analysis and the log-rank test for survival analyses. It was found that up-regulated UCHL1 expression was positively associated with both chemoresistance and poor prognosis, especially in patients with HER2+ BC. Moreover, UCHL1 expression was elevated in HER2+ BC cells (SK-BR-3 and BT474). Similarly, doxorubicin (DOX)-resistant BC cells (MCF-7/DOX) had higher UCHL1 levels than MCF-7 cells. CCK-8 assay showed that BC cells with higher UCHL1 levels were more resistant to DOX. Furthermore, by inhibiting UCHL1 in BC cells with elevated UCHL1 expression, we demonstrated that UCHL1 promoted DOX-resistance in BC. Mechanistically, UCHL1 probably promoted DOX-resistance of BC by up-regulating free fatty acid (FFA) synthesis, as exhibited by reduced FFA synthase expression and resurrected DOX-sensitivity upon UCHL1 inhibition. Overall, UCHL1 up-regulation is associated with DOX-resistance and poor prognosis in patients with HER2+ BC. UCHL1 induces DOX-resistance by up-regulating FFA synthesis in HER2+ BC cells. Thus, UCHL1 might be a potential clinical target for overcoming DOX resistance in patients with HER2+ BC.

Keywords: breast cancer, HER2+, chemoresistance, UCHL1, free fatty acid

INTRODUCTION

Breast cancer (BC) is one of the most common cancers among women worldwide. Over 1.5 million women (25% of all women with cancer) are diagnosed with BC every year (1). BC is classified into three categories depending on clinical and histopathological characteristics and the expression of progesterone receptor (PR), estrogen receptor (ER), human epidermal growth factor receptor 2-related protein (HER2), and Ki67 (2). Among these, the HER2-positive (HER2+) subtype exists in

about 20% of patients with BC; it is associated with high risk and is a significant poor prognostic factor in clinical therapy (3). Anthracyclines are the most active and widely employed chemotherapeutic drugs for BC (4), with a strong evidence showing positive impact on BC survival (5). Among them, doxorubicin (DOX) is one of the most common first-line chemotherapy in the clinical treatment of early and advanced BC. However, DOX resistance often occurs in clinical practice, which limits long-term treatment benefits in these patients (6).

There are multiple cellular mechanisms contributing to the development of DOX resistance. P-glycoprotein and breast cancer resistance protein (BCRP) are significant members of the adenosine triphosphate (ATP)-binding cassette (ABC) family and confer DOX resistance by increasing drug efflux, thereby reducing DOX concentrations within BC cells. This may also be mediated by overexpression of transcription-linked DNA repair pathways, topoisomerase II mutations, alterations in apoptotic signaling, and increased free fatty acid (FFA) synthesis. FFAs are critical to tumor growth, and it has been reported that these are highly expressed in BC cells. Similarly, inhibition of fatty acid synthase (FASN) inhibited tumor growth (7). The expression of FASN, which is an enzyme that catalyzes the terminal step of *de novo* synthesis of FFAs, was higher in BC cells than in normal cells (8). Overexpression of FFAs confers many advantages to tumor cells, such as BC cells. It also plays a critical role in chemoresistance acquisition (9, 10). Therefore, it is important to further understand the relationship between FFA synthesis and DOX resistance, which may help improve clinical employment of the drug.

Ubiquitination and deubiquitination are reversible post-translational modifications that depend on ubiquitin ligases and deubiquitinating enzymes (DUBs) (11). DUB-induced cleavage of ubiquitin chains from substrate proteins can play an important role in different cellular processes (12). The impact of DUBs on chemoresistance in cancers has been reported. Our previous studies have demonstrated that ubiquitin C-terminal hydrolase L1 (UCHL1), which is a member of the DUB family, could promote resistance to pemetrexed in non-small cell lung cancer (NSCLC) by up-regulating thymidylate synthase (13). However, the role of UCHL1 in DOX resistance in BC remains unclear.

Thus, our findings demonstrate that UCHL1 is highly expressed in BC tissues and cells, especially in the HER2+ BC type, and is positively associated with poor prognosis and DOX resistance. We also show that UCHL1 induces DOX resistance in HER2+ BC cells by promoting FFA synthesis. These data demonstrate that UCHL1 may be a potential target to overcome DOX resistance in clinical therapy of patients with HER2+ BC.

MATERIALS AND METHODS

Patients and BC Specimens

Fifty-four primary BC tissues were obtained from patients in the Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou, China) between January 2012 and March 2014. The patients with pathologically confirmed BC disease had not undergone chemotherapy or radiotherapy; they underwent postoperative chemotherapy containing DOX according to the BC guidelines provided by the National Comprehensive Cancer Network (NCCN). All patients provided informed consent for specimen collection and analysis. According to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1), the patients with BC and their specimens were considered as "chemosensitive" if they had complete or partial response or "chemoresistant" if they had progressive disease (13). All experimental protocols were approved by the ethics committee of the Affiliated Soochow Hospital of Nanjing Medical University and were in accordance with the principles of the Declaration of Helsinki.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a standard immunoperoxidase staining procedure to test the expression of UCHL1 in paraffin-embedded BC specimens. The primary antibody was a rabbit anti-human UCHL1 antibody (1:400; Cell Signaling Technology, Danvers, Massachusetts, USA), and secondary staining was performed using an anti-rabbit secondary antibody and the DAKO ChemMate TM Envision TM Detection Kit (DAKO A/S, Denmark). Positive staining for UCHL1 (brown) was largely localized in the cytoplasm. IHC staining was scored using a H-score system dependent on both the staining intensity and the percentage of UCHL1-positive tumor cells. The staining intensity was scored as negative (0), weak (1+), moderate (2+), and strong (3+). The H-score was calculated using the following formula: 1 × (percentage of cells stained weakly [1+]) + 2 × (percentage of cells stained moderately [2+]) + 3 × (percentage of cells stained intensely [3+]), with overall scores ranging from 0 to 300 (13). For the cohort dichotomization into two subgroups based on chemotherapy response, UCHL1 expression in patients with BC was assessed by the R statistical environment employing the "survival ROC" package to determine the optimal cut-off value for defining high or low UCHL1 expression.

Validation Using Human Databases

The detailed procedure has been described previously (14). Clinical data from patients with BC were obtained from the Kaplan Meier-plotter database, as a publicly acceptable BC database (https://kmplot.com/analysis/, 201387_s_at). Kaplan–Meier survival analysis was conducted in different subtype patients with BC.

Establishment of DOX-Resistant BC Cells

MCF7 cells were purchased from the American Type Culture Collection (ATCC, USA). DOX-resistant (DOX-R) cell lines were named MCF7/DOX and were established by exposing the parental cell lines to stepwise increment of DOX (Haizheng, Zhejiang, China) at a 50% inhibitory concentration (IC50) for 6 months. The DOX-R BC cells were verified to have acquired stable resistance and were employed for subsequent experiments.

Cell Proliferation

Cell proliferation was observed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), which was in accordance to the manufacturer's instructions.

Western Blot Analysis

A detailed procedure was reported in a previous study (15). Primary anti-human antibodies against UCHL1, FASN, acetyl-CoA carboxylase, and GAPDH were purchased from Cell Signaling Technology.

Real-Time Quantitative PCR

Detailed procedures have been previously reported (14). The primer sequences employed for the PCR analysis are listed in **Table S1**. All primers were synthesized by Sangon Biotech (Shanghai, China).

Cell Fatty Acid Extraction and Analysis

Fatty acid (FA) extraction: 1 ml 5% H_2SO_4/methyl alcohol was added to tubes containing cell pellets (2×10^6) and supplemented with 100 μg nonadecanoic acid methyl ester as a confidential standard. N_2 was added to exclude air from the tubes, which were heated at 80°C for 90 min. After cooling the tubes at 4°C for 10 min, 1.5 ml double distilled H_2O and 1 ml hexane were added into the tubes. Vortexed mixtures were centrifuged at 2,000 rpm for 2 min, and the upper phase was used for FA analysis.

FA analysis: Gas chromatography–mass spectrometry (GC–MS, 7890A-5975C, Agilent Technologies, Santa Clara, CA, USA) was employed to measure FA types (containing fatty acids C14:0, C16:0, C16:1, C18:0, and C18:1) and contents. FA concentrations were determined based on the confidential standard (16).

Flow Cytometry

Flow cytometry (FCM) was performed using an Annexin V/PI assay kit (Thermo Fisher, Massachusetts, USA) according to the manufacturer's protocol and analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Statistical analyses were performed using IBM SPSS software (version 20) and GraphPad Prism software (version 7). All measurement data were shown as mean ± standard error. The Mann–Whitney test and analysis of variance were employed to compare continuous variables. Relationships between the UCHL1 expression and clinicopathological characteristics were assessed using the χ^2 test or Fisher's exact test. Survival curves were created using the Kaplan–Meier method and compared using the log-rank test. Differences were considered statistically significant at a p-value of <0.05.

RESULTS

Higher UCHL1 Expression Was Associated With DOX Resistance in Patients With BC

To explore the role of UCHL1 in DOX resistance in BC, we evaluated IHC and showed that UCHL1 was differentially expressed in 54 patients with BC (**Figure 1A**), with an IHC score cut-off value of 135, which was employed to classify

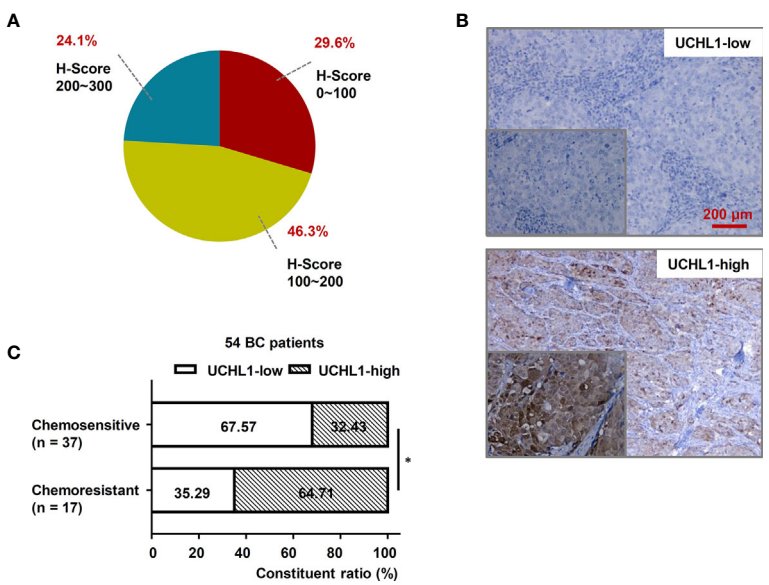

FIGURE 1 | High UCHL1 expression was associated with chemoresistance in patients with BC. **(A)** The scores (ranged from 0 to 300) of IHC staining of UCHL1 expression in 54 BC samples were calculated and its proportions are shown. **(B)** Representative images showing both UCHL1-high and UCHL1-low expression were presented (red bar: 200 μm). Comparison of chemoresistant status in 54 BC patients with different levels of UCHL1 expression **(C)**. Statistical analysis was performed with χ^2 test. *$p < 0.05$.

UCHL1-high or UCHL1-low expression (**Figure 1B**). Moreover, the patients were classified as chemosensitive (37 patients) or chemoresistant (17 patients), depending on their responses to clinical treatment based on DOX. Patients with high UCHL1 expression showed a higher rate of resistance to clinical chemotherapy (**Figure 1C**), although UCHL1 expression was not significantly associated with any other clinicopathological characteristics (**Table 1**). These results suggest that UCHL1 is a potential marker in clinical BC therapy and is positively associated with chemoresistance.

UCHL1 Expression Was Probably Positively Associated With Poor Prognosis of Patients With BC

Kaplan–Meier analysis showed that in 54 patients with BC, high UCHL1 levels had no correlation with overall survival (OS, **Figure 2A**) but correlated with poor recurrence-free survival (RFS, **Figure 2B**). Based on cases from the publicly acceptable databases (Kaplan–Meier-plotter dataset), we further ensured that there was no correlation between UCHL1 levels with OS (**Figure 2C**) and RFS (**Figure 2D**) in patients with BC. All these findings demonstrated that high UCHL1 expression was probably positively associated with poor prognosis in patients with BC.

UCHL1 Was Overexpressed and Associated With DOX-Resistance in HER2+ BC Cells

We examined UCHL1 expression in normal mammary epithelial cells (MCF-10A) and some subtypes of BC cells (ER+ HER2−: MCF-7 and T47D; HER2+: SK-BR-3 and BT474; ER− HER2−: SUM-1315 and MDA-MB-231). Compared to HER2− BC cells, the mRNA and protein levels of UCHL1 in HER2+ BC cells (SK-BR-3 and BT474) were significantly increased (**Figures 3A, B**). By detecting IC50 values of subtype representative cell lines in the presence of DOX using CCK-8, we discovered that HER2+

TABLE 1 | The association between UCHL1 expression and clinical pathologic characteristics in 54 patients with BC.

Clinical pathologic characteristics		Case No.	UCHL1 expression		*p*
			low	high	
Total cases		54	31	23	
Age (years)					
	<60	25	12	13	0.1943
	≥60	29	19	10	
Grade					
	I~II	21	13	8	0.8150
	III	29	17	12	
	unknown	4	1	3	
Regional lymph node invasion					
	N0~N1	32	21	11	0.0703
	N2~N3	20	8	12	
	Nx	2	2	0	
Chemotherapeutics (DOX-based)					
	Chemosensitive	37	25	12	0.0259*
	Chemoresistant	17	6	11	

*Analysis by χ^2 test or Fisher's exact test, *p < 0.05 value is set for highly significant difference.*

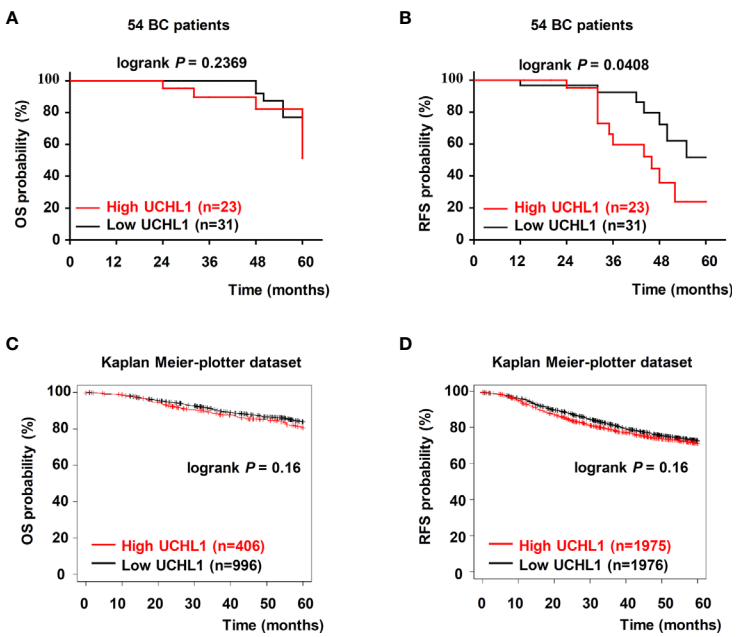

FIGURE 2 | Upregulation of UCHL1 correlated with poor prognosis in patients with BC. **(A, B)** Kaplan–Meier analysis of overall survival (OS) and recurrence-free survival (RFS) in 54 patients with BC based on UCHL1 expression are indicated. OS and RFS were determined according to UCHL1 expression in BC samples from the public Kaplan–Meier-plotter dataset **(C, D)**. Statistical analysis was carried out by Log-rank test.

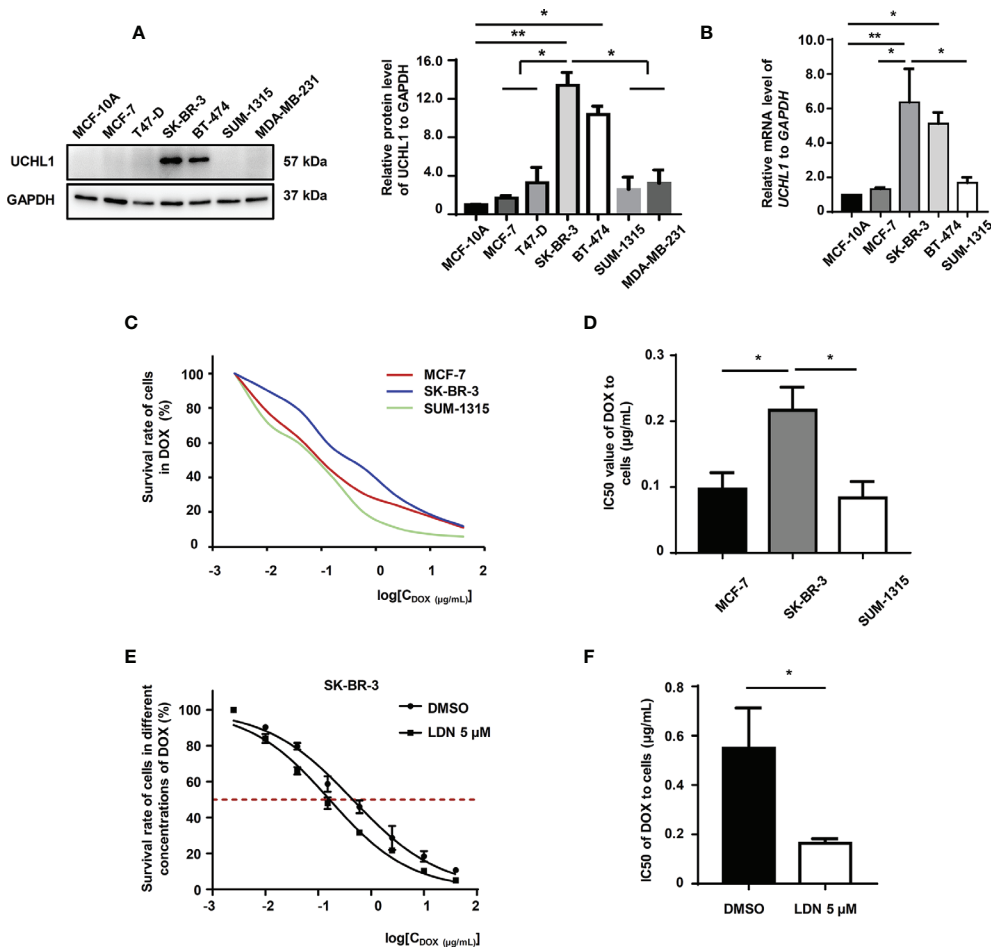

FIGURE 3 | UCHL1 level and its role in BC cells. Western blot **(A)** and real-time PCR **(B)** analysis of UCHL1 levels in BC cells are shown and were analyzed by Mann-Whitney test (n = 3). Cell viability curves of BC cells treated with DOX, as evaluated by CCK8 assay **(C)**. The IC50 values, as analyzed by Mann–Whitney test (n = 3) **(D)**. Cell viability curves and IC50 values of SK-BR-3 cells treated with DOX in the presence of 5 μM LDN or DMSO are shown (n = 3) **(E, F)**. *$p < 0.05$, **$p < 0.01$.

BC cells had a higher survival rate at different concentrations of DOX (**Figure 3C**), with a significantly increased IC50 value (**Figure 3D**). LDN-57444 (LDN), which did not significantly affect cell proliferation at 5 μM, inhibited UCHL1 (13) in SK-BR-3 cells. Specifically, the IC50 values of SK-BR-3 cells sharply decreased when treated together with LDN (**Figures 3E, F**). Thus, we confirmed that the UCHL1 level was high and positively associated with DOX-resistance in HER2+ BC cells.

UCHL1 Induced DOX-Resistance in BC Cells by Promoting FFA Synthesis

Furthermore, we explored the relationship between UCHL1 and FFA synthesis in DOX-resistance in BC cells. The level of FFAs in HER2+ BC cells was higher than that in HER2− BC cells (**Figure 4A**). The lipogenesis-associated proteins, such as acetyl-CoA carboxylase and fatty acid synthase, were up-regulated in HER2+ BC cells (**Figure 4B**). Furthermore, HER2+ BC cells (SK-BR-3), which inhibited UCHL1 activity by LDN, showed lower FFA levels (**Figure 4C**). The lipogenesis-associated proteins and genes (*ACACA*, *FASN*, and *SREBF1*) were downregulated in SK-BR-3 cells with UCHL1 inhibition (**Figures 4D, E**).

To further demonstrate the role of UCHL1 in FFA synthesis in DOX-resistance in other subtypes of BC cells, we established DOX-resistant cells (MCF7/DOX) with a resistance index (RI) of 51.6 (**Figures 5A, B**). The UCHL1 levels in MCF7/DOX were higher than the parental cells (**Figure 5C**). Moreover, the mRNA levels of lipogenesis-associated genes and the levels of FFAs in MCF7/DOX cells were significantly higher compared to the parental cells (**Figures 5D, E**). Based on these results, we then performed UCHL1 inhibition with LDN in MCF7/DOX cells. The FFA level in MCF7/DOX cells with UCHL1 inhibition was significantly reduced (**Figure 5F**), and its IC50 value to DOX was lower than that of MCF7/DOX cells with DMSO (**Figures 5G, H**). These findings suggested that UCHL1 was positively related to the FFA level in HER2+ BC cells and probably induced DOX resistance by promoting FFA synthesis.

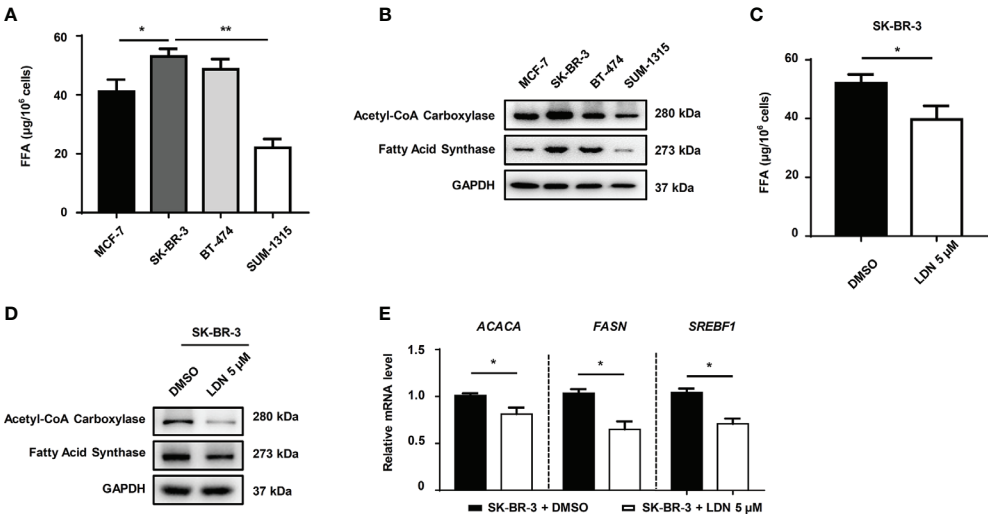

FIGURE 4 | The role of UCHL1 in fatty acid synthesis in BC cells. The level of fatty acids in different subtype BC cells **(A)** and SK-BR-3 cells in the presence of 5 μM LDN or DMSO **(C)**. Western blot **(B, D)** and real-time PCR **(E)** analysis of lipogenesis-associated proteins in BC cells are shown and were analyzed by Mann–Whitney test (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$.

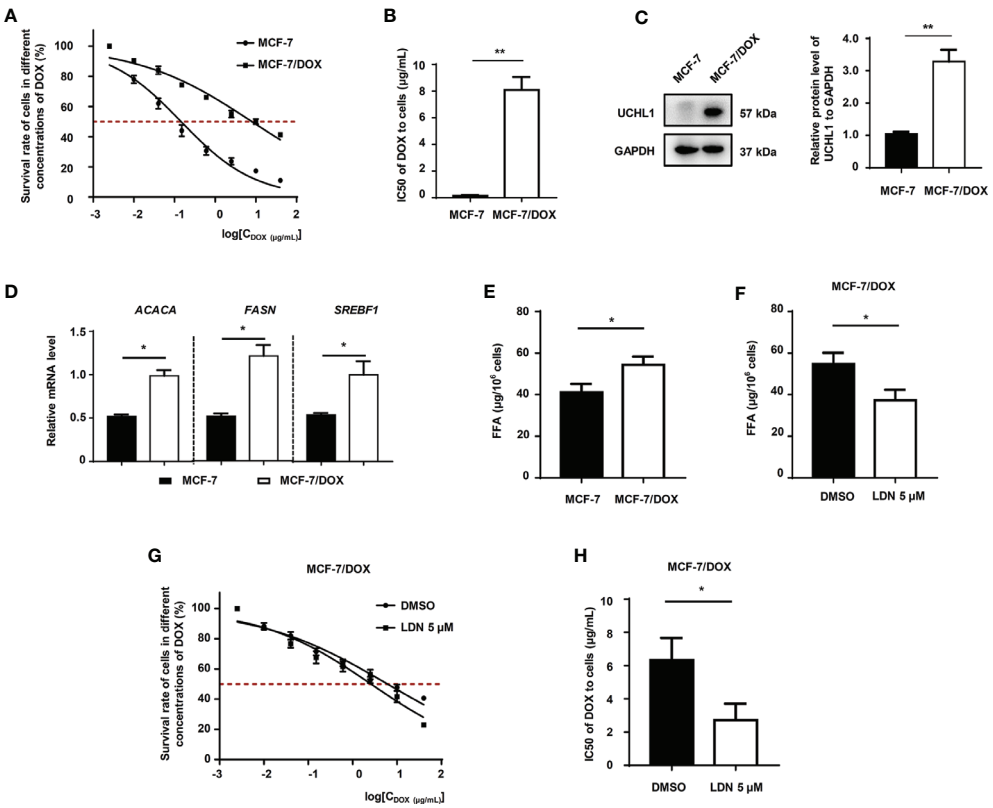

FIGURE 5 | The role of UCHL1 in fatty acid synthesis in BC cells. **(A, B)** Cell viability curves and IC50 values of MCF-7 and DOX-resistant MCF-7/DOX cells treated with DOX are shown (n = 3). Western blot analysis of UCHL1 **(C)** and real-time PCR analysis of lipogenesis-associated genes **(D)** in MCF-7 and MCF-7/DOX cells are shown and were analyzed by Mann-Whitney test (n = 3). The level of fatty acids in MCF-7 and MCF-7/DOX cells **(E)** and MCF-7/DOX cells in the presence of 5 μM LDN or DMSO **(F)**. **(G, H)** Cell viability curves and IC50 values of MCF-7/DOX cells treated with DOX in the presence of 5 μM LDN or DMSO are shown (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$.

UCHL1 Was Positively Associated With Poor Clinical Prognosis of Patients With HER+ BC

To further investigate the correlation between UCHL1 levels and poor prognosis in patients with BC, HER2+ and HER2− BC samples from the Kaplan–Meier-plotter dataset were reanalyzed. As expected, we found that ER+ HER2+ patients with high UCHL1 expression had poorer RFS (**Figures 6B, C**) and distant metastasis-free survival (DMFS, **Figures 6E, F**). However, although UCHL1 expression correlated with RFS in patients with HER2− BC, it did not correlate with DMFS (**Figures 6A, D**). These findings suggested that UCHL1 was positively associated with poor prognosis in patients with HER2+ BC.

DISCUSSION

Systemic chemotherapy has been the core treatment strategy for both postoperative and non-postoperative BC for decades, and it is still a critical component of treatment regimens. Chemotherapeutic agents, especially anthracyclines, are useful for most patients with BC, including patients with HER2+ BC (17). DOX, as an anthracycline drug, is one of the first-line chemotherapy drugs for patients with BC (18). However, DOX resistance still hinders the treatment of patients with BC (19). We found that UCHL1 expression was positively associated with poor prognosis and DOX-resistance in patients with BC. Interestingly, UCHL1 was highly expressed in HER2+ BC cells and DOX-resistant BC cells, while UCHL1 inhibition significantly improved the HER2+ cell sensitivity to DOX. Moreover, we discovered that UCHL1 conferred DOX-resistance in patients with BC by promoting FFA synthesis (**Figure 7**).

HER2, which is a protooncogene encoding epidermal growth factor receptor with tyrosine kinase activity, is amplified in 15–20% of invasive BC. HER2 amplification is a poor prognostic factor related to a high rate of mortality and recurrence. Similarly, it is also a predictive factor associated with response to anthracycline-based chemotherapies in patients with BC (20). It has been reported that increased HER2 could promote glycolysis *via* activation of the Akt/mTOR/HIF-1α axis, thus, inducing tamoxifen resistance in BC (21). DUBs are critical components of the ubiquitin-proteasome system (UPS). The basic role of DUBs is the characteristic removal of ubiquitin from substrates. Altered DUB activity is related to multiple cancers (22). Previous studies showed that ubiquitin-specific peptidase 18 (USP18) could promote HER2+ BC progression by up-regulating EGFR and activating the Akt/Skp2 pathway (23). UCHL1, which is a member of DUBs, is associated with chemoresistance in many cancers. For example, the level of UCHL1 is negatively related to cisplatin resistance in ovarian

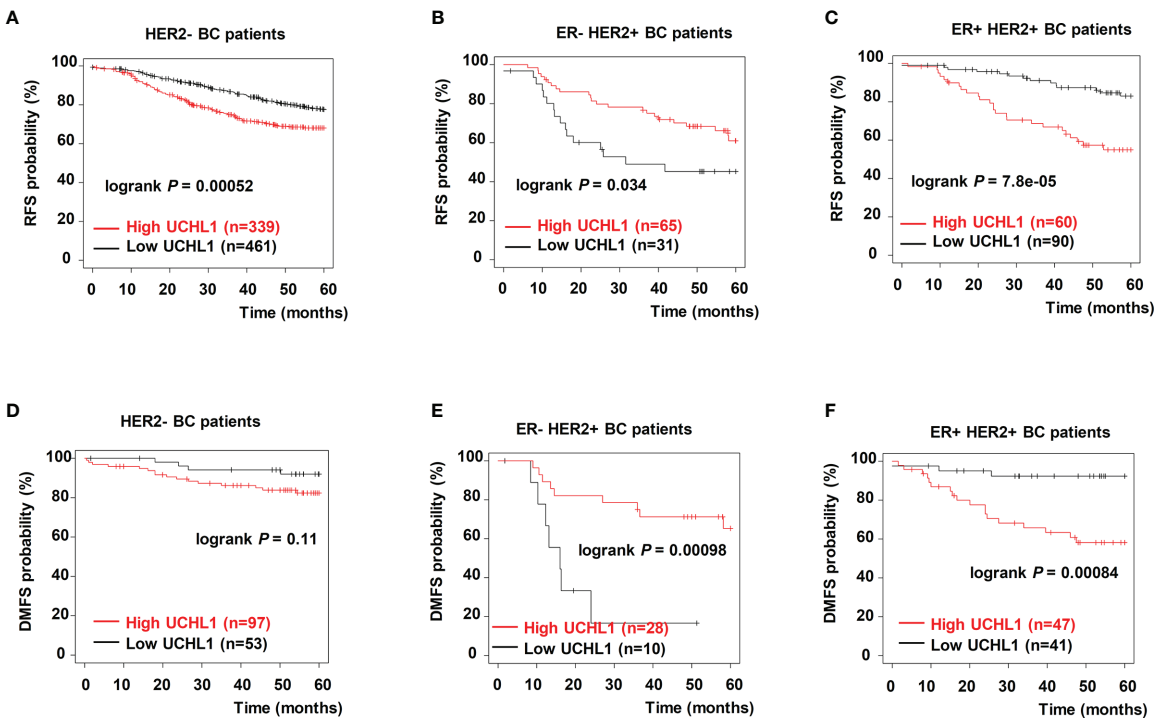

FIGURE 6 | Upregulation of UCHL1 correlated with poor prognosis in patients with different subtypes of BC. Kaplan–Meier analysis of RFS and distant metastasis free survival (DMFS) in 54 patients with BC based on UCHL1 expression and different genetic typing are indicated **(A–C)**. RFS and DMFS, as determined according to UCHL1 expression and genetic typing in BC samples from the Kaplan–Meier-plotter dataset **(D–F)**. Statistical analysis was carried out by Log-rank test.

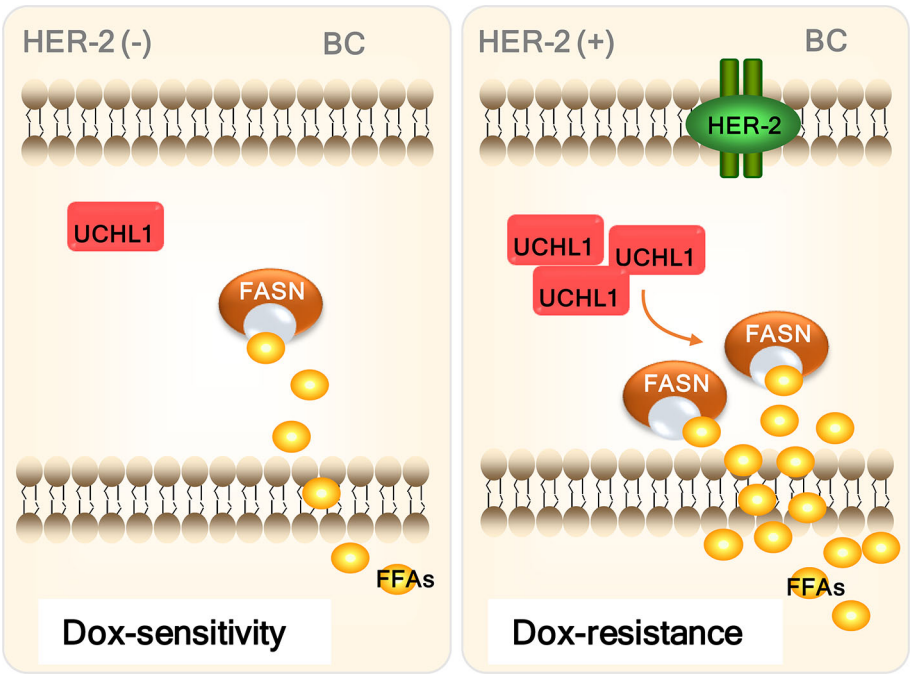

FIGURE 7 | Schematic diagram of the mechanism by which UCHL1 confers DOX-resistance in BC.

cancer, and knockdown of UCHL1 promotes cisplatin resistance (24). However, the role of UCHL1 in HER2+ BC is poorly researched. Our study found that high UCHL1 expression was associated with poor chemosensitivity of HER2+ BC cells, and Kaplan–Meier analysis based on the public dataset showed that patients with high UCHL1 expression, especially in ER+ HER2+ BC, had poorer RFS and DMFS, without any significant correlation with OS. The correlation between UCHL1 expression and RFS in patients with ER- HER2+ BC was opposite, probably owing to the limited number of patients with this subtype of BC, or the effective targeted drug (trastuzumab) for anti-HER2 treatment.

Chemoresistance in tumors can be caused by numerous factors related to either acquisition or *de novo* mechanisms (25). Accumulating evidence has demonstrated that DUBs play critical roles in chemoresistance (26). Nevertheless, whether UCHL1 plays a critical role in DOX-resistance in BC remains unclear. Our findings showed that the level of UCHL1 was positively related to chemoresistance in patients with HER2+ BC. Although it is difficult to discriminate between intrinsic resistance and acquired resistance using human specimens, UCHL1 induces acquired resistance in DOX-resistant cells, which indicates that UCHL1 plays an important role in acquired resistance.

There are various mechanisms of DOX resistance that can affect the treatment of patients with BC. Overexpression of ABC-transporters, topoisomerase II mutations, and inhibition of cell apoptosis are known to mediate DOX-resistance (27, 28). The increase in FFA utilization can also significantly promote DOX resistance (29). Significantly, the mechanisms by which UCHL1 induces chemoresistance in some cancers, such as pediatric high-grade gliomas and hepatoma cells, have been reported previously (30, 31). However, the mechanism by which UCHL1 induces DOX resistance in BC is still unknown. Here, we found that the FFA levels were increased and expression of both lipogenesis-associated proteins and genes (FASN) were higher in HER2+ BC cells with highly expressed UCHL1 than in HER2– BC cells with low-expressed UCHL1. Furthermore, the FFA levels were decreased in HER2+ BC cells with UCHL1 inhibition, and the IC50 value with DOX treatment was also decreased. It was previously reported that FFA could maintain the proliferation and aggressiveness in BC cells by activating both the ERα and mTOR pathways (32). Elevated FASN expression had also been shown to promote cell survival and contribute to DOX resistance in BC (33). The findings indicated that overexpression of UCHL1 promoted FFA synthesis to induce DOX resistance in HER2+ BC cells, and combination of UCHL1 inhibition and FASN inhibition could improve the DOX resistance.

Although we have found a correlation between UCHL1 and FFA synthesis in DOX-resistance of BC, further studies are still needed to explore the downstream target proteins to confirm our findings and provide further evidence supporting the role of UCHL1 in the chemoresistance of BC. *In vivo* experiments are also needed to support our findings and support the possibility of UCHL1 inhibition in overcoming DOX-resistance.

Our present findings revealed that DOX-resistance in HER2+ BC cells relied on UCHL1 expression. Moreover, UCHL1 induced DOX-resistance in HER2+ BC cells by promoting FFA synthesis. Therefore, UCHL1 plays an important role in the

DOX-resistance of HER2+ BC cells and may become a promising therapeutic target for overcoming DOX-resistance in patients with HER2+ BC.

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

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Suzhou Municipal Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XD and WW conceived and designed the study. GL, JL, LD, CW, LT, and XL performed the experiments and analyzed the data. GL and XD wrote the manuscript. GL, JX, QZ, and JS participated in data collection of clinical parameters. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 81902320), Wu Jieping Medical Foundation (No. 320.6750.2020-04-37), Foundation of Jiangsu Pharmaceutical Association (No. H202052), Suzhou Science and Technology Development Plan Project (No. SYSD2020188, SYSD2019183 and SYSD2019189) and the Program of Suzhou Municipal Health and Health Committee (No. KJXW2017033). The funders were not involved in study design, data collection, data analysis, nor writing of the paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.629640/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.

frontiers in Oncology

REVIEW
published: 25 February 2021
doi: 10.3389/fonc.2021.628359

Hyperglycemia and Chemoresistance in Breast Cancer: From Cellular Mechanisms to Treatment Response

Jie Qiu[1†], *Qinghui Zheng*[2†] *and Xuli Meng*[2*]

[1] Zhejiang Chinese Medical University, Hangzhou, China, [2] Department of Breast Surgery, Zhejiang Provincial People's Hospital, Hangzhou, China

Female breast cancer is a complex, multifactorial disease. Studies have shown that hyperglycemia is one of the most important contributing factors to increasing the risk of breast cancer that also has a major impact on the efficacy of chemotherapy. At the cellular level, hyperglycemia can promote the proliferation, invasion, and migration of breast cancer cells and can also induce anti-apoptotic responses to enhance the chemoresistance of tumors *via* abnormal glucose metabolism. In this article, we focus on the latest progress in defining the mechanisms of chemotherapy resistance in hyperglycemic patients including the abnormal behaviors of cancer cells in the hyperglycemic microenvironment and the impact of abnormal glucose metabolism on key signaling pathways. To better understand the advantages and challenges of breast cancer treatments, we explore the causes of drug resistance in hyperglycemic patients that may help to better inform the development of effective treatments.

Keywords: hyperglycemia, chemotherapy resistance, chemoresistance, breast cancer, glucose metabolism

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Edited by:
Xiaosong Chen,
Shanghai Jiao Tong University, China

Reviewed by:
Chuangui Song,
Fujian Medical University, China
Chaochen Wang,
Zhejiang University, China

****Correspondence:***
Xuli Meng
mxlmail@126.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *11 November 2020*
Accepted: *05 January 2021*
Published: *25 February 2021*

Citation:
Qiu J, Zheng Q and Meng X (2021) Hyperglycemia and Chemoresistance in Breast Cancer: From Cellular Mechanisms to Treatment Response. Front. Oncol. 11:628359. doi: 10.3389/fonc.2021.628359

BACKGROUND

Breast cancer (BC) is the most common malignancy in women all over the world (1) and has several known risk factors including age, sex, obesity, estrogen levels, and family history (2). Recent studies have shown that hyperglycemia is an important risk factor in the development of BC (3). In BC, hyperglycemia is associated with an increased prevalence and mortality but also has major impacts on the efficacy of chemotherapy efficacy and can lead to chemoresistance. The development and progression of BC involves the dysfunction of several molecular processes including abnormal glucose metabolism, abnormal insulin levels, insulin resistance, distorted signal pathways, oxidative stress, and enhanced inflammatory processes (4, 5).

The "Warburg" effect is considered to be the most important feature of glucose metabolism in tumors (6). Under hypoxic conditions, aerobic glycolysis in tumor cells significantly changes compared to aerobic oxidation and so cancer cells upregulate the processes of glycolysis and the catabolism of glucose to form lactate. This process is accompanied by ATP production. However, ATP produced by glycolysis is not sufficient to support the survival of cancer cells and so the rate of glucose uptake and the fermentation of glucose to lactate are both increased (7). Sufficient energy supply activates cellular signaling pathways, promotes the abnormal activity of tumor cells, and induces an anti-apoptotic response and chemotherapy resistance (8). The reprogramming of glucose metabolism accelerates the conversion of glycolysis and changes the acidity of the

microenvironment which acts to promote the expression of angiogenic factors and enhance tumor metastasis (9).

Hyperglycemia in patients is mostly accompanied by dyslipidemia. It has been reported that glycolipid metabolism may have a synergistic influence on chemotherapy resistance (10). Obesity leads to an accumulation of lipids and increases the circulating levels of fatty acids that enhance insulin resistance and hyperinsulinemia eventually leading to hyperglycemia and diabetes (1). However, glucose also can act as a substrate for fatty acid synthase which is a key enzyme responsible for the *de novo* synthesis of fatty acids (11, 12). The prognosis for women with BC is adversely affected by the comorbidities of hyperlipidemia and hyperglycemia. In this review, we primarily focus on the effects of crucial glucose metabolic pathways to explore how abnormal blood glucose levels influence the pathology of BC cells and reduce the efficacy of chemotherapy to drive chemoresistance.

THE EFFECTS OF HYPERGLYCEMIA ON TUMOR BEHAVIOR

Promotion of Proliferation and Metastasis

Hyperglycemia can provide nutrients for the rapid proliferation of breast cancer cells. Studies have reported that high concentrations of glucose significantly increase the proliferation of BC cells (such as MDA-MB-231 and MCF-7) (13, 14) through a mechanism involving activation of epidermal growth factor receptors (EGFRs) (15). EGFR is frequently overexpressed in triple-negative BC and is associated with poor prognosis. It has been reported that the GTPase activating protein (GAP) USP6NL that is involved in endocytosis and signal transduction is also overexpressed in BC, particularly the basal-like subtype (16). BC cells with high levels of USP6NL show delayed inactivation of EGFR leading to chronic activation of AKT which maintains the stability of the glucose transporter 1 (GLUT1) on the plasma membrane leading to increased glucose metabolism (17). GLUT1 transports and absorbs glucose through the plasma membrane to provide energy for BC cells and to promote cellular proliferation and invasion (18, 19). When BC cells lack sensitivity to respond to changes in glucose concentration, elevated USP6NL can compensate for this deficiency and stabilize GLUT1 by activated AKT, shows that its glycolysis ability depends on the protein.

Long-term hyperglycemia results in insulin resistance, hyperinsulinemia, and dysfunctional signaling through the insulin-like growth factor-1 pathway (20). It has been reported that IGF-1 participates in estrogen receptor signal transduction through IGF-1 receptor/ER interactions. This process is bidirectional as these interactions can regulate the proliferation, apoptosis, and differentiation of breast epithelial cells, resulting in increased risk of endocrine-related cancers particularly in post-menopausal women (21).

It has also been reported that hyperglycemia promotes the proliferation of malignant BC epithelial cells by increasing activity of the leptin/insulin-like growth factor-1 receptor signaling pathway and causing activation of the Protein Kinase B/mechanistic target of rapamycin (AKT/mTOR) pathway (22). Luey et al. found that the type I IGF receptor is expressed widely in BC cells and mediates the effects of IGFs on cell proliferation and migration. IGF-1 activates both the PI3K/Akt and Grb2/Ras/MAP-kinase pathways to increasing the invasive capacity of BC cells (23). The abnormal activation of the classical PI3K/AKT/mTOR signaling pathway leads to a poor prognosis due to increased tumor cell proliferation, metastasis, and drug resistance. Based on these data, targeting the PI3K/AKT/mTOR signaling pathway may be a potential therapeutic strategy in the treatment of BC (24).

The regulation of key transcription factors and inflammatory mediators in the glycolytic phenotype of BC remains an area of intense investigation (25). Hypoxia is a common phenomenon within the tumor environment. The cellular hypoxic response is mediated by hypoxia-inducible factor-1 (HIF-1) which is a crucial regulatory factor of glycolysis in cancer cells. HIF-1 is an oxygen-sensing transcription factor that regulates the consumption of glucose through oxidation or glycolysis (26). Using short interfering RNA (siRNA) to silence the expression of HIF-1, Chen et al. showed that HIF-1 KO significantly inhibited the extracellular acidification rate (ECAR), glucose consumption rate, and production of lactic acid in BC cells. HIF-1 silencing has also been shown to reduce the expression of metabolic enzymes and transporters in tumor cells and reversing resistance to apoptosis in BC cells following chemotherapy treatment (27).

Hypoxia-inducible factor 1α-induced glycolysis is essential for the activation of inflammatory macrophages. For example, the high infiltration of M2 tumor-associated macrophages is an extremely important feature of inflammatory BC (28). HIF-1 regulates metabolism during hypoxia and its transcriptional activity is also induced by T cell activation in response to hypoxia (29). These changes promote metabolic reprogramming and lead to the upregulation of genes encoding glycolytic promoting enzymes such as pyruvate kinase (PKM1), hexokinase 2 (HK2), and GLUT1 (30–32). Upregulated expression of these genes can also produce a false hypoxic state in tumors to promote angiogenesis, migration, and metastasis (33) that are closely related to the occurrence and development of BC tumors.

Epithelial-mesenchymal transformation (EMT) is an important mechanism that promotes the migration, invasion, and metastasis of cancer cells (29, 34). Zielinska et al. showed that hyperglycemia can induce matrix-specific EMT to promote the Warburg effect by upregulating glucose uptake, lactate release, and the expression of specific glycolytic enzymes and transporters. They also found that silencing fatty acid synthase (FASN) reversed the effects of hyperglycemia on the levels of EMT markers leading to increased expression of E-cadherin and decreased the expression of vimentin and fibronectin. Upregulation of these proteins is indicative of EMT and associated with metastatic progression (34). Taken together, these studies highlight the importance of hyperglycemia in the development and progression of BC (**Figure 1**).

Resistance to Apoptosis

P53 inhibits cellular transformation and activates tumor cell responses to chemotherapy drugs. Homeodomain-interaction

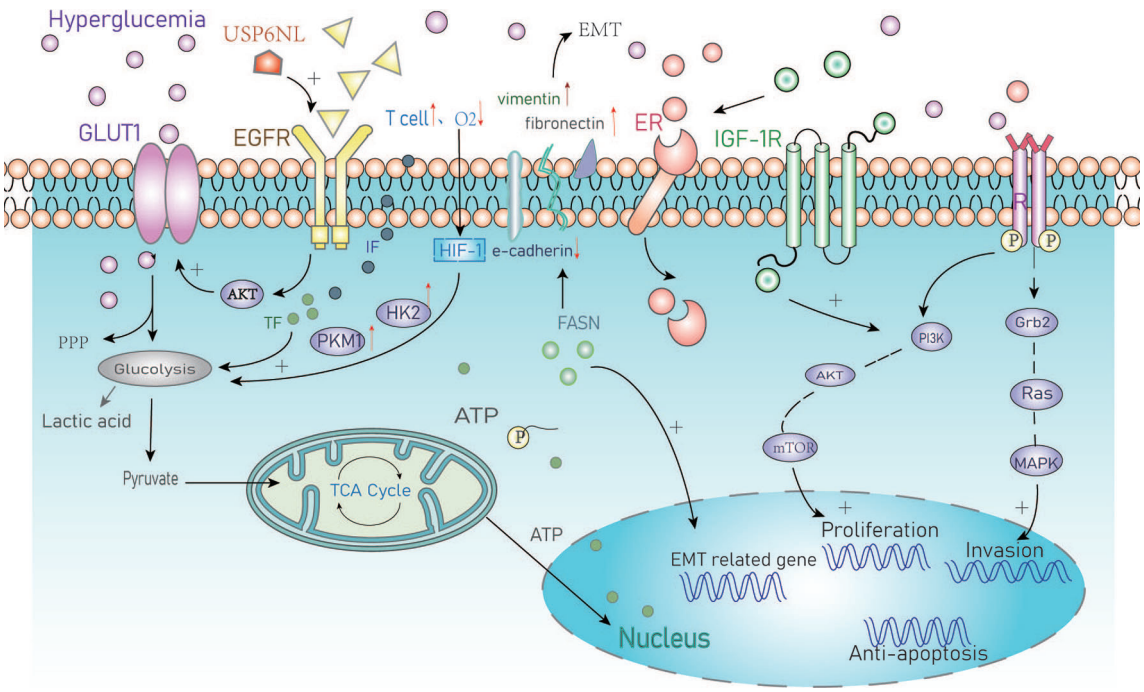

FIGURE 1 | Summary of the cellular metabolic effects of hyperglycemia in cancer. GLUT1, Glucose transporter 1; PPP, Pentose phosphate pathway; EGFR, Epidermal growth factor receptor; IF, Inflammatory factors; TF, Transcription factors; HIF, Hypoxia-inducible factor-1; HK2, Hexokinase2; PKM1, Pyruvate kinase M1; EMT, Epithelial-mesenchymal transition; FASN/FAS, Fatty acid synthase; ER, Estrogen receptor; IGF, Insulin growth factor; PI3K, Intracellular phosphatidylinositol kinase; AKT, Protein kinase B; mTOR, Mammalian target of rapamycin; Grb2, Growth factor receptor-bound protein2; MAPK, Mitogen-activated protein kinase.

protein kinase 2 (HIPK2) is a nuclear serine/threonine kinase that mediates p53-dependent apoptotic pathways in tumor cells (8). Hyperglycemic environments can trigger the degradation of the HIPK2 protein and upregulate the expression of mutant p53 to inhibit p53-induced apoptosis (35). Overexpression of mutant p53 is positively correlated with high expression of the P-glycoprotein which is a known protein biomarker of chemotherapeutic resistance. Upregulation of P-glycoprotein means can facilitate drug resistance and promote tumor progression, however, this mechanism has not been frequently reported in BC.

Rac1 is a small GTP binding protein. Li et al. found it is overexpressed and associated with multidrug resistance to neoadjuvant chemotherapy (NAC) (36). Rac1 activates aldosterone A and ERK signals and upregulates glycolysis, particularly the pentose phosphate pathway (PPP). This leads to an increase in nucleotide metabolism that can protect BC cells from DNA damage caused by chemotherapy (37). The PPP metabolite pentose phosphate is critical for nucleic acid biosynthesis and NADPH is essential for fatty acid synthesis as well as the mitigation of cellular oxidative stress (38) (**Figure 2**). The overexpression of Rac1 and the abnormal activation of the PPP pathway in BC patients with hyperglycemia can result in resistance to DNA damage as well as reduced oxidative stress. This can lead to therapeutic resistance that contributes to poor outcomes in BC patients.

Resistance to Chemotherapy

Adriamycin (ADR) resistant BC cells have increased glucose metabolism (39). It has been reported that ADR containing chemotherapy plans can effectively induce insulin resistance in cells (40). The fibroblast growth factor (FGFs)-FGFR signaling pathway is involved in many biological processes such as embryonic development, wound healing, and angiogenesis. Xu et al. postulated that high expression of FGFR4 is a key regulator in ADR resistant cells that is associated with poor survival (41). Activation of FGFR4 signaling leads to phosphorylation of FGF receptor substrate 2 (FRS2) and further activation of downstream MAPK/ERK signaling. Pharmacological inhibition of the FGFR4-FRS2-ERK signaling pathway has been shown to decrease chemoresistance and the glycolytic phenotypes of ADR-resistant cells (42). MQA et al. hypothesized that glucose metabolism has a major impact on the expression of insulin-like growth factor binding protein2 (IGFBP-2) which is an essential regulator of the IGF signaling axis. The continuous secretion of IGFBP-2 promotes chemotherapy resistance in BC and can be abrogated by silencing of IGFBP-2 expression. This results in reversing chemotherapy resistance induced by high glucose levels and resensitizes BC cells to ADR (5). The combination of 3-BRPY (a glycolysis inhibitor) and ADR has been shown to reduce total ATP and lactic acid levels (43) yet these specific mechanisms remain to be fully understood.

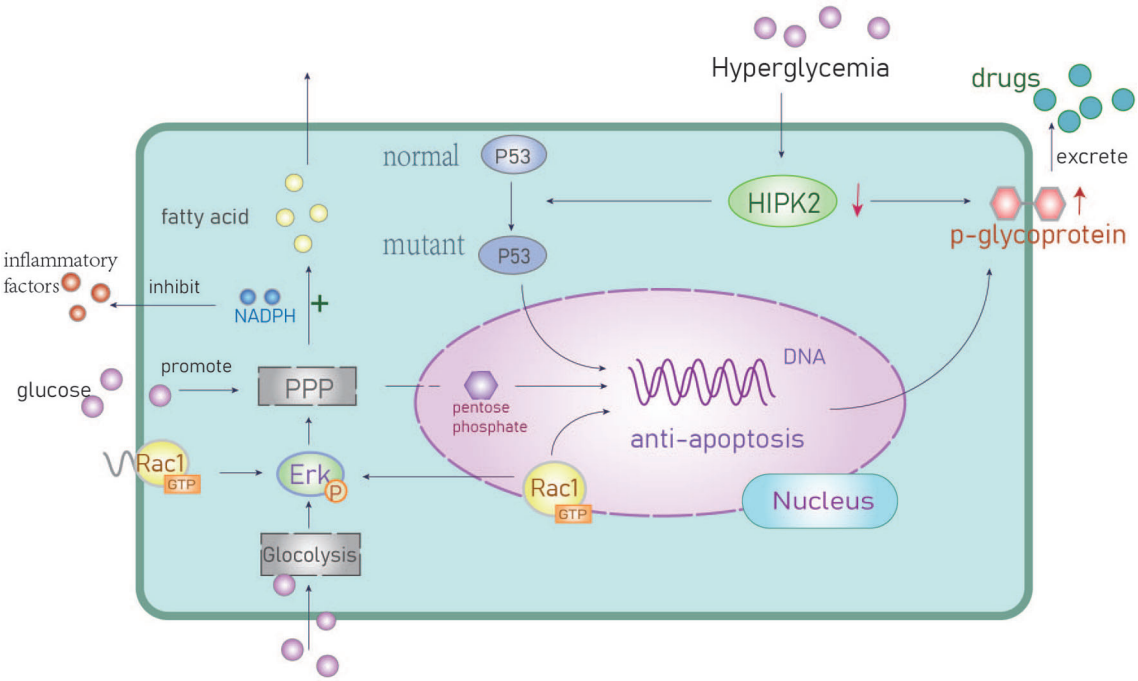

FIGURE 2 | The anti-apoptotic mechanisms of hyperglycemia. Activation of anti-apoptotic genes promotes chemotherapeutic drug resistance. PPP, Pentose phosphate pathway; HIPK2, Homologous domain interacting protein kinase1; Erk, extracellular regulated protein kinase; P53, a tumor suppressor gene.

Paclitaxel is a commonly used chemotherapy drug in the treatment of TNBC to which patients commonly develop resistance (44). Studies have reported the relationship between lactate dehydrogenase A (LDHA) and paclitaxel resistance. The downregulation of LDHA combined with paclitaxel with oxamate (an analogue of pyruvate) has been shown to result in a two-fold increase in the sensitivity to paclitaxel suggesting that targeted glycolytic enzymes may resensitize drug-resistant cells to paclitaxel. These data indicate that LDHA is a potential therapeutic target for overcoming paclitaxel resistance and in BC (45). Moreover, the most common mechanism of paclitaxel resistance is through drug efflux from the ATP binding cassette transporter. The energy required for drug efflux mainly comes from the glycolytic pathway and the P-glycoprotein is an important factor that mediates drug outflow causing drug resistance (46).

Cisplatin is a common chemotherapy drug to which BC patients often develop resistance mediated by the altered expression of glycolytic enzymes and glucose transporters (47). Considering the role of GLUT1 as an important glucose transporter (17, 18), GLUT1 may be involved in cisplatin resistance in BC cells under hyperglycemia. He et al. found that the overexpression of the oncogene TRIM59 in non-small cell lung cancer cells was related to cisplatin resistance and was mediated by the glycolysis-related gene HK2. Based on these data, we hypothesize that TRIM59 is involved in the metabolic reprogramming of cisplatin-resistant cancer cells by regulating the expression of HK2 (48), however, this has not yet been reported in the field of BC.

Resistance to Endocrine Therapy and Targeted Drugs

Tamoxifen is the most commonly used endocrine therapy in BC and patients often develop drug resistance due to enhanced glycolysis in ER-positive BC. He et al. demonstrated that activation of the EGFR signaling pathway and its downstream glycolytic genes play an important role in tamoxifen-resistant BC cells (49). Tamoxifen acts by inhibiting the mitochondrial respiratory complex I to reduce ATP levels and activate AMPK. These alterations induce apoptosis by suppressing mTOR (50). Hyperglycemia can activate the AKT/mTOR/AMPK signaling pathway which is involved in tamoxifen resistance (51). Also, Huang et al. recently reported that tamoxifen inhibits the proliferation of gallbladder cancer cells by impairing glucose metabolism (52). It is known that the occurrence of gall bladder cancer may be related to estrogen receptors suggesting a potential link with the effects of tamoxifen. This deserves in the field of breast cancer deeply.

Trastuzumab (Herceptin) is an antibody targeted against HER2. Aerobic glycolysis can be inhibited by the ErbB2 (HER2)-heat shock factor1 (HSF1)-LDHA pathway. The sensitivity to trastuzumab has been associated with LDHA activity (53). PKM2 is another key glycolytic enzyme that has been implicated in response to trastuzumab in BC (54). PKM2 is a rate-limiting enzyme in glycolysis that has been proposed as an early marker of the treatment response to trastuzumab in BC patients with metastasis (55). The PI3K/AKT signaling pathway has multiple control points in the glucose metabolism pathway

including glucose transporters and enzymes that regulate glycolysis. Studies have proposed that trastuzumab combined with PI3K/AKT inhibitors may be used to improve responses to treatment in cancer (56). In particular, one study showed differences in glucose metabolism between ER+/HER2−/+ subtypes of BC cell lines exposed to palbociclib. In ER+/HER2- palbociclib sensitive cells, aerobic glycolysis and glucose catabolism were enhanced in ER+/HER2+ Palbociclib resistance (57) (**Figure 3**). However, the mechanism through which targeted drugs promote aerobic glycolysis in BC remains to be fully determined.

THE EFFECTS OF HYPERGLYCEMIA IN THE TUMOR MICROENVIRONMENT (TME)

BC cells undergo metabolic reprogramming that usually includes enhanced glycolysis and increased activity of the tricarboxylic acid cycle (58). Scholars put forward the blood glucose changes three pathways in TME: VEGF and its receptors, cell to cell, and cell to extracellular matrix (ECM) adhesion proteins. What cause BC cells (MDA-MB-231) to metastatic mutations to bone and brain (59). It can be seen that the blood glucose load in the microenvironment is very important in tumor growth. We talked about the changes caused by hyperglycemia in the microenvironment, such as the increase in PH, the high concentration of lactic acid, the production of inflammatory factors, the imbalance of ROS, and the impact on immune cells.

The Abnormal Microenvironment

The TME is mainly composed of tumor cells, surrounding immune and inflammatory cells, fibroblasts, interstitial tissue, capillaries, various cytokines, and chemokines. The TME is characterized by high levels of H2O2 and glutathione (GSH), low pH, and hypoxia that have important implications for responses to treatment (60).

The TME plays an important role in the metabolic adaptation and survival of tumor cells. The inflammatory microenvironment can directly induce aerobic glycolysis (61). Studies have found that hyperglycemia and insulin can induce mesenchymal phenotypes through the generation of reactive oxygen species (ROS) (62). Under hyperglycemic or pathological microenvironments, imbalances between the production and removal of ROS and the production of chronic inflammatory markers (such as IL6, TNF-α, and COX-2) under hyperglycemic conditions can induce anti-apoptotic activities and EMT in cells (63).

The Warburg effect causes tumor cells to continuously export and accumulate lactic acid in the TME (5, 64). Chen and colleagues reported that high levels of lactic acid are related to the incidence of distant metastasis. LIN28B promotes the secretion of lactate and enhances the stem cell properties of cancer cells. Overexpression LIN28B increases the rate of extracellular acidification, glucose uptake, and lactic acid secretion both *in vivo* and *in vitro* (65). The impact of the acidic TME on cancer stem cells (CSCs) is thought to result in tumor relapse, therapeutic resistance, and metastasis (66). Moreover, lactic acid is also a key factor involved in

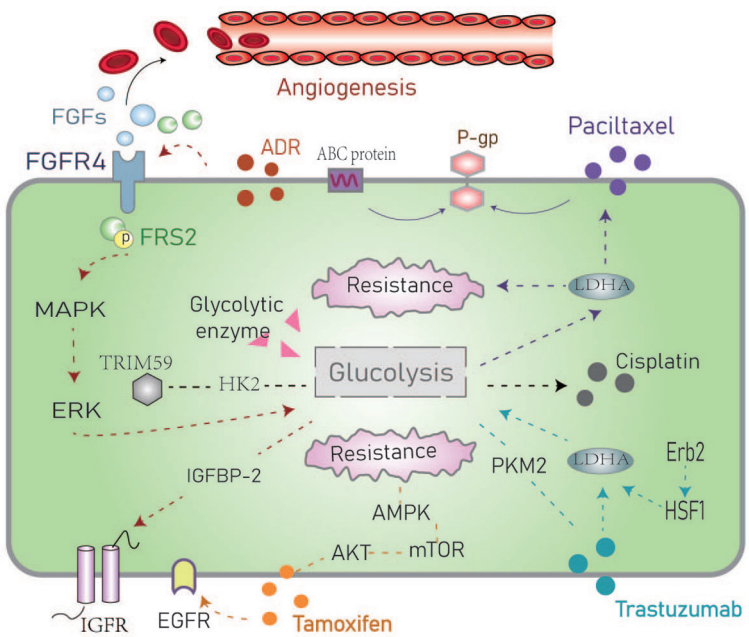

FIGURE 3 | Schematic representation of the resistance mechanisms of common chemotherapeutic drugs in the hyperglycemic environment. The dotted line represents the route in the text. ADR, Adriamycin; FGF/FGFR, Fibroblast growth factor and its receptor; FRS, Fibroblast growth factor receptor substrate; IGFBP-1, Insulin-like growth factor-binding protein-1; ABC protein, ATP binding cassette transporter; P-glycoprotein; AMPK, AMP-activated protein kinase; PKM2, Pyruvate kinase M2; HSF1, Heat shock factor1; LDHA, Lactate dehydrogenase.

angiogenesis and immune evasion. Lactic acid leads to extracellular pH acidification within the microenvironment that is also related to clinical prognosis (67). The production of lactic acid depends on the levels of key enzymes in the glycolytic pathway. LDH also plays an important role in regulating the nutritional exchange between tumor cells and stroma cells. Studies have found LDHA targeting tumor cells and LDHB targeting stromal cells influence tumor proliferation. These data suggest that it may be beneficial to block lactate exchange between tumor and stroma as a potential therapeutic strategy (68).

Uridine diphosphate glucose (UDP)-sugars are generated as intermediate products of glucose metabolism. The levels of these sugars are significantly increased in BC and have been shown to promote the accumulation of hyaluronic acid which is a known promoter of the disease. The metabolism of hyaluronic acid within the TME a key factor that drives invasive growth and metastasis (69, 70). In ductal and lobular BC, the levels of UDP-sugars are significantly increased. These observations suggest that blocking the excessive supply of UDP-sugars and reducing the content of hyaluronic acid may be potential therapeutic strategies in BC patients with glucose metabolism disorders (71, 72) (**Figure 4**).

Cancer cells within the TME have higher levels of reactive oxygen species (ROS) compared to normal cells. In basal-like and BRCA1-associated BC, it has been shown that ROS levels are associated with the expression and activity of the transcription factor aryl hydrocarbon receptor (AHR). These changes promote the transcription of antioxidant enzymes, epidermal growth factor receptor (EGFR) ligands, and the bidirectional regulatory factor AREG. AHR can attract monocytes into the TME and activate macrophages to promote angiogenesis (73, 74). Parekh et al. captured multinucleated cells in chemotherapy-resistance triple-negative BC cells. They showed that these cells are non-proliferate but can significantly regulate the TME by elevating levels of ROS and by stabilizing HIF-1. These processes contribute to increased levels of vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF) and can induce chemotherapeutic resistance by upregulating anti-apoptotic proteins through the RAS/MAPK pathway (75).

Changes in Immune Cells Within the TME

The highly acidic TME formed by glycolysis may affect the infiltration of immune cells eventually leading to immune escape and cancer progression (76). The immune infiltrates of the TME consist of lymphocytes and bone marrow cells. In this section, we briefly review the changes in immune cells in the hyperglycemia TME.

Macrophages are the most abundant type of immune cell found in the TME. M2 macrophages are closely related to the occurrence and development of tumors. Lactic acid can promote macrophages towards an M2-like phenotype that is associated with adverse clinical characteristics such as large tumor volumes, higher histological grades, ER negativity, higher recurrence rates, and lower rates of survival (76, 77). It has been demonstrated that tumor-associated macrophages (TAMs) enhance aerobic glycolysis and apoptotic resistance in BC cells *via* the transmission of extracellular vesicles (EV) that contain a myeloid-specific HIF-1α-stabilizing long noncoding RNA (HISLA). Lactic acid released by glycolytic tumor cells upregulates macrophage HISLA and forms a feed-forward loop between TAMs and tumor cells in the TME (27).

Tumor-infiltrating lymphocytes (TIL) comprise B and T cells. Cytotoxic CD8+T lymphocytes (CTL) are the most abundant TILs in the TME of BC but helper CD4+T cells and NK cells are also present (78). Tumor cells compete with these cells for glucose. Abundant lactate production by tumor cells has been shown to inhibit MCT1-mediated lactate export by TILs leading to decreased cell proliferation, cytokine production, and/or cytolysis. This phenomenon will inhibit the glycolysis of T cells and inhibit their tumor-killing function along with functional damage to NK cells (79).

Myeloid-derived suppressor cells (MDSCs) inhibit anti-tumor immunity. In TNBC mouse models, studies have found glycolysis inhibits the expression of granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) resulting in the decreased expression of MDSCs and promotion of tumor immunosuppression (80). Although other immune cells such as dendritic cells, mast cells and granulocytes are also present in the BC TME, studies are required to better define their metabolic interactions with tumor cells (**Figure 4**).

HYPERGLYCEMIA AFFECTS RESPONSE TO THERAPY IN BC PATIENTS

Anti-Sugar Drugs in BC Patients

In BC patients, hyperglycemia during chemotherapy will increase the resistance to treatment (81) and so reducing blood glucose levels is particularly important. Drugs that alter glucose metabolism can be beneficial in improving the effects of conventional chemotherapy drugs commonly used in cancer treatment. The choice of chemotherapy plan and cycle time should be carefully considered in hyperglycemic patients.

Metformin was developed in the late 1970s as an anti-hyperglycemia drug (82). It has now been widely tested for an anticancer agent along with other hyperglycemic drugs (3). Metformin can alter cancer metabolism and mitochondrial function. It can also regulate key signaling pathways such as the Ras/Raf MEK/ERK PI3K/Akt and mTOR pathways to increase cell death and inhibit many cellular processes including proliferation, migration, EMT, invasion, and metastasis (83). Metformin also changes signal transmission of the Warburg effect during tumor development and can inhibit glucose uptake by cancer cells (84). It reduces circulating hormone levels, particularly estrogens, that are associated with the development of postmenopausal BC (85).

Additionally, studies have shown that the thiazolidinediones are insulin sensitizers that can also inhibit the growth of BC cells. Pioglitazone acts as an agonist of the tumor suppressor PPAR. When PPAR is activated, the levels of free fatty acid (FFA) and eicosanoid are reduced, and VEGF-induced angiogenesis is

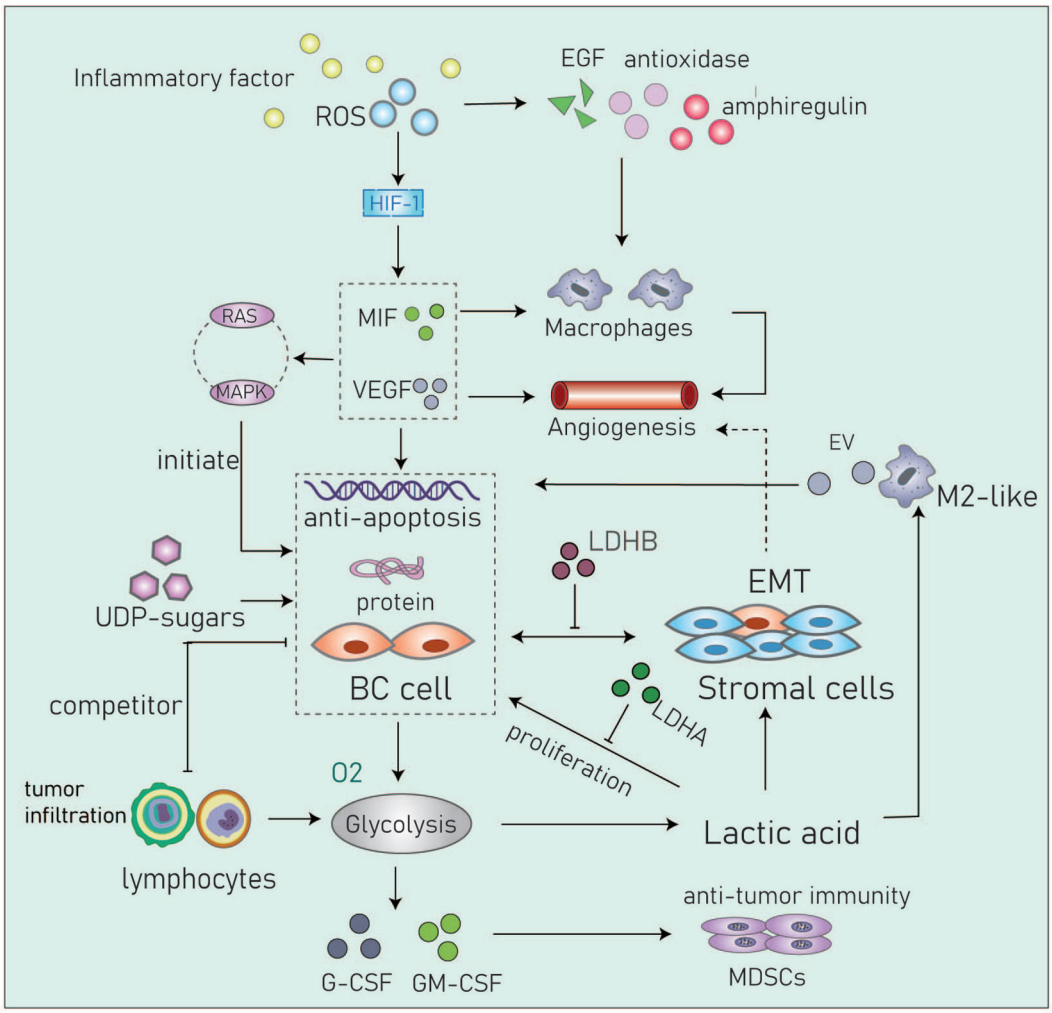

FIGURE 4 | Cancer cells in the acidic microenvironment can escape the immune system and eventually lead to tumor progression. Inflammatory factor, IL6, TNF-α, COX-2 and so on; RDS, Reactive oxygen species; MIF, Migration inhibitory factor; VEGF, Vascular endothelial growth factor; UDP-sugar, Uridine diphosphate glucose; G-CSF, Granulocyte colony stimulating factor; GM-CSF, Granulocyte macrophage colony stimulating factor; MDSCs, Myeloid-derived suppressor cells; EV, Extracellular vesicle, transmit myeloid-specific lncRNA and HIF-1α-stabilizing lncRNA.

inhibited. Also, the proliferation and migration of cancer cells are inhibited through the JAK2/STAT3 pathway (86).

Insulin and insulin analogs such as sulfonylureas and glitinides have powerful hypoglycemic effects. Studies have reported that insulin downregulates IGF-BPs and sex hormone-binding proteins (SHBGs) leading to IGF and hormone-dependent BC (21). Type 2 diabetes and insulin therapy may be independently associated with a poorer prognosis in BC. Premenopausal women with diabetes tend to develop breast tumors that do not express hormone receptors making their treatment extremely challenging (87, 88).

The antidiabetic drugs described above may also have potential roles as anti-cancer drugs. A total of 46 studies of metformin in BC patients have been registered in ClinicalTrials.gov. 17 studies have completed, 14 studies are recruiting, 4 studies were terminated due to inapplicable factors such as long rest intervals, changes in treatment methods, slow accumulation of patients' number, and data loss. And we also have listed completed in **Table 1** and recruiting trials in **Table 2**. Hope to be helpful to interested readers.

Combining the above table, we can see that the role of metformin on its anti-tumor effects are researched continuously. In addition, there are reported retrospective studies in the past have shown that the use of metformin combined with neoadjuvant chemotherapy can enable BC patients to obtain a higher pCR rate (89). The METTEN study shows that the addition of metformin plus trastuzumab to neoadjuvant chemotherapy can effectively increase the pCR rate of early her2-positive BC patients (90). The METEOR study also provides evidence of the neoadjuvant metformin plus letrozole for anti-tumor effects in non-diabetic postmenopausal ER-positive patients (91).

TABLE 1 | Completed researches on ClinicalTrials.gov. We excluded some studies that have too few participants, or inapplicable conclusions.

Phase	Study Design	Number
II	**group1**: metformin; **group2**: metformin plus chemotherapy	NCT04143282
II	**group1**: metformin; **group2**: placebo	NCT01310231
II	**group1**: exercise training; **group2**: exercise training with metformin; group3: only metformin; group4: control	NCT01340300
II	**group**: Liposomal doxorubicin +Docetaxel+Trastuzumab+Metformin	NCT02488564
II	**group1**: Letrozole with concurrent metformin; **group2**: Letrozole with placebo	NCT01589367(**94**)
II	**group1**: placebo; group2: Metformin 500 mg/d; group3: Metformin 1,000 mg/d	NCT00909506
II	**group1**: Metformin + Myocet + Cyclophosphamide; **group2**: Myocet + Cyclophosphamide	NCT01885013
I	**group1**: Exemestane alone; **group2**: Exemestane plus metformin plus rosiglitazone	NCT00933309

TABLE 2 | Recruiting researches on ClinicalTrials.gov.

Phase	Study Design	Registration no. on ClinicalTrials.gov accessed November 10, 2020
I	**metformin group**: GDC-0077+Palbociclib+Fulvestrant+Metformin; **control group**: GDC-0077 + Palbociclib + Fulvestrant	NCT03006172
II	**metformin group**: Dexamethasone and Metformin; **control group**: Dexamethasone	NCT04001725
II	**metformin group**: Toremifene and metformin; **control group**: Toremifene	NCT02506790
II	**metformin group**: 5 – Fluoruracil, doxorubicin, cyclophosphamide(FDC) ×6 cycles with metformin; **control group**: FDC ×6 cycles	NCT02506777
II	**metformin group**: Fasting-mimicking diet plus metformin plus chemotherapy **control group**: Chemotherapy plus Fasting-Mimicking Diet (FMD)	NCT04248998
II	**metformin group**: Taxotere, Carboplatin, Herceptin + Pertuzumab (TCH+P) plus metformin; **control group**: Taxotere, Carboplatin, Herceptin + Pertuzumab	NCT03238495
II	**metformin group**: receive AC-T neoadjuvant chemotherapy in addition to oral metformin HCl (850 mg tablets, twice per day, for 6 months) **control group**: receive AC-T neoadjuvant chemotherapy alone	NCT04170465
II	**metformin group**: 4 cycles (Doxorubicin+Cyclophosphamide) followed by 12 cycles Paclitaxel+ Metformin (1,000 mg twice daily) followed by surgery. **control group**: 4 cycles (Doxorubicin+Cyclophosphamide) followed by 12 cycles Paclitaxel followed by surgery.	NCT04559308
II、III	**metformin group**: metformin 850 mg once daily increased within 3 weeks to a maximum dose of 2,550 mg on three divided daily doses. Neoadjuvant cytotoxic chemotherapy as per MDT (multi-disciplinary team) decision. Patients scheduled for AC-T (adriamycin, Cyclophosphamide, paclitaxel) or AC (adriamycin, cyclophosphamide) will be eligible to randomization. **control group**: placebo plus the some neoadjuvant	NCT04387630
III	**metformin group**: receive metformin hydrochloride PO QD or BID for 24 months. Patients will continue metformin 850 mg PO BID for months 13–24; **control group**: receive placebo PO QD or BID for 12 months. Patients may crossover to Arm I for months 13–24.	NCT01905046
I	**metformin group**: metformin **control group**: Atorvastatin	NCT01980823
II	**metformin group:** Metformin Hydrochloride **control group:** Doxycycline	NCT02874430
I	**I-SPY trial**: Multi-group study, one group of metformin intervention	NCT01042379
II	**METALLICA**: Normal fasting glycemia group: Alpelisib plus metformin and fulvestrant Abnormal fasting glycemia group: Alpelisib plus metformin and fulvestrant	NCT04300790

Although metformin has shown good antitumor activity in BC, there remain many challenges concerning how best to further optimize treatment. The efficacy of metformin as an anticancer drug depends largely on the glucose concentration in the TME and so treating diabetes in cancer patients may be necessary. There are relatively few studies concerning glitazones and other antidiabetic drugs. The heterogeneity and differences in diseases mean that it is necessary to adopt personalized treatments and using precision medicine approaches to tailor treatments at the individual patient level.

Patients with hyperglycemia are resistant to chemotherapy drugs. Metformin resistance is no exception (92). Scherbakov et al. demonstrated for the first time that structural activation of Akt/Snail1/E-Cadherin signaling leads to cross-resistance of BC cells to metformin and tamoxifen (93).

Glycolysis Inhibitors in BC Patients

Reports have shown that glycolysis inhibitors such as 2-deoxy-d-glucose (2DG) combined with metformin can also significantly reduced the survival of BC cells (94). It has been reported in the

literature that the combined treatments using glycolysis inhibitors and anti-glycemic drugs are feasible in humans, however, these approaches have not yet translated to clinical evaluation.

Targeted Drugs in BC Patients

PI3K/AKT/mTOR and CDK4/6 inhibitors are emerging drugs for the treatment of ER-positive and human epidermal growth factor receptor-2 (HER2) negative metastatic BC (95). The PI3K/AKT/mTOR pathway is an important oncogenic signaling pathway in BC that can be activated by hyperglycemia to promote the proliferation of malignant BC epithelial cells (56, 95). Everolimus is an mTOR inhibitor that can target the rapamycin pathway. However, because of its toxic effect and its efficacy it has limited applications in the clinic (96). Gerke et al. have found that everolimus combined with metformin had a combined anti-cancer effect and a common inhibitory effect on glucose metabolism, tumor cell growth, and colony formation (97). However, hyperglycemia is also the most common adverse reaction of everolimus (98). Metformin may be used to prevent and/or treat everolimus-induced hyperglycemia and may enhance its anticancer effects yet there are relatively few clinical research studies in this area.

CDK4/6 inhibitors have rapidly translated from preclinical studies to clinical evaluation (99). Palbociclib can downregulate glucose uptake by GLUT-1 through the RB/E2F/C-MYC signaling pathway to reprogram glucose metabolism. It also inhibits the expression of HIF-1, a key regulator of tumor progression (100). *In vitro* experiments have shown that ER+/HER2- BC cells are sensitive to palbociclib under conditions of enhanced aerobic glycolysis whilst ER+/HER2+ cells show enhanced glycolytic catabolism with the development of palbociclib resistance. These metabolic phenotypes may have potential prognostic value (101).

CONCLUSIONS

Abnormal glucose metabolism is an important clinical problem in many types of BC. Recent studies have shown that somatic and BC cells from patients with hyperglycemia or metabolic abnormalities have elevated acidity in the TME accompanied by increased ROS and other changes in energy homeostasis. Hypoxia within the TME leads to the abnormal activation of cancer cell behaviors including enhanced proliferation, invasion, and metastasis. The reprogramming of metabolic changes in the glycolysis pathway increases the supply of substrates to cancer cells. However, tumor cells need to adapt to new conditions within the TME which can affect the survival and prognosis of BC patients.

The treatment of BCs with abnormal metabolism remains a major clinical challenge. For example, metformin can be used as an anticancer drug and can also control hyperglycemia or diabetes in patients. However, the efficacy of metformin depends on the glucose concentration within the TME and the sensitivity of patients to metformin. Other anti-sugar drugs also have anti-cancer effects but have not been studied in detail. Consequently, individualized treatment plans are required to optimize BC treatments in patients with hyperglycemia.

AUTHOR CONTRIBUTIONS

JQ and QZ contributed equally to this paper. JQ wrote the manuscript and composed the figures for this article. QZ reviewed the literature and contributed to the writing of the article. XM contributed to the editing and composition of the final version. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a special program from the Chinese National Natural Science Funds (81973861 to XM), the Key Construction Project Co-sponsored by Province and Ministry (WKJ-ZJ-2116 to XM), the Key Project for Medical and Health in Zhejiang Province (2016ZDA004 to XM), Zhejiang Medicine and Health Technology Plan Project(WKJ-ZJ-1803-01-XM), the Medical and Health Projects in Zhejiang Province (2020KY495 to QZ) and Zhejiang Traditional Chinese Medicine Project (2020 ZQ 009-QZ).

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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.

frontiers in Oncology

REVIEW
published: 11 March 2021
doi: 10.3389/fonc.2021.652266

Hypoxia in Breast Cancer—Scientific Translation to Therapeutic and Diagnostic Clinical Applications

Ying Zhang[1,2†], Hongyi Zhang[3†], Minghong Wang[4†], Thomas Schmid[5], Zhaochen Xin[6], Lora Kozhuharova[7], Wai-Kin Yu[8], Yuan Huang[8], Fengfeng Cai[3*] and Ewelina Biskup[1,2,9,10]*

[1] Department of Breast Surgery, Fudan University Shanghai Cancer Center, Shanghai, China, [2] Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China, [3] Department of Breast Surgery, Yangpu Hospital, Tongji University School of Medicine, Shanghai, China, [4] Department of Health Management, Shanghai Public Health Clinical Center, Shanghai, China, [5] Department of Medical Oncology, St. Claraspital, Basel, Switzerland, [6] Department of General Surgery, Zhongshan Hospital, Fudan University, Shanghai, China, [7] Faculty of Medicine, University of Freiburg, Freiburg, Germany, [8] Cellomics International Limited, Hong Kong, China, [9] Division of Internal Medicine, University Hospital of Basel, University of Basel, Basel, Switzerland, [10] Department of Advanced Biomedical Sciences, Federico II University of Naples, Naples, Italy

OPEN ACCESS

Edited by:
Xiaosong Chen,
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***Correspondence:**
Fengfeng Cai
caifengfeng@tongji.edu.cn
Yuan Huang
christineyuanh@gmail.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *12 January 2021*
Accepted: *12 February 2021*
Published: *11 March 2021*

Citation:
Zhang Y, Zhang H, Wang M, Schmid T, Xin Z, Kozhuharova L, Yu W-K, Huang Y, Cai F and Biskup E (2021) Hypoxia in Breast Cancer—Scientific Translation to Therapeutic and Diagnostic Clinical Applications. Front. Oncol. 11:652266. doi: 10.3389/fonc.2021.652266

Breast cancer has been the leading cause of female cancer deaths for decades. Intratumoral hypoxia, mainly caused by structural and functional abnormalities in microvasculature, is often associated with a more aggressive phenotype, increased risk of metastasis and resistance to anti-malignancy treatments. The response of cancer cells to hypoxia is ascribed to hypoxia-inducible factors (HIFs) that activate the transcription of a large battery of genes encoding proteins promoting primary tumor vascularization and growth, stromal cell recruitment, extracellular matrix remodeling, cell motility, local tissue invasion, metastasis, and maintenance of the cancer stem cell properties. In this review, we summarized the role of hypoxia specifically in breast cancer, discuss the prognostic and predictive value of hypoxia factors, potential links of hypoxia and endocrine resistance, cancer hypoxia measurements, further involved mechanisms, clinical application of hypoxia-related treatments and open questions.

Keywords: hypoxia, breast cancer, hypoxia-induced factors, biomarkers, oxidative stress, hypoxia-related treatment, precision medicine

INTRODUCTION

Breast cancer has been the most commonly diagnosed cancer and the leading cause of cancer deaths among women, accounting for ∼630,000 deaths in 2018 (1). It is a highly heterogeneous disease and clinic-pathological factors as well as multi-genomic assays allow for a sub-classification into several types and diverse subtypes, with different biological features and prognoses as well as different response to treatments (2, 3). Breast cancer mortality has decreased since the early 1990s (absolute reduction of 39% from 1989 to 2015) due to a combination of improved prevention, screening and earlier detection/diagnosis, lifestyle changes and awareness, as well as significant improvements in anti-cancer therapies. Despite these advances, worldwide every minute a woman dies from breast cancer. While mortality has been decreasing the incidence of breast cancer has been increasing. In the US, every 2 min and in the EU, 8 women are newly diagnosed every hour (4). China is another giant, modern society, where the burden of cancer is reaching epidemic proportions. In 2015, China National Cancer Center reported 12,000 per day (4.3 million) newly diagnosed cancer cases, accounting for a quarter of the global prevalence, out of which 15% are attributed to breast cancer (in women) (5).

Hypoxia is a characteristic feature of cancer. Tissue microenvironment influences tumorigenesis and tumor progression. Most solid tumor types have been shown to exhibit regions of hypoxia. The presence of a hypoxic microenvironment is a recognized event in mutagenesis and cancer development. At the same time, cancer *per se* also induces hypoxia secondary to inflammation (6, 7). It is crucial to discern the term hypoxia, which is mostly understood as a general system state which can accompany any advanced malignancy via completely different mechanisms (e.g., pathways reactive to necrosis, apoptosis, chronic permanent inflammation). The local hypoxia, however, is intentionally produced by tumor cells in order to induce angiogenesis and growth factors directed to tumor growth and metastatic features, with the healthy tissue surrounding the tumor experiencing an accompanying great structural and functional damage.

At the present time, various studies demonstrated a correlation between hypoxia and carcinogenesis, metastasis, treatment failure, and patient mortality (8–10). About 25–40% of invasive breast cancers exhibit hypoxic regions (11). Local hypoxia within the tumor and surrounding microenvironment is mainly a result of an abnormal anatomy of blood vessels, excessive angiogenesis leading to local obstructions or compressions and disturbed microcirculation (12). Generation of a hypoxic environment and the activation of its main effector, the hypoxia-inducible factor (HIF)-1, are even more common features of advanced malignancy (13, 14).

Hypoxia may retain cancer stem cells in their undifferentiated state, permitting solely cancer cells to differentiate and uninterruptedly accumulate genetic and epigenetic changes over a period of time (15, 16). Intra-tumoral hypoxia increases the number of breast cancer stem cells (BCSCs), which are essential for disease progression and recurrence (8, 17). Hypoxic breast cancer (and other) tumors are associated with a more aggressive phenotype, increased risk of metastasis and resistance to anti-cancer treatments (18, 19).

In this review, we address the role of the local hypoxia in breast cancer. We discuss unanswered questions and potential hypoxia-related treatments, in the context of relevant published literature.

HYPOXIA-INDUCIBLE FACTORS (HIFs) AND BREAST CANCER

The response of cancer cells to hypoxia is principally ascribed to HIFs, which are composed of a HIF-α (HIF-1α, HIF-2α, or HIF-3α) and a HIF-1ß subunit (8, 20, 21). These HIFs are responsible for the majority of the hypoxia-induced changes in gene expression (22, 23). HIF-1α-mediated mechanisms favor tumor growth and malignant progression, up- and down-regulation of genes, as well as pathologic modifications of the genome (24), whereas HIF-2α stimulates some, but not all genes activated by HIF-1α (25). HIF-1α responds in transient manner to severe hypoxia with rapid stabilization and activation of target genes, whereas HIF-2α responds to moderate levels of hypoxia and accumulates over time (26).

Under normoxic conditions, HIF1α is degraded by the proteasome, while under hypoxia, it translocates to the nucleus and forms a heterodimer with HIF-1β, which triggers the hypoxic response- a coordinated gene expression program (27). The hypoxic response triggers a decrease in cellular metabolism, thus inactivating the mammalian target of rapamycin (mTOR) pathway (28).

In addition, HIFs activate the transcription of a large battery of genes encoding proteins that promote primary tumor vascularization and growth, stromal cell recruitment, extracellular matrix remodeling, cell motility, local tissue invasion, metastasis, HIF-1α promotes primary breast cancer growth, vascularization (8, 29). Overexpression of HIF in breast cancer was often proposed as an unfavorable feature (30).

Hypoxia and the expression of hypoxia-mediated proteins, such as HIF-lα and VEGF, have been suggested to be negative prognostic and predictive factors, owing to its multiple contributions to chemo- and radioresistance, angiogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism, and genomic instability (10). As reported in a variety of studies, HIF-1α overexpression is significantly correlated to adverse outcomes and a poorer survival in breast cancer patients (31–34). Increased concentrations of HIF-1α have also been independently associated with a worse outcome, as demonstrated by immunohistochemistry in subsets of biopsies analyzed from both lymph node-negative (32) and lymph node-positive breast cancer patients (33, 35). Moreover, higher levels of both HIF-1α or HIF-2α in breast cancer biopsies are associated with metastasis to regional lymph nodes and distant organs, primary mammary tumor growth, as well as with an increased patient mortality (36, 37). The proposed mechanisms involve tumor-infiltrating cells (TICs). HIF-1α expression rises alongside tumor grading, being higher in less differentiated than in well-differentiated lesions (38). Finally, HIF-1α was proposed as a prognostic marker for an unfavorable outcome in those with T1/T2 tumors and positive axillary lymph nodes (29).

HIF-1β has also been reported to correlate with more aggressive cancer characteristics and poor survival, but the difference was not statistically significant in multivariate analysis (39).

HYPOXIA IN BREAST CANCER DEVELOPMENT AND PROGRESSION

Hypoxia plays an important role in tumor progression and development (13). Related processes include the meditation of angiogenesis, apoptosis, the glycolytic shift and the recruitment of tumor-associated macrophages (40).

Angiogenic growth factors and their receptors are significantly up regulated in response to hypoxia, which causes vascular effects including endothelial cell migration with increased vascular permeability and promotes tumor angiogenesis (41). During these phases of transformations and growth, as the vessels are loosening their hierarchy and become arbitrarily arranged, cancer and stromal cells have a restricted access to nutrients and oxygen. Oxygen partial pressure in the tumor

is significantly lower than in the healthy tissue at the tumor margins. The tumor cells in the vicinity of perfused vessels obviously still benefit from their oxygen supply, while cells at a greater distance are particularly hypoxic. This exacerbates even more in anemic cancer patients. Moreover, when a region of hypoxia is encountered, tumor-associated macrophages (TAMs) are induced to accumulate and exhibit a tumorigenic phenotype. TAMs can also secrete angiogenic growth factors and are associated with angiogenesis and poor prognosis in invasive breast cancer (42).

Coordinated regulation of a number of pro- and anti-apoptotic pathways by both HIF-dependent and HIF-independent mechanisms governs susceptibility to hypoxia-induced apoptosis in a cell-type-specific manner (43).

Hypoxia inhibited the pro-apoptosis effects of serum deprivation, reduced the bax/bcl-2 ratio, decreased cytochrome c release and caspase 3 activity via induction of vascular endothelial growth factor (VEGF) (44). Also, hypoxia selects for $p53^{mut}$ cells with elevated levels of apoptosis inhibitor bcl-2. The reduced ratio of p53/bcl-2 acts increases mutation rates within a clone population, promoting the oncogenesis of breast cancer (45, 46). Normally, such hypoxic state, if persistent, causes apoptosis of healthy cells, while some tumor cells stop dividing, but continue to exist and others (with a certain genetic predisposition) succeed in surviving and continue to be destructive. Out of many tumor cell populations, mechanism of selection will lead to a preference of those capable to assimilate and thrive under hypoxic conditions. These are particularly aggressive as they usually become apoptosis-resistant and thus the responsiveness to radiation or chemotherapy is reduced.

In addition, hypoxia maximizes the efficiency of the glycolytic shift via changes in the expression of glycolytic enzymes (47) and glucose transporter genes (48). Both the maximal glucose uptake and high efficiency of glucose utilization lay the basis for glycolytic respiration, which enables tumor cells to grow and proliferate under such conditions.

Extracellular matrix (ECM) is a network of proteins and proteoglycans, which supports diverse cellular functions (49). Apart from the direct increase on endothelial cells (ECs) via the expression of matrix metalloproteinase (MMPs) (50), ECM is also involved during hypoxia-driven angiogenesis (51). Numerous studies highlighted that hypoxia regulates the expression and stability of ECM proteins (collagen I and IV and laminin) in cancer cells (52). ECM deposited from co-cultures of Neonatal Fibroblasts (NuFF) with breast cancer cells supported 3-dimensional vascular morphogenesis (53, 54). Hypoxic fibers occupied a greater percent area and possessed larger diameter fibers than those deposited by co-cultures in normoxic conditions (51). It has been reported that HIF-1α is related to the changes in fiber organization, given that fiber alignment was abrogated in hypoxia-treated fibroblasts when HIF-1αwas knocked down (55). Overall, a disturbed and overloading structure results. This activates angiogenic responses by promoting up-regulated expression of vascular pro-angiogenic factors VEGFAand Ang1, proteolytic enzymes MT1-MMP, and MMP1, while leading to a down-regulation of the vascular destabilizing factor, thus altering EC responses (51). In sum, not only the architecture, content and order of the EC are modified, but the functional aspects as well.

Hypoxia and vascularity of the tumor are interrelated on various levels, including a variety of environmental and signaling components described above (56). Under hypoxia, a number of cells under lactic acid fermentation metabolism (anoxic), numerous messenger substances, including VEGF, stimulate afferent blood vessels to grow from neighboring tissues to tumor cells. Since a tumor cannot grow larger than 1 mm without neovascularization, a large number of disturbed vessels are being mobilized, to assure that the supply of nutrients to cancer cells is not relying exclusively via diffusion and the supply of oxygen the center of the grown cell cluster is adequate. Overall, different areas of a tumor are supplied with different levels of oxygen: Cancer cell clusters in place and detached CTCs with a lack of oxygen have comparatively few blood vessels. At the same time, as compared to clusters with normal oxygen content, the hypoxic clusters are significantly more aggressive, metastasizing more quickly. Thus, it becomes obvious that in order to improve the oxygen supply, the formation of blood vessels is stimulated around the primary tumor, while local hypoxia is tumor-induced in order to promote CTC detachments and thus metastasis.

HYPOXIA PROGNOSTIC FACTORS IN BREAST CANCER

Several recent studies have shown independent prognostic significance of a number of hypoxia related factors, such as PGC1, transcription intermediary factor 1γ (Tif1γ) or transforming growth factor -β (TGF-β), similarly to HIF1α, where this has been confirmed before (39).

TGFβ has been shown to have both tumor suppressive (early stages) and oncogenic (later stages, pro-metastatic and pro-EMT) effects. Especially the isoform TGFβ1 is an inhibitor of mammary gland epithelial cell proliferation and plays an important role in breast carcinogenesis.

TIF1γ contributes to breast cancer by controlling TGF-β/Smad signaling, leading to a TGF-β-induced EMT. A link was reported between TIF1γ and HIF1α in TNBC. In a study in press, we were able to show that the levels of Tif1γ were significantly lower in patients with breast cancer than in healthy controls. The average concentration of Tif1γ-discriminated between Tif1γ-positive and Tif1γ-negative patients. The latter group had a significantly worse OS ($P = 0.0174$); this was confirmed in the multivariate analysis. Tif1γ plasma level seems to be thus an independent prognostic factor for patients with breast cancer. This supports the potential of using measurements of Tif1γ plasma level to guide breast cancer therapy and monitoring.

Other proteins involved in cell homeostasis might become additional biomarkers in the early detection/diagnosis and monitoring of breast cancer if their apoptotic features react to the influence of aerobic vs. anaerobic microenvironment. Further studies are required to identify and validate new easily detectable, non-invasive biomarkers with prognostic power—studies of some such biomarkers are already ongoing, e.g., PGC1α, Tif1γ, etc.).

Increased levels of PGC1α were, similarly to HIF-1α, associated with more aggressive tumors -e.g., histologically higher grade and higher stage–and were therefore proposed as a prognostic marker for unfavorable outcomes, especially in positive axillary lymph nodes tumors (39). Recently, PGC1α was confirmed to be an independent prognostic marker, where over-expression correlates with poorer outcome in an unselected (all stages) breast cancer population (39).

All these markers (PGC1α, HIF-1α, and Tif1γ) can be measured easily from patients' plasma, which allows a simple, cost- and time-effective method for an improved clinical decision-making regarding treatment or even an early diagnosis for patients.

HYPOXIA AND BREAST CANCER METASTASES

Despite the rapid progress in breast cancer treatment, the development of metastases remains the primary reason for breast cancer mortality (57). It is a complex process, which until now is known as a series of steps: epithelial-mesenchymal transition (EMT), local tissue invasion and intravasation, extravasation and metastatic niche formation (58). As mentioned above, hypoxia contributes to cell transformations, so that they undergo fundamental functional and structural changes. In this manner, a number of those cells, who were previously sedentary cancer cells, acquire properties that are essential for mobilization, conspicuously due to genetic modifications in p53 (tumor suppressor) and modifications on chromosome level (e.g., in Chromosome 1).

EMT is characterized by cellular and molecular changes that include loss of cell-to-cell adhesions (58). Thus, phenotypically, the migratory cells possess little or no adhesion molecules, while they develop a battery of lytic enzymes to invade lymphatic and blood vessels. HIF-1 activates EMT through regulating associated signaling pathways, modulating EMT-associated inflammatory cytokines, as well as interfering in other pathways, such as epigenetics (here, concrete data are still missing) (59). Transcription factors like E-cadherin, SNAIL (zinc finger protein snail), ZEB1 (zinc finger E-box-binding homeobox 1), and TWIST are also involved in the HIF-1 induced EMT (60) (**Table 1**).

MMPs degrade many of the components of the ECM, which enables cancer cells to invade the surrounding tissues and intravasation. Hypoxia and HIF-1 up-regulate the expression levels and/or MMP-2 and MMP-9 (63, 64), which are positively correlated with a higher incidence of metastases and with a poor prognosis (64). HIF-1αplays a critical role in collagenogenesis by up-regulating the expression levels of pro-collagen prolyl (P4HA1 and P4HA2) and lysyl hydroxylases (PLOD2), which are reported to be crucial for breast cancer metastasis (55).

Hypoxic breast cancer cells produce multiple members of the lysyl oxidase (LOX) family, including LOX, LOXL2, and LOXL4, in a HIF-1-dependent manner (65). LOX remodel ECM both in the primary and the distant site, which provokes metastatic niche formation (66). Furthermore, HIF-1 induces miR-210-expression, a non-coding RNA, which contributes to tumor proliferation and forming of metastasis, alongside of other non-coding RNAs (miRNAs and lncRNAs) (67).

HYPOXIA AND BREAST CANCER STEM CELLS

Breast Cancer Stem Cells (BCSCs) are characterized by an unlimited self-renewal differentiation potential, performance of symmetrical and asymmetric cell divisions, as well as regeneration (68). In mesenchymal stem cells (MSC), it was shown that most of them are exposed to a lower oxygen concentration *in vivo*, e.g., by about 7% in the medulla or adipose tissue. *Ex vivo*, culturing of MSC under hypoxic oxygen concentrations resulted in a higher growth rate, glucose consumption and longer life at a constant level of the stem cell functionality (69–71).

The metastasis-promoting effects of HIF-1 help to maintain an expanding renewing population of BCSCs ready to be distributed much like seeds or pollen blowing in the wind (72). Increased expression of HIF-1α and HIF-2α in BCSCs lead to increased expression of pluripotency factors such as NANOG, OCT4, SOX2, and KLF4 in response to intratumoral hypoxia (73). HIFs also mediate complex and bidirectional paracrine signaling between breast cancer cells (BCC) and MSC that stimulate breast metastasis. Interactions between BCC and MSC are supposedly mediated by CXCL10→CXCR3, CCL5→CCR5, and PGF→VEGFR1 signaling in a HIF-dependent manner (66). Further research is needed to explore these interdependencies more fully.

A LINK BETWEEN OBESITY AND BREAST CANCER VIA HYPOXIA?

Despite numerous epidemiological studies illustrating the link of obesity and breast cancer, the underlying mechanisms are not elaborated. In addition, no general statements about the cancer-and-obesity relation can be made, as seen in the complexity of the breast cancer: e.g., postmenopausal obesity is correlated with an increased risk of breast cancer. Such a clear association has not been proven for obese in premenopausal females (74). Longitudinal data regarding the relapse rate link to obesity are still suboptimal, but from epidemiological observations, conclusions were already drawn and sufficient evidence is now available that a healthy body weight has a positive effect on the survival of breast cancer patients (75).

A potential causal relation is given based on the local hypoxia in breast cancer via the adenosine receptor 2B (A2BR), a modulator of glucose homeostasis and obesity (76). Elevated A2BR expression have been found in adipose tissue of obese individuals. Under hypoxic conditions in the breast cancer tissue (A2BR) is overexpressed. A2BR is linked both to adipositas and to BCSC. It primarily regulates pre-adipocyte differentiation and macrophage inflammation in adipose tissue. When activated (among others through HIF1 factors), A2BR leads to the

TABLE 1 | Role of various factors in breast cancer and hypoxia.

Factor	Role in breast cancer	Role in hypoxia
HIF-1α/ HIF-2α (Hypoxia-inducible factors) P4HA1, P4HA2	1. HIF-1α favor tumor growth and malignant progression, up- and down-regulation of genes, as well as pathologic modifications of the genome (24), whereas HIF-2α stimulates some, but not all genes activated by HIF-1α (25). 2. HIFs activate the transcription of a large battery of genes encoding proteins that promote primary tumor vascularization and growth, stromal cell recruitment, extracellular matrix remodeling, cell motility, local tissue invasion, metastasis, and maintenance of the cancer stem cell properties (29, 61) 3. Up-regulating the expression levels of pro-collagen prolyl (P4HA1 and P4HA2) and lysyl hydroxylases (PLOD2) 4. HIF-1α promotes primary breast cancer growth, vascularization and metastases to axillary lymph nodes and distant organs (61)	1. HIFs are responsible for the majority of the hypoxia-induced changes in gene expression (23, 62) 2. HIF-1α responds in transient manner to severe hypoxia with rapid stabilization and activation of target genes, whereas HIF-2α responds to moderate levels of hypoxia and accumulates over time (26)
MMPs (matrix metalloproteinase)	MMPs degrade many of the components of the ECM, which enables cancer cells to invade the surrounding tissues and intravasation	Hypoxia and HIF-1 up-regulate the expression levels and/or MMP-2 and MMP-9 (63, 64)
LOX, LOXL2, LOXL4 (lysyl oxidase)	ECM remodeling in the primary and the distant sites -> metastatic niche formation (65)	Produced by hypoxic breast cancer cells
Protein kinase C-δ, transcription factor STAT3, interleukin IL6, and NANOG	Essential for BCSC	As a linkage between obesity and breast cancer via hypoxia
PGC1 (peroxisome proliferator-activated receptorγcoactivator-1)	Prognostic and predictive marker, associated with more aggressive tumor characteristics and poorer outcomes (39)	An hypoxia factor with independent prognostic significance
Tif1γ (transcription intermediary factor 1γ)	TIF1γ contributes to breast cancer by controlling TGF-β/Smad signaling, leading to a TGF-β-induced EMT	An hypoxia factor with independent prognostic significance
TGF-β (transforming growth factor -β)	Tumor suppressive (early stages) and oncogenic (later stages, pro-metastatic and pro-EMT) effects	An hypoxia factor with independent prognostic significance
E-cadherin, SNAIL (zinc finger protein snail), ZEB1 (zinc finger E-box-binding homeobox 1), and TWIST	These factors are involved in the HIF-1 induced EMT (60)	Having some relationship with hypoxia induced EMT

activation of protein kinase C-δ, transcription factor STAT3, interleukines IL6, and NANOG. The 2 latter mediators are essential for production of BCSC, thus tumorigenesis and growth, as well as recurrence. Experiments *in vitro* showed that both a drug-related or genetic inhibition of A2BR expression or functionality lead to a decrease in BCSC enrichment, significantly reducing the tumor initiation and metastasis (68). These findings are fundamental to understand the known link between obesity and more aggressive breast cancer characteristics, as well as the higher risk of developing postmenopausal breast cancer.

HYPOXIA AND TREATMENT RESISTANCE

Hypoxia is known to directly or indirectly confer resistance to irradiation, some chemotherapeutic drugs, and endocrine therapy (24). Hypoxic tumors are less responsive to radiation therapy, mainly because the lack of oxygen causes DNA damage (77). Moreover, the responsiveness of malignancy to chemotherapeutic agents is modulated by reducing the susceptibility to DNA damage, inducing cell cycle arrest and limiting drug delivery under poor perfusion (10). The activation of ROS-shielding pathways (78) and overexpression of anti-apoptosis genes (79) mediated by hypoxia contribute to taxane resistance. As for ER-positive breast cancer patients, hypoxia has been shown to down-regulate ERα in several breast cancer cell lines and to influence the responsiveness to tamoxifen (38, 80). SNAT2, an amino acid transporter, was regulated by both ERα and HIF-1α (predominantly), leading to endocrine resistance under hypoxia (81).

While suppressing VEGF pathway initially decreases tumor progression rate and vasculature density, the activation of interrelated pathways and signaling molecules following VEGF blockade compensates the insufficiency of VEGF and the initially blocked angiogenesis, explaining part of the failure observed with bevacizumab monotherapy (82).

FUTURE MEASURING HYPOXIA IN THE BREAST

An innovative method was tested in Austria: the possibility of hypoxia measurement in the breast using magnetic resonance imaging (MRI). This opens up new avenues of research into hypoxia although at this stage MRI has clearly not

been established in the clinical approach. Besides measuring oxygen content, the MRI can also assess neovascularization in breast tumors. A significant benefit of this approach would be lower costs and greater availability compared to PET or near-infrared spectroscopy. Advanced quantitative blood oxygenation level dependent (qBOLD) imaging can directly quantify the tissue oxygen tension, while vascular architectural mapping (VAM) measures the microvascular vessel diameter and architecture (83). This approach looks potentially promising but further research is clearly required to validate any clinical utility.

GENE EXPRESSION AND HYPOXIA IN BREAST CANCER

The adaption to hypoxia is governed by multiple transcriptional and post-transcriptional changes in gene expression. Up to 1.5% of the human genome is estimated to be transcriptionally responsive to hypoxia (84). Recent years brought insights into various additional genes and pathways that have been identified as being responsive to hypoxia and which might serve as prognostic or predictive markers, and even as novel therapeutic targets. Clustering genes are chosen for their expression pattern (85–87). Since increased activity of the HIF-1a pathway is related to a more profound intratumoral hypoxia in basal-like breast tumors compared with other subtypes, gene signatures might guide treatment decisions for potential application of anti-hypoxic drugs in the future (88).

Gene signatures reflect hypoxic response at a transcriptional level, whereas microRNAs regulate it at a post-transcriptional level. Comparative analysis of hypoxia-regulated miRNAs by gene expression profiles might add additional information to target-prediction algorithms (89). Despite rapid development, this area still needs further clinical validations.

ANTI-HYPOXIC TREATMENT

HIF-1α promotes primary breast cancer growth, vascularization and metastases to axillary lymph nodes and distant organs (8). Increased HIF-1 expression shows strong correlations with poor prognostic outcomes and low survival rates of breast cancer patients (90). Therefore, targeting the HIF pathway might provide an attractive strategy to treat hypoxic tumors. Agents that inhibit HIF-1α protein accumulation and demonstrate anti-tumor effects include the topoisomerase I inhibitor, topotecan, as well as the cardiac glycoside digoxin (65, 91–93).

Since HIF-1α can be induced by hypoxia-independent signaling pathways, such as motor, ERBB2 (HER2) and MAP kinase, the therapeutic benefits of targeting these pathways may also be partially explained by a decrease in HIF-1α levels (71). Especially triple-negative breast cancers (TNBCs) have a high HIF transcriptional activity and respond poorly to currently available therapies (94). Therefore, HIF inhibitors may be particularly useful in the treatment of TNBCs. Pre-clinical studies suggest that the combination of cytotoxic chemotherapy with drugs that inhibit hypoxia-inducible factors are very promising in this group of patients (8). HIF-1 inhibitors, such as digoxin and acriflavine, showed convincing potential therapeutic effects by decreasing primary tumor growth, vascularization, invasion and metastasis in breast cancer animal models (65, 92, 95). Adding digoxin to paclitaxel or gemcitabine leads to tumor regression in TNBC by blocking HIF-dependent transcriptional responses that promote the resistance of CSCs to chemotherapy (96).

Another aspect might be hypoxia-based treatments, where a synergetic effect with drugs that cause treatment-induced hypoxia, e.g., bevacizumab, is applied (8). Despite compelling evidence-linking hypoxia with treatment resistance and adverse prognosis, the activity of hypoxia-activated drugs also depends on the coincidence of tumor hypoxia, expression of specific prodrug-activating reductases and intrinsic sensitivity of malignant clones to the cytotoxic effector (97). Hypoxia-based drugs have been tested in clinical trials to further validate their efficacy in cancer treatment. However, the failure of 2 major clinical trial efforts (tirapazamine and evofosfamide) calls for further research (97). Hypoxia itself is highly variable between and within individual tumors and is not consistent among all breast cancer subtypes.

In the era of personalized precision medicine, clinical trials are warranted to determine whether anti-hypoxia drugs may increase the survival of breast cancer patients alone or in combination with current therapeutic regimens. It is pivotal to explore the decisive influence of hypoxia on the course of breast cancer in order to gain a deeper understanding of individual disease trajectories and to better forecast them. This knowledge can then be used in the future to develop and implement adequate therapy for each individual patient.

AUTHOR CONTRIBUTIONS

YZ, HZ, MW, TS, ZX, LK, W-KY, YH, FC, and EB contributed to the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The present study was supported by the Shanghai Yangpu District Health and Family Planning Commission Fund for Hao Yi Shi Training Project (Grant nos. 201742, 2020-2023), the Natural Science Foundation of Shanghai (Grant no. 18ZR1436000). Evelyne Bischof (Ewelina Biskup) was supported by Krebsliga Schweiz, BIL KFS 4261-08-2017. EB was currently enrolled in Cardiopath Ph.D program.

ACKNOWLEDGMENTS

We would like to acknowledge Prof. John Robertson of the University of Nottingham for the critical reading of the manuscript and valuable suggestions also thank all of the participants for their participation.

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Conflict of Interest: W-KY and YH were employed by the company Cellomics International Limited.

The remaining 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.

frontiers in Oncology

REVIEW
published: 18 March 2021
doi: 10.3389/fonc.2021.629614

Potential Mechanism Underlying the Role of Mitochondria in Breast Cancer Drug Resistance and Its Related Treatment Prospects

*Yuefeng Li and Zhian Li**

Department of Oncological Surgery, Shaoxing Second Hospital, Shaoxing, China

Breast cancer incidence and mortality rates have been consistently high among women. The use of diverse therapeutic strategies, including chemotherapy, endocrine therapy, targeted therapy, and immunotherapy, has improved breast cancer prognosis. However, drug resistance has become a tremendous obstacle in overcoming breast cancer recurrence and metastasis. It is known that mitochondria play an important role in carcinoma cell growth, invasion and apoptosis. Recent studies have explored the involvement of mitochondrial metabolism in breast cancer prognosis. Here, we will provide an overview of studies that investigated mitochondrial metabolism pathways in breast cancer treatment resistance, and discuss the application prospects of agents targeting mitochondrial pathways against drug-resistant breast cancer.

Keywords: breast cancer, mitochondrial, drug resistance, chemoresistance, tumor microenvironment

OPEN ACCESS

Edited by:
Zheng Wang,
Shanghai Jiao Tong University, China

Reviewed by:
Zhichao Wang,
Shanghai Jiao Tong University, China
Wenjuan Wang,
Children's Hospital of Soochow University, China

***Correspondence:**
Zhian Li
sxeylza@163.com

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *15 November 2020*
Accepted: *03 March 2021*
Published: *18 March 2021*

Citation:
Li Y and Li Z (2021) Potential Mechanism Underlying the Role of Mitochondria in Breast Cancer Drug Resistance and Its Related Treatment Prospects. Front. Oncol. 11:629614. doi: 10.3389/fonc.2021.629614

INTRODUCTION

Breast cancer is the second most common cancer in the world and ranks first in cancer incidence in women (1). Its diagnosis rate is increasing year by year, accompanied by a long-term high mortality rate (2). Early breast cancer is usually effectively treated using surgery alone or in combination with adjuvant radiotherapy. However, most patients with advanced breast cancer undergo mastectomy combined with radiotherapy and/or chemotherapy. Notably, the growing popularity of hormone therapy and targeted drug therapy in the treatment of breast cancer has greatly improved its five-year survival rate (3). Although breast cancer treatment methods have progressively diversified, the treatment of breast cancer, especially of triple-negative breast cancer(TNBC), a highly heterogeneous tumor, remains challenging (4), primarily due to chemotherapy resistance (5). Various recent studies have focused on discovering chemotherapy targets and the mechanisms underlying chemotherapy resistance to improve breast cancer prognosis. Furthermore, molecules, such as neuropilin-1 and follistatin-like 1,are considered to be involved in breast cancer resistance to doxorubicin. Nevertheless, the role of these new molecules in specific clinical applications requires further exploration. Therefore, there is an urgent need to explore more effective methods (6, 7).

Recently, the role of mitochondria in cancer has attracted increasing attention. It is well known that mitochondria play an important role in tumor cell occurrence, proliferation and apoptosis. Interestingly, recent studies have shown that tumor chemoresistance is closely related to mitochondria (8). Studies have demonstrated that mitochondrial fission is regulated by dynamin-

related protein 1 (Drp-1) and mediated by high-mobility group box 1 protein (HMGB1), a chemotherapy-induced colon cancer product, promoting colorectal cancer tumor chemoresistance (9). Atovaquone, an antiparasitic drug, can effectively block mitochondrial respiration at clinically relevant concentrations, and further increase hepatocellular carcinoma chemosensitivity (10). These studies have laid the foundation for finding the treatments for chemoresistant cancers. In recent years, the role of mitochondria in breast cancer has received substantial attention (11, 12). However, the relationship between mitochondria and chemoresistance in breast cancer has not been systematically examined. This review focuses on studies that investigated the role of mitochondria in breast cancer chemoresistance and discusses the mechanisms and relevant treatment prospects.

Mitochondrial in Tumor Metabolism and Breast Cancer Drug Resistance

In the human body, tumor cells are found in a dynamic microenvironment, composed of complex stromal cells and extracellular matrix (13), in which mitochondria play an essential role. The original Warburg effect suggests that mitochondrial defects in tumor cells lead to impaired aerobic respiration, which renders tumor cells more prone to aerobic glycolytic metabolism (14, 15). It has been proposed that the changes in tumor cell metabolism are caused by the expression of oncogenes and hypoxia-related signal molecules that upregulate glycolytic enzymes. At the same time hypoxia inducible factor (HIF)-induced pyruvate dehydrogenase kinase (PDK) inhibits the PDH complex, and Akt, an oncogene, mediates the transcription of Glucose transporter type 1 (GLUT1) promoting the binding of hexokinase 2 to the voltage-dependent anion channels (VDAC) on the outer mitochondrial membrane to induce aerobic glycolysis. The synergistic effects of these pathways and the increase of mitochondrial autophagy lead to the glycolytic phenotype of tumor cells (16, 17). On the other hand, a series of studies on the "reverse" Warburg effect revealed that cancer-associated fibroblasts in the tumor microenvironment can change their phenotype through mitochondrial dysfunction and aerobic glycolysis, thereby providing high-energy nutrients to tumor epithelial cells promoting tumor growth, metastasis and chemoresistance (15, 18, 19). Furthermore, Sotgia, F et al. found that 15 molecular markers related to mitochondrial germination and translation were differentially expressed in the tumor microenvironment using an analysis of genome-wide transcription profile data of human breast cancer cells and immunohistochemical verification. In addition, the important role of mitochondria in the metabolic symbiosis between tumor epithelial cancer cells and their surrounding stroma, suggests that the targeting mitochondrial gene expression and translation may be a new treatment approach for breast cancer (20).

The complex metabolic network of the tumor microenvironment is regulated by a variety of molecules, many of which have been found to participate in mitochondria-related metabolic pathways (21, 22). Caveolin-1(CAV-1) is a major structural protein in small plasma membrane invaginations that maintains membrane stability and signal transduction (23). Clinical studies have shown that CAV-1 is an important predictor of breast cancer prognosis (24, 25). Furthermore, it has been found that a decrease in CAV-1 levels in stromal cells in the tumor microenvironment enhances breast cancer resistance to tamoxifen (24). Moreover, in a study using mouse xenograft models, it was found that CAV-1 expression was positively correlated with the tumor sensitivity to nab-paclitaxel (26). CAV-1 downregulation in tumor-associated stromal fibroblasts could increase reactive oxygen species (ROS) production, thus inducing oxidative stress followed by autophagy and mitochondrial dysfunction. The outcome of this metabolic change will promote mitochondrial metabolism using high-energy substances, such as L-lactic acid and ketone bodies to provide nourishment to epithelial cancer cells. The mitochondrial-targeted superoxide dismutase 2(SOD2) is a potential inhibitor that can resist to oxidative stress and block the production of molecules that can nourish tumors, thereby effectively reversing the tumor-promoting phenotype of CAV-1 in breast cancer cells (27). Monocarboxylate transporter 4 (MCT4), an independent prognostic factor for breast cancer survival, was found to be negatively correlated with the expression of CAV-1 (28, 29). Thus, the combined analysis of MCT4 and CAV-1 expression levels in the matrix can improve the accuracy of breast cancer prognosis. The antioxidant N-acetyl-cysteine has been shown to inhibit the oxidative stress-induced formation of MCT4. Furthermore, MCT4 inhibitors can effectively inhibit the influx of L-lactic acid and ketone bodies into the tumor microenvironment, constituting a novel strategy for tumor treatment. However, MCT1 inhibitors are currently considered to have similar efficacy to that of MCT4 and are employed in the clinical studies (29, 30). Corresponding to these findings, CAV-1 was found to be overexpressed in drug-resistant breast cancer cells (31). Astragaloside IV (AS-IV), a biologically active substance purified from Astragalus, can work synergistically with paclitaxel to trigger the mitochondrial apoptosis pathway and effectively induce drug-resistant breast cancer cell death. This process involves AS-IV activation of eNOS/NO/$ONOO^-$ signaling by CAV-1 inhibition that enhances the chemosensitivity of breast cancer cells to paclitaxel (32) (**Figure 1**). Intriguingly, the expression level of CAV-1 in TNBC has also been found to be negatively correlated with cancer cell radiation sensitivity (33).

Mitochondria in Tumor Apoptosis and Breast Cancer Drug Resistance

B-cell lymphoma 2 (BCL-2) family proteins play a crucial role in the process of regulating cell apoptosis and have both pro-apoptotic and anti-apoptotic activities (34). Typical pro- and anti-apoptotic members are BAX, BCL2, and BCL-XL, respectively (35, 36). Pro-apoptotic proteins such as BAX can promote the release of cytochrome C and second mitochondria-derived activator of caspases (Smac) from mitochondria, leading to cysteinyl aspartate specific proteinase (caspase)-induced cell apoptosis. BCL-2 and BCL-XL inhibit the pro-apoptotic effects of BAX and other molecules (37). In this way, the anti-apoptotic BCL-2 family proteins help breast tumor cells escape apoptosis and acquire drug resistance (38). Thus, inhibiting anti-apoptotic BCL-2 family proteins is a potentially valuable therapeutic strategy against breast cancer drug resistance. Studies have

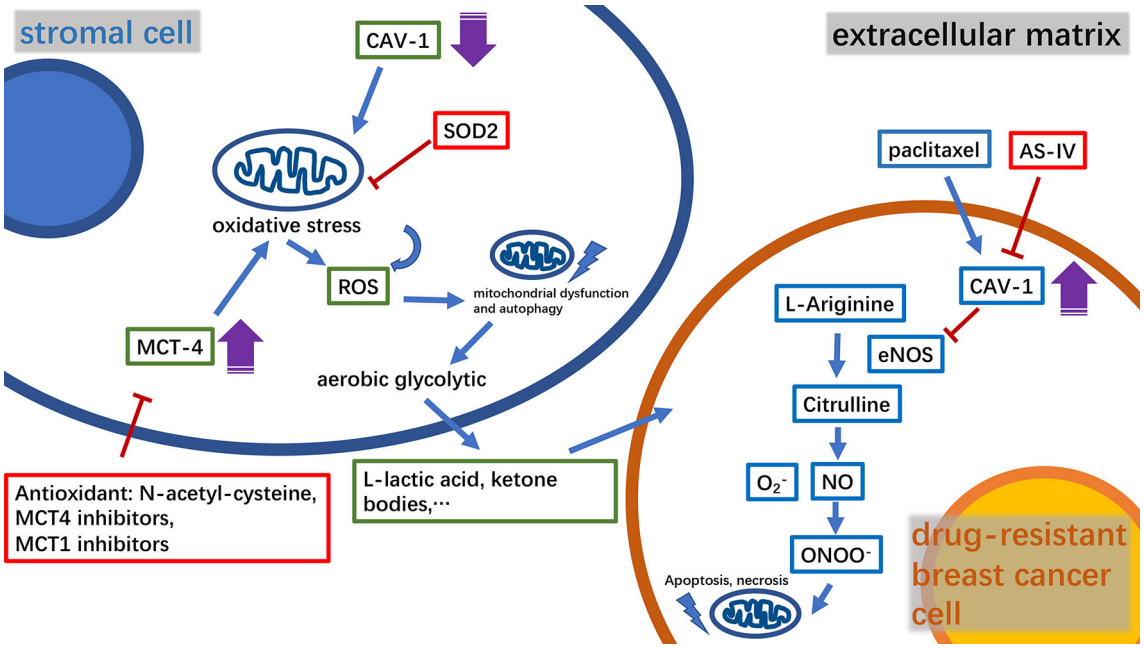

FIGURE 1 | A diagram of the relationship between key molecules in the microenvironment of drug-resistant breast cancer tumors and mitochondria, and the mechanism of the relevant drugs. CAV-1, caveolin-1; MCT4, monocarboxylate transporter 4; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; AS-IV, astragaloside IV; NO, nitric oxide.

found that microRNA-195 can target BCL-2 to trigger mitochondrial dysfunction and then cause apoptosis, thereby enhancing the therapeutic efficacy of the chemotherapy drug etoposide in breast cancer (39). Via another mechanism, Sabutoclax (BI-97C1), a BCL-2 homology domain 3 (BH3) mimetic, acts as a pan-BCL-2 inhibitor (40). Sabutoclax has shown potent cytotoxicity against drug-resistant breast cancer *in vivo* and *in vitro*. Furthermore, Sabutoclax not only causes the release of caspase from mitochondria to cause cancer cell apoptosis, but also blocks the interleukin 6/signal transducers and activators of transcription (IL-6/STAT) pathway to eliminate the breast cancer stem cells. Interestingly, it has also been successfully used in combination with standard chemotherapy to treat chemoresistant breast cancer (41). Consistent with their principle of action, drugs, such as ABT-737, ABT-263 (Navitoclax) and α-tocopheryl succinate (α-TOS), target BCL-2 in the mitochondria (**Figure 2**). Among them, ABT-737 has been confirmed to improve the docetaxel resistance in TNBC cell lines overexpressing BCL-2 using cytology experiments. Furthermore, using a new technology the drug is encapsulated in poly lactic-co-glycolic acid nanoparticles (NPs) to accumulate drugs in xenograft TNBC tumors and exert effective anti-tumor effects, thus providing a good foundation for successful drug targeting to human breast tumors in the future to avoid systemic side effects (42, 43). It is worth mentioning that Navitoclax has passed phase I of a clinical trial in refractory chronic lymphocytic leukemia, and the optimal drug concentration has also been further explored in a phase II clinical trial. In breast cancer treatment, Navitoclax has been proven to enhance the effectiveness of epidermal growth factor receptor (EGFR)-targeted antibody-drug conjugates for TNBC treatment in animal experiments. In addition, recent studies have also revealed the inherent drug resistance of TNBC to Navitoclax. Therefore, it is necessary to evaluate the specific value of the drug in conjunction with TNBC genomic research (44–46). The combination of α-TOS and high-dose tamoxifen can effectively inhibit the proliferation activity of TNBC, and improve the anti-cancer effect of pterostilbene in breast cancer xenograft mice. In a recent study, pluronic polymer (P123) was modified into ortho ester End-capping (P123-OE) and bridged with a-TOS to form a copolymer (POT), and then doxorubicin-loaded POT micelles (POT-DOX) were used in breast cancer animal models, which effectively increased the accumulation of drugs in multi-drug-resistant breast cancer cells and enhanced the drug's anti-cancer effects, potentially providing alternative clinical treatment options (47–49). Myeloid cell leukemia-1 (MCL1) is another typical anti-apoptotic protein belonging to the BCL-2 family (50). The myelocytomatosis oncogene (*MYC*), a proto-oncogene, encodes a transcription factor involved in cancer cell proliferation and apoptosis (51, 52). The mRNA and protein levels of these two molecules were co-amplified in paclitaxel-resistant breast cancer cell lines. MYC and MCL-1 mediate the enrichment of breast cancer stem cells(CSCs) (53, 54). Among them, MCL2 has also been confirmed to be located in the mitochondrial matrix to enhance mitochondrial oxidative phosphorylation (mtOXPHOS), which in turn increases the ROS generation, activates hypoxia stress, and causes drug resistance-mediated

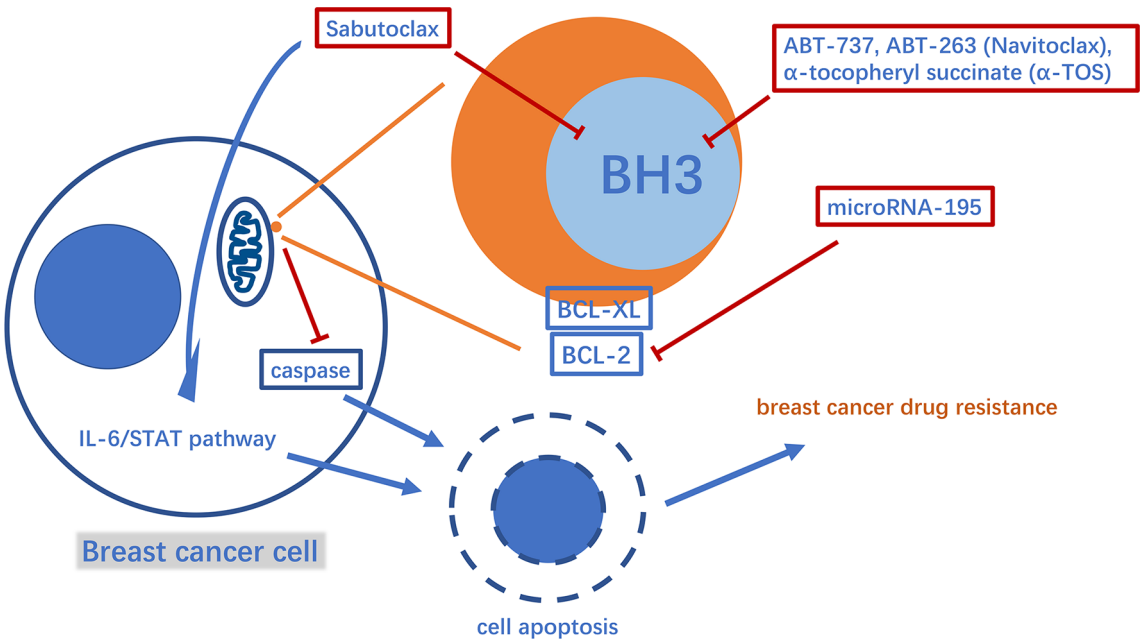

FIGURE 2 | A diagram of some mechanism of mitochondria involved in apoptosis-mediated breast cancer drug resistance and related drugs. BH3, BCL-2 homology domain 3.

CSC enrichment (53, 55). Therefore, drugs that inhibit HIF-1a, such as N-acetylcysteine, oligomycin and digoxin, may provide direction in the treatment of drug-resistant breast cancer (56–58).

Mitochondrial Dynamics and Breast Cancer Drug Resistance

Mitochondria are highly dynamic organelles that change their shape, size, and distribution to adapt to changes in different cell states *via* the coordinate action of fission and fusion (59). Dynamin-related protein 1 (Drp1), a classical mitochondrial fission protein, can cause mitochondria to divide into two to form a small circular mitochondrial fragment network. Mitochondrial fusion protein (MFN) and optic atrophy 1(OPA1), can elongate or cluster together through fusion (59–61). A study of tamoxifen-resistant breast cell lines found that the drug-resistant cell lines have a more fragmented mitochondrial network. In these cell lines, serine 637, an essential phosphorylation site of DRP1, was activated, while serine 616, another vital phosphorylation site, was not, which increased the mitochondrial fission activity of DRP1, causing mitochondrial fragmentation. Based on this, changes in mitochondrial dynamics that are closely related to breast cancer drug-resistance can be improved (62). Current research on drugs targeting mitochondrial dynamics is mostly focused on breast cancer growth and proliferation rather than breast cancer drug resistance (63–65). Mitochondrial transplantation is a new type of biological technology that gradually extends from animal models to human clinical applications (66). Using this technology, exogenous healthy mitochondria are transplanted into cells with damaged mitochondria to achieve the treatment purpose (67). A study on mitochondrial transplantation in breast cancer cell lines found that the protein levels of MFN2 and OPA1 in the cells significantly increased after the transplantation of exogenous healthy mitochondria into breast cancer cells, while the protein level of drp1 dramatically decreased. Interestingly, the morphology of the mitochondria in the cell was mostly elongated to tubular, while the fragmented mitochondria were obviously inhibited. In addition, the resistance of breast cancer cells to the anticancer drugs doxorubicin and paclitaxel was also significantly reduced (68). Recent studies have also suggested that mitochondrial transplantation can change the tumor microenvironment to combat breast cancer, and demonstrated the therapeutic effect of mitochondrial transplantation on breast cancer in animal models (69). In these studies, we observed that while mitochondrial transplantation brings about changes in mitochondrial morphology, it also regulates functions, such as oxidative respiration. The morphology and function of mitochondria are inextricably linked (68, 69). Mitochondrial transplantation is a new approach for the treatment of drug-resistant breast cancer.

Mitochondrial DNA and Breast Cancer Drug-Resistance

Mitochondrial DNA(mtDNA), a double-stranded circular DNA, contains 37 genes encoding rRNA, tRNA, and oxidative phosphorylation complex-related proteins (70). A variety of treatments that target mitochondrial gene expression have been explored in a variety of diseases, including breast cancer, with proven therapeutic benefits (71). The link between mitochondrial gene copy number and breast cancer treatment resistance has attracted increasing attention (72, 73). Metformin

can stunt breast cancer progress by inhibiting complex I encoded by the electron transport chain gene in mtDNA (74, 75). The decrease in BTB and CNC homology 1(BACH1), a hemin-binding transcription factor, can promote the expression of the electron transport chain(ETC) genes to increase the sensitivity of breast cancer to metformin. The specific degradation of BACH1 by panhematin (an FDA-approved drug) can effectively enhance the sensitivity of breast cancer cells to metformin treatment *in vitro* and *in vivo* (76). Not long ago, another extraordinary study found that cancer-related fibroblast-derived exosomes with complete genomic mitochondrial DNA can be obtained by breast stem cell-like cancer cells and display mtOXPHOS-dependent breast cancer endocrine-resistant therapy (77). Hitherto, drugs that target mtDNA, such as vitamin K3 (menadione), are often used in combination with other drugs in the treatment of breast cancer (78, 79). However, whether they improve resistance to breast cancer treatment remains unclear. This is worth exploring in future studies.

CONCLUSIONS

The problem of drug resistance in the comprehensive treatment model of breast cancer has been extensively investigated, and mitochondria have been found to play a subtle role in the process of breast cancer drug resistance. Exploration of the role of mitochondria in breast cancer drug resistance in the tumor microenvironment and cancer cell interior indicates that: 1) numerous molecules in the tumor microenvironment can mediate the production of a variety of metabolites to induce drug resistance through the action of mitochondria, 2) mitochondria can regulate cell apoptosis and affect breast cancer resistance, 3) morphological and functional changes in the mitochondria can promote breast cancer resistance, and 4) the expression level of mtDNA can mediate breast cancer resistance. These studies have proposed the use of effective molecular targeted drugs or new treatments to sensitize breast cancer cells to drugs, and some drugs are already used in clinical research. Exploring the role of mitochondria in breast cancer chemoresistance is expected to open up novel ways for breast cancer treatment.

AUTHOR CONTRIBUTIONS

YL: literature search, writing, and editing of the manuscript. ZL: Analysis, design, writing-review and edit, and supervision. All authors contributed to the article and approved the submitted version.

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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.

frontiers in Oncology

REVIEW
published: 25 March 2021
doi: 10.3389/fonc.2021.629666

Metabolic Syndrome and Breast Cancer: Prevalence, Treatment Response, and Prognosis

Shuwen Dong[†], *Zheng Wang*[†], *Kunwei Shen*[*] *and Xiaosong Chen*[*]

Department of General Surgery, Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Metabolic syndrome is a type of multifactorial metabolic disease with the presence of at least three factors: obesity, diabetes mellitus, low high-density lipoprotein, hypertriglyceridemia, and hypertension. Recent studies have shown that metabolic syndrome and its related components exert a significant impact on the initiation, progression, treatment response, and prognosis of breast cancer. Metabolic abnormalities not only increase the disease risk and aggravate tumor progression but also lead to unfavorable treatment responses and more treatment side effects. Moreover, biochemical reactions caused by the imbalance of these metabolic components affect both the host general state and organ-specific tumor microenvironment, resulting in increased rates of recurrence and mortality. Therefore, this review discusses the recent advances in the association of metabolic syndrome and breast cancer, providing potential novel therapeutic targets and intervention strategies to improve breast cancer outcome.

Keywords: breast cancer, metabolic syndrome, obesity, incidence, treatment response, prognosis

OPEN ACCESS

Edited by:
Carmine De Angelis,
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Reviewed by:
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***Correspondence:**
Kunwei Shen
kwshen@medmail.com.cn
Xiaosong Chen
chenxiaosong0156@hotmail.com

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *15 November 2020*
Accepted: *11 March 2021*
Published: *25 March 2021*

Citation:
Dong S, Wang Z, Shen K and Chen X (2021) Metabolic Syndrome and Breast Cancer: Prevalence, Treatment Response, and Prognosis. Front. Oncol. 11:629666. doi: 10.3389/fonc.2021.629666

INTRODUCTION

Breast cancer is a malignant tumor with the highest incidence in women of all ages in the world and is associated not only with hormones or factors related to reproduction but also with environmental factors in general (1). Epidemiological studies have shown that early menarche, postmenopausal weight gain, a high-fat diet, and long-term use of exogenous estrogen are associated with a high risk of breast cancer (2). Recent studies have also shown that a specific lifestyle characterized by reduced physical activity and fat-rich dietary habits, refined carbohydrates and animal protein, which consequently causes metabolic syndrome (MetS), plays a crucial role in breast cancer initiation (1, 3). Metabolic syndrome, also known as insulin resistance syndrome or syndrome X, is a type of multifactorial metabolic disease. The definition of MetS takes into account the presence of at least three factors, namely, abdominal obesity/high body mass index (BMI), insulin resistance, hypertension, hypertriglyceridemia, and low high-density lipoprotein (HDL) (3–5). The prevalence of obesity has increased rapidly in recent years with the number of overweight/obese people almost doubling since the 1980s, representing one-third of the world's population, and the proportion may reach 57.8 percent by 2030 (6, 7). In Western countries, the prevalence of MetS in the adult population is between 20% and 25% (8). Notably, the incidence rate increases significantly with age, resulting in the prevalence of people aged over 50 reaching 40-45% (8). Moreover, changes in the balance between insulinotropic and anti-inflammatory cytokines driven by abdominal obesity

may lead to insulin resistance, which is a core component of MetS. Asian women are particularly vulnerable to these diseases because they have greater abdominal and visceral fat than white women with similar BMI (9). Previous studies have confirmed that elderly and postmenopausal women are more susceptible to MetS (3). Several cohort studies and meta-analyses have also highlighted the link between MetS and its components and the prevalence, recurrence, and mortality of various cancers, including breast cancer (4, 10, 11).

MetS has become a significant public health problem worldwide, and in-depth research on MetS and breast cancer is increasing. However, there is a paucity of systematic reviews focusing on breast cancer and metabolic syndrome and a comprehensive understanding of this topic. Thus, in this review, we will systematically discuss the latest research advances on MetS and their associations with the incidence, treatment response, prognosis and progression mechanism of breast cancer, which will help provide new therapeutic targets and strategies to improve the prognosis of breast cancer patients.

METABOLIC SYNDROME AND BREAST CANCER RISK

A population-based study by Russo et al. (12) found that MetS and its components are associated with an increased risk of breast cancer. This connection has been proven in many other studies (13, 14), and it is more pronounced in postmenopausal women regardless of race (odds ratio [OR] = 1.75, 95% confidence interval [CI] 1.37-2.22) (15). With the increase in the number of MetS components, the risk of breast cancer increases for postmenopausal women (P = 0.01) (16). Factors related to the risk of breast cancer are discussed in the following four sections (**Figure 1**). **Table 1** summarizes reports about the correlation between metabolic syndrome and its components and the risk of breast cancer with different subtypes.

Obesity and Breast Cancer Risk

Obesity is associated with the risk of postmenopausal breast cancer (26, 27) as well as greater tumor burden and higher

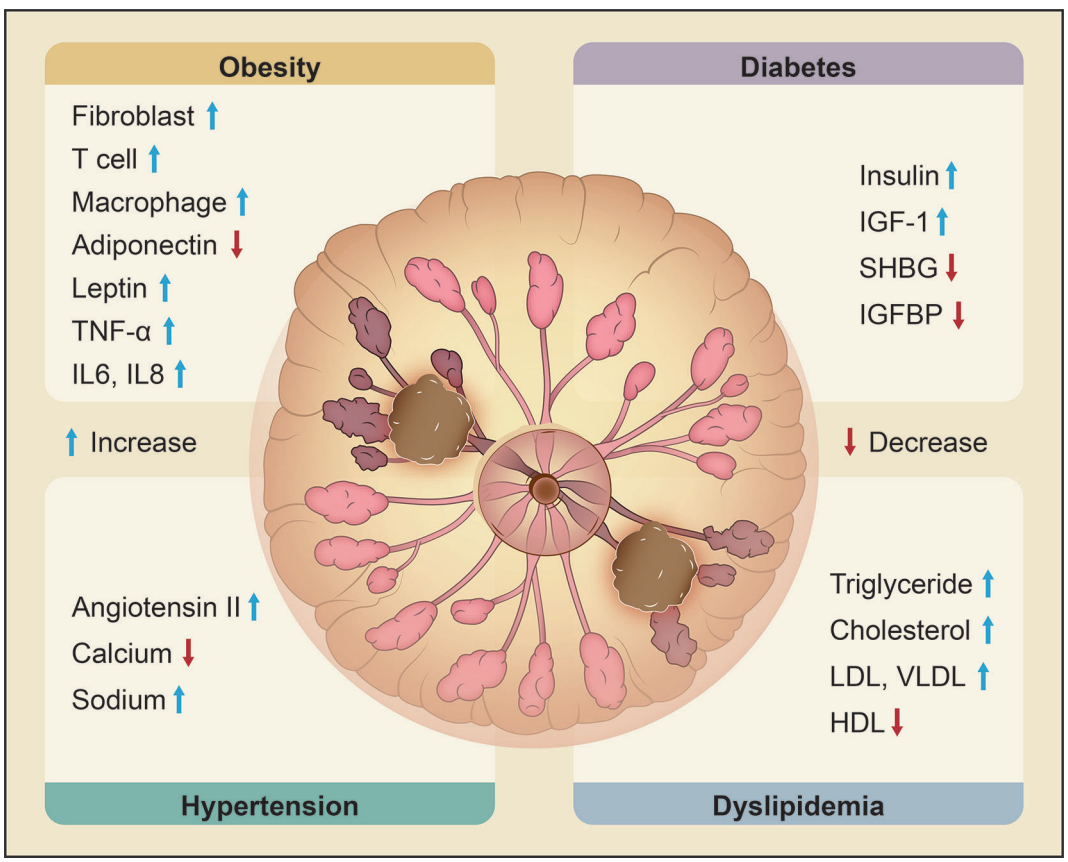

FIGURE 1 | Metabolic syndrome and its molecular changes related to the risk of breast cancer. Obesity, diabetes, hypertension and dyslipidemia are the main components of metabolic syndrome and are all significantly related to the risk of breast cancer. Obesity increases fibroblasts, T cells, macrophages, leptin, TNF-α, IL-6 and IL-8 and decreases adiponectin. Diabetes is characterized by upregulation of insulin and IGF-1 and downregulation of SHBG and IGFBP. Hypertension is associated with increased ANG II and sodium and decreased calcium. Dyslipidemia leads to high levels of TG, TC, LDL, and VLDL and low levels of HDL. Changes in the expression of these key molecules are correlated with an elevated risk of breast cancer. ('the blue arrow' means increase and 'the red arrow' means decrease).

TABLE 1 | Obesity and its measurement indexes with the risk of different breast cancer subtypes.

No.	Author	Patients(N)	Region	Menopausal status	Comparison	Molecular subtype	Measurement	95%CI	*P*	Ref
1	Agresti R. et al.	1779	Italy	Pre-menopausal	BMI≥25 vs. BMI<25	LuminalB(HER2-)	OR=1.30	0.79-2.18	N/A	(13)
						LuminalB(HER2+)	OR=1.78	0.88-3.63	N/A	
						HER2+(non-luminal)	OR=1.89	0.78-4.60	N/A	
						TNBC	OR=3.04	1.43-6.43	N/A	
					WC≥80cm vs. WC<80cm	LuminalB(HER2-)	OR=2.55	1.53-4.24	N/A	
						LuminalB(HER2+)	OR=2.11	1.03-4.35	N/A	
						HER2+(non-luminal)	OR=1.28	0.50-3.27	N/A	
						TNBC	OR=1.03	0.42-2.53	N/A	
				Post-menopausal	BMI≥25 vs. BMI<25	LuminalB(HER2-)	OR=1.51	1.14-2.00	N/A	
						LuminalB(HER2+)	OR=1.20	0.76-1.92	N/A	
						HER2+(non-luminal)	OR=1.43	0.79-2.57	N/A	
						TNBC	OR=1.11	0.65-1.90	N/A	
					WC≥80cm vs. WC<80cm	LuminalB(HER2-)	OR=1.17	0.75-1.81	N/A	
						LuminalB(HER2+)	OR=0.82	0.41-1.63	N/A	
						HER2+(non-luminal)	OR=1.36	0.50-3.69	N/A	
						TNBC	OR=0.89	0.41-1.95	N/A	
2	Chen H. et al.	4557	USA	Both	Type 2 DM vs. non-DM	ER+/HER2+	OR=0.77	0.40-1.48	<0.05	(17)
						TNBC	OR=1.38	1.01-1.89	<0.05	
						H2E	OR=1.38	0.93-2.06	<0.05	
3	Maskarinec G. et al.	681	USA	Both	Type 2 DM in subtypes	ER+/PR+	HR=1.17	1.05-1.29	N/A	(18)
						ER-/PR-	HR=1.03	0.83-1.26	N/A	
						ER+/PR- or ER-/PR+	HR=1.01	0.81-1.28	N/A	
4	Michels K.B. et al.	116488	USA	Both	Type 2 DM vs. non-DM	ER+	HR=1.22	1.01-1.47	N/A	(19)
						ER-	HR=1.13	0.79-1.62	N/A	
5	Millikan R.C. et al.	3446	USA	Pre-menopausal	WHR≥0.84 vs. WHR<0.84	LuminalB	OR=0.90	0.40-1.80	N/A	(20)
						HER2+(non-luminal)	OR=0.90	0.40-2.20	N/A	
						TNBC	OR=1.90	1.00-3.60	N/A	
				Post-menopausal	WHR≥0.84 vs. WHR<0.84	LuminalB	OR=0.50	0.20-0.90	N/A	
						HER2+(non-luminal)	OR=0.50	0.30-1.00	N/A	
						TNBC	OR=1.40	0.70-2.70	N/A	
6	Munsell M.F. et al.	/	/	Pre-menopausal	BMI≥30 vs. BMI<25	ER+/PR+	RR=0.78	0.67-0.92	0.67	(21)
						ER-/PR-	RR=1.06	0.70-1.60	0.004	
				Post-menopausal	BMI≥30 vs. BMI<25	ER+/PR+	RR=1.39	1.14-1.70	0.001	
						ER-/PR-	RR=0.98	0.78-1.22	0.02	
7	Palmer J.R. et al.	1851	USA	Both	Type 2 DM vs. non-DM	ER+	HR=1.02	0.80-1.31	N/A	(22)
						ER-	HR=1.43	1.03-2.00	N/A	
8	Pierobon M. et al.	3845	USA	Pre-menopausal	BMI≥30 vs. BMI<30	TNBC	OR=1.43	1.23-1.65	N/A	(23)
9	Rosner B. et al.	77232	USA	Pre-menopausal	Per 25 lbs weight gain	ER+/PR+	RR=1.13	0.89-1.43	0.32	(24)
						ER+/PR-	RR=2.19	1.33-3.61	0.002	
						ER-/PR-	RR=1.61	1.09-2.38	0.016	
10	Suzuki R. et al.	41594	Japan	Post-menopausal	Per increment of 5kg/m2	ER+/PR+	RR=2.24	1.50-3.34	N/A	(25)
						ER+/PR-	RR=0.63	0.31-1.27	N/A	
						ER-/PR-	RR=0.67	0.38-1.17	N/A	

BMI, Body mass index; ER, Estrogen receptor; HER2, Human epidermal growth factor 2; N/A, Not applicable; OR, Odds ratio; PR, Progesterone receptor; RR, Relative risk; TNBC, Triple negative breast cancer; WC, Waist circumference.

histopathological grade (28). BMI is routinely used to measure obesity but lacks information about actual body composition. Waist circumference (WC) and waist-to-hip ratio (WHR) are more commonly adopted for evaluating body fat distribution. Compared with patients with BMI < 25 kg/m^2, patients with BMI ≥ 30 kg/m^2 have larger tumors, poorer differentiation, a higher frequency of lymph node invasion, and more advanced disease (29). According to The International Agency for Research on Cancer, ample evidence suggests that increasing weight gain is a risk factor for postmenopausal breast cancer (30). A small case-control study by Schapira et al. (31) found that breast cancer patients had 45% more visceral fat tissue than the control group. In addition, epidemiological studies have shown that WC/WHR increases are related to breast cancer (10, 32, 33). However, some studies have also reported that obesity may have a protective effect on the risk of premenopausal breast cancer (8, 34), which requires further evidence.

Given that excessive estrogen stimulates breast tissue proliferation (35), the increase in estrogen levels in the body caused by obesity is considered to be one of the mechanisms associated with breast cancer because adipose tissue is the main source of estrogen in postmenopausal women (34). Chronic inflammation caused by the imbalance of fat homeostasis due to obesity also promotes the development of tumors. Aromatase is a kind of cytochrome P450 enzyme mainly located in the adipose tissue of breast, abdomen, thigh and buttocks, but it may also be present in tumor tissue. It can catalyze the formation of estrone and estradiol from androstenedione and testosterone (34).

Obese adipocytes upregulate the expression of pro-inflammatory factors such as TNF-α and IL-6 through the obesity-inflammation-aromatase axis (36), resulting in enhanced transcription of CYP19 gene encoding aromatase, thereby promoting the production of aromatase. Adipose tissue not only stores adipocytes, it is also an endocrine organ producing biologically active molecules called adipokines. These adipokines bind to specific receptors on the surface of target cells and affect the metabolism of tissues and organs (37). Among the adipokines, leptin increases the risk of disease (38), whereas adiponectin may have a protective effect. Some case-control studies have indicated that low adiponectin levels are associated with increased breast cancer risk and a more aggressive phenotype (39, 40).

For premenopausal women, obesity has a protective effect on hormone receptor-positive breast cancer but increases the risk of estrogen receptor (ER)+/progesterone receptor (PR)- and ER-/PR- breast cancer (24). A meta-analysis conducted by Munsell et al. (21) indicated a summary risk ratio (RR) of 0.78 (95% CI 0.67-0.92) for hormone receptor-positive breast cancer and 1.06 (95% CI 0.70-1.60) for hormone receptor-negative breast cancer in premenopausal women associated with obesity. A positive correlation is noted between obesity and triple-negative breast cancer (TNBC) (23). A multiple logistic regression analysis of 1,779 patients with primary invasive breast cancer in Italy showed that premenopausal women with WC ≥ 80 cm were prone to luminal B breast cancer, including HER2-negative (OR = 2.55, 95% CI 1.53-4.24) and HER2-positive women (OR = 2.11, 95% CI 1.03-4.35), whereas women with BMI ≥ 25 kg/m^2 (OR = 3.04, 95% CI 1.43-6.43) were significantly related to TNBC compared with other subtypes (13).

For postmenopausal women, obesity is strongly associated with the risk of ER+/PR+ breast cancer but has a weak association with PR- breast cancer (25). Munsell et al. (21) showed in their meta-analysis that obesity was significantly associated with hormone receptor-positive breast cancer in postmenopausal women (RR = 1.39, 95% CI 1.14-1.70), but the RR for hormone receptor-negative breast cancer in postmenopausal women was 0.98 (95% CI 0.78-1.22). A study on the association between BMI and breast cancer subtypes in postmenopausal women in the Mediterranean found that BMI > 25 kg/m^2 was positively correlated with the risk of luminal breast cancer but not TNBC (41). In a case-control study of a large population conducted by the Carolina Breast Cancer of the United States (20), women with a large WHR regardless of menopausal status (premenopausal OR = 1.9, 95% CI 1.0-3.6; postmenopausal OR = 1.4, 95% CI 0.7-2.7) were more prone to TNBC compared with other breast cancer subtypes.

Therefore, these results indicate that obesity plays a potential role in the prevalence of breast cancer. Obesity may be more likely to increase the risk of hormone receptor-negative breast cancer in premenopausal women and hormone receptor-positive breast cancer in postmenopausal women. In different phenotypes, the risk correlation also varies with measurements of obesity, among which WC and WHR were two important values. Abdominal obesity is correlated with more aggressive molecular types regardless of menopausal status.

Diabetes Mellitus and Breast Cancer Risk

Diabetes is one of the most frequent chronic diseases in the global population. Insulin resistance is the key factor in the pathogenesis of type 2 diabetes and the most typical and serious phenomenon (42). It is defined as decreased sensitivity to insulin-mediated glucose disposal and inhibition of hepatic glucose production (43) and presents as dysfunction of insulin transduction in glucose uptake and utilization in body skeletal muscles, adipocytes and hepatocytes (44), which leads to hyperglycemia, hyperinsulinemia and various disorders. Insulin resistance is the core part of MetS, and the corresponding increase in fasting blood glucose levels and the effects of hyperinsulinemia in breast cancer are also frequently studied. A meta-analysis (45) showed that compared with the nondiabetic group, the hazard ratio (HR) of the diabetes group was 1.23 (95% CI 1.12-1.34), which indicates that diabetes is a risk factor for breast cancer. In addition, hyperinsulinemia associated with insulin resistance is also correlated with a high risk of breast cancer. Zhu et al. (46) found that the OR for breast cancer associated with the highest quartile versus the lowest quartile of insulin was 1.45 (95% CI 1.20-1.75). Elevated insulin levels can cause high insulin growth factor-1 (IGF-1) bioavailability, leading to the occurrence and proliferation of breast cancer (10). The insulin receptor and IGF-1 receptor are widely expressed in breast cancer cells and promote cell proliferation mainly *via* the insulin receptor substrate (IRS)/phosphatidylinositol 3-kinase (PI3K) and Ras/mitogen-activated protein kinase (MAPK) pathways (8, 39).

Research data have yielded inconsistent reports on the relationship between diabetes mellitus (DM) and the risk of different types of breast cancer. Michels et al. (19) and Maskarinec et al. (18) found that type 2 DM was strongly associated with ER-positive breast cancer (HR for Michels et al. = 1.22, 95% CI 1.01-1.47; HR for Maskarinec et al. = 1.17, 95% CI 1.05-1.29). The main mechanism may be due to crosstalk between estrogen and the insulin/IGF-1 signaling pathway. The activation of ERα is influenced by the insulin/IGF-1 pathway, and estrogen may increase the expression and activity of certain proteins in the pathways that promote signal transduction (47). Palmer et al. (22) observed opposite results. Specifically, a medical history of diabetes was positively correlated with the risk of ER- breast cancer (HR = 1.43, 95% CI 1.03-2.00) rather than ER+ breast cancer. Clinical data indicate that the diabetic state may promote a more aggressive cancer subtype. A retrospective study (17), including 4,557 cases, showed that women diagnosed with type 2 DM have a higher risk of TNBC (OR = 1.38, 95% CI 1.01-1.89) or human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancer (OR = 1.38, 95% CI 0.93-2.06) than patients without a history of diabetes. Several studies also supported that type 2 DM is associated with the risk of TNBC. Regarding antidiabetes treatment, metformin has been indicated to block breast cancer cell cycle progression and selectively induce apoptosis. Previous

studies showed that patients treated with metformin had a better breast cancer prognosis (48, 49). Liu et al. (49) reported that metformin has a tumor suppressive effect on breast cancer through a variety of molecular effects, especially on TNBC. They observed that compared with the controls, tumor growth ($P = 0.0066$) and cell proliferation ($P = 0.0021$) in tumor-bearing nude mice treated with metformin were significantly inhibited. Metformin-induced apoptosis, proteolysis of polyadenosine diphosphate-ribose polymerase (PARP) and reduction of epidermal growth factor receptor (EGFR) (a key receptor in TNBC cells) were not observed in phenotypes other than TNBC. Chen et al. (17) found that compared with nondiabetic people, the use of metformin was associated with an increased risk of TNBC (OR = 1.54, 95% CI 1.07-2.22). This may be due to the relatively small sample of women with DM enrolled in the study, and the limited collection of data on history of DM and drug use was only two years before the diagnosis of breast cancer, which limits the evaluation of the impact of long-term use of metformin on the risk of TNBC.

DM and its related hyperinsulinemia and insulin resistance are risk factors for breast cancer. However, the correlation between DM and breast cancer subtypes has inconsistent conclusions among studies and requires further exploration and research. The use of metformin may improve the prognosis of breast cancer patients with diabetes or a prediabetic state, but solid clinical evidence is needed.

Dyslipidemia and Breast Cancer Risk

Dyslipidemia, including high total triglyceride (TG) levels, high total cholesterol (TC) levels and low serum HDL levels, is also considered to be associated with the occurrence of breast cancer, but the results are inconsistent. A prospective study launched by His et al. (50) showed that TC (HR 1 mmol/L increment = 0.83, 95% CI 0.69-0.99, $P = 0.04$) and HDL (HR 1 mmol/L increment = 0.48, 95% CI 0.28-.83, $P = 0.009$) were inversely associated with the risk of breast cancer. Low serum HDL was independently correlated with an increased risk of breast cancer, especially after menopause (51, 52). However, Kitahara et al. (53) found that TC was positively correlated with breast cancer risk (HR = 1.17, 95% CI 1.03-1.33) in the Korean population. Katzek et al. (54) found that TGs (HR for highest vs. lowest quartile = 0.65, 95% CI 0.46-0.92) were negatively associated with breast cancer risk, but HDL (HR for highest vs. lowest quartile = 1.39, 95% CI 1.01-1.93) was positively associated with breast cancer risk, which differed from previous results. A significant increase in TGs and a decrease in HDL have been observed in TNBC patients (4). Therefore, more clinical data and reliable meta-analyses are needed to confirm the relationship between lipid metabolism and breast cancer risk. A case-control study on Chinese women (33) showed that among all MetS components, the hypertriglyceridemia waist circumference phenotype (HTWC), namely, elevated waist circumference and triglycerides, significantly increased the risk of breast cancer (OR = 1.56, 95% CI 1.02-2.39) regardless of menopausal status. Pelton et al. (55) found that mice fed a high-fat/high-cholesterol diet had significantly higher percentages of tumor cell proliferation and higher microvessel density in preclinical models. Furberg et al. (56) suggested that low serum HDL in overweight and obese women was associated with higher levels of breast mitogens and estrogens; thus, HDL might represent a biological marker of breast cancer risk. In particular, Boyd et al. (57) observed a positive relationship between low HDL levels and atypical hyperplasia of the mammogram.

These results suggest that lipid disorders are a risk factor for breast cancer and may promote tumor cell proliferation and blood vessel formation. However, the relationship between different lipids and the risk of different subtypes of breast cancer still needs to be confirmed through more clinical evidence, so these lipids may be used as biologically reasonable markers to identify and intervene in high-risk individuals. Regarding the possible mechanism by which triglycerides, cholesterol and lipoprotein affect the occurrence and progression of breast cancer, further exploration is needed. Then, new lipid-related strategies can be launched for the prevention and treatment of breast cancer based on the findings.

Hypertension and Breast Cancer Risk

Regarding hypertension and the risk of breast cancer, the results of studies are not consistent. A cohort study in Finland (58) found no correlation between hypertension and breast cancer (standardized incidence ratio = 0.94, 95% CI 0.84-1.04). Some studies also showed that hypertension was not associated with TNBC (4). A case-control study by Pereira et al. (59) found that women with hypertension (blood pressure ≥ 140/90 mmHg) had a higher risk of breast cancer (OR = 4.18; 95% CI 1.81-9.64), and a significant association was observed in postmenopausal women (OR = 2.84, 95% CI 1.09-7.39). A meta-analysis (60) also showed a significant association between hypertension and increased breast cancer risk (risk ratio [RR] = 1.15, 95% CI 1.08-1.22), especially for postmenopausal women. In contrast, there was no significant correlation with premenopausal women (RR = 0.97, 95% CI 0.84-1.12) or the Asian population (RR = 1.07, 95% CI 0.94-1.22).

Furthermore, Largent et al. (61) additionally studied the impact of the use of antihypertensive drugs on the risk of breast cancer among people with hypertension. Compared to people not receiving antihypertensive treatment, those who used antihypertensive drugs for more than 5 years had a significantly increased risk of invasive breast cancer (RR = 1.18, 95% CI 1.02-1.36), especially ER+ breast cancer (RR = 1.21, 95% CI 1.03-1.42). Among the drugs, people who used diuretics for more than 10 years showed a significant association with the occurrence of breast cancer (RR = 1.16, 95% CI 1.01-1.33), especially ER+ breast cancer (RR = 1.21, 95% CI 1.03-1.42). The possible mechanism is that breast cancer and hypertension have a common pathophysiological pathway mediated by adipose tissue causing chronic inflammation to form metabolic syndrome (62), and hypertension may increase the risk of disease by blocking and changing apoptosis (63). More research is needed to clarify the relationship between hypertension and breast cancer.

METABOLIC SYNDROME AND BREAST CANCER PROGRESSION

Molecular Changes in Patients With Metabolic Syndrome

As the body's metabolic state changes, the corresponding molecular level also changes, leading to proliferation, invasion and metastasis of breast cancer *via* various signaling pathways and target genes (**Figure 2**). Under an obese state, the tumor microenvironment changes and produces more fibroblasts and immune cells, such as T cells, macrophages and endothelial cells (64). In the mammary gland, the interaction between obese adipocytes and breast cancer cells leads to the transformation of mammary adipocytes into cancer-associated adipocytes (CAAs) (3), which secrete more leptin and reduce the production of adiponectin. Obese adipose tissue is associated with chronic inflammation, which promotes the production of proinflammatory factors, such as TNF-α, IL-6, and IL-8, and inhibits the secretion of anti-inflammatory factors, such as adiponectin (3, 8). In addition, obese adipose tissue is also associated with increased aromatase activity, which promotes the conversion of androgens to estrogen (65).

Diabetes, especially type 2 diabetes, is often accompanied by insulin resistance, leading to hyperinsulinemia. Diabetes also promotes the production of IGF-1 in the liver (3) and inhibits the secretion of sex hormone-binding globulin (SHBG) (51) and IGF-binding protein (IGFBP) (66).

The renin-angiotensin system (RAS) is the main pathway for regulating blood pressure, and an increase in ANG II (67) leads to hypertension. Serum hypocalcemia, one of the characteristics of hypertension, is also related to the occurrence of tumors.

Dyslipidemia is mainly characterized by high TG and high TC. Elevated cholesterol and increased very-low-density lipoprotein (VLDL) and LDL promote tumor proliferation through different pathways (68).

We reviewed the main findings recent years on the principal molecular actors that are involved in the interactions between MetS and breast cancer biology, including leptin, adiponectin, insulin and IGF-1, Angiotensin II and Calcium and, cholesterol and lipoprotein.

Leptin

Leptin (**Figure 2A**) is a hormone secreted by adipose cells that acts on the hypothalamus to suppress appetite, increase basal metabolism and inhibit the synthesis of adipocytes by binding to leptin receptors. Leptin is also considered an important biomarker of metabolic syndrome. Elevated levels of leptin are related to obesity, and obese people may exhibit leptin resistance (35, 69). Leptin levels are also proportional to the degree of insulin resistance.

Sieminska et al. (70) found that the leptin level in postmenopausal women was positively correlated with the number of metabolic syndrome components. Leptin is expressed in normal breast tissue, breast cancer cells and solid tumors. Recent research shows that leptin can upregulate aromatase expression by regulating the p53-hypoxia inducible factor 1α/pyruvate kinase M2 axis in breast adipose stromal cells, increasing the risk of breast cancer (71) and promoting the growth of breast tumors. Pan et al. (72) demonstrated that serum leptin levels were related to overall breast cancer risk (standardized mean difference = 0.46, 95% CI 0.31-0.60), especially in Chinese women (standardized mean difference = 0.61, 95% CI 0.44-0.79). Some *in vitro* experiments have shown that leptin can increase the expression of cox-2 and members of the PI3K/Akt pathway in cocultures of leptin and breast cancer cells, such as MCF-7, MDA-MB-231 and BT474, to promote tumor growth (73–75). Leptin has also been found to promote breast cancer invasion and metastasis through the upregulation of procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) and IL-18 (76, 77). In *in vivo* experiments, tumor-bearing mouse models showed that leptin increased the volume of tumors and promoted lung metastasis (76). A case-control study by Maccio et al. (78) showed that leptin levels are associated with higher TNM staging and an increased risk of distant metastasis ($P < 0.05$) in postmenopausal breast cancer patients. Regarding potential mechanisms (79), leptin can activate the JAK2/STAT3, PI3K/Akt/mTOR, and extracellular regulated protein kinase (ERK) pathways by binding to its own receptors expressed in tumor cells and stromal components, including immune cells, endothelial cells and tumor-associated fibroblasts. Moreover, it may stimulate the epithelial mesenchymal transition (EMT) (80), which has been confirmed to activate the EMT of breast cancer cells by upregulating pyruvate kinase M2 to activate the PI3K/Akt pathway (81), increasing the activity of matrix metalloproteinases (MMPs), promoting the formation and maintenance of breast cancer stem cell (BCSC) survival, inducing the activation and proliferation of endothelial cells and modulating tumor-immune cell cross talk. Sabol et al. (82) found through molecular experiments that adipose stem cells modified by obesity promoted the secretion of leptin and upregulated and activated the IL-6 and Notch signaling pathways in ER+ breast cancer cells, resulting in ER+ breast cancer radiation resistance. Leptin signaling affects the progression of ER- breast cancer (83). Recent *in vitro* experiments have also found that the expression of leptin receptor and leptin-targeting genes is associated with reduced survival rate of ER- breast cancer and chemotherapy resistance, indicating that the coexpression of leptin receptor and leptin targeting genes can be used as a prognostic indicator of ER-breast cancer patients (84).

Numerous *in vivo* and *in vitro* studies confirmed the role of leptin in recurrence and metastasis. However, relevant clinical evidence is relatively lacking, so more research is needed to investigate leptin as a predictive biomarker and a novel therapeutic target in the clinic.

Adiponectin

Adiponectin (**Figure 2A**) is a protein synthesized and secreted by white adipocytes. Obese patients have lower levels of adiponectin compared with normal patients. In contrast to leptin, the concentration of adiponectin decreases as the number of metabolic syndrome components increases (85–88). Adiponectin activates various signaling pathways (AMPK,

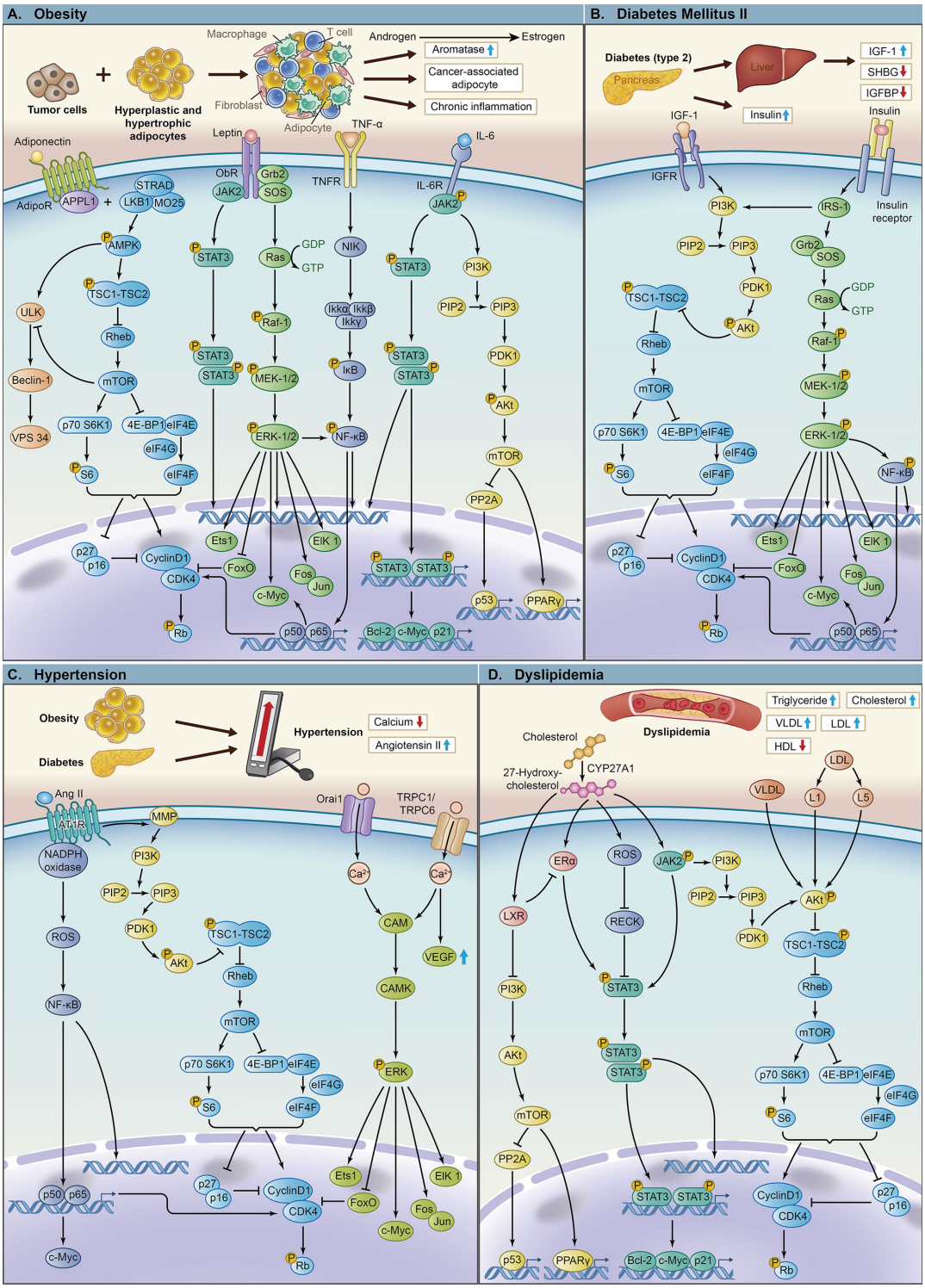

FIGURE 2 | The key pathways and molecular mechanisms of components of metabolic syndrome leading to tumor proliferation, invasion and metastasis. **(A)** The mechanism of obesity and its related key molecules leading to breast cancer with important signaling pathways, including LKB1/AMPK/mTOR, AMPK/ULK, JAK2/STAT3, Ras/Raf/MEK/ERK, NF-κB and PI3K/Akt/mTOR. **(B)** The mechanism of diabetes mellitus type 2 and its related key molecules leading to breast cancer with important signaling pathways, including PI3K/Akt/mTOR, Ras/Raf/MEK/ERK and NF-κB. **(C)** The mechanism of hypertension and its related key molecules leading to breast cancer with important signaling pathways, including NF-κB, PI3K/Akt/mTOR and CAM/CAMK/ERK. **(D)** The mechanism of dyslipidemia and its related key molecules leading to breast cancer with important signaling pathways, including PI3K/Akt/mTOR, JAK2/STAT3 and ERα/STAT3.

PI3K/Akt, MAPK, PPAR-α, STAT3 and NF-κB) (39) and exerts various cellular functions by binding to two receptors, adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2). Adiponectin improves insulin sensitivity *in vivo* by activating PPAR-α (89). Based on the role of adiponectin in the abovementioned signaling pathways, the short peptide ADP355 based on adiponectin can exert antiproliferative effects in breast cancer cells through the STAT3 and ERK1/2 signaling pathways (39, 90). Adiponectin also increases insulin sensitivity in muscles and liver through the AMPK pathway and improve insulin resistance (91) as an endogenous insulin sensitizer. Adiponectin deficiency causes downregulation of the activity of PTEN and activation of the PI3K/Akt signaling pathway, thereby promoting breast tumors (92). In addition, adiponectin controls inflammation, inhibits angiogenesis (93), reduces proliferation, and promotes apoptosis. It inhibits the expression of adhesion molecules in endothelial cells, suppresses the growth of macrophage precursors and downregulates TNF-α and IL-6/-8 to control inflammation (94). Adiponectin potentially inhibits angiogenesis through the AMPK pathway to enhance nitric oxide production, activate endothelial nitric oxide synthase (95), and suppress the expression of pro-angiogenesis factors, such as vascular endothelial growth factor (VEGF) and IL-8. *In vitro* experiments further demonstrated that in MCF-7 and MDA-MB-231 cell lines, the proliferation of endothelial cells expressing adiponectin receptors is inhibited by adiponectin, thereby reducing VEGF expression and inhibiting metastasis, invasion, and angiogenesis (96). Moreover, adiponectin may inhibit TNF-α-induced activation of the NF-κB pathway to promote apoptosis.

Adiponectin is negatively related to the risk of breast cancer. Many studies have shown that low adiponectin levels are associated with the risk of breast cancer and the progression of more aggressive subtypes (40), which is more common in ER-/PR- breast cancer. *In vivo* experiments showed that in animals receiving ER- MDA-MB-231 cells, the tumor volume was significantly reduced after adiponectin pretreatment (97). Moreover, Oh et al. (98) found that adiponectin may protect against recurrence in ER-/PR- breast cancer patients. Although adiponectin exhibits antiproliferative effects on cell growth in both ER+ and ER- cell lines, the main mechanisms may be different. For ER+ breast cancer, a low adiponectin concentration can amplify ER signaling to increase the proliferation of ER+ breast cancer cells, leading to the progression of breast tumors (40, 99, 100). Low adiponectin levels may mediate the proliferation of ER- breast cancer cells by regulating genes involved in the cell cycle (such as p53, Bax, Bcl-2, c-myc and Cyclin D1) (101), which indicates that adiponectin can be used as adjuvant therapy for ER- breast cancer, and its local and systemic application can reduce tumor size and inhibit tumor metastasis.

The possible mechanism by which adiponectin exerts antitumor growth and proautophagy effects is the LKB1-AMPK-mTOR/ULK pathway (102). *In vitro* experiments have shown that adiponectin increases the expression of the tumor suppressor gene LKB1 in breast cancer cells and subsequently reduces tumor adhesion, migration and invasion through the AMPK-p70S6 kinase (S6K) axis (103). Adiponectin binds to the C-terminus of AdipoR, and its N-terminus binds to the PTB domain of signal adaptor protein (APPL1), affecting LKB1 expression. In obese patients, adiponectin production is reduced such that binding to AdipoR is inhibited. The binding of the domain of APPL1 to LKB1 is suppressed, so AMPK and ULK expression is consequently inhibited (104). Some studies have summarized that the *in vitro* data of adiponectin's antitumor proliferation effect in breast cancer are mainly limited to full-length adiponectin (fAd) (105, 106), one of the forms of adiponectin. The other form of adiponectin, globular adiponectin (gAd), may have the opposite effect. It can activate AMPK through its high-affinity receptor AdipoR1 acting on aggressive tumor cells, and AMPK upregulates autophagy by activating ULK1 to enhance tumor metastasis (106). Mauro et al. further found that gAd inhibits the growth of ER+ breast cancer but promotes ER+ breast cancer proliferation (107).

Based on adiponectin-mediated cytotoxic autophagy, we can reasonably hypothesize that adiponectin usage represents a new adjuvant therapy strategy for obese breast cancer patients and that combining adiponectin with chemotherapeutic drugs may therefore reduce the dose of chemotherapeutic drugs (108). Studies have concluded (109) that leptin and adiponectin receptors seem to be the most promising molecular targets for the treatment of metabolic syndrome-related cancers. In the future adjuvant therapy of breast cancer, for patients with metabolic syndrome, it may be possible to improve the efficacy by adjusting the circulating levels of the above molecules to obtain a better prognosis.

Insulin and IGF-1

Insulin (**Figure 2B**) is an anabolic hormone. It has mitogenic, antiapoptotic and angiogenic effects, which are partially related to cancer progression and mortality. Hyperinsulinemia can also affect the prognosis of breast cancer mainly through the following mechanisms. Insulin itself promotes the synthesis of DNA, RNA, and ATP, induces mitosis and angiogenesis, and inhibits apoptosis. High insulin levels induce aromatase activity (110), increase the chance of conversion of androgen to estrogen, and promote mitosis. Moreover, high insulin levels also reduce the production of SHBG synthesized by the liver (111) and increase the proportion of circulating estradiol, leading to the growth of breast cancer. *In vitro* experiments proved that insulin induces EMT, invasion and migration of tumor cells (112). In a chronic hyperinsulinemic state, the ER is activated to promote tumor growth by regulating the cell cycle, apoptosis factors, and nutrient metabolism (113). This mechanism provides a basis for using metformin to treat ER+ breast cancer patients with diabetes. In addition, hyperinsulinemia mediates the production of IGF-1 (**Figure 2B**), inhibits the production of IGFBP in the liver, increases the bioavailability of IGF-1 and stimulates further tumor growth through excessive activation of the PI3K-Akt-mTOR pathway and Ras-MAPK pathway (114).

MetS patients with insulin resistance have high circulating insulin levels. Insulin binds to the insulin receptor on the cell membrane and activates IRS1 and subsequently activates the Ras/Raf/MEK/ERK pathway to regulate cell proliferation and

differentiation. Insulin also stimulates the production of IGF-1 in the liver and downregulates the secretion of IGFBP1 and 2 in the liver, leading to increased bioavailability of circulating IGF-1 (66, 115). IGF-1 receptor (IGF-1R) is a transmembrane receptor tyrosine kinase that is similar in structure to the insulin receptor. It is often overexpressed in breast cancer cells and regulates cell proliferation, survival, differentiation, and transformation (116–119). IGF-1 combines with IGFR, which activates PI3K and thereby activates Akt. Activated Akt further induces the mTOR pathway, leading to cell proliferation and protein synthesis in tumor cells.

Therefore, improving insulin resistance through the above pathways can inhibit tumor growth and proliferation directly or indirectly, and it may also relieve other metabolic abnormalities that can improve the curative effect of adjuvant therapy, leading to better disease prognosis.

Angiotensin II and Calcium

RAS and calcium (**Figure 2C**) are hypertension-related molecules. Studies have reported the relationship and possible mechanism between these molecules and breast cancer. RAS is an important physiological mechanism of hypertension, and studies have shown that it also plays an important role in the progression of breast cancer. It has been observed in some *in vitro* and *in vivo* experiments that all components of the RAS are overexpressed in breast cancer cells (67). Among them, angiotensin II (Ang II) (**Figure 2C**) is a well-known hypertension hormone that binds to angiotensin II type 1 receptor (AT1R) and angiotensin II type 1 receptor (AT2R). Ang II-AT1R is related to breast cancer proliferation, and its mechanism has been extensively studied. After Ang II binds to AT1R, AT1R interacts with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to activate AT1R and promotes the infiltration of macrophages and the production of VEGF and reactive oxygen species (ROS). ROS further promote members of the downstream NF-κB pathway to bind to target genes and mediate cell transcription. AT1R also promotes the secretion of matrix metalloproteinases and then activates the PI3K/Akt/mTOR pathway to promote tumor growth and inhibit cell apoptosis.

Calcium ions are also an important part of the pathogenesis of hypertension. Studies have found that calcium ion channels are associated with tumor proliferation, angiogenesis, apoptosis and metastasis. In hypertensive patients, calcium ions flow through calcium channels, resulting in intracellular high calcium and extracellular low calcium. A meta-analysis by Wulaningsih et al. (120) found that serum calcium had a protective effect on breast cancer (RR 0.80, 95% CI 0.66-0.97). Deliot et al. (121) showed that calcium ions flood into cells through the Orai1 channel (**Figure 2C**), phosphorylating ERK *via* the calcium/calcium-dependent calmodulin (CAM)/calmodulin kinase II (CAMK) pathway, which regulates cell proliferation. In addition, Orai1 levels in TNBC were increased compared with that in non-TNBC (122). Orai1 is a 33kDa protein with four transmembrane domains and its functional channel is a hexamer composed of 6 Orai1 subunits (123). Orai1 forms a highly selective calcium ion channel in the plasma membrane to mediate the transmission of calcium ions (124). The antiproliferative and antimigratory effects of Orai1 silencing were observed in the MDA-MB-231 basal breast cancer cell line. This finding reveals that the Orai1 channel may represent a therapeutic target for TNBC. Calcium can also stimulate breast cancer cell proliferation and activate VEGF through the (transient receptor potential-canonical) TRPC6/TRPC1 channel (**Figure 2C**). The use of calcium channel blockers may inhibit the growth of breast cancer tumors. The use of calcium-channel blockers for 10 or more years was associated with a greater than twofold increase in the risk of ductal breast cancer (OR 2.4, 95% CI 1.2-4.9) and lobular breast cancer (OR 2.6, 95% CI 1.3-5.3) according to Li et al. (125).

Cholesterol and Lipoprotein

High cholesterol (**Figure 2D**) is an important feature of dyslipidemia in obese patients and one of the side effects of adjuvant therapy in patients. It has a certain impact on the pathophysiology and progression of breast cancer. Nelson et al. (126) found that 27-hydroxycholesterol (27HC), a primary metabolite of cholesterol and an ER and liver X receptor (LXR) ligand, might play an important role in the progression of breast cancer. Cholesterol is converted into 27HC by cytochrome P450 oxidase. Then, 27HC activates ER and LXR to promote breast cancer cell growth and metastasis. Nelson et al. further confirmed that in mouse models of breast cancer, 27HC significantly reduced tumor latency ($P < 0.05$) and accelerated tumor growth ($P < 0.05$). Increases in markers, such as cell proliferation, angiogenesis, and macrophage infiltration, were also observed in mice treated with 27HC. In higher grade tumors, increased expression of cytochrome P450 oxidase was observed (OR 6.7, 95% CI 1.7-27, $P = 0.0007$), indicating that cancer cells could increase 27HC levels through autocrine signaling. In addition, 27HC activates the STAT3 pathway by reactive oxygen species (ROS) methylation and activation of JAK and extracellular ERα. LXR suppresses the PI3K/Akt pathway to inhibit cell proliferation and downregulate ER expression (127), and ER enhances STAT3 activation (128).

Epidemiologically, abnormal lipoprotein levels (**Figure 2D**) have also been shown to be significantly associated with breast cancer. Data from Lu et al. (68) provide a new mechanism by which lipoproteinemia promotes tumor development. They found that the VLDL, L1 and L5 subfractions of LDL **(Figure 2D)** activated Akt to promote cell migration by Ser473 phosphorylation. VLDL, L1, and L5 also increase mesenchymal markers, such as vimentin and β-catenin, to induce EMT and enhance angiogenic factors in breast cancer to promote angiogenesis. Therefore, reducing circulating cholesterol or inhibiting its conversion to 27HC and abnormal lipoprotein levels may represent new strategies for the prevention and/or treatment of breast cancer. However, the effect of lipid composition on the growth of breast cancer cells and its mechanism still require more research.

METABOLIC SYNDROME AND TREATMENT EFFECT ON BREAST CANCER

Metabolic Syndrome and Adjuvant Therapy Efficacy

Healy et al. (129) reported that MetS is associated with more aggressive tumor biology, such as later tumor stage ($P = 0.022$)

and lymph node invasion (P = 0.028). Compared with breast cancer patients who do not have metabolic abnormalities, those with MetS and its components show worse treatment responses to various therapies.

Regarding chemotherapy, a study by Stebbing et al. (130) on the relationship between MetS and response to chemotherapy for breast cancer found that among patients with metabolic syndrome, the proportion of progression, stability and response to treatment after chemotherapy ranged from 61.1% to 42.9% to 33.3% (P for trend = 0.03). This finding indicated that the MetS-mediated risk of nonresponse to chemotherapy was higher and that treatment efficacy was worse. Litton et al. (131) found that overweight (BMI = 25-29 kg/m^2) and obese (BMI ≥ 30 kg/m^2) groups exhibited more difficulties than normal or underweight (BMI < 25) groups in achieving pathologic complete response (pCR) (OR = 0.67, 95% CI 0.45-0.99) with neoadjuvant chemotherapy. This unsatisfactory treatment response may be due to underdose in obese patients (132). In the guidelines issued by the American Society of Clinical Oncology, full-dose chemotherapy based on body weight was recommended to obese patients because their worsening survival may be related to the insufficient dose of cytotoxic therapy (41, 133). However, considering safety and therapeutic benefits, overweight and obese patients worldwide are still receiving reduced doses of chemotherapy (23, 134).

DM can also affect the efficacy of chemotherapy. The elevated concentration of IGF-1 under hyperglycemia can specifically inhibit cell death induced by anticancer drugs in MCF-7 cells, which suggests its involvement in the mechanism of drug resistance. Zeng et al. (135) showed that hyperglycemia-induced resistance to chemotherapy was only observed in ER+ breast cancer cells, indicating that antiestrogen may promote the effectiveness of chemotherapy in such patients.

For endocrine therapy, Ewertz et al. (29) followed up on 18,967 Danish women with BMI information who received early breast cancer treatment found that after one decade, in contrast to the patients with BMI < 25 kg/m^2, endocrine therapy in patients with BMI ≥ 30 kg/m^2 had a worse response. Compared to normal weight women, the disease-free survival (DFS) rate (HR = 1.78, 95% CI 1.12-2.83) and overall survival (OS) rate (HR = 2.28, 95% CI 1.16-4.51) are worse in obese women receiving tamoxifen (TAM) combined with aromatase inhibitors (AIs) (136). In this regard, they further studied the role of fulvestrant and TAM in chemotherapy resistance. Fulvestrant is a selective estrogen receptor degrader that can not only competitively bind to ER, but also induce ER degradation and down-regulate ER levels. Fulvestrant blocked hyperglycemia-mediated chemotherapeutic resistance, but TAM did not (135). Diabetes is another major factor affecting endocrine therapy, which may lead to a worse treatment response (8, 137). For DM patients, IGF-1 receptor or IRS-1 overexpression increases the resistance of breast cancer cells to antiestrogen therapy (138).

Trastuzumab is a widely used targeted medicine. It is a monoclonal antibody against HER2, affecting the transmission of growth signals through specific binding to the HER2 receptor, and at the same time kill tumor cells through antibody-dependent cell-mediated cytotoxicity pathway. For targeted therapy, obese adipocytes secrete more IGF-1, thereby increasing drug resistance, such as trastuzumab resistance (139), but the underlying mechanism remains unclear. Lee et al. (140) demonstrated in their research that in HER2+ breast cancer patients receiving trastuzumab treatment, DM was a significant unfavorable prognostic factor for DFS (P = 0.006) and OS (P = 0.017), which was consistent with previous reports (141).

For radiation therapy, in the study of Sabol et al. (82), when breast cancer cells were cocultured with obesity-altered adipose stem cells, breast cancer cell apoptosis decreased and survival increased after radiotherapy. Research has suggested that obesity-altered adipose stem cells upregulate IL-6 and activate the Notch signaling pathway to induce radiation tolerance. Fang et al. (142) found that higher BMI was associated with worse quality of life for breast cancer patients before, during, and after radiotherapy even after adjusting for other factors. DM patients also showed worse response and radiation tolerance through elevation of IGF-1R and IRS-1 (47).

Adjuvant Therapy and Metabolic Changes

A published observational study (143) showed that after patients completed corresponding chemotherapy, metabolic syndrome and its components were significant risk factors (P < 0.01). Among them, the fasting blood glucose level changed by 20.3% and the triglyceride level changed by 18.4%, the deterioration of which were more significant. Several studies have demonstrated that breast cancer patients who have received chemotherapy are more likely to gain weight than those who have not (144–146). Fredslund et al. (147) also observed consistent results. Changes were evident in body fat (P = 0.01), triglycerides (P = 0.03), WC (P = 0.008), glucose (P = 0.02) and diastolic blood pressure (P = 0.04) of premenopausal women, whereas changes in WC (P = 0.03), HDL (P = 0.05) and glucose (P = 0.02) were observed in postmenopausal women. These findings suggested that chemotherapy may have greater adverse effects in premenopausal breast cancer patients because this group may be induced to undergo either transient or permanent early menopause that promotes the rapid decline of estradiol levels in the body, leading to weight gain and a rapid and continuous increase in fat mainly in the abdomen, which causes a series of changes in metabolism-related components. Such weight gain may also increase the risk of chemotherapy-related diabetes. Glucocorticoids used in combination with chemotherapeutic drugs or chemotherapeutic drugs themselves, such as platinum and cyclophosphamide, can cause abnormal glucose metabolism through direct or indirect mechanisms and worsen the pre-existing DM state in susceptible individuals (148). Among the three breast cancer treatments, including radiotherapy, chemotherapy, and endocrine therapy, evaluated in the analysis conducted by Bordelea et al. (149), only chemotherapy (P = 0.03) was related to new-onset diabetes.

Studies have shown that endocrine therapy, such as estrogen inhibitors, may also lead to worse metabolic status. TAM is a commonly used antiestrogen drug that can cause dyslipidemia, a well-known side effect with a worsening HDL level and circulating TG level, in women with breast cancer (150).

However, TAM was reported to lower circulating TC and (low-density lipoprotein) LDL (151). Fatty liver (P = 0.000) and visceral adipose tissue (P = 0.000) are also significant side effects of receiving TAM (152).

Furthermore, the use of TAM can lead to increased fasting blood glucose levels and insulin resistance even when administered in low doses. A population-based cohort study launched by Sun et al. (153) demonstrated that TAM users showed a significantly increased risk of DM compared with non-TAM users among breast cancer patients (adjusted HR = 1.31, 95% CI 1.19-1.45). TAM also leads to a decrease in insulin sensitivity. Johansson et al. (150) observed a 7-fold decrease in insulin sensitivity among breast cancer patients using TAM (OR = 0.15, 95% CI 0.03-0.88) compared with nonusers. The underlying mechanism of the link between TAM and diabetes is still unclear. A possible explanation is that estrogen maintains steady blood glucose, and TAM may affect the interaction between estrogen and insulin by inhibiting estrogen. Additionally, hypertriglyceridemia and fatty liver caused by TAM are features of insulin resistance and glucose intolerance (153).

AIs, including letrozole, anastrozole and exemestane, are another important hormone therapy. Similar to TAM, abnormal lipid metabolism is one of the most significant side effects of AI, but the specific impact is different. Compared with TAM, AI may not change TG levels but increase TC and LDL-C levels. Among AIs, anastrozole and letrozole increase TC and LDL-C levels, whereas exemestane decreases TC, LDL-C, TG and HDL-C levels (151, 154). An early *in vitro* study showed that 17-hydroxy exemestane, a metabolite of exemestane, may elicit androgenic effects by binding to the androgen receptor. Bell et al. (154) hypothesized that this may be a potential mechanism by which exemestane could reduce HDL cholesterol. However, less is known about nonsteroidal aromatase inhibitors, such as anastrozole and letrozole. Hong et al. (155) showed in their cohort study that the incidence rate of fatty liver was increased in the TAM group than in the AI group (P = 0.021). The reason may be that TAMs increase circulating TG levels and promote insulin resistance, thereby promoting susceptibility to fatty liver. These findings also suggested that TAMs have direct liver toxicity, but more evidence is needed to confirm this hypothesis (155).

In addition, hypertension is a characteristic adverse event of bevacizumab therapy. The incidence was 17.9% in a clinical study (156). The possible mechanism is that bevacizumab inhibits vascular endothelial growth factor (VEGF), which enhances endothelial nitric oxide synthase activity. These effects lead to decreased production of vasodilator nitric oxide, which causes hypertension (157).

Thus, an interaction exists between adjuvant therapy and human body metabolic status. Patients with unstable metabolic status will receive unfavorable results from adjuvant therapy. Conversely, adjuvant therapy will also cause deterioration of the human body's metabolic status.

Obesity and Cardiotoxicity of Trastuzumab and Anthracyclines

Compared with chemotherapy alone, the combination of targeted medicine and chemotherapy has clinical advantages in improving the DFS and OS rates of breast cancer patients (158–160). Anthracyclines and trastuzumab are widely used in the treatment of breast cancer patients, and both can induce acute or chronic dose-dependent cardiotoxicity, especially acute cardiac insufficiency, which is the most serious side effect. This condition is characterized by reduced left ventricular ejection fraction (LVEF), which is typically asymptomatic or associated with heart failure (161). A meta-analysis of 8,754 breast cancer patients treated with anthracyclines, sequential anthracyclines (anthracyclines followed by trastuzumab), and trastuzumab launched by Guenancia et al. (162) showed that in an unadjusted analysis, overweight plus obesity was obviously related to the risk of cardiotoxicity of the above therapies. The OR was 1.38 (95% CI 1.06-1.80) for overweight plus obesity, 1.47 (95% CI 0.95-2.28) for obese individuals and 1.15 (95% CI 0.83-1.58) for overweight individuals. Subgroup analysis showed that the risk of cardiotoxicity gradually increased with increasing BMI (P = 0.05). For different administrations, the rate of cardiotoxicity of obese patients treated with anthracyclines only was 20% (95% CI 5%-43%), and obese patients treated with trastuzumab with or without anthracyclines had a 16% (95% CI 10%-24%) rate of cardiotoxicity.

Gunaldi et al. (163) found an association between postmenopausal women (P = 0.01) and cardiotoxicity caused by trastuzumab. Obesity (P = 0.0001) and hypertension (P 0.002) were related to lower LVEF in patients, whereas diabetes (P = 0.766) was not statistically significant. However, a retrospective study (164) observed a correlation between a history of diabetes and trastuzumab-related cardiotoxicity (P = 0.01). Therefore, evaluation of heart function or appropriate dose reduction is needed for obese breast cancer patients before treatment with anthracycline or trastuzumab. During and after treatment, LVEF should also be evaluated regularly to prevent heart insufficiency and heart failure. The relationship between people with DM and the incidence of cardiotoxicity caused by trastuzumab requires further exploration.

Obese patients or mice fed a high-fat diet were more sensitive to the cardiotoxicity caused by anthracyclines (165, 166). The mechanism may be due to downregulation of cardiac peroxisome proliferator-activated receptor-α, decreased mitochondrial adenosine monophosphate (AMP)-α2 protein kinase and a reduction in cardiac adenosine triphosphate (ATP) levels after doxorubicin administration according to Mitra et al. (166). Therefore, high-fat diet-induced obese rats are highly sensitized to anthracycline-induced cardiotoxicity by downregulating cardiac mitochondrial ATP generation, increasing oxidative stress and downregulating the Janus kinase (JAK)/signal transducers and activators of transcription 3 (STAT3) pathway. Additionally, Maruyama et al. (167) found that adiponectin-knockout mice showed aggravated left ventricular systolic dysfunction after injection of doxorubicin, whereas exogenous adiponectin improved this condition in wild-type and adiponectin-knockout mice. These results indicate that downregulation of adiponectin levels partly affects adverse cardiac reactions, suggesting that adiponectin may be used as a therapy to prevent cardiotoxicity caused by anthracyclines in

obese breast cancer patients. More evidence is needed to confirm the underlying mechanism of trastuzumab-induced cardiotoxicity in obese patients.

METABOLIC SYNDROME AND PROGNOSIS OF BREAST CANCER

Recurrence

Current evidence suggests that metabolic syndrome and its components are associated with an increased risk of breast cancer recurrence (3, 168). A retrospective study conducted by Ewertz et al. (29) assessed the events of locoregional recurrence and distant metastasis among 53,816 women with early-stage breast cancer up to 10 years after diagnosis and showed that obesity may have no effect on the risk of local recurrence ($P > 0.05$) but had a significant association with distant metastasis 5 to 10 years after diagnosis. The HR of distant metastasis in patients with a BMI of 25 to 29 kg/m^2 was 1.42 (95% CI 1.17-1.73, $P < 0.001$). The HR in patients with a BMI of 30 kg/m^2 or more was 1.46 (95% CI 1.11-1.92, $P = 0.007$). In particular, adiponectin is an important adipocytokine, and its expression is reduced in obese people. Oh et al. (98) found that compared to the highest quartile of serum adiponectin levels, the lowest quartile showed a 2.82-fold increased risk.

Goodwin et al. (169) demonstrated that, regardless of menopausal status, fasting plasma insulin levels are associated with high tumor grade, axillary lymph node involvement, and risk of recurrence. Patients in the highest quartile of insulin level had an increased risk of distant recurrence (HR 2.0, 95% CI 1.2-3.3, $P = 0.007$). However, in different molecular types of breast cancer, the correlation between insulin resistance and recurrence risk shows different results (98). In contrast with ER-/PR- patients, a negative association is noted between insulin resistance and tumor recurrence in ER+/PR+ people, which is consistent with serum insulin levels. Moreover, ER+/PR+ patients with hyperglycemia exhibited a lower risk of recurrence (HR = 0.48, 95% CI 0.26-0.89, $P = 0.020$) (**Table 2**).

The impact of dyslipidemia on the prognosis of breast cancer has demonstrated conflicting results. In general, breast cancer patients with high levels of TC, low density lipoprotein-cholesterol (LDL-C) and TG and low levels of HDL-cholesterol (HDL-C) show poor prognosis (170, 172). However, a retrospective study by Jung et al. (173) observed opposite results. In the study, compared to high levels of LDL-C (quartile IV) and TGs (quartile IV), lower levels of LDL-C (quartile III) (HR 1.88, 95% CI 1.09-3.27, $P = 0.02$) and abnormally low levels of TGs (quartile I) (HR 1.88, 95% CI 1.07-3.29, $P = 0.03$) showed obviously higher risks of recurrence. Ozdemir et al. (174) also found that the risk of recurrence was increased in patients with normocholesterolemia compared with patients with hypercholesterolemia ($P = 0.001$). However, Bahl et al. (172) found no statistically significant relationship between lipids and breast cancer outcome other than a trend toward a risk of recurrence with higher TC (HR 1.62 for the fourth to first quartile, 97.5% CI 0.98-2.69, $P = 0.03$) in multivariate analysis. Therefore, the potential role of lipids in the recurrence of breast cancer should be further studied.

Regarding hypertension, Braithwaite et al. (175) showed that hypertension was another independent predictor of prognosis in breast cancer. Interestingly, hypertension was associated with recurrence among African Americans (HR 1.60, 95% CI 1.07-2.40) but not their white counterparts. However, the reason was not clear, and more studies are needed to explain the distinction. The association among other ethnic groups requires more solid results.

The above findings indicate that MetS and its composition can be regarded as predictors of recurrence. Among them, evaluating adiponectin and insulin concentrations can help determine the prognosis in ER-/PR- patients, and corresponding interventions can be implemented to improve the prognosis and reduce the risk of recurrence. In addition, more research is needed to prove the impact of serum lipid levels and hypertension on the recurrence of breast cancer and the relationship between MetS and its components and breast cancer subtypes. Therefore, it could provide new and specific prognostic detection methods to monitor and prevent recurrence in breast cancer patients.

Mortality

For patients with early-stage breast cancer, abnormal metabolic status will bring unsatisfactory outcomes. MetS is widely thought to be associated with a risk of mortality for breast cancer patients, and the risk sharply increases as the number of its components increases. Compared with individuals without any metabolic syndrome-related components, patients with 1-2 components have a 5-fold higher risk of death (HR = 4.90, 95% CI 1.47-16.35, $P = 0.01$) and a 6-fold higher risk of breast cancer-specific death (HR = 6.07, 95% CI 1.41-26.21, $P = 0.02$) (**Table 2**); patients with 3-5 components have a 12-fold higher risk of death (HR = 12.2, 95% CI 3.49-43.01, $P < 0.0001$). In addition, the risk of breast cancer-specific death is 16-fold higher (HR = 15.97, 95% CI 3.49-73.16, $P < 0.0001$) (3).

Epidemiologists show that the mortality rate of breast cancer among Asians is the lowest in the world, but it has rapidly increased in recent years due to the sharp increase in obesity and consequent metabolic disorders (176, 177). It was proven that obese women show a worse survival rate regardless of menopausal status. A study from the United States in 2009 found that after diagnosis with breast cancer, every gained 5 kg of body weight could increase breast cancer-specific mortality by 13% (66). Cho et al. (146) found that in all patients, BMI ≥ 25 kg/m^2 was an unfavorable factor for OS ($P = 0.030$). In a Danish retrospective study of 18,967 early breast cancer patients (29), patients with BMI ≥ 30 kg/m^2 at the time of diagnosis had a 38% increase in breast cancer mortality compared with patients with a BMI < 25 kg/m^2 (HR = 1.38, 95% CI 1.11-1.71). In addition, studies have also found that obesity was significantly associated with adverse outcomes in women with ER+ tumors, and obesity had an impact on ER- or HER2+ tumors (40, 178). The possible mechanisms may be due to obesity-related molecules, such as leptin, adiponectin, TNF-α, and IL-6, and their subsequent signal pathways. These findings also suggested that obese patients often have tumors detected later along with more aggressive tumor biological characteristics; therefore, fewer

TABLE 2 | The effect of metabolic syndrome and its components on recurrence and survival of breast cancer.

No.	Author	Pts (N)	Region	Study design	Molecular subtype	Comparison	Outcome	HR	95%CI	*P*	Ref
1	Buono G. et al.	717	Italy	prospective observational study	All	1-2MetS components vs. 0MetS component	OS	4.90	1.47-16.35	0.01	(3)
							BCSS	6.07	1.41-26.21	0.02	
						3-5MetS components vs. 0MetS component	OS	12.20	3.49-43.01	<0.0001	
							BCSS	15.97	3.49-73.16	<0.0001	
2	Cho W. K. et al.	5668	Korea	retrospective cohort study	All	BMI≥25 vs. BMI<25	OS	1.356	1.038-1.773	0.03	(146)
							DFS	1.248	1.038-1.502	0.076	
						Non-hyperlipidemia vs. Hyperlipidemia	OS	3.085	1.836-5.183	<0.001	
							DFS	1.447	1.080-1.937	0.013	
3	Emaus A. et al.	1364	Norway	retrospective cohort study	All	BMI≥30 vs. BMI=18.5-25	OS	1.47	1.08-1.99	N/A	(170)
						highest tertile of cholesterol vs. lowest	OS	1.29	1.01-1.64	N/A	
						highest tertile of blood pressure vs. lowest	OS	1.41	1.09-1.83	N/A	
4	Ewertz M. et al.	18967	Denmark	retrospective cohort study	All	BMI≥30 vs. BMI<25	BCSS	1.38	1.11-1.71	0.003	(29)
5	Minicozzi P. et al.	1607	Italy	retrospective cohort study	ER/PR+	High glucose(>94.0mg/dl) vs. reference (84.1-94.0mg/dl)	BCSS	5.49	1.56-19.31	N/A	(171)
					ER-/PR-		BCSS	0.77	0.15-4.17	N/A	
6	Oh S. W. et al.	747	Korea	retrospective cohort study	ER/PR+	Hyperglycemia vs. non-hyperglycemia	Recurrence	0.48	0.26-0.89	0.02	(98)
					ER-/PR-	Serum adiponectin	Recurrence	N/A	N/A	0.009	

BCSS, Breast cancer specific survival; BMI, Body mass index; DFS, Disease free survival; ER, Estrogen receptor; HR, Hazard ratio; MetS, Metabolism Syndrome; N/A, Not applicable; OS, Overall Survival; PR, Progesterone receptor; Pts, patients.

treatment opportunities with poorer therapeutic effects are available, which leads to an increased risk of death.

Diabetes is also associated with high breast cancer mortality, and a meta-analysis showed a significantly higher all-cause mortality risk (HR 1.49, 95% CI 1.35-1.65) of patients with breast cancer and diabetes (179). Another meta-analysis also indicated that HR was 1.51 (95% CI 1.34-1.70) for OS and 1.28 (95% CI 1.09-1.50) for DFS in breast cancer patients with diabetes compared to those without (180). However, its effects differ among subtypes. ER+/PR+ women with hyperglycemia are more likely to die of breast cancer (HR = 5.49, 95% CI 1.56-19.31), whereas ER-/PR- patients show no significant association (171). The underlying reasons may be that patients with diabetes receive less aggressive treatment because they are vulnerable to related comorbidities and may have a greater risk of chemotherapy-related toxicity. Additionally, diabetes can directly affect breast cancer by altering related molecules, such as insulin, IGF-1 and inflammatory markers. Bozcuk et al. (181) and Pasanisi et al. (182) found that fasting serum insulin levels are an independent predictor of OS in breast cancer patients, which may be related to the high expression of insulin receptors in breast cancer tissues.

Notably, in the study of Cho et al. (146), the absence of hyperlipidemia is an unfavorable prognostic factor for DFS (HR 1.447, 95% CI 1.080-1.937, $P = 0.013$) and OS (HR 3.085, 95% CI 1.836-5.183, $P < 0.001$) in breast cancer patients, which is perhaps related to the use of statins in patients with hyperlipidemia. A Norwegian research team found (170) that breast cancer patients with high total cholesterol levels (HR for OS is 1.29, 95% CI 1.01-1.64, $P = 0.03$) had a high risk of overall mortality. This finding may be attributed to the fact that cholesterol contributes to the progression and metastasis of tumors *via* several pathways, such as Akt and EGFR. Fan et al. (14) found that in the TNBC group, patients with low HDL showed worse relapse-free survival (RFS) (HR 3.266, 95% CI 2.087-5.112, $P < 0.0001$) and OS (HR 3.071, 95% CI 1.732-5.445, $P < 0.0001$). The possible mechanism is that HDL negatively correlates with angiotensin (ANG) II, which is positively associated with VEGF pathways in TNBC cells.

Therefore, MetS and its components play a profound role in the survival of breast cancer patients. In individuals, positive associations are observed between obesity as well as diabetes and mortality. Hyperlipidemia shows a protective effect on the mortality of breast cancer. The exact association between MetS and its related components and different phenotypes and underlying mechanisms remain unclear and require further study.

Anti-MetS and Prognosis

Healy et al. (129) reported that MetS is associated with more aggressive tumor biology, such as later tumor stage ($P = 0.022$) and lymph node invasion ($P = 0.028$). Interventions, including medications, such as metformin and statins, low-calorie and low-fat diets and appropriate exercise to improve metabolic disorders, are needed to reduce the risk of comorbidities in breast cancer patients. The measures to treat MetS may consequently have a positive impact on the prognosis of patients to a certain extent.

Metformin is widely used in the treatment of hyperglycemia. Jiralersong et al. (183) showed that during neoadjuvant chemotherapy for breast cancer, patients with diabetes who

received metformin had a higher pathologic complete response (pCR) rate (24%, 95% CI 13%-34%) compared with those who did not (8%, 95% CI 2.3%-14%). These researchers additionally indicated that metformin use during neoadjuvant chemotherapy was an independent predictor of pCR (OR 2.95, 95% CI 1.07-8.17, P = 0.04). Given the differences in insulin usage among the enrolled population, they further analyzed the effect of insulin usage on pCR. In the metformin group, the rate of pCR was not different for insulin use vs. no insulin use (27% vs. 23%, P = 0.75). Metformin plays a role in inhibiting the proliferation, invasion and angiogenesis of tumor cells by reversing hyperinsulinemia (183) and improving insulin resistance (34). Some *in vitro* studies have shown that the antitumor effect of metformin *via* epidermal growth factor receptor (EGFR)-mediated pathways (49) is most prominent in TNBC cell lines. In addition, metformin may target the immune microenvironment of tumors to inhibit tumor proliferation (184).

It was reported that breast cancer patients without hyperlipidemia had worse DFS (HR 1.447, 95% CI 1.080-1.937, P = 0.013) and OS (HR 3.085, 95% CI 1.836-5.183, P < 0.001) (146), which may be due to the use of statins. The retrospective analysis of Li et al. (185) found that long-term use of statins (> 5 years) was associated with improvements in OS (HR = 0.38, 95% CI 0.17-0.85, P < 0.018) and DFS (HR = 0.15, 95% CI 0.05-0.48, P < 0.001), even after adjusting for metabolic comorbidities. There was no significant difference in OS between patients taking statins for less than 5 years and those who did not take statins. A randomized phase III trial (186) conducted by the International Breast Organization on 8,010 patients with early postmenopausal hormone receptor-positive invasive cancer indicated that compared with patients who did not use cholesterol-lowering drugs, patients who received cholesterol-lowering drugs before endocrine therapy had better DFS (HR = 0.82, 95% CI 0.68-0.99). *In vitro*, statins inhibit the proliferation of the breast cancer cell line MCF-7, which may be due to the blockade of hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase (187). Ghosh-Choudhury et al. (188) found that simvastatin significantly inhibits the phosphorylation of Akt kinase in MDA-MB-231 breast cancer cells and further inhibits the mammalian target of rapamycin (mTOR) pathway. Statins also induce apoptosis in a variety of cancer cell lines, including colon, prostate, and breast cancer cells (185). Additionally, statins are well tolerated, and their drug interactions are limited. Among different statin types, lipophilic drugs show direct inhibition of breast cancer cell growth *in vitro* and *in vivo*, whereas, hydrophilic drugs such as pravastatin, have no effect (187, 189, 190).

For a long time, aerobic exercise has been widely believed to be effective in improving the abnormal metabolic state of the body, such as reducing fasting blood sugar, HDL, TGs, and WC. Resistance exercise can induce changes in insulin sensitivity by maintaining and/or increasing lean body mass, increasing glucose storage, reducing circulating glucose levels, and promoting a decrease in the amount of insulin required by obese people. A prospective study of 1,490 women with breast cancer conducted by Women's Healthy Eating and Living reported that the equivalent of walking 30 minutes a day for 6 days a week plus eating at least 5 servings of fruit and vegetables a week significantly benefited survival (HR = 0.56, 95% CI 0.31-0.98). However, in the analysis of subtypes, this lifestyle intervention can only reduce the mortality of ER+ tumors (P < 0.05).

Adhering to the Mediterranean diet is another option to reduce the occurrence of metabolic syndrome, preventing the prevalence of breast cancer and improving its prognosis. Some cohort studies have observed a corresponding risk reduction (191–193). The main characteristics of the Mediterranean diet are extensive consumption of fruits, vegetables, unrefined grains, legumes, fish, cereals, nuts, olive oil, and moderate drinking of wine during the main meal (194). Shifting to a Mediterranean diet can improve the imbalance of body metabolism (195) and prevent intractable diseases related to insulin resistance, such as obesity and breast cancer (196). A systemic review published in 2008 confirmed that the Mediterranean diet has a significant negative correlation with the risk of postmenopausal ER- breast cancer (197). Reducing inflammation may be a possible mechanism for the anticancer effect of the Mediterranean diet (1).

Therefore, medications for metabolic disorders and specific lifestyles may be effective strategies to improve the outcome of breast cancer patients, especially those with MetS, after diagnosis to obtain a better prognosis. These research conclusions may provide us with innovative treatments.

CONCLUSION

Metabolic syndrome and its components have been widely considered to be correlated with the initiation and progression of breast cancer, which is due to obesity and its related adipokines, insulin and IGFs, abnormal serum lipids and lipoproteins and the molecules leading to hypertension. These molecular changes partly exert a profound influence on the tumor and its microenvironment. Metabolic syndrome is significantly associated with an increased risk, worse treatment response, invasive progression and poor prognosis of breast cancer. Notably, we systematically reviewed the mechanisms and pathways of the highlighted molecules affecting disease progression and summarized several potentially novel treatment targets. In the future, new treatment strategies can be prospectively performed based on the above findings to improve prognosis and improve quality of life for breast cancer patients.

AUTHOR CONTRIBUTIONS

SD, ZW, and XC designed this study. SD performed the search and analysis. SD and ZW wrote the manuscript. XC and KS helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (81772797, 82072897, and 82002773), Shanghai Municipal Education Commission—Gaofeng Clinical Medicine Grant Support (20172007), Ruijin Hospital, Shanghai Jiao Tong University School of Medicine-Guangci Excellent Youth Training Program (GCQN-2017-A18), and Ruijin Youth NSFC Cultivation Fund (2019QNPY01046).

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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.

GLOSSARY

27HC	27-hydroxycholesterol
AdipoR	adiponectin receptor
AI	aromatase inhibitor
AMP	adenosine monophosphate
AMPK	adenosine monophosphate kinase
ANG	angiotensin
APPL1	adaptor protein containing the pleckstrin homology domain, phosphotyrosine-binding domain, and leucine zipper motif 1
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
ATP	adenosine triphosphate
BMI	body mass index
BCSC	breast cancer stem cell
CAA	cancer-associated adipocytes
CAM	calmodulin
CAMK	calmodulin kinase
CI	confidence interval
DFS	disease-free survival
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ER	estrogen receptor
ERK	extracellular regulated protein kinases
fAd	full-length adiponectin
gAd	globular adiponectin
HDL	high-density lipoprotein
HER2	human epidermal growth factor receptor 2
HMG-CoA	hydroxy methyl glutaryl coenzyme A
HTWC	hypertriglyceridemia waist circumference
IGF-1	insulin growth factor-1
IGFBP	insulin growth factor binding protein
IL	interleukin
IRS	insulin receptor substrate
JAK	Janus kinase
LDL	low density lipoprotein
LKB1	liver kinase B1
LVEF	left ventricular ejection fraction
LXR	liver x receptor
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
MetS	metabolic syndrome
MMP	matrix metalloproteinases
mTOR	mammalian target of rapamycin
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa-B
OR	odds ratio
OS	overall survival
PARP	poly adenosine diphosphate-ribose polymerase
pCR	pathologic complete response
PI3K	phosphatidylinositol 3-kinase
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
PPAR	peroxisome proliferators-activated receptors
PR	progesterone receptor
PTB	phosphotyrosine binding
RAS	renin-angiotensin system
RFS	relapse-free survival
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	risk ratio
S6K	p70S6 kinase
Ser	serine
SHBG	sex hormone binding globulin
STAT3	signal transducers and activators of transcription 3
TAM	tamoxifen
TC	total cholesterol
TG	total triglyceride
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
TRPC	transient receptor potential-canonical
ULK	unc-51-likekinase
VEGF	vascular endothelial growth factor
VLDL	very-low-density-lipoprotein
WC	waist circumference
WHR	waist-to-hip ratio

frontiers in Oncology

ORIGINAL RESEARCH
published: 26 March 2021
doi: 10.3389/fonc.2021.619840

Comprehensive Association Analysis of 21-Gene Recurrence Score and Obesity in Chinese Breast Cancer Patients

Yiwei Tong, Weiqi Gao, Jiayi Wu, Siji Zhu, Ou Huang, Jianrong He, Li Zhu, Weiguo Chen, Yafen Li, Kunwei Shen and Xiaosong Chen**

Department of General Surgery, Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

OPEN ACCESS

Edited by:
San-Gang Wu,
First Affiliated Hospital of Xiamen University, China

Reviewed by:
Patrick Neven,
University Hospitals Leuven, Belgium
Can Zhou,
The First Affiliated Hospital of Xi'an Jiaotong University, China

****Correspondence:***
Xiaosong Chen
chenxiaosong0156@hotmail.com
Kunwei Shen
kwshen@medmail.com.cn

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *21 October 2020*
Accepted: *25 February 2021*
Published: *26 March 2021*

Citation:
Tong Y, Gao W, Wu J, Zhu S, Huang O, He J, Zhu L, Chen W, Li Y, Shen K and Chen X (2021) Comprehensive Association Analysis of 21-Gene Recurrence Score and Obesity in Chinese Breast Cancer Patients. Front. Oncol. 11:619840. doi: 10.3389/fonc.2021.619840

Purpose: A center-specific 21-gene recurrence score (RS) assay has been validated in Luminal-like, HER2-, pN0-1 Chinese breast cancer patients with both predictive and prognostic value. The association between RS and host factors such as obesity remains unclear. The objectives of the current study are to comprehensively analyze the distribution, single gene expression, and prognostic value of RS among non-overweight, overweight and obese patients.

Patients and methods: Luminal-like patients between January 2009 and December 2018 were retrospectively reviewed. Association and subgroup analysis between BMI and RS were conducted. Single-gene expression in RS panel was compared according to BMI status. Disease-free survival (DFS) and overall survival (OS) were calculated according to risk category and BMI status.

Results: Among 1876 patients included, 124 (6.6%), 896 (47.8%) and 856 (45.6%) had RS < 11, RS 11-25, and RS ≥ 26, respectively. Risk category was significantly differently distributed by BMI status (P=0.033). Obese patients were more likely to have RS < 11 (OR 2.45, 95% CI 1.38-4.35, P=0.002) compared with non-overweight patients. The effect of BMI on RS significantly varied according to menstruation (P<0.05). Compared to non-overweight patients, obese ones presented significantly higher *ER*, *PR*, *CEGP1*, *Ki67*, *CCNB1* and *GSTM1* (all P<0.05) mRNA expression, and such difference was mainly observed in postmenopausal population. After a median follow-up of 39.40 months (range 1.67-119.53), RS could significantly predict DFS in whole population (P=0.001). RS was associated with DFS in non-overweight (P=0.046), but not in overweight (P=0.558) or obese (P=0.114) population.

Conclusions: RS was differently distributed among different BMI status, which interacted with menopausal status. Estrogen receptor and proliferation group genes were more expressed in obese patients, especially in postmenopausal population.

Keywords: breast cancer, body mass index, obesity, prognosis, recurrence score

INTRODUCTION

Breast cancer is the most frequent malignancy reported in women worldwide (1). About 70% of breast cancer patients are of Luminal-like, human epidermal growth factor 2 (HER2)-negative subtype, which is characterized by the expression of hormone receptor (HR), and the absence of HER2 amplification (2). Over the past decade, in addition to traditional tumor anatomic, biologic features, genetic factors have been integrated to guide treatment decisions as well as predict disease outcomes in these patients. The Oncotype Dx is the most common multigene panel to predict chemotherapy benefit and prognosis for HR-positive, HER2-negative, node-negative patients, based on the findings of the prospective TAILORx trial (3, 4). In order to facilitate the application of genetic panel in the management of Chinese breast cancer patients, a center-specific 21-gene recurrence score (RS) panel was developed based on quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique. Our RS panel has previously been validated in both node-negative (5) and node-positive (6) patients with two large cohorts of Chinese patients. Increased RS was associated with poor differentiation, PR-negative or high-proliferation characteristics in Chinese early breast cancer patients, as indicated in our former work (5), which was comparable to the findings for Oncotype Dx in NSABP B-14 study population (4). In addition, our RS panel showed similar prognostic value in node-negative and positive diseases (6). With the help of RS testing, selective low RS patients can be spared from adjuvant chemotherapy, while chemotherapy is recommended for high RS patients.

Apart from tumor-intrinsic factors, the microenvironment in which tumors arise and progress substantially varies between individuals, calling for the necessity to identify host determinants for tumor behaviors (7). Obesity is a well-established risk factor for multiple cancers including breast cancer (7, 8). The evidence for the effect of obesity on breast cancer is generally based on studies using body mass index (BMI) as an alternative for total adiposity (9). The effect of obesity and overweight on breast cancer incidence differs before and after menopause (8). Several large meta-analyses have showed an inverse association between obesity and breast cancer risk in premenopausal population, with breast cancer risk being reduced by 8% per 5 kg/m^2 BMI increase (8–11). On the other hand, for postmenopausal women, obesity is positively associated with both increased overall and increased HR-positive breast cancer risk (8, 10). With regards to clinical outcomes, obesity is related to higher risk of disease recurrence and mortality for both premenopausal and postmenopausal breast cancer, with every 5 kg/m^2 increase in BMI augmenting the risk of breast cancer-specific death by 18% (8).

However, how obesity or overweight interacts with patient genetic profiles remains uncertain for breast cancer patients. In a retrospective study including 534 women with HR-positive, HER2-negative disease, Muniz et al. found that neither metabolic syndrome, nor any individual criterion including central obesity, had significant association with 21-gene RS group after stratification by menstrual status (12). It is also unclear whether RS can accurately predict disease outcomes in patients with different BMI status. One retrospective study involving 940 HR-positive breast cancer patients from the transATAC trial showed that Oncotype Dx had the highest prognostic effect in patients with BMI ≤ 25 kg/m^2, but decreasing effect size with increasing BMI (13). Evidence is still limited with regards to the relationship between genetic risk score and host BMI status in Luminal-like patients.

Therefore, in the current study, we aim to analyze the distribution of RS category and gene expression level among non-overweight, overweight and obese Chinese patients, to identify potential impact factors for the association of RS and BMI in HR-positive, HER2-negative breast cancer patients, and to explore the prognostic value of RS in Chinese patients with different BMI status.

MATERIALS AND METHODS

Study Population

Consecutive breast cancer patients receiving surgery in Comprehensive Breast Health Center, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, between January 2009 to December 2018 were retrospectively reviewed. The inclusion criteria were as listed below: 1) female gender; 2) invasive breast cancer; 3) HR-positive, HER2-negative disease; 4) available 21-gene RS result with cycle threshold (C_T) values for each gene. Exclusion criteria were as follows: 1) patients receiving preoperative systemic treatment; 2) *de novo* stage IV disease. The current study was reviewed and approved by the independent Ethical Committees of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from each participant. All procedures were in accordance with the ethical standards of national research committee and with the 1964 Helsinki declaration and its later amendments.

Data Collection

Patient clinical information was retrieved from Shanghai Jiao Tong University Breast Cancer Database (SJTU-BCDB). Patients aged no less than 60 years, <60 years and amenorrheic for ≥ 36 months, or with prior bilateral oophorectomy were considered postmenopausal. Patient's height and weight were measured on the day of hospital admission for surgical treatment and BMI was calculated by dividing weight (kg) by the square of height (m^2). Patients were then classified into non-overweight (BMI <24.0 kg/m^2), overweight (BMI ≥ 24.0 and <28.0 kg/m^2) and obese (BMI ≥ 28.0 kg/m^2) subgroups, according to the recommended cutoffs for Chinese population from the Guidelines for Prevention and Control of Overweight and Obesity in Chinese Adults (Ministry of Health of the People's Republic of China, People's Medical Publishing House, 2006) (14, 15).

The histo-pathologic evaluation of the tumor was accomplished in the Department of Pathology, Ruijin Hospital by at least two independent, experienced pathologists (C Wang, X Fei, X Jin and J Xie). The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP)

guidelines were adopted for the immunohistochemistry (IHC) assessment of estrogen receptor (ER), progesterone receptor (PR), HER2 and Ki-67, as described in our previous studies (5, 6). HR positive was defined as no less than 1% invasive tumor cells with positive nuclear staining (16). The cut-off point for ER high and low expression was set at 50% (17, 18). HER2 negative was defined as IHC 0 or 1+, and IHC 2+ with fluorescence *in situ* hybridization negative (19). According to the 2013 St. Gallen Consensus, tumors were classified into two molecular subtypes, which were Luminal A-like (ER+/PR≥20%/Ki-67<14%), and Luminal B-like (ER-/PR+/any Ki-67, or ER+/PR<20%/any Ki-67, or HR+/Ki-67≥14%) (18).

Patient follow-up was accomplished by specialized breast cancer nurses in our center. Clinical outcomes were analyzed according to the STEEP system (20). Disease-free survival (DFS) was calculated from the date of surgery to the recurrence of tumor including ipsilateral, local/regional or distant recurrence, second non-breast malignancy, and death attributable to any cause. Overall survival (OS) was calculated from the date of surgery till death of any cause. Last follow-up was completed by February 2020.

21-Gene Recurrence Score Evaluation

The 21-gene assay testing was conducted in the Department of Clinical Laboratory, Ruijin Hospital by Lin L, Lin J and Meng J, as described in our previous work (5, 6). RNA extraction and reverse transcription were performed with RNeasy FFPE RNA kit (Qiagen, 73504, Germany) and Omniscript RT kit (Qiagen, 205111, Germany), respectively. Quantitative RT-PCR was accomplished in Applied Biosystems 7500 Real-Time PCR System (Foster City, CA) using Premix Ex TaqTM (TaKaRa Bio, RR390A). C_T value, defined as the number of cycles required for the fluorescent signal to cross a certain threshold, was verified in triplicate, and then normalized to reference genes β-*actin*, *GAPDH*, *GUS*, *RPLPO* and *TFRC*. The relative expression level of each target gene, in form of $-\Delta C_T$ value, was defined as $C_{T\ reference} - C_{T\ gene}$. The 21-gene RS was calculated from the reference gene-normalized formula, then applied to classify patients into low risk (RS ≤ 11), intermediate risk (RS 11-25), and high risk (RS ≥ 25) groups. For those with multifocal diseases, the highest RS was recorded.

Statistical Analysis

Chi-square test and multivariate logistic regression were applied to compare the distribution of categorical variables by BMI status in the study population. T-test was adopted to compare the distribution of RS by BMI intervals. Subgroup analysis of interacting factors with BMI and 21-gene RS was accomplished using stratified Mantel-Haenszel test to estimate odds ratio (OR) with 95% confidence interval (CI). The comparison of gene expression in 21-gene RS panel by BMI status was demonstrated in terms of violin plots. Univariate survival analyses were conducted using Kaplan–Meier curves. Data analysis and image production were performed using IBM SPSS statistics software version 23 (SPSS, Inc., Chicago, IL) and GraphPad Prism version 8.0 (GraphPad Software, CA, USA). Two-sided *P* value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics Stratified by Body Mass Index Status

Overall, 1876 Luminal-like breast cancer patients were enrolled in the current study (**Supplementary Figure S1**). The baseline clinical pathological characteristics of the participants were presented in **Table 1**. The average age was 57 ± 12.50 (range 24-92) years. All but four patients had ER-positive disease, among whom 98 had ER ≤ 50%. PR staining was positive in 88.2% of the population. Luminal A-like and Luminal B-like subtypes were found in 32.0% and 68.0% cases.

Among patients included, 1139 (60.7%) patients were non-overweight, including 84 underweight (BMI ≤ 18.5 kg/m^2), while 551 (29.4%) were overweight, and 186 (9.9%) obese. Univariate analysis (**Table 1**) and multivariate analysis (**Table 2**) demonstrated that the overall distribution of age (*P*=0.005), grade (*P*=0.030), tumor size (*P*=0.009), and PR status (*P*=0.041) were significantly distinguishable among three BMI subgroups. Compared to non-overweight patients, overweight ones were less likely to be young (≤50 *vs* >65: OR 0.43, 95% CI 0.26-0.74, *P*<0.001), to have low tumor grade (I *vs* III: OR 0.48, 95% CI 0.30-0.78, *P*=0.003), smaller tumor size (≤2.0 *vs* >2.0: OR 0.68, 95% CI 0.54-0.87, *P*=0.002), and negative PR status (OR 0.68, 95% CI 0.47-0.98, *P*=0.037). Meantime, obese patients were less likely to be <50 years (*vs* >65: OR 0.33, 95% CI 0.14-0.76, *P*=0.009), while tumor grade, size and PR status were similarly distributed compared to non-overweight ones.

Association Between 21-Gene Recurrence Score and Body Mass Index Status

Among the included population, 124 (6.6%), 896 (47.8%) and 856 (45.6%) were classified into low, intermediate and high risk groups, with an average 21-gene RS of 25.77 (95% CI 25.20-26.33; **Table 1**). As shown in **Figure 1**, RS distribution was significantly different in patients with various BMI status (*P*=0.006), with an average RS of 28.48 ± 11.91 in underweight, 26.05 ± 11.74 in non-overweight, 25.15 ± 11.59 in overweight, and 23.17 ± 12.26 in obese population. After adjusting for clinico-pathologic confounders, multivariate analysis demonstrated that RS category was independently significantly associated with BMI status (*P*=0.033, **Table 2**). Obese patients were more likely to have an RS ≤ 11 (OR 2.45, 95% CI 1.38-4.35, *P*=0.002) compared with non-overweight patients.

Further subgroup analysis was conducted comparing the odds of having higher RS (RS ≥ 26) between different BMI status, which identified menstruation status as the only interacting factor on the association of BMI and 21-gene RS (**Figure 2**). In detail, overweight patients were significantly less likely to have

TABLE 1 | Baseline characteristics of study participants (N = 1,876).

Characteristics	Total N = 1,876	Non-overweight N = 1,139 (%)	Overweight N = 551 (%)	Obese N = 186 (%)	*P* value
Age, years					**<0.001**
<50	573	436 (38.3)	110 (20.0)	27 (14.5)	
50-65	812	451 (39.6)	271 (49/2)	90 (48.4)	
>65	491	252 (22.1)	170 (30.9)	69 (37.1)	
Menopausal status					**<0.001**
Premenopausal	654	482 (42.3)	138 (25.0)	34 (18.3)	
Postmenopausal	1222	657 (57.7)	413 (75.0)	152 (81.7)	
Breast surgery					0.470
BCS	827	515 (45.2)	233 (42.3)	79 (42.5)	
Mastectomy	1049	624 (54.8)	318 (57.7)	107 (57.5)	
ALN surgery					**0.027**
SLNB	879	562 (49.3)	237 (43.0)	80 (43.0)	
ALND	997	577 (50.7)	314 (57.0)	106 (57.0)	
Histology					0.434
IDC	1616	977 (85.8)	473 (85.8)	166 (89.2)	
Non-IDC	260	162 (14.2)	78 (14.2)	20 (10.8)	
Tumor grade					**0.014**
I	175	123 (10.8)	31 (5.6)	21 (11.3)	
II	1122	667 (58.6)	339 (61.5)	116 (62.4)	
III	369	215 (18.9)	120 (21.8)	34 (18.3)	
NA	210	134 (11.8)	61 (11.1)	15 (8.1)	
Tumor size, cm					**<0.001**
≤2.0	1303	831 (73.0)	345 (62.6)	130 (69.9)	
>2.0	570	308 (27.0)	206 (37.4)	56 (30.1)	
ALN					**0.030**
Negative	1563	968 (85.0)	440 (79.9)	155 (83.3)	
Positive	313	171 (15.0)	111 (20.1)	31 (16.7)	
ER, %					0.078
≥50	1778	1069 (93.9)	531 (96.4)	178 (95.7)	
<50	98	70 (6.1)	20 (3.6)	8 (4.3)	
PR status					**0.026**
Negative	222	152 (13.3)	56 (10.2)	14 (7.5)	
Positive	1654	987 (86.7)	495 (89.8)	172 (92.5)	
Ki-67, %					0.346
<14	886	549 (48.2)	246 (44.6)	91 (48.9)	
≥14	990	590 (51.8)	305 (55.4)	95 (51.1)	
Molecular subtype					0.204
Luminal A-like	601	362 (31.8)	169 (30.7)	70 (37.6)	
Luminal B-like	1275	777 (68.2)	382 (69.3)	116 (62.4)	
21-gene RS[a]					**0.002**
Low risk	124	67 (5.9)	34 (6.2)	23 (12.4)	
Intermediate risk	896	529 (46.4)	270 (49.0)	97 (52.2)	
High risk	856	543 (47.7)	247 (44.8)	66 (35.5)	

[a]*The cut-off for RS category was <11, 11–25, >25.*

BCS, breast conserving surgery; ALN, axillary lymph node; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; IDC, invasive ductal carcinoma; NA, not available; ER, estrogen receptor; PR, progesterone receptor; RS, recurrence score.

Bold values mean statistically significant.

RS ≥ 26 compared to those non-overweight after menopause (OR 0.64, 95% CI 0.51-0.80, *P*<0.001), while such difference no longer held in premenopausal population (OR 1.25, 95% CI 0.88-1.77, *P*=0.216; *P* for interaction=0.002; **Figure 2A**). Alternatively, obese patients had lower odds for high risk RS than non-obese ones, but the significance was only observed in postmenopausal subgroup (OR 0.49, 95% CI 0.34-0.70, *P*<0.001), not in premenopausal women (OR 1.38, 95% CI 0.69-2.75, *P*=0.366; *P* for interaction=0.009; **Figure 2B**). In addition, average RS score decreased with increasing BMI in postmenopausal patients (*P*=0.020, **Figure 1**), but not in premenopausal patients (*P*=0.843).

Single Gene Expression in 21-Gene Recurrence Score Panel by Body Mass Index Status

Supplementary Table S1 summarized the gene expression and gene group score in the 21-gene RS panel of the study population. Single gene expression was further compared according to BMI status. Compared to normal weight patients, obese patients presented significantly higher ER group score (*P*=0.002), with higher *ER* (*P*<0.001; **Figure 3**), higher *PR* (*P*=0.004), higher *CEGP1* (*P*<0.001) expression, and tended to have higher proliferation group score (*P*=0.060), with higher *Ki67* (*P*=0.006), and higher *CCNB1* (*P*=0.020). In addition,

TABLE 2 | Multivariate analysis of factors associated with BMI status.[a]

Characteristics	Overweight (N = 551)		Obese (N = 186)		*P* value
	OR	95% CI	OR	95% CI	
Age, years					**0.005**
<50 *vs* >65	0.43	0.26-0.74	0.33	0.14-0.76	
50-65 *vs* >65	0.91	0.69-1.19	0.80	0.55-1.16	
Menstruation status					0.413
pre- *vs* post-	0.91	0.58-1.42	0.63	0.31-1.28	
Grade					**0.030**
I *vs* III	0.48	0.30-0.78	0.90	0.48-1.67	
II *vs* III	0.91	0.69-1.20	0.96	0.62-1.47	
Tumor size, cm					**0.009**
≤2.0 *vs* >2.0	0.68	0.54-0.87	0.87	0.60-1.26	
ALN status					0.529
Negative *vs* Positive	0.88	0.66-1.18	1.12	0.72-1.76	
PR status					**0.041**
Negative *vs* Positive	0.68	0.47-0.98	0.59	0.33-1.084	
RS category[b]					**0.033**
Low *vs* High	1.05	0.65-1.70	2.45	1.38-4.35	
Intermediate *vs* High	1.13	0.89-1.43	1.40	0.97-2.03	

[a]*The reference category for subtype characteristics is BMI < 24 kg/m^2 (N = 1,139).*

[b]*The cutoff for RS category was <11, 11–25, >25.*

BMI, body mass index; OR, odds ratio; CI, confidence interval; ALN, axillary lymph node; PR, progesterone receptor; RS, recurrence score.

Bold values mean statistically significant.

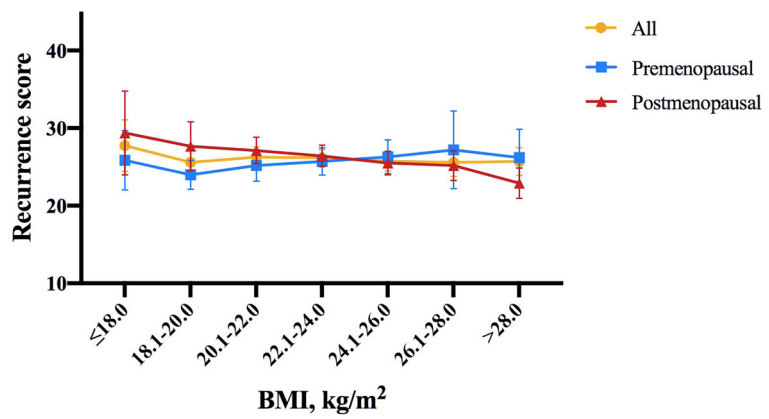

FIGURE 1 | Distribution of 21-gene RS by BMI intervals of 2.0 according to menopausal status in the study population. In whole population, RS distribution was significantly different in patients with different BMI status (P = 0.006). Average RS score tended to decrease with increasing BMI in postmenopausal patients (P = 0.020), but not in premenopausal patients (P = 0.843). The symbols refer to average score, error bars refer to standard deviation (yellow: all patients, blue: premenopausal population, red: postmenopausal population). RS, recurrence score; BMI, body mass index.

GSTM1 was significantly elevated in the obese group (P=0.001). On the other hand, overweight patients had generally similar gene expression compared to non-overweight ones, except for significantly higher *ER* (P<0.001). The HER2 group score (P=0.467) and invasion group score (P=0.210) were comparable among BMI groups.

When stratified by menstrual status, no significant difference in single gene expression among different BMI subgroups was observed in premenopausal population (**Supplementary Figure S2**). ER group (P=0.814), HER2 group (P=0.826), proliferation group (P=0.539), and invasion group (P=0.386) scores were comparable by BMI status. However, for postmenopausal population, ER group (P<0.001) and proliferation group (P=0.044) scores were significantly distinguishable among various BMI status, while HER2 group (P=0.252) and invasion group (P=0.892) scores were identical. BMI ≥ 28 kg/m^2 was associated with considerably higher *PR* (P<0.001; **Supplementary Figure S3**), higher *CEGP1* (P<0.001), and higher *GSTM1* (P=0.005) expression compared to normal weight group. Overweight patients expressed higher *GRB7* (P=0.033) and higher *PR* (P<0.001) than those with BMI ≤ 24 kg/m^2.

Clinical Outcomes by Body Mass Index Status and Recurrence Score Category

After a median follow-up of 39.40 months (range 1.67-119.53), 109 (5.81%) DFS events were observed, including 22 local

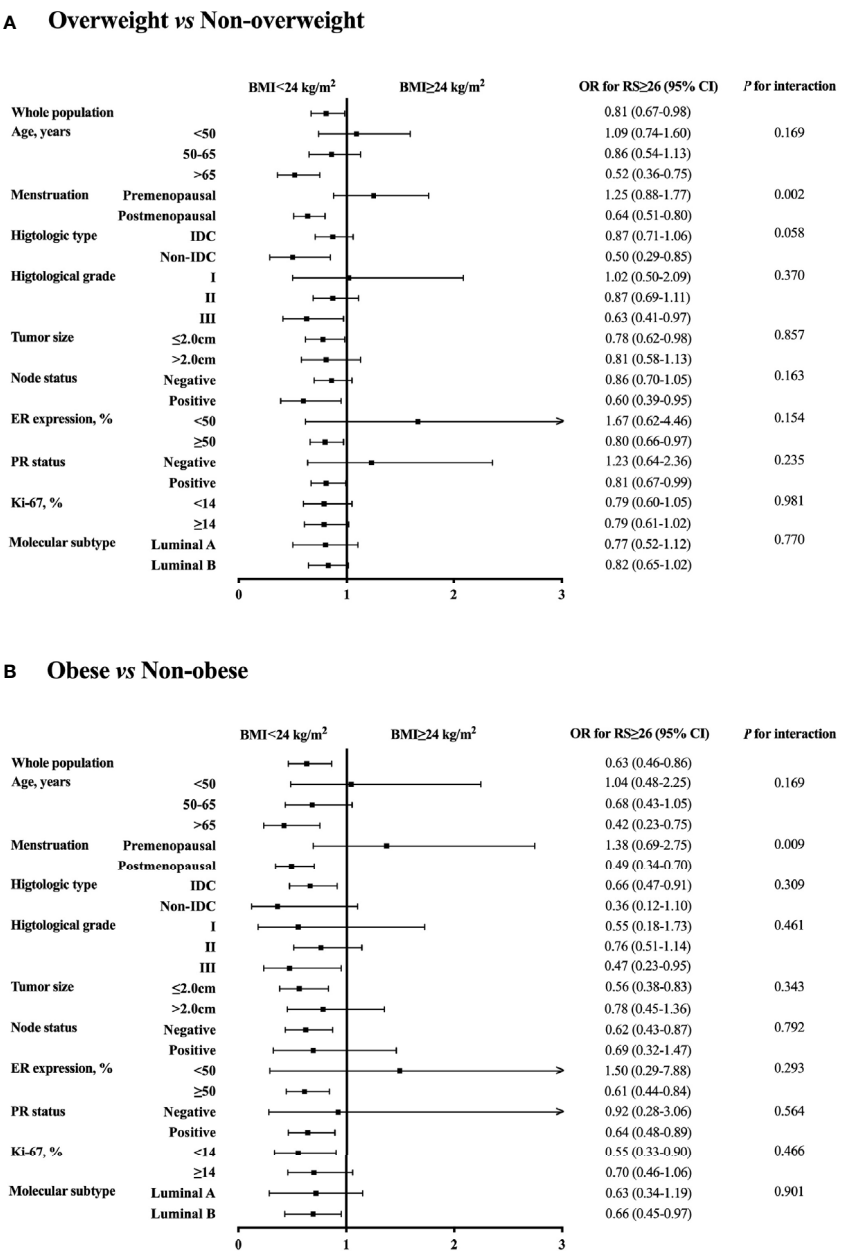

FIGURE 2 | Subgroup analysis of interacted factors with BMI and 21-gene RS. The odds with 95% CI for RS ≥ 26 were compared between **(A)** overweight *vs* non-overweight, and **(B)** obese *vs* non-obese patients by each subgroup. BMI, body mass index; OR, odds ratio; RS, recurrence score; CI, confidence interval; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor.

regional recurrences, 13 contralateral breast cancer, 27 distant metastases, 25 second non-breast malignancy, and 22 deaths.

In all, non-overweight, overweight, and obese patients had similar DFS in whole population (5-year DFS 91.61% *vs* 92.60% *vs* 89.41%, *P*=0.227; **Supplementary Figure S4**), low RS group (5-year DFS 95.36% *vs* 100.00% *vs* 83.33%, *P*=0.225), intermediate RS group (5-year DFS 93.70% *vs* 94.01% *vs* 94.88%, *P*=0.996), and high RS group (5-year DFS 88.97% *vs* 89.91% *vs* 83.45%, *P*=0.090), respectively. OS was also comparable among different BMI subgroups in the whole population (*P*=0.178), low RS (*P*=0.167), intermediate RS (*P*=0.809), and high RS (*P*=0.331) groups. In addition, when applying different BMI cutoff values, we also found comparable disease outcomes between patients with BMI ≥ 24 kg/m^2 and <24 kg/m^2 (DFS: *P*=0.933; OS: *P*=0.104; **Supplementary Figures S5A, S5B**), between patients with BMI ≥ 28 kg/m^2 and <28 kg/m^2 (DFS: *P*=0107; OS: *P*=0.138; **Supplementary Figures S5C, S5D**) and between patients with BMI ≥ 30 kg/m^2

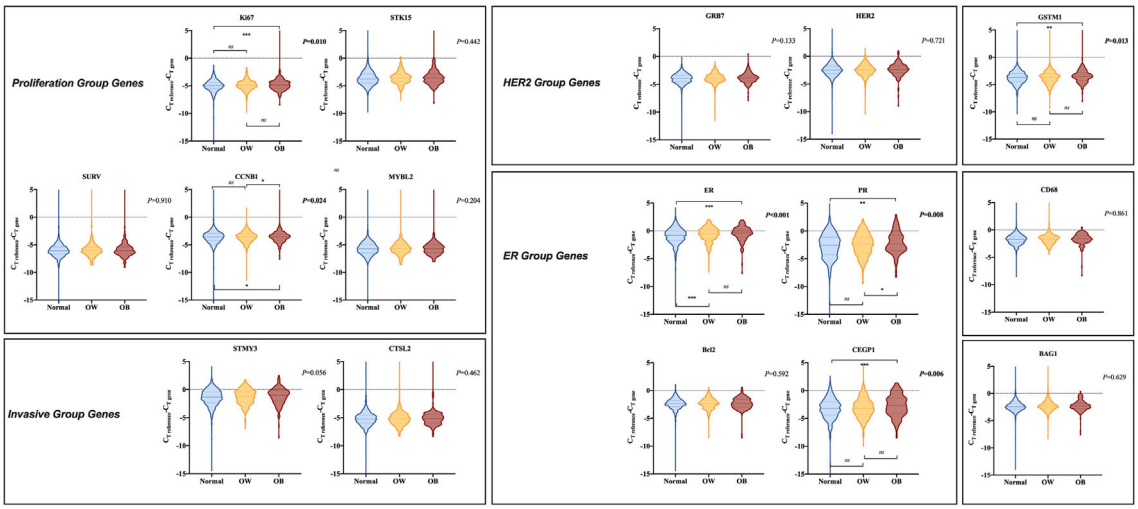

FIGURE 3 | Comparison of gene expression in 21-gene RS panel by BMI status. The violin plots refer to the expression of a certain gene. The dashed lines and the dotted lines refer to the median and quartiles, respectively. BMI, body mass index; C_T, cycle threshold; OW, overweight; OB, obese; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor.

and <30 kg/m^2 (DFS: *P*=0.198; OS: *P*=0.231; **Supplementary Figures S5E, S5F**).

RS category significantly predicts DFS in the whole population (5-year DFS 95.41% for low RS *vs* 93.90% for intermediate RS *vs* 88.94% for high RS, *P*=0.001; **Figure 4**). Other impact factors on DFS identified in the univariate analysis included tumor grade, size, ER, Ki-67, molecular subtype, and adjuvant endocrine therapy usage (all *P*<0.05; **Supplementary Table S2**). In patients with normal weight, RS category (5-year DFS 95.36% *vs* 93.70% *vs* 88.97%, *P*=0.046), together with histology, tumor grade, size, and adjuvant endocrine therapy (all *P*<0.05) was associated with DFS. However, RS category was not associated with DFS in overweight (*P*=0.558) or obese (*P*=0.114) population. Furthermore, no statistically significant difference was found with regards to OS in the whole population (*P*=0.194), non-overweight (*P*=0.404), overweight (*P*=0.530) or obese (*P*=0.219) patients.

DISCUSSION

In this study, which involved 1876 HR-positive, HER2-negative breast cancer patients with 21-gene RS records, we found that RS

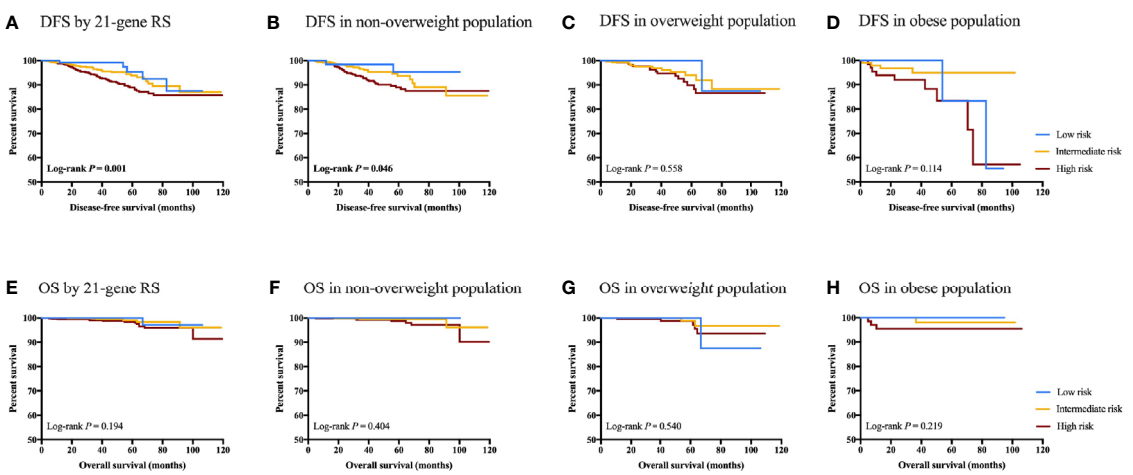

FIGURE 4 | Clinical outcomes of breast cancer patients with different RS category stratified by BMI status. DFS in **(A)** DFS by 21-gene RS; **(B)** DFS in non-overweight population; **(C)** DFS in overweight population; **(D)** DFS in obese population; **(E)** OS by 21-genes RS; **(F)** OS in non-overweight population; **(G)** OS in overweight population; and **(H)** OS in obese population.

category was significantly differently distributed among patients with different BMI status. Menstrual status was an interacting factor for the association of BMI and 21-gene RS. With regards to the gene expression of 21-gene RS panel, obese patients presented significantly higher *ER*, *PR*, *CEGP1*, *Ki67*, *CCNB1* and *GSTM1* (all $P<0.05$) than non-overweight ones in the whole population. Overweight patients had generally similar gene expression pattern except for higher *ER* ($P<0.001$) than non-overweight ones. In terms of disease outcome, BMI was not an independent factor for DFS ($P=0.227$) or OS ($P=0.178$) in HR-positive, HER2-negative patients. The prognostic value of RS was decreased in patients overweight or obese. To our knowledge, this is the largest study, as well as the first in Chinese population, to focus on the comprehensive association of 21-gene RS and BMI in Luminal-like patients, which provides evidence of association between tumor genetic profile and host metabolic factor.

Previous studies have shown that obesity leads to increased free fatty acid release, hyperinsulinemia, persistent low-grade inflammation, and abnormal secretion of adipokines, resulting in disease development or progression (8). Overwhelming consensus has been made with regards to the adverse effects of obesity on breast cancer prognosis (8, 21). For HR-positive patients, an analysis from the NSABP B-14 trial demonstrated that obese women had a 30% increased mortality risk compared to non-obese ones (22). In a joint analysis of 6885 women from E1199, E5188, and E3189 clinical trials, Sparano et al. showed that BMI ≥ 30 kg/m^2 was associated with inferior DFS and OS in Luminal-like patients (23). Another meta-analysis of 21 trials indicated that obesity was associated with higher breast cancer-specific mortality for HR-positive patients, regardless of menopausal status (24). In spite of prior studies showing impaired prognosis with obesity in breast cancer patients, our study demonstrated no significant difference in time to recurrence or mortality based on BMI status. Such discrepancy may be attributed to the different study population, different BMI cutoffs, the overall low event incidence in our cohort, and rather inadequate follow-up of 39.40 months. Moreover, when applying different BMI cutoff values, we also found comparable disease outcomes between patients with BMI ≥ 24 kg/m^2 *vs* <24 kg/m^2, BMI ≥ 28 kg/m^2 *vs* <28 kg/m^2, and BMI ≥ 30 kg/m^2 *vs* <30 kg/m^2. Along with our finding, one study of Cespedes Feliciano et al. found that among women with PAM50 Luminal A disease, those who had BMI ≥35 kg/m^2, but not BMI 30-35 kg/m^2 or overweight, had worse prognosis, while no association between BMI and prognosis was observed for Luminal B subtype (25).

The association between host obesity and genetic profile of Luminal-like breast cancer patients remains indeterminate. Several studies found a limited correlation between obesity and breast cancer genomics. For example, Muniz et al. revealed that central obesity was not associated with 21-gene RS category after stratification by menopausal status in a cohort of 534 HR-positive, HER2-negative patients (12). A lifestyle study of MINDACT trial-enrolled population involving 1555 patients showed that BMI was not an independent impact factor for the prognostic 70-gene expression signature MammaPrint, regardless of menstrual status (26). Nevertheless, our cohort of 1876 HR-positive, HER2-negative patients revealed a significant association between BMI status and RS category, which was only established in postmenopausal population. The inconsistency between previous findings and ours may be due to the BMI cutoff as well as the RS cutoff applied in the study. To note, one strength of our study was that we managed to conduct a subgroup analysis, showing that the association of BMI and RS was substantially influenced by menopausal status. After menopause, the possibility for obese or overweight patients to have a lower RS than non-overweight ones significantly rose. This is to our knowledge the first study presenting the interaction between menstruation and the correlation of BMI and 21-gene RS.

Another highlight was that we revealed, for the first time, the potential influence of BMI on single gene expression in the 21-gene RS panel. Overall, ER group genes including *ER*, *PR*, *CEGP1*, had substantially higher expression in obese patients compared to non-overweight ones, which was mainly found in the postmenopausal population. Overweight patients also expressed higher level of *ER* than those non-overweight. This finding added to the previous notion that for postmenopausal women, obesity is correlated with higher plasma levels of estradiol derived from adipose tissue (27) and increased risk of ER-positive breast cancer (28). In addition, we also found that the expression of proliferation group genes *Ki67* and *CCNB1* was significantly elevated in obese population. Meanwhile, the difference in proliferation group gene expression was less obvious between overweight *vs* non-overweight patients. As shown by Kwan et al., patients with BMI ≥ 35 kg/m^2 had higher expression of proliferation genes compared with normal weight women (29). This might be due to the link between obesity and pro-inflammatory microenvironment, insulin resistance, the abnormal activation of insulin-like growth factor pathway, and altered adipokines, which results in more aggressive behavior of breast tumors.

Another issue to resolve is whether RS has identical prognostic value in patients with different BMI status. Sestak et al. found that 21-gene RS was most predictive in the lowest BMI tertile, and significantly less predictive in obese women, indicating an interaction of BMI and RS on the prediction of disease outcomes (13). Meanwhile in the same cohort, the prognostic value of Prosigna Risk of Recurrence Score was the greatest for women with a BMI 25 to 30 kg/m^2 (13), suggesting that the effect of BMI on genetic assay varied across panels. In consistent with previous evidence, here we demonstrated that RS category significantly predict DFS in the whole population ($P=0.001$), and for non-overweight patients ($P=0.046$). RS category was not associated with clinical outcomes in overweight ($P=0.558$) or obese ($P=0.114$) population. The prognostic value of RS might be decreased in patients overweight or obese, but our results should be validated with longer follow-up and more events.

Apart from the strengths, there are still some limitations. First of all, given the retrospective design of the study, selection biases might be inevitable. Secondly, as a result of inadequate follow-up time and relatively superior disease outcomes, limited events

were observed, so that our findings on clinical outcomes should be further validated. In addition, the current study was carried out in Chinese population, and the optimal BMI and RS cutoffs should be tested in the future to gain a better understanding of the association of obesity and 21-gene RS.

In conclusion, 21-gene RS category and gene expression were significantly differently distributed among patients with various BMI status, especially in postmenopausal patients. The prognostic value of RS might be influenced by host obesity, which warranted further validation.

DATA AVAILABILITY STATEMENT

Datasets are available on reasonable request from the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committees of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YT analyzed and interpreted the patient data and was a major contributor in writing the manuscript. WG and JW contributed to the data collection of 21-gene recurrence score. SZ, OH, JH, LZ, WC, and YL were responsible for the clinical data collection. XC substantially contributed to the conception of the work and revised the manuscript. KS substantively revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The authors appreciated the financial support from the National Natural Science Foundation of China (Grant Number: 81772797), Shanghai Municipal Education Commission—Gaofeng Clinical Medicine Grant Support (20172007), Ruijin Hospital, Shanghai Jiao Tong University School of Medicine—"Guangci Excellent Youth Training Program" (GCQN-2017-A18). All these financial sponsors had no role in the study design, data collection, analysis, or interpretation.

ACKNOWLEDGMENTS

The authors thank Dr. Yitian Xiao from the University of California, Davis, for language polishing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.619840/full#supplementary-material

Supplementary Figure S1 | Study population flowchart. HER2, human epidermal growth factor receptor 2; RS, recurrence score; CT, cycle threshold.

Supplementary Figure S2 | Comparison of gene expression in 21-gene RS panel by BMI status in premenopausal population. The violin plots refer to the expression of a certain gene. The dashed lines and the dotted lines refer to the median and quartiles, respectively. BMI, body mass index; CT, cycle threshold; OW, overweight; OB, obese; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor.

Supplementary Figure S3 | Comparison of gene expression in 21-gene RS by BMI status in postmenopausal population. The violin plots refer to the expression of a certain gene. The dashed lines and the dotted lines refer to the median and quartiles, respectively. BMI, body mass index; CT, cycle threshold; OW, overweight; OB, obese; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor.

Supplementary Figure S4 | Clinical outcomes of breast cancer patients with different BMI statuses stratified by 21-gene RS category. DFS in **(A)** whole, **(B)** low risk, **(C)** intermediate risk, and **(D)** high risk population; OS in **(E)** whole, **(F)** low risk, **(G)** intermediate risk, and **(H)** high risk population. Navy, yellow and red lines refer to non-overweight, overweight and obese group, respectively. DFS, disease-free survival; OS, overall survival; BMI, body mass index; RS, recurrence score.

Supplementary Figure S5 | Clinical outcomes of breast cancer patients with different BMI cutoffs. Disease-free survival and overall survival **(A, B)** whole, **(B)** low risk, **(C)** intermediate risk, and **(D)** high risk population; OS in **(E)** whole, **(F)** low risk, **(G)** intermediate risk, and **(H)** high risk population.

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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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 21 May 2021
doi: 10.3389/fonc.2021.616443

The Synergistic Effects of Pyrotinib Combined With Adriamycin on HER2-Positive Breast Cancer

Chaokun Wang[1], Shuzhen Deng[2], Jing Chen[1], Xiangyun Xu[1], Xiaochen Hu[1], Dejiu Kong[1], Gaofeng Liang[2], Xiang Yuan[1], Yuanpei Li[3] and Xinshuai Wang[1]**

[1] Henan Key Laboratory of Cancer Epigenetics, Cancer Hospital, The First Affiliated Hospital, College of Clinical Medicine, Medical College of Henan University of Science and Technology, Luoyang, China, [2] Medical College, Henan University of Science and Technology, Luoyang, China, [3] Department of Internal Medicine, UC Davis Comprehensive Cancer Center, University of California Davis, Sacramento, CA, United States

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***Correspondence:**
Xinshuai Wang
xshuaiw@haust.edu.cn
Yuanpei Li
lypli@ucdavis.edu

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: 12 October 2020
Accepted: 19 April 2021
Published: 21 May 2021

Citation:
Wang C, Deng S, Chen J, Xu X, Hu X, Kong D, Liang G, Yuan X, Li Y and Wang X (2021) The Synergistic Effects of Pyrotinib Combined With Adriamycin on HER2-Positive Breast Cancer. Front. Oncol. 11:616443. doi: 10.3389/fonc.2021.616443

Pyrotinib (PYR) is a pan-HER kinase inhibitor that inhibits signaling *via* the RAS/RAF/MEK/MAPK and PI3K/AKT pathways. In this study, we aimed to investigate the antitumor efficacy of pyrotinib combined with adriamycin (ADM) and explore its mechanisms on $HER2^+$ breast cancer. We investigated the effects of PYR and ADM on breast cancer *in vitro* and *in vivo*. MTT assay, Wound-healing, and transwell invasion assays were used to determine the effects of PYR, ADM or PYR combined with ADM on cell proliferation, migration, and invasion of SK-BR-3 and AU565 cells *in vitro*. Cell apoptosis and cycle were detected through flow cytometry. *In vivo*, xenograft models were established to test the effect of PYR, ADM, or the combined therapy on the nude mice. Western blotting was performed to assess the expression of Akt, p-Akt, p-65, p-p65, and FOXC1. The results indicated that PYR and ADM significantly inhibited the proliferation, migration, and invasion of SK-BR-3 and AU565 cells, and the inhibitory rate of the combination group was higher than each monotherapy group. PYR induced G1 phase cell-cycle arrest, while ADM induced G2 phase arrest, while the combination group induced G2 phase arrest. The combined treatment showed synergistic anticancer activities. Moreover, PYR significantly downregulated the expression of p-Akt, p-p65, and FOXC1. In clinical settings, PYR also exerts satisfactory efficacy against breast cancer. These findings suggest that the combination of PYR and ADM shows synergistic effects both *in vitro* and *in vivo*. PYR suppresses the proliferation, migration, and invasion of breast cancers through down-regulation of the Akt/p65/FOXC1 pathway.

Keywords: HER2 positive breast neoplasm, pyrotinib, adriamycin, synergistic, Akt

INTRODUCTION

Breast cancer (BC) is the most common malignant tumor among women in the world, with 2.1 million new patients and 626,679 deaths in 2018 (1). The overexpression of HER2 or gene amplification accounts for about 15-20% of all BC cases, which is related to the invasiveness of the tumor, and the prognosis is worse without appropriate therapy (2, 3). HER2, encoded by

oncogene ErbB2, is a transmembrane protein in human cells (4), which is involved in regulating cell proliferation, differentiation, and apoptosis through the activation of signal transduction by homo- or hetero-dimerization (5). Therefore, blocking the HER2 pathway is considered a potential therapy for BC. At present, several HER2-targeted agents are available to treat HER2-overexpressing BC. Five drugs were approved by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-positive BC, known as trastuzumab, pertuzumab, TDM-1, lapatinib, and neratinib (6–10). Furthermore, the Chinese State Drug Administration recently authorized a new tyrosine kinase inhibitor (TKIs), pyrotinib, for the treatment of patients with HER2-positive recurrence and metastasis breast cancer (11).

Pyrotinib is an oral, irreversible pan-ErbB receptor TKI with activity against HER1, HER2, and HER4 (12). Previous studies have suggested that pyrotinib can irreversibly inhibit multiple ErbB receptors and effectively inhibit the proliferation of HER2-overexpressing BC cells *in vivo* and *in vitro* (13, 14). By covalently binding with the ATP binding sites of intracellular kinase regions, pyrotinib inhibits the formation of homo- or hetero-dimerization and auto-phosphorylation of the HER family, thus blocking the activation of the RAS/RAF/MEK/MAPK and PI3K/AKT signaling pathways. AKT is activated *via* several mechanisms such as recruitment to the membrane by PIP3, PDK1, and mTORC2 (15–17). Activated phospho-AKT can phosphorylate a number of proteins including GSK-3b, 6-phosphofructo-2-kinase, and IκB (18, 19). The phosphorylation of IκB frees NF-κB and allows it to translocate to the nucleus to bind and subsequently activate target genes (20). The best characterized subunit of NF-κB is p65. This heterodimer is a potent activator of gene expression, where p65 is responsible for this activation (21–23). It was previously shown that activation of the NF-κB can upregulate FOXC1 expression. Overexpressed FOXC1 has been demonstrated in many different types of cancers (24–28). Recent studies have suggested that upregulation of FOXC1 may exacerbate cell invasion and indicate a poor prognosis due to EMT and drug resistance (24, 29, 30). It is unknown whether combination with adriamycin treatment inhibited BC cell proliferation, migration, and invasion through down-regulation of the Akt/p-65/FOXC1 signaling pathway.

In $HER2^+$ cancers, the usefulness of pyrotinib has been shown in preclinical and clinical reports (31–35). However, the clinical efficacy of pyrotinib alone is limited, and it is anticipated that development trends will involve combining it with chemotherapy for anti-HER2 treatments in the future. Currently, several ongoing clinical trials are exploring the combination of anti-HER2 agents with chemotherapy in HER2-positive BC and the clinical benefit of combined therapy of pyrotinib and chemotherapy is expected (36).

Adriamycin, also known as doxorubicin, is an anthracycline antibiotic that functions by intercalating DNA and inhibiting topoisomerase II. It has been widely used in the combination therapy of first-line antitumor agents and other antitumor agents for breast cancer (37–39). Until now, there was no more powerful evidence for the combined effect of pyrotinib and chemotherapy. Therefore, we hypothesize that the combination of both drugs would show synergistic anticancer activities against HER2-positive BC. In the present study, we investigated the antitumor efficacy of pyrotinib in combination with adriamycin and explored its related mechanisms as new therapeutics for HER2-positive BC. These results suggest that the combination of pyrotinib and adriamycin show strong synergistic antitumor effects both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

HER2-positive breast cancer cell lines SK-BR-3 and AU565 cells were maintained in DMEM medium supplemented with 10% FBS. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Chemicals and Antibodies

PYR was acquired from Hengrui Medicine Co. Ltd. ADM was acquired from Pfizer. Drugs were dissolved to a concentration of 200μg/ml in dimethyl sulfoxide (DMSO), diluted with PBS, and then stored at -80°C until use. The following antibodies: Akt, p-Akt, p65, p-p65, GAPDH, FOXC1 were obtained from Abcam (Abcam Trading Co., Ltd, Shanghai, China).

Cell Viability Assay

Cell viability was assayed by the MTT assays. SK-BR-3 or AU565 cells were seeded in 96-well plates at a density of 3000–5000 cells/well and were treated for 12 h with PBS, PYR, ADM, or both drugs in combination. The treatments continued for 48 h. The MTT solution (0.1 mg/ml) was added and then cultured for another 4 h, and the medium was subsequently removed. Next, 150 μl of DMSO was added to dissolve the formed formazan crystals. The absorbance of each well was measured at 570 nm by a microplate reader (Bio-Tek, Norcross, GA, U.S.A.). The mean IC50 values were calculated by SPSS. CompuSyn (ComboSyn Inc.) was used to calculate the combination index (CI) values (40).

Wound Healing Assay

Wound-healing assay was used to analyze the migration ability of SK-BR-3 and AU565 cells. We plated $1x10^6$ cells/well in 6-well plates and cultured them overnight until the cells reached 90% confluence. A straight scratch was created by a sterile pipette tip. The destroyed cells were rinsed off gently with PBS 3 times and followed by incubation with PBS, PYR (0.3, 3 μg/ml), ADM (0.1, 0.3μg/ml) or both drugs in combination (PYR 3 μg/ml + ADM 0.3μg/ml) for 24 h. Cell migration was observed and imaged at 0 h and 24 h with a digital camera. ImageJ software (NIH, Bethesda, MA, U.S.A.) was used to quantitatively analyze cells migrated to the denudated regions of each petri dish in the field of view. The experiments were independently performed three times.

Transwell Invasion Assays

The aperture of the bottom membrane of the Transwell chambers or wells (Corning Inc., Corning, NY, USA) was 8 μm. The chambers were coated with Matrigel (Sigma-Aldrich,

St. Louis, MO, USA), and were used for detecting the cell invasive ability. SK-BR-3 and AU565 cells were harvested and resuspended in serum-free DMEM, and 200 µl of cell suspension (5×10^5 cells/ml) containing PBS, PYR (0.3, 3 µg/ml), ADM (0.1, 0.3µg/ml) or both drugs in combination(PYR 3 µg/ml + ADM 0.3µg/ml). The cells were cultured in an incubator at 37˚C with 5% CO_2 for 24 h. The cells on the upper surface of the membrane were removed with cotton swabs. The migrated or invaded cells were fixed in 95% ethanol, stained with Hematoxylin. Cell numbers were counted in ten randomly selected fields under a light microscope at × 100 magnification. The cell numbers were counted by ImageJ software.

Cell Cycle Analysis

For cell cycle arrest assay, SK-BR-3 and AU565 cells were starved for 24 h before treatments. Then cell were treated with PBS, PYR (0.3, 3 mg/ml), ADM (0.1, 0.3mg/ml) or both drugs in combination (PYR 3 mg/ml + ADM 0.3mg/ml). After treatment for 24 h, The treated cells were washed with PBS and fixed in darkness at -20°C with 70% pre-cooled ethanol for 1h. Then the fixed cells were washed with PBS and treated with RNase I at 37°C for 30 minutes. Finally, the cells were stained with PI at 4°C for an additional 30 minutes and measured by BD FACS caliber. Each experiment was independently repeated in triplicate.

Apoptosis Analysis

SK-BR-3 and AU565 cells (2.5×10^5 cells/well) were plated in 6-well plates for the apoptosis assay and treated with PBS, PYR (0.3, 3 µg/ml), ADM (0.1, 0.3µg/ml), or both drugs in combination (PYR 3 µg/ml + ADM 0.3µg/ml) for 48h. Then, cells were washed with PBS, adjusted to 1×10^6 cells/ml, and treated with Annexin V-FITC/PI apoptosis detection kit based on the manufacturer's protocol. Finally, the cells were detected by BD FACS caliber, using the BD CellQuest Pro software for analysis.

Animals and Tumor Model

Female nude mice (4-5 weeks old) were raised in a specific pathogen-free animal facility (Temperature, 20-26°C; humidity, 40-60%; 12/12-h light/dark cycle; free access to food and water). The back of each mouse was subcutaneously inoculated with SK-BR-3 cells (1×10^7) suspended in 0.2 mL PBS. When the tumor volume reached nearly $60mm^3$, mice were randomly assigned into 4 groups. Mice in each group (n=6) were treated *via* daily oral gavage with PYR (30 mg/kg/d), intravenous injection ADM (5 mg/kg/w) every week, or a combination of both drugs for 27 days. The weight and tumor size were measured twice a week. Tumor volume was calculated as $V = (\text{length} \times \text{width}^2)/2$. All of the animal experiments conformed to the requirements of the ethics committee.

Western Blotting

The cells were treated with 0.3, 3, 10µg/ml of pyrotinib or 0.1, 0.3, 3µg/ml of adriamycin for 48h. Then, the cells were harvested and prepared for cytosolic and nuclear protein extraction using a cytoplasmic and nuclear protein extraction kit (Cowin Bio., Beijing, China) according to the manufacturer's instructions. Equal amounts of protein were employed in 12% SDS-PAGE (Cowin Bio., Beijing, China) followed by transfer to PVDF membrane. After blocking with 5% skim milk powder in 0.1% Tween in phosphate buffered saline (PBST) at room temperature for 2 h, the membrane was incubated with primary antibody (Akt, p-Akt, p65, p-p65, FOXC1) at 4°C overnight. Subsequently, secondary antibodies were incubated at 37°C for 1 h. Finally, the membranes were washed with PBST and detected in Tanon 2500 chemiluminescence imaging system (Tanon, Shanghai, China). Image J software (NIH, Bethesda, MA, U.S.A.) was used for density analysis and quantitative analysis of protein level.

Clinical Application of Pyrotinib

To evaluate the efficacy of PYR treatment, we retrospectively screened the breast cancer patient who was given PYR treatment, collected clinical data, chest computerized tomography (CT) scans, and paraffin-embedded pathological specimens. This study was approved by the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology.

Statistical Analysis

Statistical analysis was performed using IBM SPSS 23.0 software (SPSS, Chicago, IL, USA). All the data were presented as mean value ± SD, and statistically significant differences between different experimental groups and control groups were examined using one-way analysis of variance (ANOVA). $P<0.05$ was considered a statistically significant difference.

RESULTS

Effects of Pyrotinib and Adriamycin on the Proliferation of Breast Cancer Cells

To assess the cytotoxicity of pyrotinib and adriamycin on SK-BR-3 and AU565 cells, cell viability was detected by MTT assays after treatment with various concentrations of pyrotinib or/and adriamycin for 48 h. As shown in **Figure 1**, we demonstrated that SK-BR-3 and AU565 cell growth was significantly inhibited at concentrations ranging from 3 to 200µg/ml of pyrotinib and 0.3 to 10µg/ml of adriamycin, and the inhibitory effect was positively correlated with the pyrotinib or adriamycin concentration. The mean IC_{50} values of pyrotinib for SK-BR-3 and AU565 cells were 3.03µg/ml, 3.82µg/ml. The mean IC_{50} values of adriamycin for SK-BR-3 and AU565 cells were 0.31µg/ml, 0.62µg/ml. Different concentrations of pyrotinib (0.3 and 3µg/ml) and adriamycin (0.1 and 0.3µg/ml) were used for the remaining experiments. To determine the synergistic antitumor effects, the combination index for SK-BR-3 and AU565 cells were calculated using the method of Chou and Talalay. As shown in **Figures 1G, H**, **Tables 1** and **2**. The results showed that co-treatment with pyrotinib and adriamycin was more effective in SK-BR-3 and AU565 cells.

Effects of Pyrotinib and Adriamycin on the Migration and Invasion of Breast Cancer Cells

The effects of pyrotinib and adriamycin on the migration of SK-BR-3 and AU565 cells were analyzed by wound-healing assays.

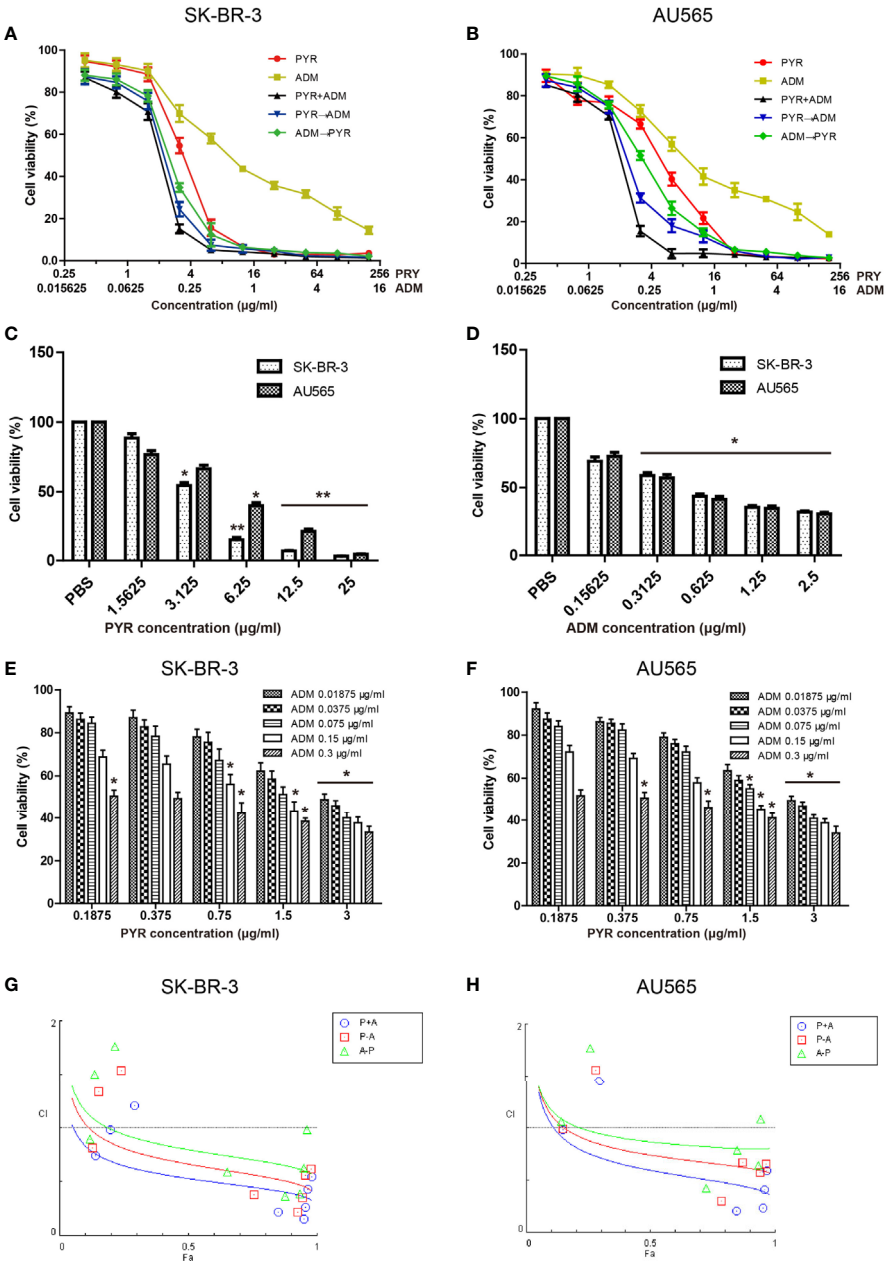

FIGURE 1 | Effects of PYR and ADM on the viability of breast cancer cells. Proliferation activity of SK-BR-3 or AU565 cells was determined by the MTT assay after incubation for 48h with different concentrations of PYR or ADM. **(A, B)** SK-BR-3 or AU565 cells were treated with PYR or ADM alone or in combination or in sequences (PYR first for 6 h followed by ADM or PYR first for 6 h followed by ADM). **(C, D)** The histogram represents the statistical analysis for SK-BR-3 or AU565 cells were treated with PYR or ADM alone. **(E, F)** Proliferation activity of SK-BR-3 or AU565 cells was determined after incubation for 48h with different concentrations of PYR combination with 0.3, 0.15, 0.075, 0.0375, 0.01875μg/ml of ADM. **(G, H)** The combination index (CI) v.s. fraction affected (Fa) affected plot was calculated by Compusyn and depicted the combination effects. Synergistic growth inhibitory effects of PYR combined with ADM on SK-BR-3 or AU565 cells. Synergy is defined as CI values < 1.0, antagonism as CI values > 1.0, and additivity as CI values=1.0. *p<0.05 and **p<0.01 compared with the PBS group.

As shown in **Figure 2**, after treatment with pyrotinib (0.3, 3μg/ml) and adriamycin (0.1, 0.3μg/ml) for 24 h, the migration rate of SK-BR-3 and AU565 cells decreased with the increase of the concentration of pyrotinib and adriamycin ($p<0.05$), and SK-BR-3 and AU565 cells were treated with both drugs (pyrotinib in combination with adriamycin), the migration rates significantly decreased compared with those cells treated with single drugs ($p<0.01$). Similar conclusions were found in the transwell invasion assay. The invasion of SK-BR-3 and AU565 cells were dose-dependently inhibited by pyrotinib and adriamycin.

TABLE 1 | Combination Index Value and Fraction Affected of PYR and ADM on AU565 cells.

PYR c(μg/ml)	ADM c(μg/ml)	PYR+ADM		PYR-ADM		ADM-PYR	
		Fa	CI	Fa	CI	Fa	CI
0.39063	0.01953	0.12968	0.81322	0.12968	0.81322	0.11942	0.89319
0.78125	0.03906	0.15348	1.33928	0.15348	1.33928	0.13879	1.50464
1.5625	0.07813	0.24210	1.54313	0.24210	1.54313	0.21765	1.76330
3.125	0.15625	0.75557	0.37870	0.75557	0.37870	0.65233	0.59323
6.25	0.3125	0.92628	0.22036	0.92628	0.22036	0.87669	0.36236
12.5	0.625	0.94279	0.34830	0.94279	0.34830	0.93604	0.38605
25	1.25	0.95460	0.56432	0.95460	0.56432	0.94900	0.62706
50	2.5	0.97698	0.61692	0.97698	0.61692	0.96100	0.98412

Synergy is defined as CI values < 1.0, antagonism as CI values > 1.0, and additivity as CI values = 1.0.

TABLE 2 | Combination Index Value and Fraction Affected of PYR and ADM on AU565 cells.

PYR c(μg/ml)	ADM c(μg/ml)	PYR+ADM		PYR-ADM		ADM-PYR	
		Fa	CI	Fa	CI	Fa	CI
0.39063	0.01953	0.14822	0.97918	0.13676	1.09062	0.12551	1.22266
0.78125	0.03906	0.20782	1.12981	0.17232	1.24982	0.16783	1.43563
1.5625	0.07813	0.29695	1.45174	0.27342	1.64753	0.26393	1.73746
3.125	0.15625	0.84571	0.20532	0.68732	0.51277	0.58526	0.80749
6.25	0.3125	0.93675	0.14231	0.84872	0.43752	0.78971	0.79078
12.5	0.625	0.95175	0.23492	0.87114	0.66727	0.85085	0.78898
25	1.25	0.95805	0.40789	0.94091	0.57803	0.93436	0.64443
50	2.5	0.96962	0.59104	0.96616	0.65782	0.94444	1.08499

Synergy is defined as CI values < 1.0, antagonism as CI values > 1.0, and additivity as CI values = 1.0.

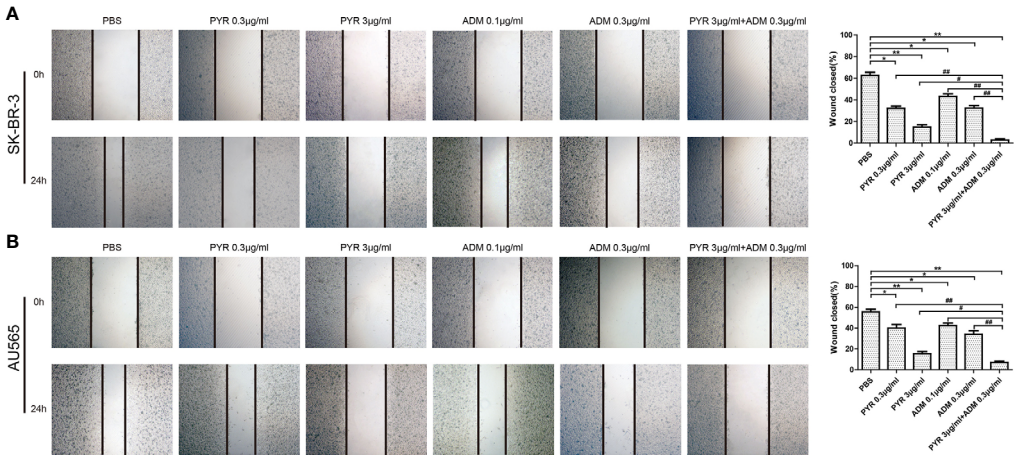

FIGURE 2 | Effects of PYR and ADM on cell migration. The monolayers of SK-BR-3 and AU565 cells were scratched with a pipette tip, and incubated with PBS, different concentrations of PYR (0.3, 3μg/ml), ADM (0.1, 0.3μg/ml) or a combination treatment (PYR+ADM) for 24 h. **(A)** Wound healing assay assessed the effect of PYR and ADM on SK-BR-3 cell migration ability and histogram represents the statistical analysis. **(B)** Wound healing assay assessed the effect of PYR and ADM on AU565 cell migration ability and histogram represents the statistical analysis. Original magnification was ×100. Data represent the mean ± S.D. of three independent experiments. *p<0.05 and **p<0.01 compared with the PBS group, $^{\#}$p<0.05 and $^{\#\#}$p<0.01 compared with the combination group.

The statistical results showed that the number of invasive cells in the pyrotinib and adriamycin group, and decreased remarkably compared with the control group. The inhibitory effect of pyrotinib in combination with adriamycin was more significant than single drugs (p<0.01, **Figure 3**).

Effects of Pyrotinib and Adriamycin on the Cell Cycle and Apoptosis of Breast Cancer Cells

To further investigate the mechanism of the proliferation inhibition on SK-BR-3 and AU565 cells, the effect of pyrotinib

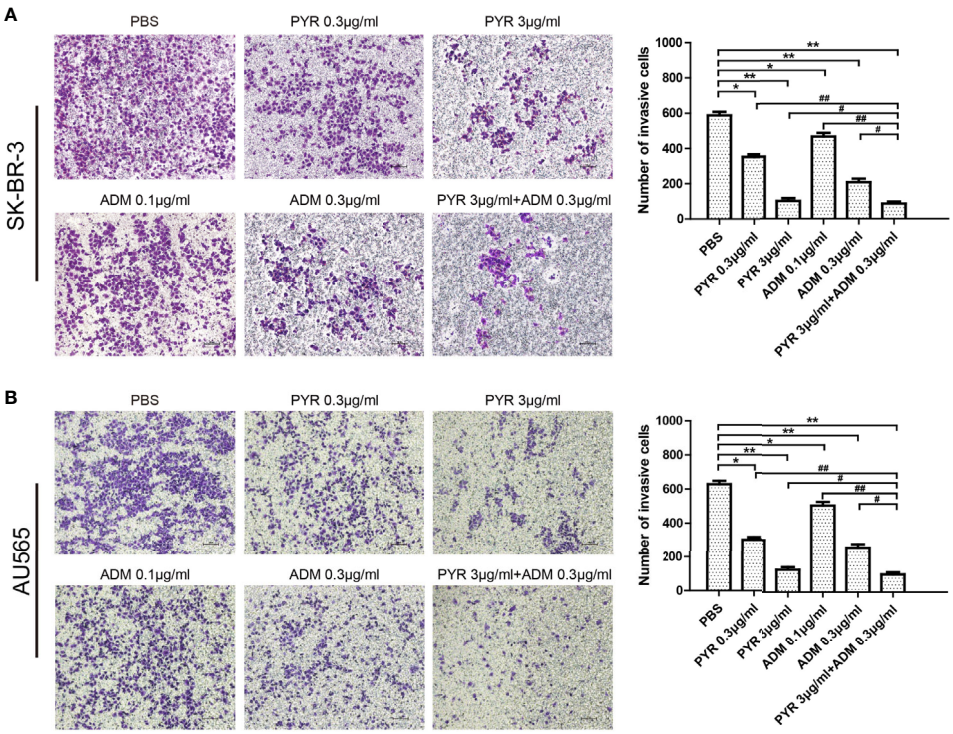

FIGURE 3 | Effects of PYR and ADM on cell invasion. Cell invasion was analyzed with a Matrigel-coated Boyden chamber. SK-BR-3 and AU565 cells were treated with PBS, different concentrations of PYR (0.3, 3μg/ml), ADM (0.1, 0.3μg/ml) or a combination treatment(PYR+ADM) for 24 h. **(A)** Transwell invasion assays assessed the effect of PYR and ADM on SK-BR-3 cell invasion ability and histogram represents the statistical analysis. **(B)** Transwell invasion assays assessed the effect of PYR and ADM on AU565 cell invasion ability and histogram represents the statistical analysis. Original magnification was ×100. Data represent the mean ± S.D. of three independent experiments. *p<0.05 and **p<0.01 compared with the PBS group, #p<0.05 and ##p<0.01 compared with the combination group.

and adriamycin on apoptosis was conducted by Annexin V-FITC/PI double staining followed by flow cytometer analysis (**Figure 4**). The increase in the percentage of apoptotic cells was dependent on the increase in concentration. At 24 hours, in SK-BR-3 cells, the early apoptotic cells increased from 7.26% at 0.3 μg/ml to 9.33% at 3 μg/ml of pyrotinib compared to 0.843% of the control group. The number of apoptotic cells increased from 6.12% at 0.1 μg/ml to 8.97% at 0.3 μg/ml of adriamycin compared to 0. 843% of the control group. The percentage of apoptotic cells was 10.2% in the combined group (**Figures 4A, C**). Similarly, in AU565 cells, the early apoptotic cells increased from 5.96% (PBS treated) to 12.1%, 15.2% when cells were treated with 0.3 and 3 μg/ml PYR, respectively. The early apoptotic cells increased from 5.96% (PBS treated) to 18.7%, 23.0% when cells were treated with 0.1 and 0.3 μg/ml ADM, respectively. The percentage of apoptotic cells was 30.00% in the combined group (**Figures 4B, D**).

The distribution of the cell cycle phase in SK-BR-3 and AU565 cells treated with pyrotinib and adriamycin at 24h was depicted in **Figure 5**. The cell cycle arrest by pyrotinib and adriamycin was concentration-dependent. Compared with the untreated control group, treatment with pyrotinib resulted in the significant cell stagnation of SK-BR3 and AU565 cells in the G1 phase of the cell cycle. On the other hand, adriamycin treatment makes SK-BR3 and AU565 cell cycles arrest in the G2 phase. As shown in **Figure 5**, compared to the PBS group, the percentage of SK-BR-3 and AU565 cells arrested in the G2 phase was increased in the combination group.

In Vivo Anticancer Effect of Pyrotinib and Adriamycin in SK-BR-3 BC Xenograft Models

After confirming the inhibitory effect of pyrotinib combined with adriamycin on cell proliferation and cell growth *in vitro*, The tumor model was established to further evaluate the inhibitory effect of the two drugs on tumor growth either individually or synergistically. Mice with SK-BR-3 xenografts were randomly divided into 4 treatment groups. These groups included the PBS control group, pyrotinib (30 mg/kg every day), adriamycin (5 mg/kg weekly), or the combination of pyrotinib (30 mg/kg every day) and adriamycin (5 mg/kg weekly). The relative tumor volume and body weight were measured twice per week. The results showed that pyrotinib was more effective than adriamycin in inhibiting tumor growth ($p<0.05$, **Figures 6A, C, D**). Nevertheless, the combined drug group (pyrotinib and adriamycin) had a stronger inhibitory effect on SK-BR-3 xenograft growth than any drug alone (**Figures 6A, C, D**). All the treatments caused a significant inhibition in tumor growth as compared with the PBS control group ($p<0.05$).

No general toxicities were noted in all groups, as all groups showed a slightly steady increase in body weight without

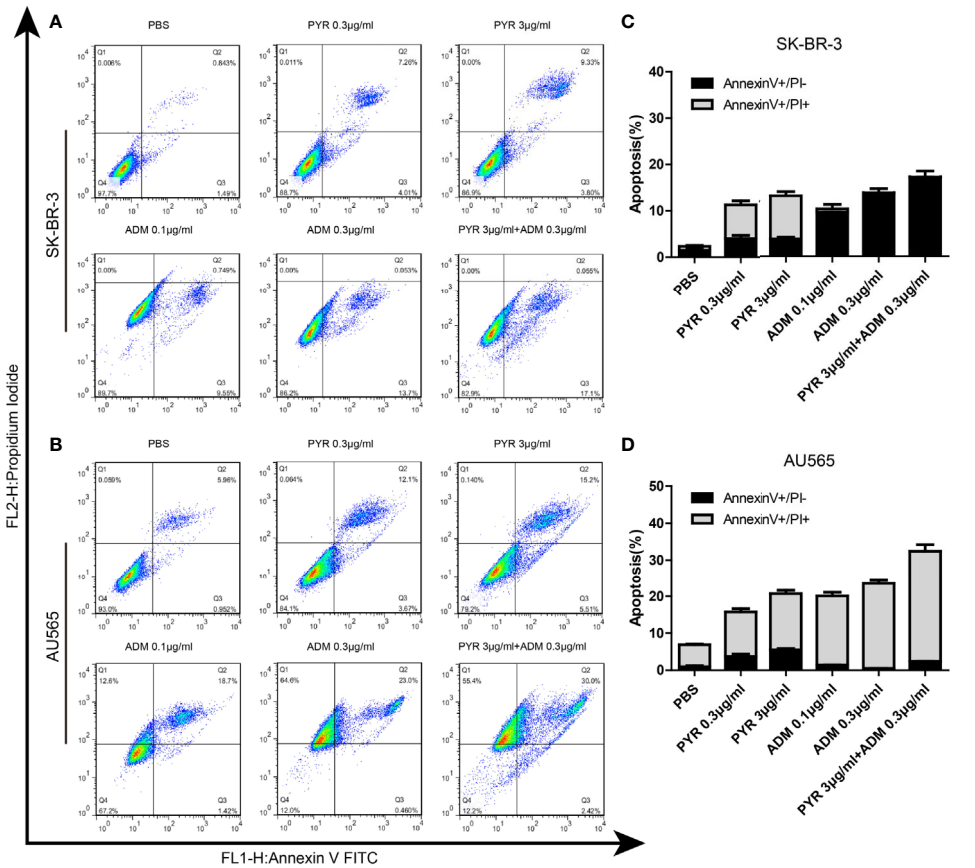

FIGURE 4 | Effects of PYR and ADM on cell apoptosis. **(A, B)** Cell apoptosis was detected through Annexin V-FITC/PI double staining and following flow cytometry for SK-BR-3 cells after incubated with PBS, different concentrations of PYR(0.3, 3μg/ml), ADM(0.1, 0.3μg/ml) or a combination treatment(PYR+ADM) for 24 h. **(C, D)** The histograms were the representative results, while the figure in the right. Compared to the control, PYR combined with ADM induces apoptosis in SK-BR-3 and AU565 cells. Data represent the mean ± S.D. of three independent experiments.

significant difference (**Figure 6B**). These data indicate that the combination of pyrotinib and adriamycin can augment anticancer activity without increased toxicity.

Effects of Pyrotinib and Adriamycin on the Expression of Akt, p-Akt, p65, p-p65 and FOXC1

To investigate the mechanism of pyrotinib and adriamycin on BC cells, Western blots were used to assess the protein content of SK-BR-3 cells after treatment with PBS, pyrotinib (0.3, 3, 10μg/ml) or adriamycin (0.1, 0.3, 3μg/ml). We evaluated several key molecules in cell signaling pathways. The results showed that p-Akt, p-p65, FOXC1 activity were tremendously decreased by pyrotinib treatment at 3, 10μg/ml after 48 h incubation, whereas no significant effect on p-Akt, p-p65, FOXC1 activity was observed in the adriamycin group (**Figure 7**).

Antitumor Activity of Pyrotinib in BC Patients

A 52-year-old female patient was clinically diagnosed with right breast cancer and lymph node metastasis in 2017. The histopathological diagnosis was HER2 amplification, ER negative, PR negative, Ki-67 20%. Subsequently, the patient received chemotherapy and trastuzumab treatment. However, the patient still suffered from disease progression after multiline treatment. As shown in **Figures 8A, B**, breast and lymph node had progressed significantly. Since June 16, 2019, the patient has been taking 400 mg of pyrotinib orally on a daily basis. In July 2019, the chest CT showed that the right breast lump and lymph node were all smaller than before (**Figures 8C, D**). The efficacy evaluation confirmed partial response (PR) compared with the baseline computed CT scan.

DISCUSSION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death among women. HER2 is overexpressed in about 15-20% of breast cancer patients. This transmembrane receptor tyrosine kinase promotes abnormal cell growth and proliferation in human breast cancer, resulting in aggressive tumor cells and poor prognosis. With the further understanding of the molecular mechanism of HER2-positive

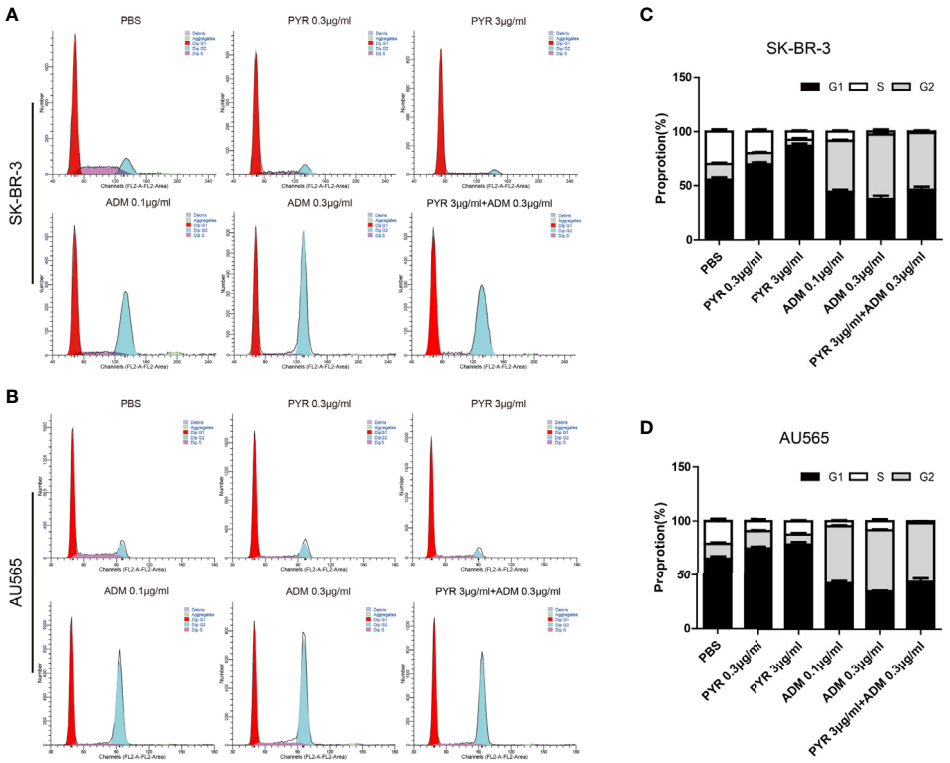

FIGURE 5 | Effects of PYR and ADM on the cell cycle. **(A, B)** Cell cycle analysis through PI staining and following flow cytometry for SK-BR-3 cells after incubated with PBS, different concentrations of PYR(0.3, 3μg/ml), ADM(0.1, 0.3μg/ml) or a combination treatment (PYR+ADM) for 24 h. ModFit was used to perform cell cycle analysis. **(C, D)** The histograms were the representative results, while the figure in the right. Compared with the PBS group, PYR caused significant G1 phase arrest, while ADM caused significant G2 phase arrest, PYR combined with ADM induces G1/S arrest in SK-BR-3 and AU565 cells. Data represent the mean ± S.D. of three independent experiments.

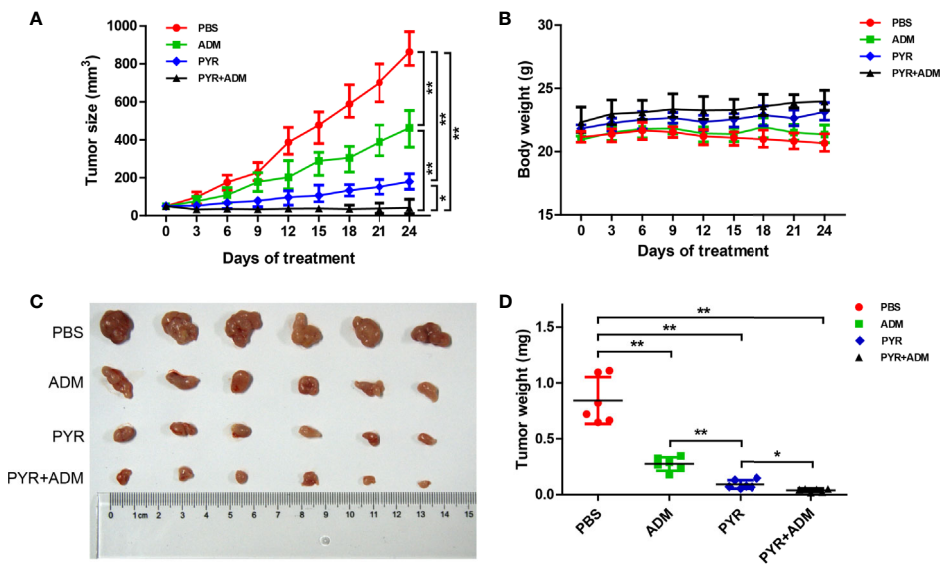

FIGURE 6 | In vivo anticancer effect of PYR and ADM in breast cancer xenograft models. Randomly grouped nude mice were treated with PBS, PYR(30 mg/kg), ADM(5 mg/kg), or a combination treatment (PYR+ADM) for 27 days. **(A, B)** Tumor growth ratio curve and body weight changes every three days after the onset of treatment. **(C, D)** Photos of the excised tumors and weight obtained on day 27 after treatment. *p<0.05 and **p<0.01.

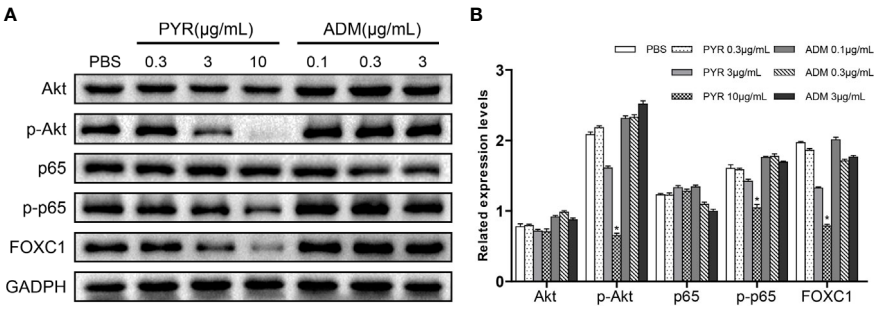

FIGURE 7 | Molecular mechanism studies in breast cancer cells after treatment of PBS, PYR, ADM, or a combined treatment. SK-BR-3 cells were treated with PBS, different concentrations of PYR (0.3, 3, 10μg/ml), ADM (0.1, 0.3, 3μg/ml) for 24 h, respectively. Nuclear and cytosolic protein extracts were subjected to Western blot analysis. **(A)** The results of Western blot for Akt, p-Akt, p65, p-p65, and FOXC1 in the nuclear fractions and cytosolic extracts, respectively. GADPH served as the loading control. **(B)** Quantitative analysis of the Western blotting results. Data represent the mean ± S.D. of three independent experiments. *p<0.05 with the PBS group.

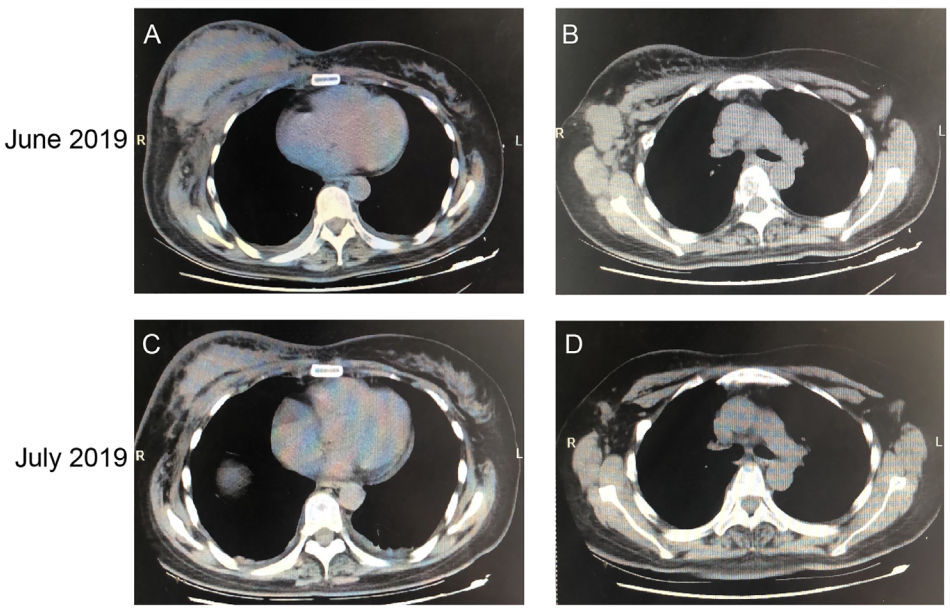

FIGURE 8 | CT Imaging of the breast and lymph node before and after pyrotinib treatment. CT scans before the administration of pyrotinib (images **A**, **B**) and after one month of treatment (images **C**, **D**), showing the tumor mass and lymph node.

breast cancer, a series of HER2-targeted drugs have been developed, including trastuzumab, pertuzumab, lapatinib, neratinib, T-DM1, and pyrotinib have been approved for the treatment of HER2-positive breast cancer.

Pyrotinib is an irreversible dual pan-ErbB receptor tyrosine kinase inhibitor developed for the treatment of HER2-positive advanced malignant solid tumors. In August 2018, the Chinese State Drug Administration first conditionally approved pyrotinib for use in combination with capecitabine for the treatment of HER2-positive, advanced or metastatic breast cancer in patients previously treated with anthracycline or taxane chemotherapy (11). In a randomized, open, controlled I/II clinical study, the efficacy and safety of pyrotinib plus capecitabine in contrast with lapatinib plus capecitabine were evaluated in the treatment of HER2-positive recurrent or metastatic BC. Compared with lapatinib combined with capecitabine, pyrotinib combined with capecitabine has a higher objective response rate (79 vs. 57%; p = 0.01). Pyrotinib combined with capecitabine significantly prolonged median progression-free survival versus lapatinib combined with capecitabine (18.1 vs. 7.0 months p<0.0001) (11, 36). The result of the phase III trial assessing pyrotinib versus placebo both in combination with capecitabine in women with HER2-positive

metastatic BC who received prior taxanes and trastuzumab therapy was reported at ASCO in June 2019. Patients were randomly assigned to be administrated with pyrotinib plus capecitabine (*n*=185) or placebo plus capecitabine (n=94). The median PFS for the combination group was 11.1 months, and that for the placebo group was 4.1 months. Furthermore, 71 patients in the placebo group whose disease progressed received pyrotinib monotherapy afterward, revealed a single drug response rate of 38.0% and the median PFS of 5.5 months (41). It is anticipated that combining pyrotinib with chemotherapy will be a trend for anti-HER2 therapy in the future.

In this study, we examined the effect of pyrotinib and adriamycin on cell lines and xenograft models. MTT assay and flow cytometry showed that pyrotinib and adriamycin significantly inhibited the growth of the breast cancer cell line and induced cell apoptosis in a concentration-dependent manner. We also observed that co-treatment with pyrotinib and adriamycin led to growth inhibition in breast cancer cells. Although previous studies revealed that pyrotinib displayed cytotoxic effects and induced apoptosis in breast cancer cell lines *via* different molecular mechanisms, there is no research on the effect of pyrotinib on HER2-positive breast cancer cell migration and invasion. The present study found that pyrotinib and adriamycin significantly inhibited cell migration and invasion in the breast cancer cells, which has a strong ability in cell migration and invasion. Furthermore, the inhibitory effect of pyrotinib was more significant than adriamycin.

The combined effect of pyrotinib and adriamycin was demonstrated *in vivo*, the results indicated that the anticancer effect of the combinatorial treatment was higher than any other single drug, which was consistent with *in vitro*. In addition, nude mice were treated with combinatorial treatment but did not show worse body weight than the patients in the groups treated with pyrotinib or adriamycin alone (**Figure 6B**). Hence, our data show that a combination of pyrotinib and adriamycin led to enhanced antitumor activity without extended toxicity. To evaluate the efficacy of pyrotinib treatment in a clinical setting, we observed the effect of HER2-positive breast cancer patients before and after treatment with pyrotinib, the results show that pyrotinib also has good antitumor activity (**Figure 8**). Nevertheless, more clinical trials are needed to confirm the efficacy of pyrotinib on HER2-positive breast cancer.

To further explore the potential molecular mechanisms and signaling pathways involved in the anticancer effects of pyrotinib and adriamycin, western blotting was used to evaluate molecular changes upon pyrotinib or adriamycin therapy. We found that pyrotinib treatment significantly down-regulated Akt, p-65 phosphorylation and reduced the protein level of FOXC1 in the breast cancer cells, but adriamycin had no significant effect on the expression and phosphorylation of Akt, p65, and FOXC1 in breast cancer cells. These findings indicate that pyrotinib inhibited cell proliferation, migration, and invasion possibly through inactivation of Akt/p-65/FOXC1 signaling in HER2-positive breast cancer cells. Consistent with our findings, Zhang et al. also reported that pyrotinib treatment down-regulated Akt phosphorylation in breast cancer cells (31). Previous studies also showed that NF-κB-p65 enhances FOXC1 promoter activity in basal-like breast cancer cells (MDA-MB-468) (42). According to our findings, we proposed a schematic presentation of possible mechanisms for the suppressive effects of pyrotinib on proliferation, migration, and invasion in HER2-positive breast cancer cells (**Figure 9**).

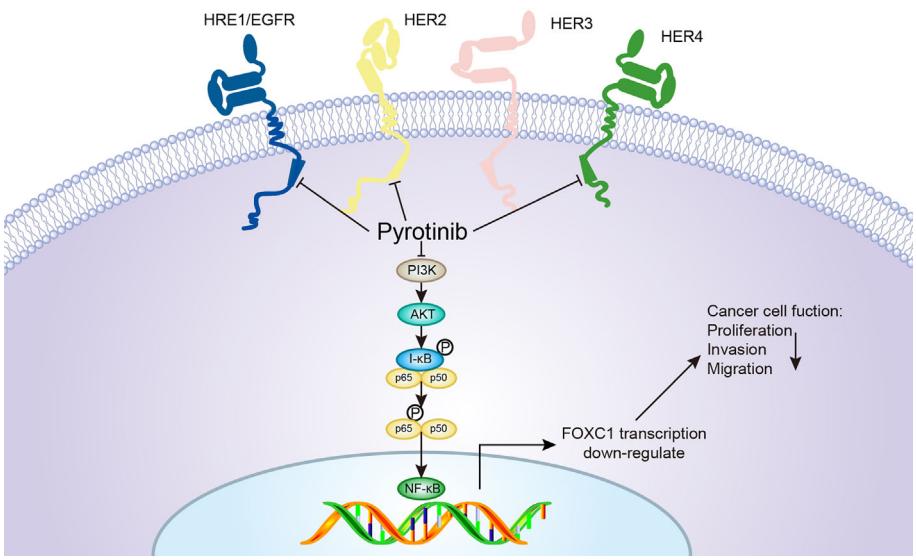

FIGURE 9 | A proposed model for the pyrotinib-mediated inhibitory effects on the apoptosis, migration, and invasion of BCs. Pyrotinib is an irreversible HER 1, 2, and 4 inhibitor that inhibits the downstream signals of the Akt/p-65/FOXC1 pathway, resulting in a significant efficacy in cell proliferation, migration, and invasion of HER2-overexpressing cancer cells.

In summary, this is the first work to reveal the antitumor activity of pyrotinib combined with adriamycin on HER2-positive breast cancer *in vitro* and *in vivo*, which may be more effective than pyrotinib or adriamycin alone. This study reveals the molecular mechanisms of pyrotinib in the treatment of breast cancer, which provides a theoretical basis for the comprehensive treatment of breast cancer in clinics.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology.

AUTHOR CONTRIBUTIONS

CW and XW designed and carried out the experiments. SD and XY analyzed the data. CW and YL wrote the manuscript. JC, XX, and XH performed MTT assay, wound-healing, and transwell invasion assays, flow cytometry, and Western blotting assays. CY and FS collected the clinical samples. DK and GL performed animal models. XW provided supervision and guidance. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by grants from the project of the Science and Technology Department of Henan Province (2018010019).

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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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 27 May 2021
doi: 10.3389/fonc.2021.668624

Enhanced Susceptibility to Breast Cancer in Korean Women With Elevated Serum Gamma-Glutamyltransferase Levels: A Nationwide Population-Based Cohort Study

*Aeran Seol[1], Wenyu Wang[2,3], Se Ik Kim[1], Youngjin Han[2,4], In Sil Park[2,5], Juhwan Yoo[6], HyunA Jo[2,4], Kyung-Do Han[7] and Yong Sang Song[1,2,4,5]**

[1] Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, South Korea, [2] Cancer Research Institute, College of Medicine, Seoul National University, Seoul, South Korea, [3] Interdisciplinary Program in Cancer Biology, Seoul National University, Seoul, South Korea, [4] WCU Biomodulation, Department of Agricultural Biotechnology, Seoul National University, Seoul, South Korea, [5] Department of Agricultural Biotechnology, Seoul National University, Seoul, South Korea, [6] Department of Biomedicine & Health Science, The Catholic University of Korea, Seoul, South Korea, [7] Department of Statistics and Actuarial Science, Soongsil University, Seoul, South Korea

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Edited by:
Zheng Wang,
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Oslo University Hospital, Norway
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Government Medical College (GMC), India

***Correspondence:**
Yong Sang Song
yssong@snu.ac.kr

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *16 February 2021*
Accepted: *22 April 2021*
Published: *27 May 2021*

Citation:
Seol A, Wang W, Kim SI, Han Y, Park IS, Yoo J, Jo H, Han K-D and Song YS (2021) Enhanced Susceptibility to Breast Cancer in Korean Women With Elevated Serum Gamma-Glutamyltransferase Levels: A Nationwide Population-Based Cohort Study. Front. Oncol. 11:668624. doi: 10.3389/fonc.2021.668624

Background: The incidence of breast cancer has been gradually increasing in Korea. Recently, the elevated level of serum gamma-glutamyltransferase (GGT) has emerged to be associated with the development and progression of some malignancies. This study aimed to determine the effect of serum GGT levels on the risk of developing breast cancer in Korean women.

Methods: We used National Health Insurance Service Health Checkup data to examine the association between serum GGT levels and breast cancer development in Korean women. Women aged 40 years or older who participated in the Korean National Health Screening Examination between January 2009 and December 2009 and who did not develop any cancer within 1-year post examination were included in this analysis (n = 3,109,506). Cox proportional hazard regression analysis was conducted to calculate hazard ratios (HRs) with 95% confidence intervals (CIs).

Results: Overall, an elevated serum GGT level was associated with the increased risk of developing breast cancer; compared to the Q1 group, the Q4 group showed a significantly increased breast cancer risk (HR: 1.120,95% CI: 1.08–1.162). Such a relationship was stronger in post-menopausal women than pre-menopausal women (HR: 1.173, 95% CI: 1.107–1.243; HR: 1.070, 95% CI:1.019–1.124). Women with a high GGT level (Q4) were also at an increased risk of developing carcinoma *in situ* (CIS) (HR: 1.114, 95% CI: 1.04–1.192). In post-menopausal women, the Q4 group also exhibited higher CIS risk (HR: 1.266, 95% CI: 1.132–1.416). However, no significant difference in the risk of developing CIS was observed between the Q1 and Q4 groups in

pre-menopausal women. Further analysis revealed that obese, post-menopausal women with a high GGT level (Q4) were associated with an increased risk of developing breast cancer (HR: 1.214, 95% CI: 1.125–1.31) and CIS (HR: 1.348, 95% CI: 1.159–1.569).

Conclusions: Our study results demonstrate that increased serum GGT level is a risk factor for developing breast cancer. The post-menopausal women group with obesity and elevated serum GGT level showed the highest incidence of breast cancer. Thus, serum GGT concentration could be a novel and potential risk factor for breast cancer. Further validation in different ethnic groups would be warranted.

Keywords: breast cancer, gamma-glutamyltransferase (GGT), menopause, cancer incidence, biomarker, obesity, body mass index (BMI)

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in most countries and the leading cause of cancer death in over 100 countries (1, 2). There will be approximately 2.3 million new female breast cancer cases in 2020 (2). Incidence rates of breast cancer have been rising for most countries over the last decade (2). In the United States, breast cancer accounts for 30% of female cancers. Incidences and deaths were estimated to be 276,480 and 42,170 cases respectively in 2020. In addition, approximately 48,530 cases of ductal carcinoma *in situ* (DCIS) of the female breast are expected to be diagnosed in 2020 (3). In Korea, the incidence of breast cancer has been steadily increasing over the past 10 years. 25,452 new cases and 2,748 cancer deaths are expected to occur in Korea in 2020 (4).

Obesity is on the rise worldwide, and the importance is increasing as the association with cancer is revealed including breast cancer (5, 6). For breast cancer, several large studies have shown an increased risk of breast cancer with weight gain in post-menopausal women (7, 8). In Korea, nationwide studies have confirmed that obesity increases the risk of breast cancer in post-menopausal women (9). Notably, the peak age for breast cancer is between 40 and 50 years in Asian countries, whereas in the western countries, itis between 50 and 70 years (10). For instance, the peak age of breast cancer in the United States was 61 years while that was 47 years in Korea (10, 11). Therefore, the different epidemiologic background in different countries should not be neglected in cancer prevention.

Serum gamma-glutamyl transferase (GGT) is a serum marker for alcoholic liver disease, alcohol consumption, and bile duct condition (12, 13). In addition, it is an indicator of oxidative stress caused by factors including cardiovascular disease, diabetes, and stroke (14–16). GGT differs according to gender, ethnicity, and region. In Korea, the GGT level is an upward trend as in USA which might be related to changing environmental factors such as excessive iron intake, exposure to xenobiotics and impairment of cell membranes caused by nutrition (12). Recent studies have been reported that elevated GGT level is associated with the occurrence of several cancers such as prostate cancer, liver cancer including hepatocellular carcinoma (17–19). The association with elevated GGT levels in breast cancer has been studied in the UK, but a cut-off value for this study was not suggested (20).

In this study, we analyzed the association between GGT and breast cancer risk by using a large cohort data from the Korean insurance registry by including 3,109,506 women (≥40 years old) who underwent health check-ups in 2009.

METHODS

Study Participants

This study was a nationwide population-based cohort study using a Health Check-up database from the National Health Insurance Service (NHIS) of Korea. The NHIS is a single universal insurance service covering approximately 97% of the entire Korean population. In Korea, breast cancer screening tests are provided free of charge to all women over 40 years of age every 2 years. This test result is registered in the health check-up database of the NHIS. From the NHIS cohort, we identified women aged 40 years and older who received health examinations and completed the cancer-screening questionnaire between January 2009 and December 2009 (n = 3,109,506). Among them, we excluded women with the following conditions: (1) who reported that they had received a hysterectomy before; (2) who had been diagnosed with any cancer before health examination and had been registered with cancer registration code; or (3) those with insufficient data. If any cancer occurred within one year from the day of screening, it could have been caused by other cause than GGT. So we applied a 1-year lag period to minimize detection bias about the cancer diagnosis. Finally, 2,066,998 women were included in this analysis (**Figure 1**). The follow-up period is from the date of the NHIS Health Screening in 2009 to the onset of breast cancer or December 31, 2018, whichever comes first. In Korea, the national cancer registration project is in progress, when cancer or CIS is diagnosed, it is registered in the NHIS database. Women who met the inclusion criteria were followed up for breast cancer or CIS through the NHIS database.

Data Collection

We retrieved detailed health check-up data, such as comorbidities, smoking and drinking status, and other lifestyle factors. Participants' laboratory test results were also collected. Based on body mass index (BMI)at the time of health examination, we defined obese women as BMI ≥25.0 kg/m^2 (21). Development of breast cancer and CIS was identified when participants made a

documented visit to hospitals during the observation period with the registration code, V193 and the International Statistical Classification of Diseases, tenth revision (ICD-10) codes. The registration code of breast cancer is C50 and that of CIS is D05.

Statistical Analysis

Incidences of breast cancer and CIS were calculated by dividing the number of events by 1000 person-years. Between the women who developed breast cancer (study group) and those who did not (control group), baseline demographic characteristics were compared using Student's t test for continuous variables and chi-square test for categorical variables. Based on the quartiles of serum GGT levels, we stratified participants into each quartile group (Q1 to Q4) with an increasing GGT level.

Investigating the relationship between breast cancer and serum GGT levels, we used Cox proportional hazard regression models for multivariate analysis; the hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated through cox proportional hazard regression using Q1 as a reference. The risk of breast cancer was evaluated in total women, pre- and post-menopausal groups, respectively. While none of the confounding factors were adjusted in Model 1, participants' age was adjusted in Model 2. Model 3 adjusted for age, smoking status (3 levels), alcohol consumption (3 levels), regular physical activity and diabetes. Adding to this, Model 4 further adjusted for the number of parities, menopausal status, and age of menarche. For post-menopausal women, model 4 also included duration of hormonal replacement therapy as a confounder.

All statistical analyses were performed using R statistical software (version 3.4.4; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). Statistical and SAS version 9.4 (SAS Institute, Cary, NC, USA) and a p value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics of the Study Cohort

The mean follow-up periods was 8.37 years. Among the study cohort (n=2,066,998), 25,182 individuals (1.22%) developed breast cancer and assigned to the study group while 2,041,816 individuals (98.78%) were assigned to the control group (**Figure 1**). To analyze the association between GGT level and incidence of breast cancer in different menopausal status, each group was divided into pre- and post-menopausal subgroups (**Figure 1**).

Baseline characteristics of the two groups were displayed in **Tables 1** and **2** according to the menopausal status. Post-menopausal women who developed breast cancer showed higher proportions of diabetes mellitus, dyslipidemia, and smoking history compared to those who did not. Obesity was more prevalent in the study group than in the control group. Age was lower and the physical activity was higher in the breast cancer group. Serum GGT level is significantly higher in the post-menopausal breast cancer group (median 20.91 vs. 21.98, p<0.0001) while the GGT levels of pre-menopausal women did not differ between the two groups (median 17.29 vs. 17.46, p=0.1902). So we studied the effect of GGT on breast cancer incidence depending on whether menopause or not. We selected model 4 to adjust for other parameters that showed differences between the two groups.

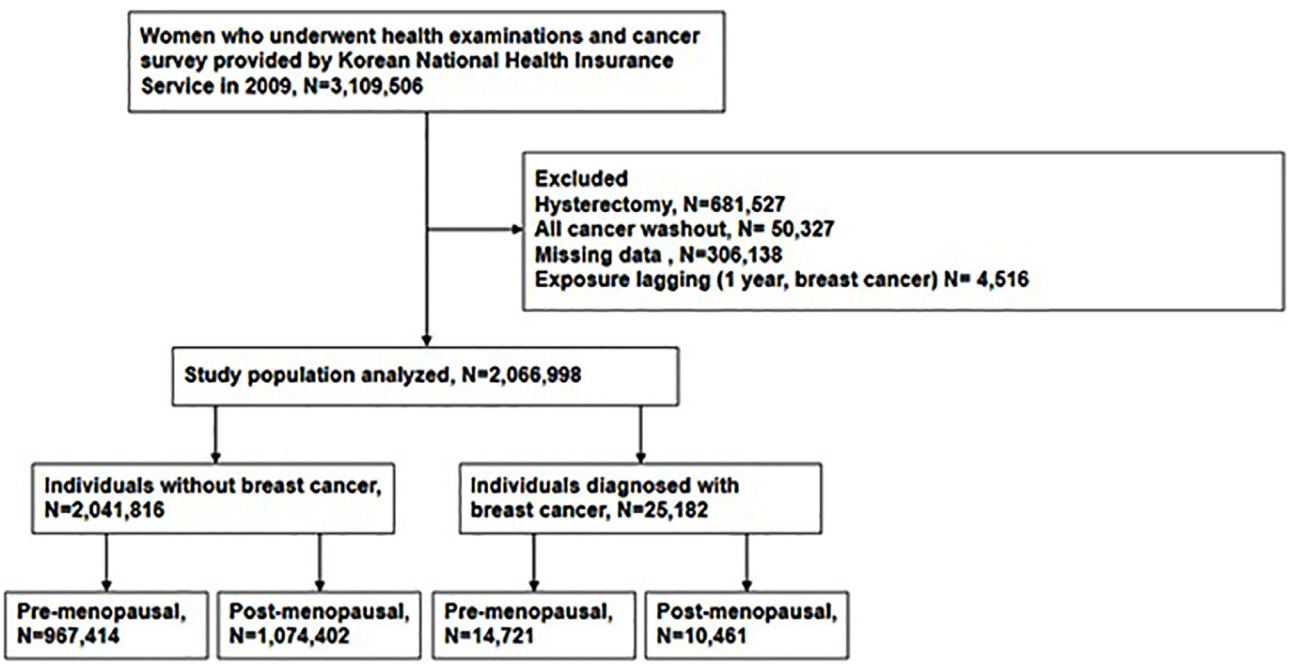

FIGURE 1 | Flow chart depicting the design of the study participants by Korean National Health Insurances Service.

TABLE 1 | Baseline demographic and clinical data according to female cancer status in pre-menopausal women.

	Breast cancer		p-value
	No (N = 967414, %)	Yes (N = 14721, %)	
*Age (years)	45.04 ± 3.96	44.91 ± 3.92	<.0001
Smoking			0.4637
Never	918726 (94.97)	13947(94.74)	
Past smoker	15511(1.6)	246 (1.67)	
Current smoker	33177(3.43)	528 (3.59)	
Alcohol consumption			0.4158
No	692678(71.6)	10609 (72.07)	
Mild	263501(27.24)	3938 (26.75)	
Heavy	11235(1.16)	174 (1.18)	
Regular physical activity	166893 (17.25)	2503(17)	0.4283
Hypertension	138721(14.34)	2065 (14.03)	0.284
Diabetes mellitus	29311(3.03)	412(2.8)	0.1043
Dyslipidemia	110474(11.42)	1697(11.53)	0.682
*BMI (kg/m^2)	23.24 ± 3.07	23.13 ± 3.02	<.0001
*Cholesterol	192.33 ± 39.16	192.07 ± 35.86	0.4253
*LDL	114.74 ± 71.38	113.69 ± 47.11	0.0746
*HDL	60.46 ± 35.65	60.56 ± 34.28	0.5615
**GGT	17.29(17.29, 17.31)	17.46(17.31, 17.61)	0.1902

Parameter was indicated as mean +/- SD.
***Geometric means(95% C.I).*

TABLE 2 | Baseline demographic and clinical data according to female cancer status in post-menopausal women.

	Breast cancer		p-value
	No (N = 1074402, %)	Yes (N = 10461, %)	
*Age (years)	58.43 ± 5.8	57.76 ± 5.55	<.0001
Smoking			0.0375
Never	1035039 (96.34)	10035 (95.93)	
Past smoker	11137(1.04)	132 (1.26)	
Current smoker	28226 (2.63)	294 (2.81)	
Alcohol consumption			0.0655
No	924240(86.02)	8916(85.23)	
Mild	143794(13.38)	1481(14.16)	
Heavy	6368 (0.59)	64 (0.61)	
Regular physical activity	213260 (19.85)	2176 (20.8)	0.0152
Hypertension	438793(40.84)	4364(41.72)	0.0696
Diabetes mellitus	82266 (7.66)	877(8.38)	0.0054
Dyslipidemia	365533(34.02)	3733 (35.68)	0.0004
*BMI (kg/m^2)	24.23 ± 3.09	24.49 ± 3.14	<.0001
*Cholesterol	209.1 ± 43.65	208.99 ± 42.87	0.7919
*LDL	127.99 ± 70.43	128.55 ± 69.55	0.4225
*HDL	58.11 ± 34.62	58.31 ± 36.69	0.5615
**GGT	20.91(20.88, 20.93)	21.98 (21.73,22.22)	<0.0001

Parameter was indicated as mean +/- SD.

Higher Serum GGT Level Increases the Risk of Breast Cancer

Individual data were categorized into quartiles to investigate the association between GGT levels. The GGT ranges of each quartile (Q1-Q4) were displayed in **Table 3**. The normal cut-off value for serum GGT in Korean is 35 IU/L. Only a portion of women in the quartile group 4 were above the normal serum GGT value. The Q1 was set as a reference for further analysis to investigate the relationship between serum GGT level and breast cancer risk.

Overall, women with higher GGT levels (Q4) had an increased risk of developing breast cancer, compared to those with Q1 (HR: 1.120, 95% CI: 1.080–1.162) regardless of the participants' menopausal status (**Figure 2**). Both Q2 and Q3 groups also showed increased breast cancer risk than the Q1 group (**Figure 2**).

Similar results were also obtained in pre-menopausal subgroup and post-menopausal subgroup (**Figure 2**). Moreover, the Q4 group's HR for breast cancer was higher in the post-menopausal group (HR: 1.173, 95% CI: 1.107–1.243)

TABLE 3 | Ranges of GGT levels in each quartile.

Quartile	Serum GGT levels (IU/L)
Q1	<14.0
Q2	≥14.0 and <18.0
Q3	≥18.0 and <26.0
Q4	≥26.0

than that in the pre-menopausal group (HR: 1.070, 95% CI: 1.019–1.124) (**Figure 2**).

Further investigation was done to ascertain whether serum GGT level is associated with the development of CIS and invasive breast cancer. Compared to the Q1 group, the Q4 group showed an increased risk of developing CIS (HR: 1.114; 95% CI: 1.040–1.192) (**Figure 3**). However, no difference was observed among the Q2-3 group in the total women group (HR for Q2: 1.006, 95% CI: 0.948–1.08; HR for Q3: 0.994, 95% CI: 0.931–1.062) in the CIS risk (**Figure 3**).

Next, we performed subgroup analysis according to the menopausal status. In the post-menopausal women, the Q4 group exhibited a higher risk of CIS than the Q1 group (HR: 1.266, 95% CI: 1.132–1.416) (**Figure 3**). However, no difference was observed the Q2, Q3, and Q4 group in the pre-menopausal women (HR: 1.035, 95% CI: 0.959–1.119; HR: 0.988, 95% CI: 0.911–1.072; HR: 0.992, 95% CI: 0.905–1.087) (**Figure 3**). These results showed that elevated serum GGT level was associated with a higher risk of developing CIS in post-menopausal women.

Subsequently, the effects of serum GGT levels in post-menopausal women were analyzed by age (**Figure S1**). The breast cancer incidence of Q1 was set as a reference for the analysis. In post-menopausal women, women with elevated serum GGT level (Q4) had a high risk of developing breast cancer at all ages except for women aged between 55 and 60, with the highest risk in women over 65 years (HR:1.272, 95% CI:1.085-1.49) (**Figure S1**).

Additionally, post-menopausal women with elevated serum GGT level (Q4) had a high risk of developing CIS at all ages except for women aged less than 55, also with the highest risk in women over 65 years(HR 1.508, 95% CI 1.084-2.099) (**Figure S2**).

High Serum GGT Levels Increase Breast Cancer Risk Particularly in Obese Women

We conducted the further analysis with additional discussion of the presence of obesity. The Q1-Q3 with BMI less than 25 kg/m^2 was set as a reference for the analysis. The relationship between serum GGT level and the breast cancer risk in obese and non-obese women was assessed. Our result showed that the breast cancer risk of women with BMI more than 25 kg/m^2 (HR 1.165, 95% CI 1.103-1.231) was higher than those of women with BMI less than 25 kg/m^2 (HR 1.054, 95% CI 1.014-1.096) (**Figure 4**). There was no significant association of serum GGT level and breast cancer risks in pre-menopausal women when subcategorized into obese- and non-obese groups (**Figure 4**). However, the subgroup analysis for post-menopausal women revealed that elevated serum GGT level was associated with increased breast cancer risk in obese women (**Figure 4**). HRs for the quartiles Q1-3 was 1.042 (95% CI 0.973-1.115) and HR for the quartiles Q4 was HR 1.214 (95% CI 1.125-1.31) (**Figure 4**).

Meanwhile, an analysis was also performed about the incidence of CIS (**Figure 5**). In obese women, the risk of CIS was significantly increased in the high GGT level group (HR 1.226, 95% CI 1.101-1.365) (**Figure 5**). No significant association of serum GGT level and CIS risks when pre-menopausal women were subcategorized into obese- and non-obese groups (**Figure 5**). However, subgroup analysis for post-menopausal

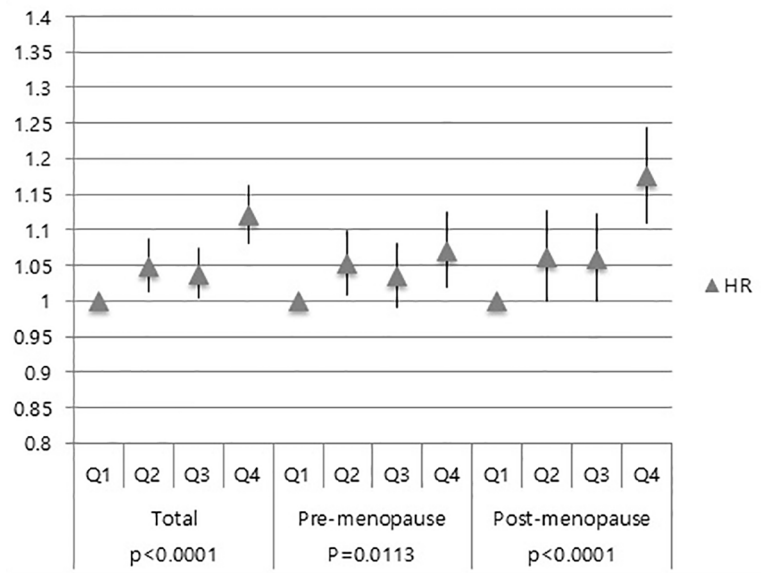

FIGURE 2 | Association of GGT levels with breast cancer risk in total subjects, pre-menopausal women and post-menopausal women.

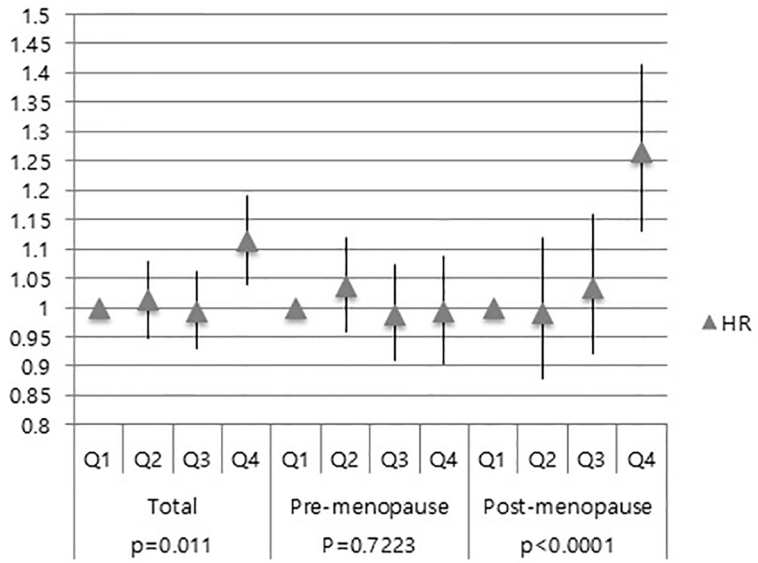

FIGURE 3 | Association of GGT levels with CIS risk in total subjects, pre-menopausal and post-menopausal women.

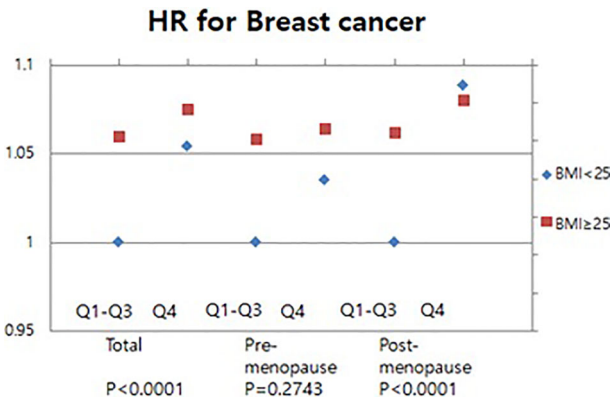

FIGURE 4 | Association of serum GGT levels with breast cancer in pre-and post-menopausal women by BMI.

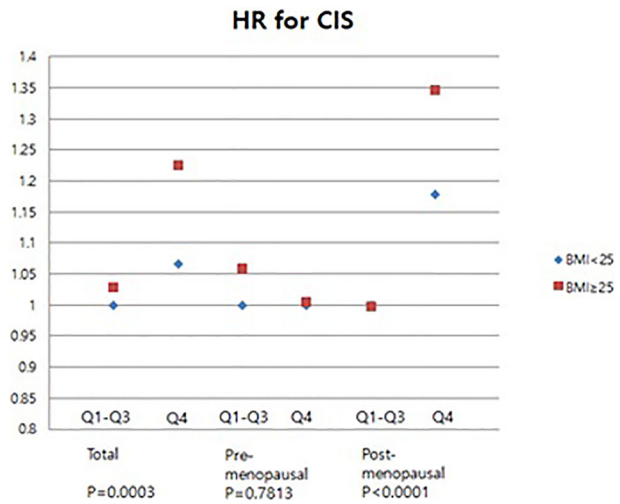

FIGURE 5 | Association of levels of GGT with carcinoma *in situ* of breast in pre- and post-menopausal women by BMI.

women revealed that in the Q4 group, the CIS risk was higher in obese women (HR: 1.346; 95% CI: 1.157-1.566) than that in non-obese women (HR: 1.18; 95% CI: 1.059-1.316) (**Figure 5**). These results indicate that high serum GGT level related to the risk of developing breast cancer and CIS particularly in obese women.

DISCUSSION

In this nationwide cohort study, we suggest serum GGT as a novel biomarker of breast cancer risk. Our results mainly showed that women exhibiting higher serum GGT levels had a high risk of developing breast cancer later according to the menopausal status, obesity status, age and conducted subgroup analyses. Additionally, subgroup analysis was conducted according to the menopausal status, age and obesity status. The subgroup analysis suggests that obese post-menopausal elderly women were more susceptible to breast cancer and CIS with elevated GGT levels.

A previous study analyzing the data from a large cohort in Sweden showed a positive correlation between GGT levels and overall primary cancer risk. The cancer-site specific analysis showed that digestive organ, respiratory system/intra-thoracic

organs, female genital organ/breast, urinary organs, and male genital organs cancer risk was significantly elevated in people with high serum level of GGT(>36U/L) (22). Another population-based cohort study in Austria showed that elevated serum GGT(≥72 U/L) increased cancer risk (HR:1.43,95% CI:1.28-1.61). In cancer-site specific analysis, high level of GGT significantly increased the risk for cancer in digestive organs, the respiratory/intra-thoracic organs, female genital organ/breast and lymphoid/hematopoietic cancer (19). Despite that these large cohort studies have been conducted in western countries, large cohort studies were also needed in Asia since breast cancer in Asia has a different epidemiologic characteristics compared to western countries. To the best of our knowledge, this is the first study in a large data set reporting the association between high levels of GGT and breast cancer incidence in Asia.

GGT is a main enzyme related to glutathione metabolism (23). GGT has been reported about involvement of a pro-oxidant activity and its early recognized contributions to cellular antioxidant defenses (13). Pro-oxidants derived from GGT can regulate important redox-sensitive processes and functions of the cell with particular reference to proliferative/apoptotic balance (24). It has obvious and important implications in tumor progression and drug resistance (24). Some recent studies have suggested that the serum GGT level is associated with cancer prognosis. Grim m et al. reported that high pre-therapeutic serum GGT level (> 72 IU/L) was significantly related to the worse overall survival of epithelial ovarian cancer patients and also associated with elevated GGT expression in the epithelial ovarian tissue (25). Another study demonstrated that the pre-treatment elevated serum GGT was associated with low 5-year progression-free survival in endometrial cancer (26). These results are explained by the study that the high expression of GGT in cancer cells is significantly related to drug resistance (27). Through the studies so far, elevated serum GGT levels seem to have an effect on various aspects from cancer development and progression.

We analyzed a large cohort covering almost the entire Korean women aged over 40 years old who were eligible for national breast cancer screening. The median follow-up period of the current study was 8.37 years, which is a relatively long period for monitoring the incidence of cancer. The cut-off value of quartile group4, serum GGT level of 26U/L, is suggested as a new cut-off value for Korean women for predicting the breast cancer risk. In addition, obesity was shown as a co-factor with the high serum GGT level in contributing the increasing breast cancer risk. However, the role of GGT in breast tumorigenesis remains largely uninvestigated. Therefore, future studies elucidating GGT on the breast cancer etiology are warranted.

CONCLUSIONS

To summarize, our study elucidated the association between serum GGT and the risk of developing breast cancer by utilizing a big dataset from the Korean health insurance database. Elevated serum GGT is associated with increased breast cancer incidence particularly in post-menopausal women with obesity. We believe that our study could provide a new insight into the strategy of breast cancer prevention.

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 below: the Korea National Health Insurance Sharing Service homepage (nhiss.nhis.co.kr).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of Seoul National University Hospital (IRB number: 2101-003-1184). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AS, WW, SK, YH, IP, JY, HJ, K-DH, and YS: design, collection of data, manuscript, editing, approval of final version, and accountability. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by BK21 Plus Program of the Department of Agricultural Biotechnology, Seoul National University (Seoul, Korea), a grant of Health Technology R&D Project through the Korea Health Industry Development R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea [grant number: HI16C2037] and a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea [grant number: HA17C0037].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.668624/full#supplementary-material

Supplementary Figure 1 | Association of GGT levels with breast cancer risk by age in post-menopausal women.

Supplementary Figure 2 | Association of GGT levels with carcinoma *in situ* of breast risk by age in post-menopausal women.

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

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ORIGINAL RESEARCH
published: 09 June 2021
doi: 10.3389/fonc.2021.654472

The lncRNA ADAMTS9-AS2 Regulates RPL22 to Modulate TNBC Progression *via* Controlling the TGF-β Signaling Pathway

Kan Ni[1†], *Zhiqi Huang*[2†], *Yichun Zhu*[1], *Dandan Xue*[1], *Qin Jin*[3], *Chunhui Zhang*[1*] *and Changjiang Gu*[1*]

[1] Department of General Surgery, Affiliated Hospital of Nantong University, Nantong, China, [2] Department of General Surgery, Nantong First people's hospital, The Second Affiliated Hospital of Nantong University, Nantong, China, [3] Department of Pathology, Affiliated Hospital of Nantong University, Nantong, China

Background: Long non-coding RNAs (lncRNAs) are key regulators of triple-negative breast cancer (TNBC) progression, but further work is needed to fully understand the functional relevance of these non-coding RNAs in this cancer type. Herein, we explored the functional role of the lncRNA ADAMTS9-AS2 in TNBC.

Methods: Next-generation sequencing was conducted to compare the expression of different lncRNAs in TNBC tumor and paracancerous tissues, after which ADAMTS9-AS2differential expression in these tumor tissues was evaluated *via* qPCR. The functional role of this lncRNA was assessed by overexpressing it *in vitro* and *in vivo.* FISH and PCR were used to assess the localization of ADAMTS9-AS2within cells. Downstream targets of ADAMTS9-AS2 signaling were identified *via* RNA pulldown assays and transcriptomic sequencing.

Results: The expression ofADAMTS9-AS2 was decreased in TNBC tumor samples ($P < 0.05$), with such downregulation being correlated with TNM stage, age, and tumor size. Overexpressing ADAMTS9-AS2 promoted the apoptotic death and cell cycle arrest of tumor cells *in vitro* and inhibited tumor growth *in vivo.* From a mechanistic perspective, ADAMTS9-AS2 was found to control the expression of RPL22 and to thereby modulate TGF-β signaling to control TNBC progression.

Conclusion: ADAMTS9-AS2 controls the expression of RPL22 and thereby regulates TNBC malignancy *via* the TGF-β signaling pathway.

Keywords: TNBC (Triple negative breast cancer), TGFb (transforming growth factor-beta), lncRNA, ADAMTS9-AS2, signaling pathway

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***Correspondence:**
Changjiang Gu
changjiang058@foxmail.com
Chunhui Zhang
783624840@qq.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *16 January 2021*
Accepted: *08 April 2021*
Published: *09 June 2021*

Citation:
Ni K, Huang Z, Zhu Y, Xue D, Jin Q, Zhang C and Gu C (2021) The lncRNA ADAMTS9-AS2 Regulates RPL22 to Modulate TNBC Progression via Controlling the TGF-β Signaling Pathway. Front. Oncol. 11:654472. doi: 10.3389/fonc.2021.654472

INTRODUCTION

Breast cancer (BC) is a leading cause of cancer-related death in women (1). While many advances in BC diagnosis and treatment have been made in recent years, high rates of tumor chemoresistance and metastasis often result in a poor prognosis (2). There are many molecular subtypes of BC, with triple-negative BC (TNBC) being the most aggressive subtype, accounting for ~20% of BC diagnoses (3). TNBC tumors are so-named owing to their lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and these tumors are not susceptible to therapies targeting these receptor signaling pathways (4–6). Further work is needed to improve the 5-year survival rate of BC patients (7), and as such, more work is needed to clarify the molecular mechanisms governing BC progression and recurrence in order to guide appropriate patient treatment.

Approximately 80% of transcripts in cells are non-coding RNAs (ncRNAs) (8), including long ncRNAs (lncRNAs) > 200 nucleotides in length (9). Despite not encoding proteins, lncRNAs are able to control transcription and translation (10–12), thereby regulating the progression of many tumor types including BC (13) by controlling cell survival, proliferation, metastasis, and chemoresistance (14, 15). ADAMTS9-AS2 is a lncRNA encoded on chr3:64684720-64809891 that is differentially expressed in many tumor types (16). When upregulated, ADAMTS9-AS2 has previously been shown to suppress miR-223-3p expression and to thereby control TGFBR3 expression, ultimately inhibiting lung cancer growth (17). In BC, ADAMTS9-AS2 downregulation is linked to miR-130a-5p upregulation and tamoxifen resistance (18), while in ovarian cancer this lncRNA targets the miR-182-5p/FOXF2 signaling axis to control tumor progression (19). As such, ADAMTS9-AS2 likely serves as a tumor suppressor in several cancer types, but the mechanistic basis for ADAMTS9-AS2 regulatory activity in TNBC remains to be defined.

Herein, we employed a bioinformatics approach to evaluate differential lncRNA expression profiles in TNBC, and found ADAMTS9-AS2 to be downregulated in this cancer type. We then explored the functional role of this lncRNA in this oncogenic context.

MATERIALS AND METHODS

Clinical Tissues

Sixty-two pairs of TNBC and matched adjacent normal tissue samples were obtained from patients surgically resected at the Affiliated Hospital of Nantong University from January 2018 to July 2020. None of the patients received preoperative chemotherapy or radiotherapy. The tissue samples were stored at -80°C. This study was approved by the Clinical Research Ethics Committee of the Affiliated Hospital of Nantong University.

Cell Culture

The TNBC cell lines MDA-MB-231 and HCC1937 were acquired from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and HCC1937 cells were cultured in DMEM (Gibco, Grand Island, NY, USA) and RPMI-1640 (Gibco) media, respectively. Both media were supplemented with 10% fetal bovine serum (GIBCO-BRL, Invitrogen, USA), 100 ug/mL penicillin and 100 U/mL streptomycin (Shanghai Genebase Gen-Tech, Shanghai, China) The cells were maintained at 37°C in a 5% CO2 incubator.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from tissues and cells according to the manufacturer's instructions. RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) was then performed to reverse transcribe total RNA(1 μg) to cDNA. A Roche LightCycler 480 (Roche, Basel, Switzerland) was used to conduct qRT-PCR analyses. Target primers were amplified by SYBR Green Mix (Roche) according to the manufacturer's instructions. Sequences of the primers Primers used in this study were as follows: ADAMTS9-AS2 (F: 5'-CTACCCCCTCC CAGTCTTCA-3'; R: 5'-GGTCTTGCTCTTTCCTTATCCTCA-3'), RPL22 (F: 5'- GACAACTGAAAGGGGCTACCAAGG-3'; R: 5'- GCACCACAAGGCACCAGAGTC-3'); GADPH (F: 5′-CGCTGAGTACGTCGTGGAGTC-3′; R: 5′-GCTGATGATC TTGAGGCTGTTGTC-3′). All results were calculated and expressed as 2-ΔΔCt. GAPDH was used as endogenous control.

Cell Transfection

TNBC cells in the logarithmic growth phase were cultured in a 6-well plate (5 × 104 cells/well) for one day. When the cells had settled, the cells were infected with lentivirus with ADAMTS9-AS2 overexpression. The cells were then cultured in the presence of puromycin for 5 days to screen for positively infected cells. When the infection efficiency exceeded 90% under fluorescence microscope and the efficiency was proved by qRT-PCR, the cells were collected for use in experiments. The siRPL22 was transfected into TNBC cells by using Lipofectamine 3000 (Invitrogen). The manufacturer's instructions were strictly followed.

Cell Counting Kit-8 (CCK8), Colony Formation and EdU Assay

2000 cells were seeded onto 96-well plates and placed in an incubator with 5% CO2 at 37°C, 10 μl of CCK8 solution was added to each well every 24h. The cell viability was evaluated in a absorbance values of 450 nm. For colony formation assay, 1000 cells were seeded in to 6-well plates for 2 weeks, then fixed in methanol and stained with 0.1% crystal violet. EdU assay (5-ethynyl-20-deoxyuridine) was performed with a commercial kit (Ribobio, Guangzhou, China). The cells in logarithmic growth phase were inoculated in 24 well plates with 5×10^4 cells per well. 200 μL 50 μM EdU medium added to each well for incubation for 2 hours, then the cells were washed 2 times by PBS. 200 μl of 4% paraformaldehyde was added to each well and incubated at room temperature for 30 minutes. 200 μL PBS was used to wash each well. 0.5% TritonX-100 was added to each well and shaked

for 10 min. 100 μL of 1× Apollo® Staining reaction solution was added into each well and incubated in dark, with shaking for 30 minutes. 0.5% TritonX-100 was added to each well and shaked for 10 min 2 times. 200 μL methanol was used to wash each well. 100 μL 1 x Hoechst 33342 reaction solution was added to each well and incubated in dark for 30 min. The well was washed by PBS. The rate of proliferating cells was counted by fluorescence microscope. Triplicate is required for each experiment.

Wound Healing, Migration and Invasion Assays

The transfected TNBC cells were seeded in 6-well plate and scratched with a10 μL pipette tip, then cultured in serum-free medium for 24 h, the width of wounds was examined and normalized to control group. For invasion assays, 5×10^4 TNBC cells were suspended in serum-free medium and placed into the upper chambers (BD BioCoat, MA, USA) coated with matrigel (BD Biosciences, NJ, USA) in a volume of 200 μL, and then 600 μL 10% FBS medium was added into the bottom chambers. After 24 h, the cells on the upper chambers were removed and cells on the lower compartment were fixed with ethanol and stained by crystal violet, then photographed and counted with microscope. For migration assay, Matrigel was not needed and the left steps were the same as above.

ADAMTS9-AS2 Subcellular Location Analysis

Living TNBC cell lines were fixed in 4% paraformaldehyde firstly for FISH assay. Then the cells were treated with triton X-100 (Solarbio, China) and subsequently treated with Fluorescent In Situ Hybridization Kit (RiboBio, China) following the manufacturers' instructions. RNA FISH probes were designed by GenePharma (GenePharma, Shanghai, China). Cytoplasmic and nuclear separation was used by the PARIS Kit (Life Technologies, Carlsbad, CA, USA). 10^7 living cells were collected and washed by cold PBS. 500 μL of ice-cold Cell Disruption Buffer was added to the cells. Then the cells was lysed by vortex. The lysate was transferred to a tube containing 2X Lysis/Binding Solution for RNA isolation at room temp and then mixed gently. ACS grade 100% ethanol equal was added to the mixture and then the sample mixture was transferred to Filter Cartridge in Collection Tube for centrifugation until all the mixture was through the filter. The flow-through was discarded and the filter was washed by Wash Solution. Elution Solution was preheated to 95°C and added to the filter. the RNA was recovered by centrifugation for 30 sec. qRT-PCR was used to detect the relative expression.

Tumor Xenografts in Animals

The xenograft mice *in vivo* assays were performed with four-week-old female BALB/c-nude mice (about 18 g), which were purchased from the animal center of Nantong University (Nantong, China). according to the institutional guidelines and approved by the Animal Ethics Committee of Afliated Hospital of Nantong University. The mice were injected with MDA-MB-231 cells (1×107) with LV NC or ADAMTS9-AS2 subcutaneously. The volume of xenograft tumors was measured every 5 days. After 25 days, the mice were executed and tumors were taken out for weighing and subjected to H&E and immunohistochemical staining assay.

RNA Pulldown Assay

Cell protein was collected from MDA-MB-231. Pierce RNA 3′ End Desthiobiotinylation Kit (Thermo Fisher Scientific, USA) and Pierce Magnetic RNA-Protein PullDown Kit (Thermo Fisher Scientific) were used for RNA pulldown following the manufacturer's instructions. After Pre-Washing the beads, 50 μl beads was added for the assay. The supernatant was discarded by a magnetic stand and was added an equal volume of 1X RNA Capture Buffer. 50pmol of labeled RNA (GenePharma, Shanghai, China) was added to the beads and incubated for 30 minutes at room temperature with agitation. The supernatant was discarded and washed with 20mM Tris. 100μL of 1X Protein-RNA Binding Buffer (Mixed buffer with collected cell protein) was added to the beads and mixed well. The supernatant was discarded and 100μL of Master Mix was added to the RNA-bound beads for incubating 60 minutes at 4°C with agitation. The mixture was washed with 1X wash buffer twice and the supernatant was discarded. Then 50μL of Elution Buffer was added to the beads and mixed well by vortexing and incubated 30 minutes at 37°C with agitation. Finally the tube was put into a magnetic stand to collect supernatant for downstream analysis. The samples to be tested were identified by mass spectrometry performed by the Shanghai Applied Protein Technology Co, Ltd. Western blot assay was used to prove the downstreamed protein.

Assays of Production of Lactate and ATP

Lactate Assay Kit II and ATP Colorimetric Assay Kit were used to measure the production of lactate and ATP according to the manufacturer's instructions (Beyotime, Shanghai, China). Appropriate amount of cells were homogenized in 100 μl corresponding assay buffer provided by the kits. The homogenized cells were centrifuged, and the soluble fraction was analyzed and measured the RLU value with a luminometer (Promega, Madison, WI, USA). ATP levels was estimated based on the standard curve, and normalized to the cell number.

Western Blot Analysis

The total protein of TNBC cells was exacted with RIPA buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, then electransferred onto a PVDF membrane (Bio-Rad, CA, USA). The membranes were blocked with 5% skim milk and incubated with antibodies against Cyclin D1 (1:1000, Proteintech, USA), p27 (1:1000, Proteintech), Bax (1:1000, Proteintech), Bcl-2 (1:1000, Proteintech), E-cadherin (1:2000, Proteintech), Vimentin (1:1000, CST, Danvers, USA), N-cadherin(1:1000, Proteintech), p-ERK1/2 (1:1000, Abcam, Cambridge, UK); ERK1/2 (1:2000, Abcam), Smad2(1:500, Proteintech), Smad7(1:500, Proteintech),TGFBR1(1:500,

Proteintech) and GADPH (1:5000, CST) at 4°C overnight and then incubated with secondary antibodies (1:5000, Proteintech) at room temperature for 2 h. Finally, the bands were examined by Immobilob™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and were detected by Immunoblots visualized by ECL detection system (Quantity One software, BioRad).

Bioinformatics Analysis

ADAMTS9-AS2 expression levels in a large BC patient cohort were assessed using the Ualcan database (http://ualcan.path.uab.edu) using the search terms "ADAMTS9-AS2," "Cancer VS. Normal/Cancer Analysis," and "Triple Negative Breast Cancer." Data were compared based upon log2 median-centered intensity in TCGA microarray datasets. Kaplan-Meier Plotter (http://kmplot.com/analysis/) was further used to assess the relationship between ADAMTS9-AS2 expression levels and TNBC patient survival outcomes.

Microarray Analysis

Total RNA were isolated from the paired tissue samples of five TNBC patients using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched by Oligo(dT) beads. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and buffer. Then the cDNA fragments were purified with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end repaired, poly(A) added, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Statistical Analysis

Statistical analyses were performed by SPSS 20.0 (IBM, SPSS, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., USA). Data were showed as mean ± standard deviation (SD). The differences between groups were assessed by Student's t test, one-way ANOVA or $\chi 2$ test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

ADAMTS9-AS2 Is Downregulated and Associated With Poor Prognosis in TNBC Patients

We began by screening the most different lncRNAs and GO pathways that were differentially expressed (FC > 1.5, P < 0.01) in TNBC tissues relative to matched healthy control samples (**Figures 1A–C**). This analysis revealed significant ADAMTS9-AS2 downregulation in TNBC tumor tissues, and such downregulation was confirmed *via* qPCR (**Figures 1D, E**). Such ADAMTS9-AS2 downregulation was correlated with decreased TNBC patient survival (**Figure 1F**), and with TNM stage, age and tumor size (**Table 1**). Consistent with these patient findings, ADAMTS9-AS2 was downregulated in the MDA-MB-231 and HCC1937 TNBC cell lines relative to normal MCF-10A control cells (**Figure 1G**). The results of these qPCR analyses were validated *via* agarose gel electrophoresis and sequencing, revealing the presence of a single 104 bp product consistent with the length of the predicted sequence of this lncRNA (**Figures 1H, I**). As such, the downregulation of ADAMTS9-AS2 may play an important role in TNBC progression.

ADAMTS9-AS2 Suppresses TNBC Cell Proliferation

The functional role of ADAMTS9-AS2 was next evaluated by utilizing pcDNA vectors to overexpress this lncRNA in MDA-MB-231 and HCC1937 cells (**Figure 2A**). CCK-8 assays revealed that ADAMTS9-AS2 overexpression decreased the viability of these two BC cell lines, and EdU uptake assays confirmed this finding (**Figures 2B–E**). A cell cycle analysis subsequently revealed that ADAMTS9-AS2 overexpression increased the frequency of TNBC cells in the G0-G1 phase and reduced the frequency of cells in the S phase, suggesting that this lncRNA induced G1 phase arrest in these cancer cells (**Figures 2F, G**). A colony formation assay further revealed that ADAMTS9-AS2 overexpression markedly impaired colony formation (**Figures 2H, I**). Consistent with the cell cycle influence, the expression levels of cell cycle proteins were markedly changed when ADAMTS9-AS2 was enhanced (**Figures 2J, K**). As such, ADAMTS9-AS2 can suppress TNBC cell proliferation.

ADAMTS9-AS2 Regulates TNBC Cell Invasion, Metastasis, and Cell Cycle Progression and Inhibited Warburg Effect

The role of ADAMTS9-AS2 as a regulator of TNBC cell invasion was next evaluated through Transwell and wound healing assays, which demonstrated that the upregulation of this lncRNA impaired both of these activities in MDA-MB-231 and HCC1937 cells (**Figures 3A–D**). Flow cytometry analyses further revealed that MDA-MB-231 and HCC1937 cells overexpressing ADAMTS9-AS2 exhibited higher rates of apoptotic cell death relative to cells transfected with negative control constructs (**Figures 3E, F**). Western blotting also revealed that ADAMTS9-AS2 overexpression increased the levels of Bax in these tumor cells, whereas such overexpression suppressed Bcl-2 expression and EMT-related protein relative to control cells (**Figure 4G**). The glycolytic pathway is the main metabolic pathway for tumor cells to perform energy metabolism. In this process, every molecule of glucose taken by cancer cells can quickly generate 2 molecules of ATP to meet their own energy needs. ADAMTS9-AS2 decreased the production of lactate and ATP in MDA-MB-231 cells (**Figures 3H, I**). Since LDHA is a marker of glycolytic pathway, we detected the expression of LDHA in TNBC after transfection and ADAMTS9-AS2 inhibited the expression of LDHA

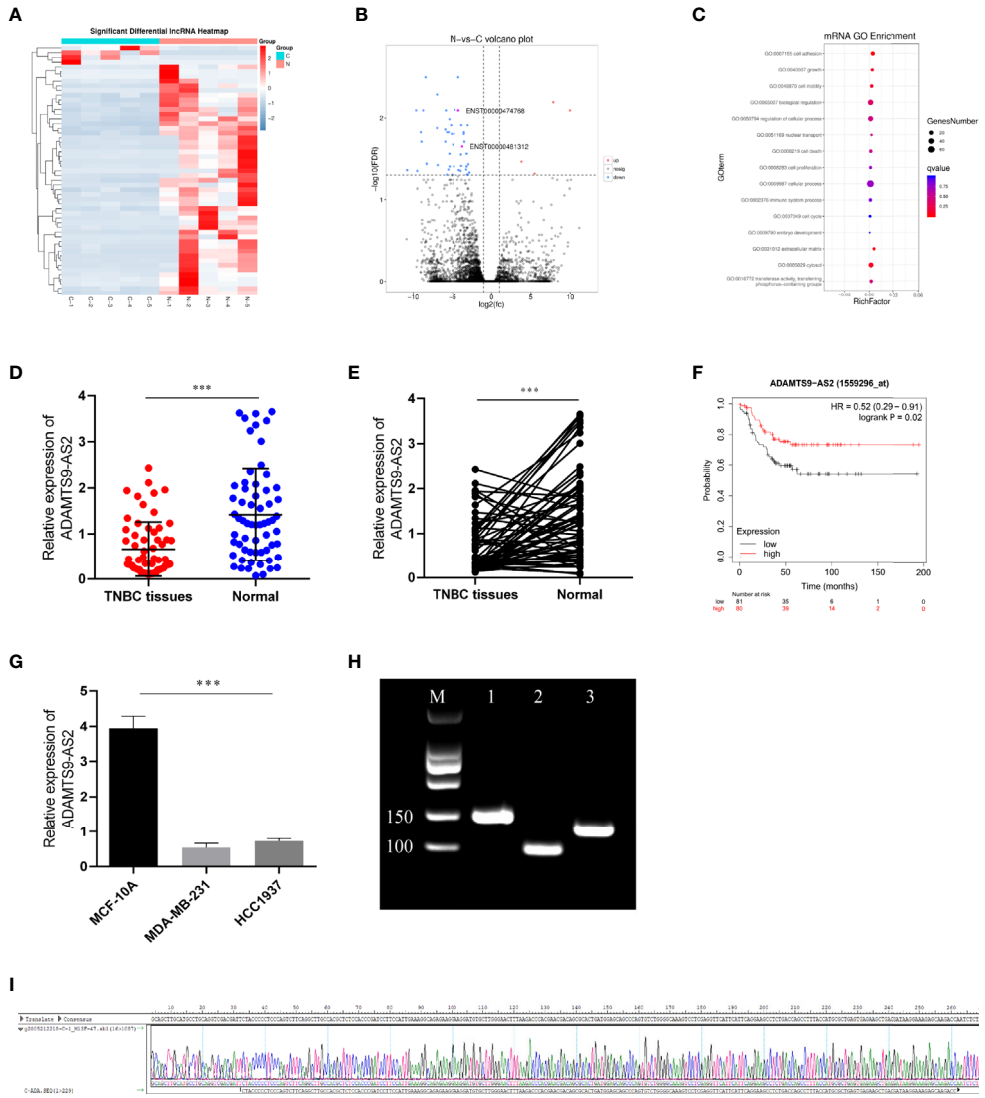

FIGURE 1 | ADAMTS9-AS2 is downregulated and associated with poor prognosis in TNBC patients. **(A, B)** The cluster heat maps and the volcano plot visualize the expression of lncRNA between TNBC tissues and adjacent non-tumor tissues. The red dots and green dots represent upregulated and downregulated LncRNAs with statistical significance, respectively. **(C)** GO pathway analysis of TNBC tissues. **(D, E)** Relative expression of ADAMTS9-AS2 in 62 TNBC tissues and adjacent normal tissues. **(F)** Kaplan–Meier survival curve of patients with TNBC downloaded from TCGA database. **(G)** Relative expression of ADAMTS9-AS2 in TNBC cell lines and normal breast cell line. **(H, I)** PCR product in agarose gel electrophoresis and the splicing site verified by DNA sequencing. ***P < 0.001.

(**Figure 3J**). As such, ADAMTS9-AS2 may suppress TNBC progression *in vitro*.

Overexpression of ADAMTS9-AS2 Suppresses *In Vivo* BC Tumor Growth

To evaluate the ability of ADAMTS9-AS2 to impact TNBC progression *in vivo*, we next transduced MDA-MB-231 cells with LV-NC or LV-ADAMTS9-AS2 and then implanted these tumor cells subcutaneously in mice. Tumors overexpressing ADAMTS9-AS2 were smaller than those transduced with the control lentivirus (**Figures 4A–C**), and these tumors weighed significantly less than control tumors (**Figure 4D**). As expected, ADAMTS9-AS2 was expressed at a higher level in tumors from the overexpression group relative to the NC group (**Figure 4E**). Subsequent immunohistochemical staining revealed that Ki-67 AND MMP9 expression levels were decreased in tumors overexpressing ADAMTS9-AS2 (**Figure 4F**). Together, these findings thus indicated that ADAMTS9-AS2 can suppress *in vivo* tumor growth.

ADAMTS9-AS2 Interacts With RPL22 in TNBC Cells to Suppress Tumor Progression

FISH and subcellular localization analyses were next confirmed to evaluate the localization of ADAMTS9-AS2 in TNBC cells, revealing it to be present within both the cytoplasm and nucleus

TABLE 1 | Associations between ADAMS9-AS2 expression and clinicopathological parameters.

Clinicopathological parameters	Case No.	ADAMTS9-AS2		*P* value
		Low	High	
Total	62	30	32	
Age, yr				0.003**
<50	29	8	21	
≥50	33	22	11	
Tumor diameter, mm				0.011*
≤20	13	2	11	
>20	49	28	21	
Tumor stage(TNM)				0.04*
I	15	3	12	
II	36	21	15	
III	11	6	5	
Histological grade				0.876
I	14	5	9	
II	26	11	15	
III	22	14	8	
Lymph node metastasis				0.423
negative	41	18	23	
positive	21	12	9	
Ki67				0.102
low	19	6	13	
high	43	24	19	

(Continued)

TABLE 1 | Continued

Statistical analysis were carried out using Person χ^2 test.
**P < 0.05, **P < 0.01 have statistical significances.*

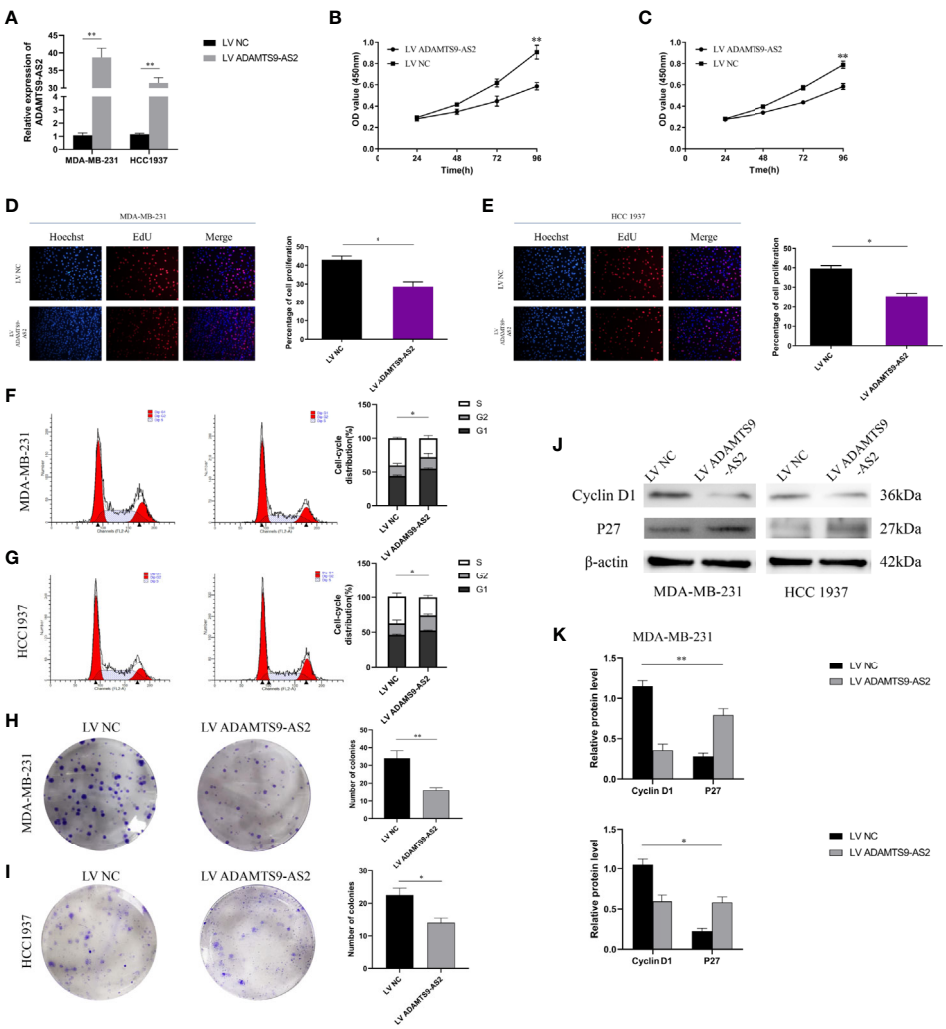

FIGURE 2 | ADAMTS9-AS2 suppresses TNBC cell proliferation. a: qRT-PCR analysis of LncRNA ADAMTS9-AS2 expression in TNBC cells transfected with ADAMTS9-AS2 overexpression vector or NC. **(B, C)** Growth curves of cells transfected with indicated vectors were evaluated by CCK8 assays. **(D, E)** EdU assays were conducted in cells after transfection with LV ADAMTS9-AS2 or LV NC. **(F, G)** The cell cycle progression was analyzed by flow cytometry after indicated transfected. **(H, I)** Colony formation assays were executed to detect the proliferation of TNBC cells transfected with indicated vectors. **(J, K)** Western blot was used to detect the influence of ADAMTS9-AS2 on cell cycle markers. *P < 0.05, **P < 0.01.

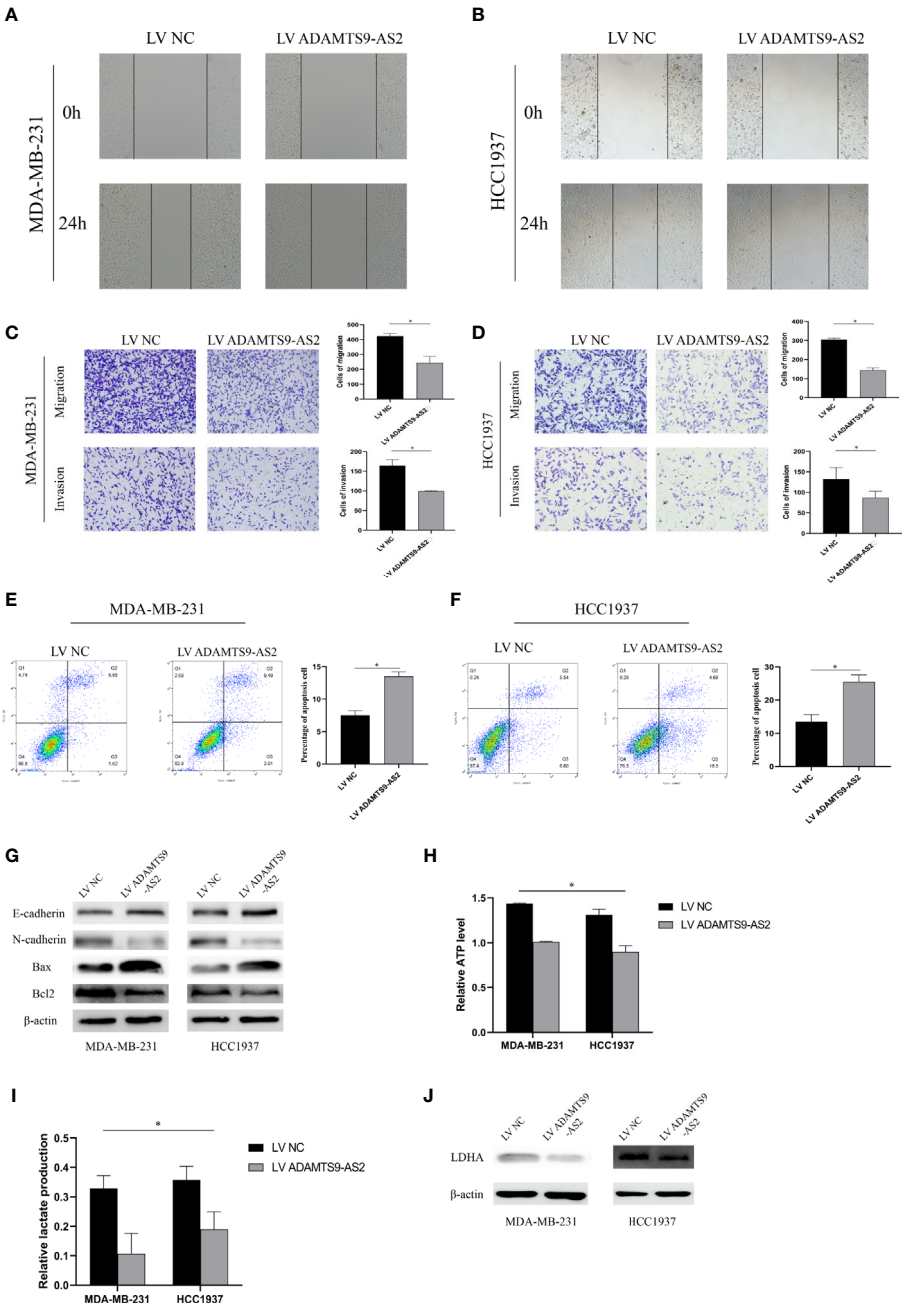

FIGURE 3 | ADAMTS9-AS2 regulates TNBC cell invasion, metastasis, and cell cycle progression and inhibited Warburg effect. **(A, B)** Cell migration capacities were detected by wound healing assays after transfected with indicated vectors. **(C, D)** Cell migration and invasion abilities were determined by transwell assays after transfection. **(E, F)** Apoptosis rate of TNBC cells was analyzed by flow cytometry after LV ADAMTS9-AS2. **(G)** The expression levels of apoptosis-related and epithelial-mesenchymal transition process marker proteins were determined by western blot. **(H, I)** Production of lactate and ATP were examined in TNBC cells transfected with LV ADAMTS9-AS2 or LV NC as indicated. **(J)** related LDHA expression was detected by western blot. *$P < 0.05$.

(**Figures 5A, B**). Given that we were able to detect this lncRNA in the nucleus, we next utilized biotinylated ADAMTS9-AS2 to conduct a pull-down assay using MDA-MB-231 cell lysates, which were then separated *via* SDS-PAGE. Subsequent mass spectrometry (MS) analyses led to the identification of RPL22, which is a key ribosomal protein (RP) associated with ribosome biogenesis and protein translation, as being specifically present within the ADAMTS9-AS2 pulldown lane. This pulldown assay

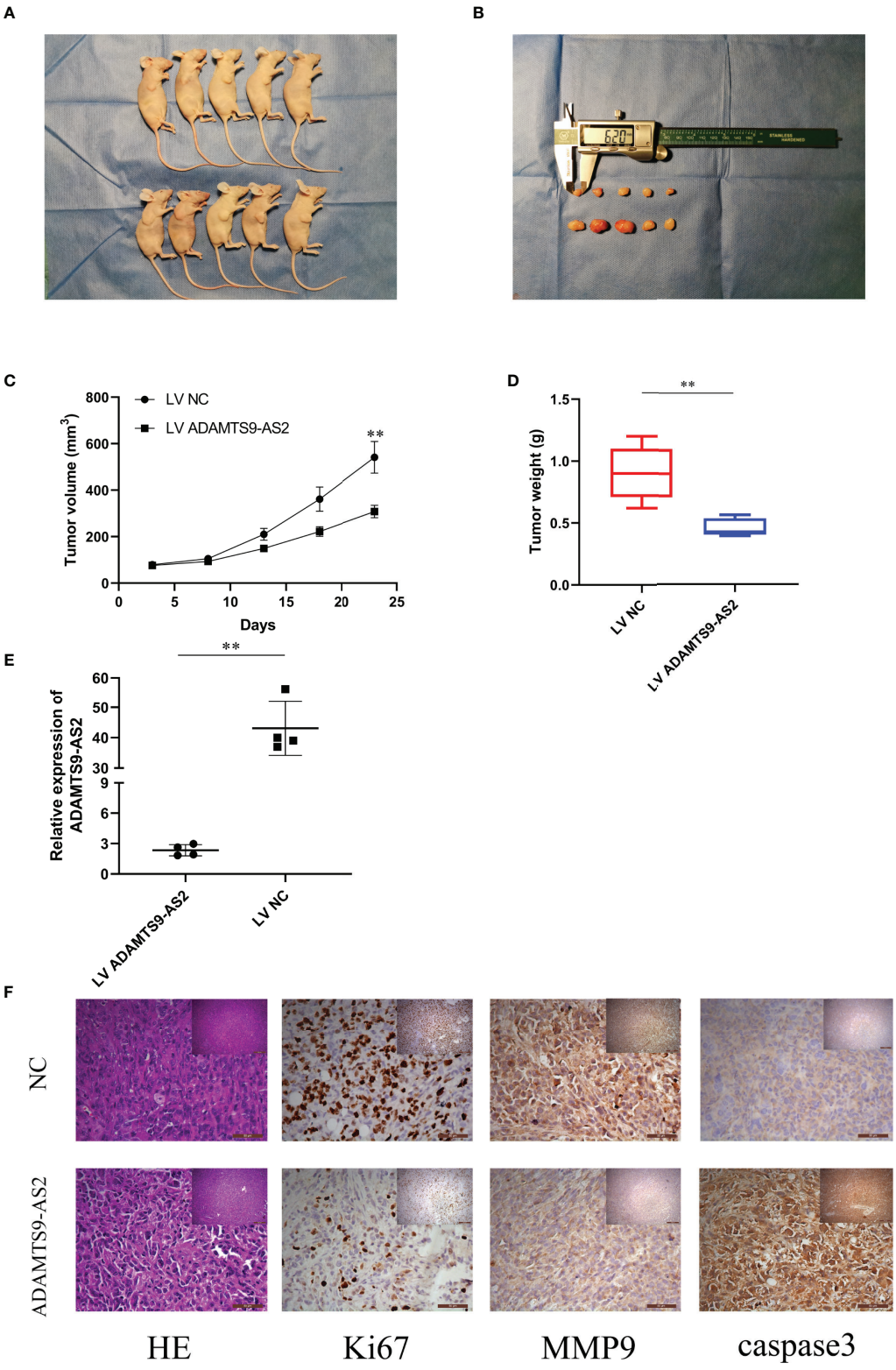

FIGURE 4 | Overexpression of ADAMTS9-AS2 suppresses *in vivo* BC tumor growth. **(A, B)** Representative images of xenograft tumors of each group. **(C)** Growth curves of xenograft tumors which were measured every 5 days. **(D)** Tumor weights from two groups are represented. **(E)** qRT-PCR detected relative expression in two groups. **(F)** IHC staining was applied to analyze the protein levels of Ki67, MMP9 and cleaved-caspase 3. **P < 0.01.

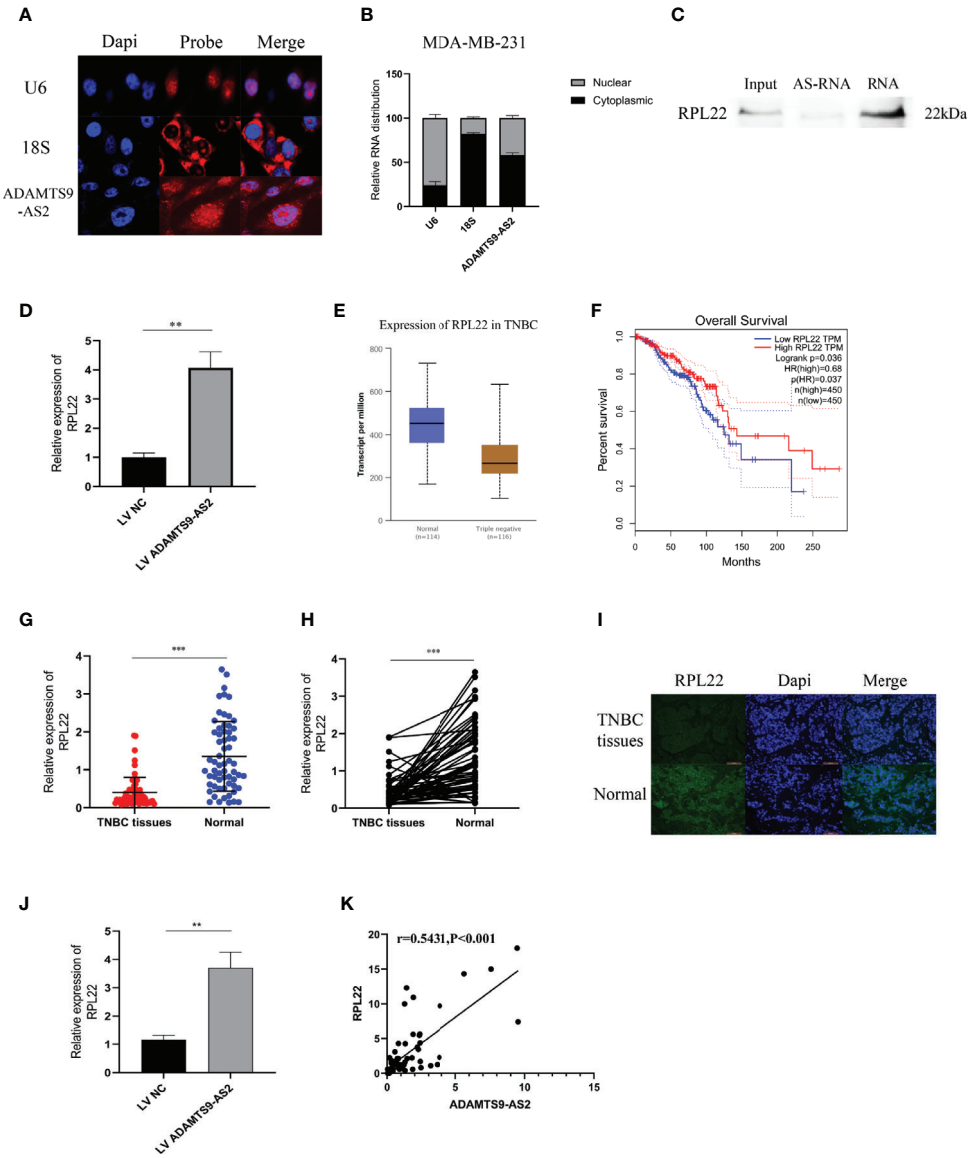

FIGURE 5 | ADAMTS9-AS2 interacts with RPL22 in TNBC cells to suppress tumor progression. **(A)** FISH analysis of the location of ADAMTS9-AS2 in the cytoplasm and nuclear fractions of MDA-MB-231 cells. **(B)** qRT-PCR was used to detect ADAMTS9-AS2 subcellular fractionation. **(C)** RNA pull-down assay indicated that ADAMTS9-AS2 interacted with RPL22. **(D)** Western blot proved the relationship between ADAMTS9-AS2 interacted with RPL22. **(E)** Relative expression of RPL22 in TNBC tissues (Tumor) compared with normal tissue (normal) was analyzed using TCGA data. **(F)** Kaplan-Meier survival analysis of overall survival based on TCGA data. **(G)** Relative expression of RPL22 in TNBC tissues (Tumor) and adjacent non-tumor tissues (Normal) was determined by qRT-PCR (n = 62). **(I)** IF was used to demonstrate the relative expression of RPL22 in TNBC tissues. **(J)** Relative expression of RPL22 in xenograft tumors of each group was determined by qRT-PCR. **(K)** Spearman–Pearson correlation of ADAMTS9-AS2 and RPL22. **P < 0.01, ***P < 0.001.

thus confirmed the ability of ADAMTS9-AS2 and RPL22 to interact with one another (**Figure 5C**). RPL22 mRNA and protein levels were significantly increased in TNBC following ADAMTS9-AS2 overexpression (**Figure 5D**). In the TCGA database, RPL22 was shown to be expressed at lower levels in tumor relative to control breast tissue samples, with such downregulation being correlated with poorer prognosis (**Figures 5E, F**). When we assessed RPL22 levels *via* Immunofluorescence of TNBC patient tumor and control tissues and *via* qPCR in 62 TNBC tissue samples, we confirmed that this RP was present at lower levels in tumor tissues **(Figures 5G–I)**. qRT-PCR further confirmed higher levels of RPL22 expression in xenograft tumors overexpressing ADAMTS9-AS2 (**Figure 5J**). ADAMTS9-AS2 was also positively correlated with RPL22 in TNBC tissues ($r = 0.5431$, $P < 0.001$, **Figure 5K**).

ADAMTS9-AS2 Regulates RPL22 to Control TNBC Progression Through RPL22

We next conducted rescue experiments to confirm whether RPL22 was involved in ADAMTS9-AS2-mediated suppression of TNBC tumor growth. Through CCK-8 and colony formation assays, we confirmed that ADAMTS9-AS2 overexpression impaired the viability of these cells, whereas knocking down RPL22 reversed this effect (**Figures 6A–D**). Similarly, Transwell assays revealed that ADAMTS9-AS2 overexpression was able to suppress the migrative activity of TNBC cells, while si-RPL22 co-transfection reversed these phenotypic changes (**Figures 6E, F**).

ADAMTS9-AS2 Regulates the TGF-β Signaling Pathway to Control TNBC Progression

Lastly, we performed RNA-seq analyses of MDA-MB-231 cells overexpressing ADAMTS9-AS2 to further explore its functional role in this oncogenic context. Heatmap and KEGG analyses revealed that ADAMTS9-AS2 overexpression impacted the TGF-β signaling pathway (**Figures 7A, B**). Given previously reported interactions between RPL22 and Smad2 (20), we next assessed the ability of RPL22 to suppress TGF-β signaling. Following ADAMTS9-AS2, siRPL22, and RPL22 transfection into TNBC cell lines, we evaluated changes in TGF-β-related gene expression. Western blotting revealed that ADAMTS9-AS2 and RPL22 controlled TGFBRI, smad2, smad7, and p-ERK1/2 protein levels (**Figures 7C, D**). Together, these findings suggest that ADAMTS9-AS2 controls the survival, proliferation, and malignancy of TNBC cells *via* the TGF-β pathway (**Figure 7E**).

DISCUSSION

The advent of next-generation sequencing has led to the identification of many differentially regulated lncRNAs in TNBC (21), some of which have been identified as key diagnostic or prognostic biomarkers of this disease type (22). For example, the M2 macrophage-induced lncRNA PCAT6 regulates VEGFR2 expression and thereby controls TNBC growth and angiogenesis (23). Li. et al. found that the MNX1-AS1 lncRNA was able to enhance STAT3 phosphorylation,

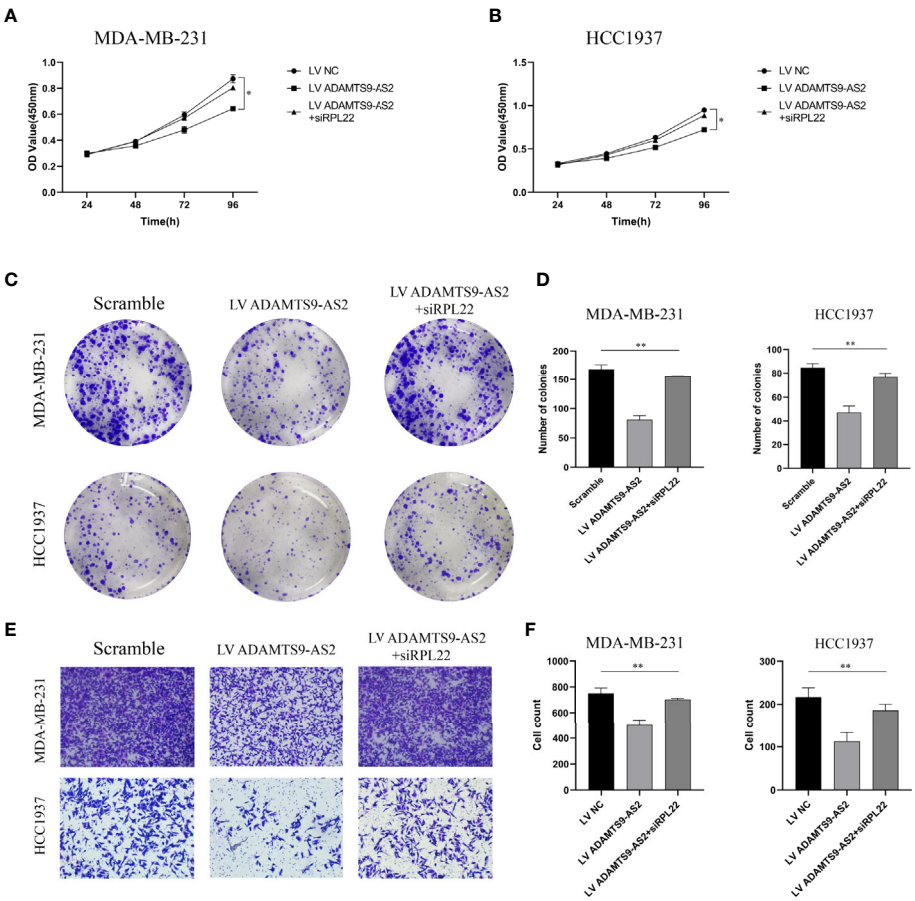

FIGURE 6 | ADAMTS9-AS2 regulates RPL22 to control TNBC progression through RPL22. **(A–D)** The cell proliferation was determined after transfection with LV ADAMTS9-AS2, ADAMTS9-AS2+siRPL22 and LV NC by CCK-8 and Colony formation assay. **(E, F)** The cell migration was determined after transfection by Transwell assay. *P < 0.05, **P < 0.01.

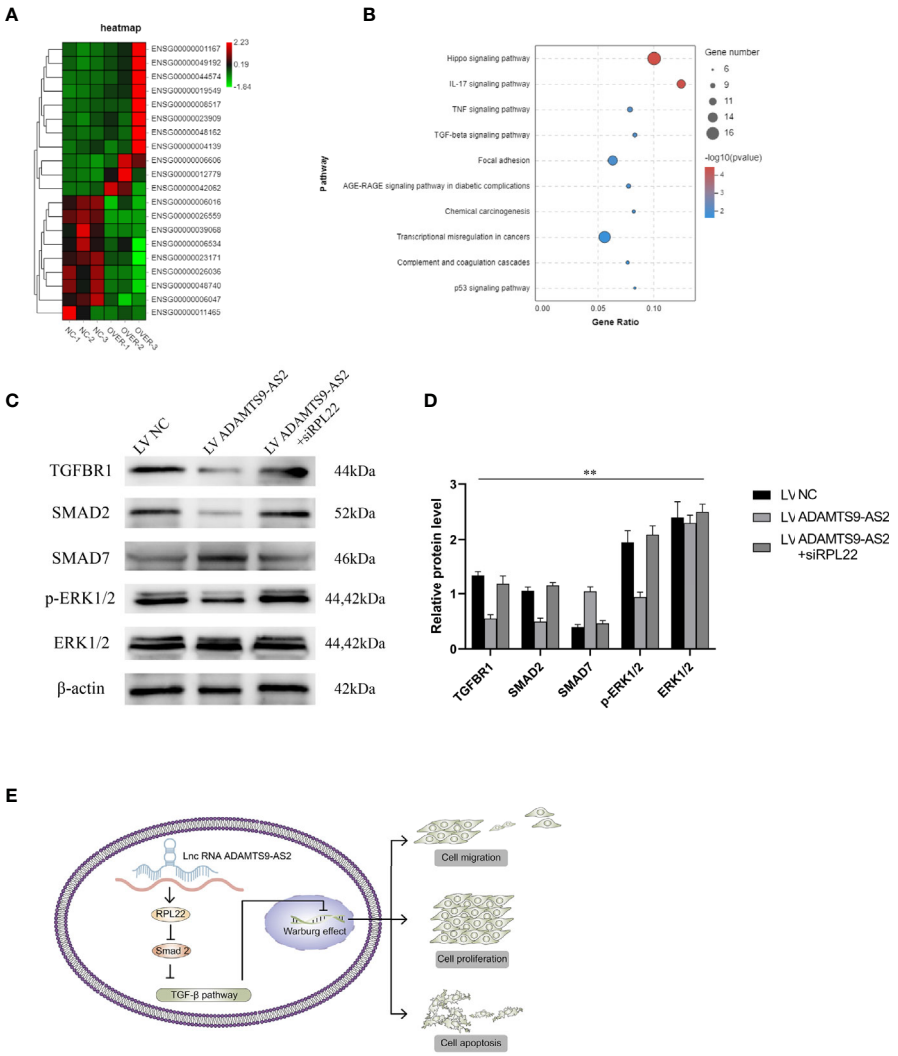

FIGURE 7 | ADAMTS9-AS2 regulates the TGF-β signaling pathway to control TNBC progression. **(A)** The cluster heat maps displayed the 20 most representative differentially expressed mRNAs with transcriptomic sequencing. **(B)** KEGG pathway analysis of differentially expressed mRNAs. **(C, D)** Western blot assay determined the total and active protein level of TGF-β signaling pathway related proteins in MDA-MB231 cell lines. **(E)** Summary of the mechanism of ADAMTS9-AS2 in TNBC cell lines. **P < 0.01.

thereby driving TNBC progression (24). ADAMTS9-AS2 has also previously been shown to suppress lung and ovarian cancer growth (17, 19), but its functional role in TNBC has not been previously clarified.

Herein, we established that ADAMTS9-AS2 is an important regulator of TNBC. RNA sequencing and subsequent bioinformatics analyses identified ADAMTS9-AS2 as being among the lncRNAs that were most significantly downregulated in TNBC tumor samples relative to matched paracancerous tissue samples. The downregulation of this lncRNA was associated with patient age, tumor size, lymph node status, higher TNM stage, and a poorer prognosis. When functional experiments were conducted, we determined that ADAMTS9-AS2 was able to suppress the proliferative and invasive activity of TNBC cells *in vitro* and *in vivo*, instead promoting their apoptotic death. There are multiple mechanisms whereby lncRNAs can modulate cellular physiology, including by sequestering miRNAs (25), controlling protein stability (26), or regulating chromatin structure (27). ADAMTS9-AS2 is an antisense transcript derived from the ADAMTS9 protein-coding gene. Such antisense RNAs have been shown to function as post-transcriptional regulators of RNA and protein stability and of promoter activation. As we found that ADAMTS9-AS2 was largely localized within BC cell nuclei, this suggested that it may influence the biology of these cells by interacting with specific proteins. Pull-down assays revealed that ADAMTS9-AS2 was able to regulate RPL22 expression, and this regulatory relationship was confirmed *via* qPCR and Western blotting.

Ribosomes contain essential RPs that are necessary for facilitating translation in the cytoplasm. RPs are important RNA-binding proteins that are present at high levels in all cell

types (28). Altered ribosomal biogenesis and protein translation can profoundly shape tumor progression. BC tumors exhibit the upregulation of RPL19 (29), whereas RPL7A is downregulated in osteosarcoma (30), and RPL41 is downregulated in BC (31). Such changes in RP expression can alter tumor cell malignancy by modulating their proliferative activity (28). However, relatively little is known about the prognostic relevance of specific RPs in BC. Herein, we found that BC tumors exhibit significant RPL22 downregulation as compared to healthy tissue samples at the mRNA and protein levels. RPL22 is a 60S large ribosomal subunit component, and mutations in this gene have been linked to bacterial macrolide resistance (32). This RP has also been linked to viral infections, protecting against EBV-infected cell transformation (33), and controlling the development of T cells by controlling apoptotic cell death (34). RPL22 mutations may enhance cellular proliferation (35) and promote oncogenesis. Herein, we found that RPL22 expression to be positively correlated with that of ADAMTS9-AS2, and rescue experiments confirmed the ability of ADAMTS9-AS2 to regulate TNBC cell progression. In prior reports, RPL22 was shown to control TGF-βpathway activation (36), with TGF-β signaling being closely linked to cancer progression and metastasis (37, 38). Among them, TGFBR, SMAD2 and SMAD7 were the most known regulators, so we examined whether ADAMTS9-AS2 could affect their expression. In the KEGG map, the TGF-βinduced the activation of MAPK/ERK pathway, which promote cancer progression in TNBC, so we detected relative protein and found the possible mechanism ADAMTS9-AS2 may involve in.

In brief, we have discussed the function and mechanism of ADAMTS9-AS2. However, some questions remained for further study. Firstly, the clinical samples are not enough. As the expression was associated with age, we wondered whether there is any relationship between cell aging and ADAMTS9-AS2, resulting in a new therapeutic target for elderly TNBC patients. What's more, we could see a tendency between the LncRNA and lymph node metastasis and Ki67, making our theory more persuasive. Then, based on our experiment, about 40% of the LncRNA was located in cytoplasm, whether ADAMTS9-AS2 could play the anti-tumor role through the cytoplasmic LncRNA remained to be studied.

As such, we hypothesized that the downregulated ADAMTS9-AS2 levels were associated with a poorer TNBC patient prognosis. ADAMTS9-AS2 could regulate TNBC cells proliferation, apoptosis, metastasis and Warburg effect by ADAMTS9-AS2/RPL22 axis *via* modulation of the TGF-β pathway. We identified ADAMTS9-AS2 as a potential therapeutic target in patients with TNBC, although further studies will be needed to validate this possibility.

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 Affiliated Hospital of Nantong University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Affiliated Hospital of Nantong University. 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

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

FUNDING

This study was partially supported by a Nantong City-level Science and Technology Plan Project fund from KN. (Number GJZ17086.)

ACKNOWLEDGMENT

We would like to thank Kan Ni and Zhiqi Huang for their support in data collection and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.654472/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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 10 June 2021
doi: 10.3389/fonc.2021.654452

Lactate Dehydrogenase-A (LDH-A) Preserves Cancer Stemness and Recruitment of Tumor-Associated Macrophages to Promote Breast Cancer Progression

Shengnan Wang[1,2], Lingyu Ma[1,2], Ziyuan Wang[1,2], Huiwen He[1,2], Huilin Chen[1,2], Zhaojun Duan[1,2], Yuyang Li[1,2], Qin Si[1,2], Tsung-Hsien Chuang[3], Chong Chen[1,2†] and Yunping Luo[1,2*†]*

[1] Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, China, [2] Collaborative Innovation Center for Biotherapy, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, China, [3] Immunology Research Center, National Health Research Institutes, Zhunan, Taiwan

Increasing evidence reveals that breast cancer stem cells (BCSCs) subtypes with distinct properties are regulated by their abnormal metabolic changes; however, the specific molecular mechanism and its relationship with tumor microenvironment (TME) are not clear. In this study, we explored the mechanism of lactate dehydrogenase A (LDHA), a crucial glycolytic enzyme, in maintaining cancer stemness and BCSCs plasticity, and promoting the interaction of BCSCs with tumor associated macrophages (TAMs). Firstly, the expression of LDHA in breast cancer tissues was much higher than that in adjacent tissues and correlated with the clinical progression and prognosis of breast cancer patients based on The Cancer Genome Atlas (TCGA) data set. Moreover, the orthotopic tumor growth and pulmonary metastasis were remarkable inhibited in mice inoculated with 4T1-shLdha cells. Secondly, the properties of cancer stemness were significantly suppressed in MDA-MB-231-shLDHA or A549-shLDHA cancer cells, including the decrease of $ALDH^+$ cells proportion, the repression of sphere formation and cellular migration, and the reduction of stemness genes (SOX2, OCT4, and NANOG) expression. However, the proportion of $ALDH^+$ cells (epithelial-like BCSCs, E-BCSCs) was increased and the proportion of $CD44^+$ $CD24^-$ cells (mesenchyme-like BCSCs, M-BCSCs) was decreased after LDHA silencing, suggesting a regulatory role of LDHA in E-BCSCs/M-BCSCs transformation in mouse breast cancer cells. Thirdly, the expression of epithelial marker E-cadherin, proved to interact with LDHA, was obviously increased in LDHA-silencing cancer cells. The recruitment of TAMs and the secretion of CCL2 were dramatically reduced after LDHA was knocked down *in vitro* and *in vivo*. Taken together,

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Wake Forest Baptist Medical Center, United States
Jiabao He,
University of Aberdeen, United Kingdom

***Correspondence:**
Chong Chen
chenchong86@ibms.pumc.edu.cn
Yunping Luo
ypluo@ibms.pumc.edu.cn

†These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *16 January 2021*
Accepted: *11 May 2021*
Published: *10 June 2021*

Citation:
Wang S, Ma L, Wang Z, He H, Chen H, Duan Z, Li Y, Si Q, Chuang T-H, Chen C and Luo Y (2021) Lactate Dehydrogenase-A (LDH-A) Preserves Cancer Stemness and Recruitment of Tumor-Associated Macrophages to Promote Breast Cancer Progression. Front. Oncol. 11:654452. doi: 10.3389/fonc.2021.654452

LDHA mediates a vicious cycle of mutual promotion between BCSCs plasticity and TAMs infiltration, which may provide an effective treatment strategy by targeting LDHA for breast cancer patients.

Keywords: metabolism, cancer stem cells, tumor-associated macrophages, lactate dehydrogenase A, E-cadherin, CCL2

INTRODUCTION

Breast cancer stem cells (BCSCs), a small population of tumor cells with self-renewal ability and differentiation potential, have been proposed as a driving force in breast cancer initiation and dissemination (1, 2). Substantial evidence indicates that BCSCs are not homogeneous but compose of stem cells with different phenotypes and functions, which is known as BCSCs heterogeneity (3, 4). At present, BCSCs are mainly divided into two subtypes: one is mesenchymal, quiescent type marked by $CD44^+/CD24^-$ (M-BCSCs); the other is epithelial, proliferative type marked by $ALDH^+$ (E-BCSCs) (5). During tumor progression, BCSCs convert between quiescent mesenchymal-like and proliferative epithelial-like states to acquire specific phenotypes stimulated by their niche cells, ultimately cause tumor relapse and metastasis. Therefore, intensive study on BCSCs heterogeneity may provide a more precise strategy to targeting BCSCs for breast cancer therapy.

Cancer cells exhibit high glycolysis even in the presence of sufficient oxygen, which is known as Warburg effect (6). This abnormal metabolism promotes proliferation and survival of tumor cells with elevated glucose uptake and lactate production, and agents targeting glycolysis may offer a therapeutic opportunity in clinical practice (7–9). Interestingly, recent work has shown that distinct BCSCs states (M- and E-BCSCs) response markedly different to the antiglycolytic treatment (10). Lactate dehydrogenase A (LDHA), a class of 2-hydroxy acid oxidoreductase, mediates the conversion of lactate from pyruvate, NADH from NAD^+ in the last step of glycolysis (11). Intensive studies documenting that elevated LDHA has been associated with the progression of aggressive cancers in a variety of tumor types and different responses to LDHA targeted therapy are shown in clinical settings (12–14). Although LDHA is indicated to have an essential role in survival and proliferation of tumor cells, relatively little is known about the mechanisms of it on regulating stemness and BCSCs heterogeneity.

Tumor-associated macrophages (TAMs) account for 30% to 50% of all infiltrating inflammatory cells in the tumor microenvironment of breast cancer, which have significant impact on the tumor occurrence, development, and metastasis (15–18). Stemness-related properties of BCSCs, for example self-renewal, drug-resistance and metastasis initiation are intensively supported by their suitable niche and dynamic interplay between BCSCs and TAMs is one of the most important manner (19). However, the potential molecular link between BCSCs metabolism and tumor immune evasion is not well established in breast cancer. In the present study, using orthotopic syngeneic immunocompetent mouse model, we investigate the fundamental role of LDHA, a metabolic enzyme, in BCSCs maintenance and TAMs recruitment. We demonstrate a fundamental role of LDHA in negative regulation of epithelial marker E-cadherin during E/M-BCSCs transition, and increased tumoral infiltration of CCL2-responding TAMs.

MATERIALS AND METHODS

Cell Lines and Reagents

MDA-MB-231, 293T, 4T1, and RAW264.7 were obtained from ATCC. All cell lines were cultured according to guidelines from ATCC. The medium was supplemented with 10% FBS (ThermoFisher Scientific, Cat. No. 10099-141) and 100 U/ml penicillin-streptomycin (ThermoFisher Scientific, Cat. No. 15140122). Bone marrow–derived macrophage (BMDM) was extracted from femur of 6- to 8-week-old female Balb/c mice and cultured with RPMI 1640 (Biological Industries, Cat. No. 01-100-1A, containing 30 ng/ml M-CSF (Peprotech, 315-02) for 6 days.

Animal Experiments

The animal study was reviewed and approved by the Ethics Review Committee of Peking Union Medical College. Six- to 8-week-old female Balb/c mice were provided from the Institute of Basic Medical Science of Peking Union Medical College. 10^5 4T1 cells resuspended in 100-μl PBS were injected into the fourth mammary fat pad of each mouse and treated with oxamate or vehicle after tumor formation. The tumor size was measured and recorded on the 7th day after injection. On day 21 after injection, tumor, lung, and spleen in each mouse were harvested for tested by flow cytometry, hematoxylin, and eosin (H&E) staining and immunohistochemical (IHC) analyses.

Western Blotting

The cells were lysed with protein extraction reagent (ThermoFisher Scientific, USA) and total protein was harvested after centrifuging at 14,000*g* for 10 min. The concentration of protein was measured by NanoDrop (ThermoFisher Scientific, USA). After being separated in Bis-Tris Gels (180-8008H, Tanon), total protein was transferred to 0.22-μm PVDF membranes (1620177, Bio-Rad), which were then incubated in 5% bovine serum albumin (BSA) for 1 h. Primary antibodies against LDHA (2012, CST), OCT4 (2840, CST) were used to incubate the membranes at 4°C overnight. After washing, the membranes were incubated with secondary antibodies (goat anti-rabbit IgG-HRP; 40295, Bioss) for 1 h and then detected using high-sig ECL Western Blotting

Substrate (180-5001, Tanon). The bandings were quantified by ImageJ software.

RNA Experiments

Total RNA was extracted by using Trizol reagent (ThermoFisher Scientific, USA) and 1 μg of total RNA was used for the synthesis of first-strand cDNA with HiScript II Q RT SuperMix for qPCR (+gDNA wiper) reagent (R223-01, Vazyme). Real-time PCR was performed with ChamQ SYBR qPCR Master Mix Reagent (Q311-02, Vazyme) and detected by CFX96 Real-Time PCR Detection System (Bio-Rad).

Generation of Stable Cancer Cell Lines

Mouse shNC and shLdha PLKO.1 plasmid were designed and produced by Beijing Yimeiang Biotechnology Co. Ltd (sequence shLdha 1, 5′-CCGGGUUCCCAGUUAAGUCGUAUAACUCG AGUUAUACGACUUAACUGGGAACUUUUUG-3′; sequence shLdha 2, 5′-CCGGCGUCUCCCUGAAGUCUCUUAACUCG AGUUAAGAGACUUCAGGGAGACGUUUUUG-3′; sequence shLdha 3, 5′-CCGGCGUGAACAUCUUCAAGUUCAUCUCG AGAUGAACUUGAAGAUGUUCACGUUUUUG-3′). The plasmids were amplified by transformed into E. coli and then extracted with TIANprep Mini Plasmid Kit (DP106, TIANGEN). Objective plasmids and packaging plasmids were co-transfected into 293T cells by Lipofectamine™ 2000 Transfection Reagent (ThermoFisher Scientific, USA) and lentiviruses produced by 293T cells were collected. After infection with lentiviruses and 1-week puromycin treatment, stable cancer cell lines were generated successfully.

Cell Migration Assay

2×10^6 cells/ml tumor cells resuspended in 100 μl RPMI1640 (containing 1% FBS) were seeded into 8-μm pore polycarbonate filters, and complete medium (containing 10% FBS) was added into the bottom well. After 16 h culture, the non-migratory cells on the top of the filters were removed by cotton swab, and the migratory cells on the bottom of filters were dyed with 0.1% crystal violet for 10 min. The crystal violet could be eluted by 33% acetic acid, and the absorbance value of eluent was read out by Elisa (Biotec) at the wavelength of 570 nm.

Flow Cytometry

The $ALDH^+$ cells were detected by using ALDEFLUOR assay kit (01700, Stem Cell). 5×10^5 cells were suspended in ALDH assay buffer containing substrate and then incubated at 37°C for 40 min with or without diethylaminobenzaldehyde (DEAB) reagent. The $CD44^+CD24^-$ CSCs were detected by using CD44-PE and CD24-APC antibodies incubated with tumor cells for 30 min at 4°C from light. Macrophages in tumor tissues were marked by $CD45^+F4/80^+CD11b^+$ after single cell preparation. Suspensions of single cells were detected by flow cytometer (Millipore, Guava 5HT), and the data were analyzed by FlowJo software.

Tumorsphere Formation Assay

Tumor cells were suspended in Mammocult medium (Stem Cell, Canada) at appropriate concentration and added into ultra-low plates (Corning, USA). The tumorspheres (sphere diameter ≧70 μm) were counted after 5 to 7 days of culture.

ELISA

Culture supernatants of tumor cells was collected after centrifugation, and the concentration of CCL2 was measured by Mouse Uncoated ELISA Kit (88-7391-88, ThermoFisher Scientific, USA) according to providing protocol.

Bioinformatics Analysis

From the GDC of TCGA, the clinical information and LDHA sequencing data of breast cancer patients were downloaded and sorted into a matrix. Patients were divided into tumor and paratumor groups according to source of tissue and compared in pair. Furthermore, patients were divided into three groups according to the expression of LDHA and then classified into four groups characterized by cancer clinical stage. In addition, patients were divided into negative and positive groups according to the distant metastasis information. With the statistical analysis, the correlation between LDHA expression and clinical stage or metastasis were obtained.

Statistical Analysis

All results were presented as mean ± SD. Correlation between LDHA and clinical stage and metastasis of breast cancer in TCGA database was analyzed by one-way ANOVA test. Other results were analyzed with the Student's t-test (unpaired). The data normality was established before the application of the statistical analysis. $P < 0.05$ was used as the criterion for statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

LDHA Positively Correlated With Clinical Stages and Cancer Metastasis, and Might Be Involved in the Regulation of Tumor Immune Microenvironment

To investigate the role of LDHA in breast cancer progression, we analyzed the correlation of LDHA expression with the tumor clinical stages, metastasis, and survival according to TCGA database. The results showed that LDHA expression was markedly increased in patients at tumor advanced-stage or in metastatic tumors (**Figure 1A**), and high expression of LDHA was significantly associated with short overall survival of breast cancer patients (**Figure 1B**, $P < 0.0001$). In addition, with TCGA data set, we analyzed the relationship between LDHA expression and macrophages/$CD8^+$ T lymphocytes infiltration in tumor microenvironment for the first time. It was meaningful to find that LDHA expression was positively correlated with macrophages infiltration but negatively related with that in $CD8^+$ T lymphocytes in breast cancer (**Figure 1C**). Abovementioned results suggested that LDHA played an important role in the development of human breast cancer, as well as the immunoregulation of tumor microenvironment.

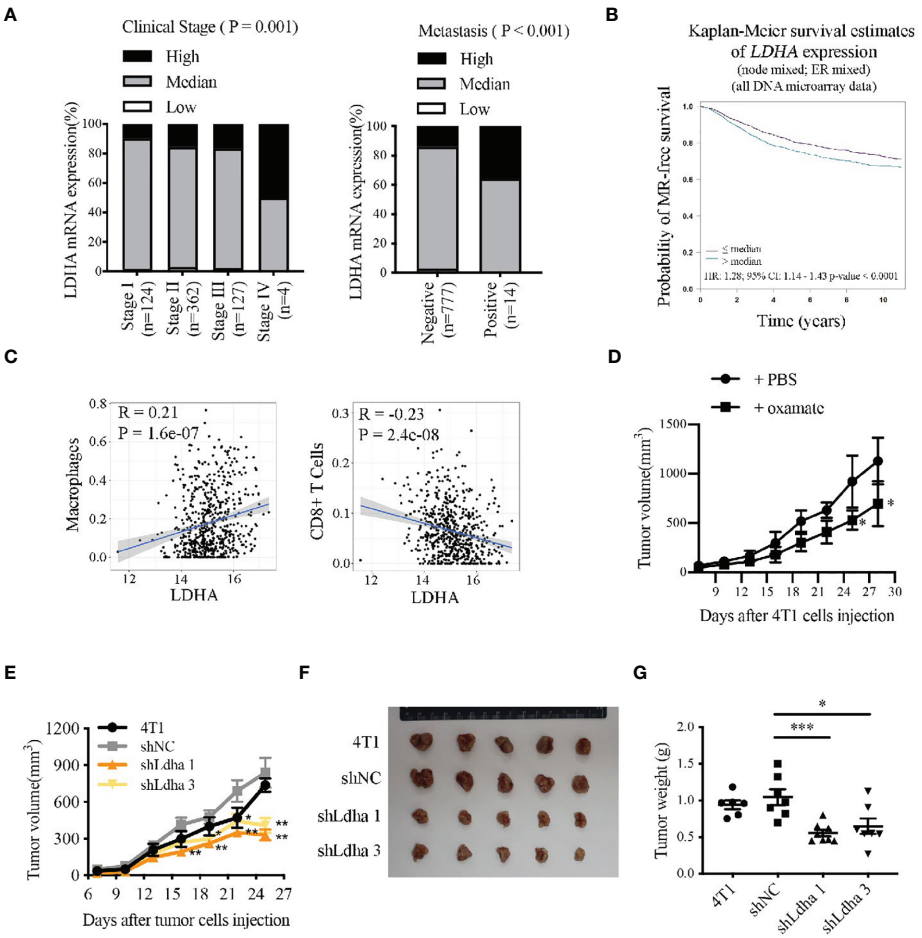

FIGURE 1 | LDHA predicted progression and prognosis of breast cancer patients, correlated with immunocytes infiltration in TME and promoted 4T1 tumor growth in mouse. **(A)** Proportion of patients depend on the LDHA expression in each clinical stage. Breast cancer samples were classified as high, medium, or low LDHA expression according to log2 (FPKM of LDHA). **(B)** Kaplan-Meier analysis of the overall survival of breast cancer patients with LDHA high or low expression. The analysis was performed by Breast Cancer Gene-Expression Miner v4.4 in Integrated Center for Oncology. **(C)** Bioinformatics analysis of the correlation between LDHA expression and infiltration of macrophages or CD8 T cells in breast cancer based on TCGA data set. **(D)** Tumor growth curve of mice inoculated with 4T1 cells treated with PBS (n = 4) or oxamate (n = 5). **(E)** Tumor growth curve of mice inoculated with 4T1 wild type, shNC or shLdha cells (n = 8 in each group). 1×10^5 cells were injected per mice. **(F)** Tumors were harvested on 25th day after tumor cells inoculation and tumor picture was shown (n = 5). **(G)** Tumors weight were measured following tumor harvest (n = 8 in each group initially). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

LDHA Was Essential for the Growth of Murine Breast Cancer in an Orthotopic Syngeneic Immunocompetent Mouse Model

Oxamate, a specific LDHA inhibitor, which could suppress LDHA activity (**Supplementary Figure 1A**) was used to verify the effect of LDHA on tumor generation and progression in mice. For the treatment strategy, oxamate, dissolved in PBS, was intraperitoneally injected into the Balb/c mice xenografted with 4T1 tumor every day for 3 weeks (**Supplementary Figure 1B**). During the treatment, tumor size was measured every 3 days and as expected, tumor growth was obviously restricted in mice treated with oxamate (**Figure 1D**). It is important to note that by using two stable 4T1 cell lines with Ldha knock-down (4T1-shLdha1 and 4T1-shLdha3), we found that tumor grew significantly more slowly in both 4T1-shLdha groups compared with the control mice after tumor cells inoculated in the 4th breast fat pad of Balb/c mice, and tumor weight at 25th day was much lower as well (**Figures 1E–G**). Taken together, growth of 4T1 tumors was significantly suppressed in mice by using LDHA-specific inhibitor or shRNA, which indicated an important role of LDHA in mouse breast cancer progression.

LDHA Maintained Stemness-Related Properties of Breast Cancer Stem Cells

In consideration of elevated glycolysis in BCSCs from our previous study (20), and the important role of LDHA in maintaining glycolysis (**Figure 2A**), we presumed that LDHA was critical for keeping functionality of BCSCs. To demonstrate the role of LDHA in stemness maintenance, the expression of LDHA was detected in

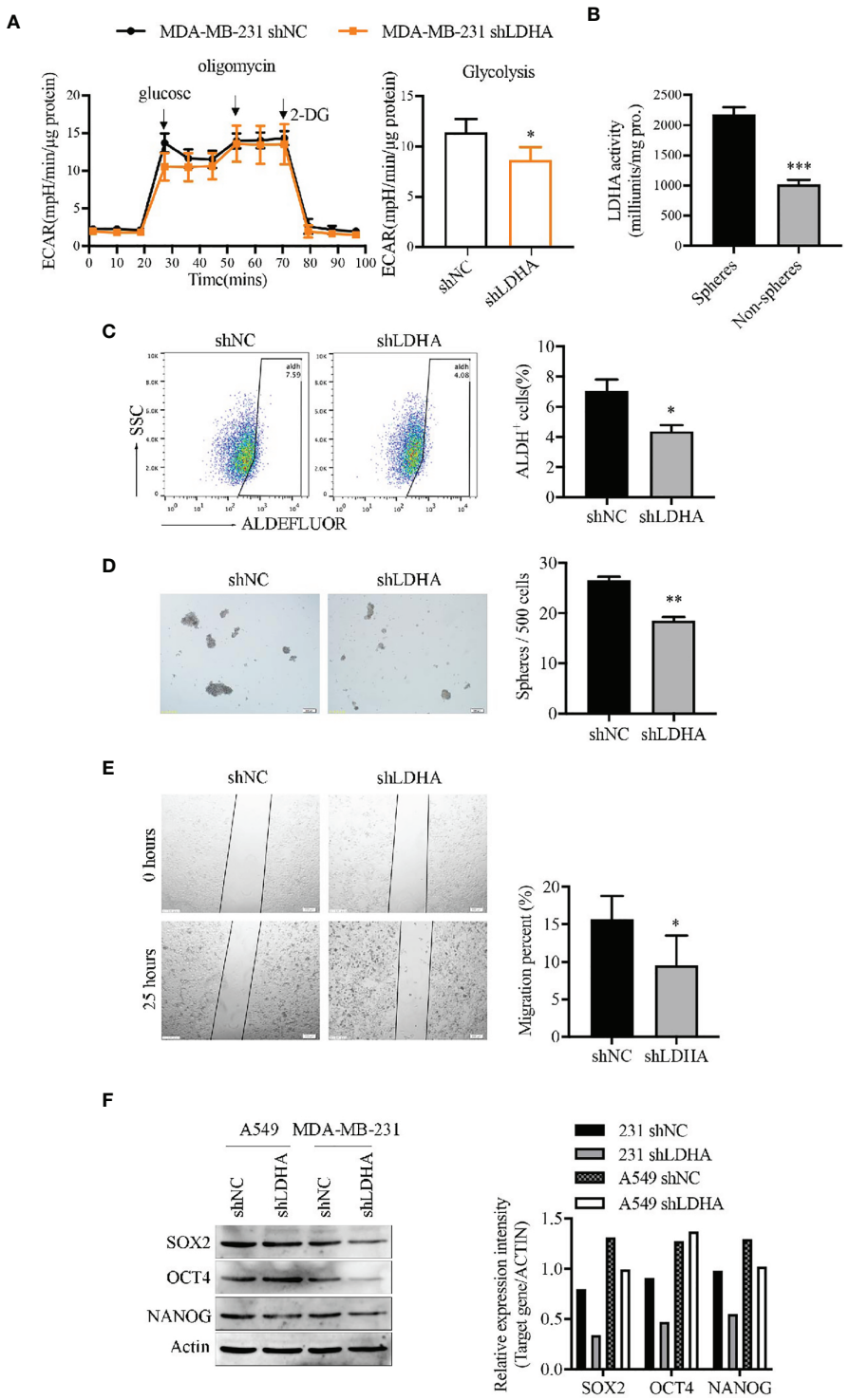

FIGURE 2 | LDHA maintained stemness properties of human breast cancer cells. **(A)** Extracellular acidification rate (ECAR) of MDA-MB-231-shLDHA or control cells was detected by Seahorse Cellular Bioenergetics Analyzer. **(B)** LDH activity was detected in spheres or non-spheres cancer cells of MDA-MB-231 by colorimetry. **(C)** Percentage of $ALDH^+$ cells in MDA-MB-231-shLDHA or control cells was analyzed by flow cytometry. **(D)** MDA-MB-231-shLDHA or control cells was cultured in sphere formation medium for 5-7 days, and the numbers of tumor-spheres were counted under a microscope. The representative images of spheres were shown on the left. Scale bar, 200 μm (insets). **(E)** Ability of cellular migration was measured by wound healing assay in MDA-MB-231 shNC and shLDHA cells. **(F)** Western blot analysis for SOX2, OCT4 and NANOG in A549-shLDHA, MDA-MB-231-shLDHA and their control cells. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

different breast cancer cell lines, and results have shown cells with higher degree of malignancy ($HER2^+$ or basal–like type) have relative higher LDHA level (**Supplementary Figures 2A, B**). Tumor spheres of MDA-MB-231 cells cultured *in vitro* was enriched of CSCs population that was determined to have higher LDH activity compared with their adherent cells (**Figure 2B**). Next, stable cancer cell lines of MDA-MB-231-shLDHA, A549-shLDHA, and 4T1-shLdha were established, and both protein level and enzyme activity of LDHA/Ldha were significantly attenuated in LDHA knockdown cells (**Supplementary Figure 3**). Thereafter, the tumor spheres' number, $ALDH^+$ cell proportion, stemness genes (SOX2, OCT4, and NANOG), expression, and migratory cells were all reduced in MDA-MB-231-shLDHA cells (**Figures 2C–F**). Similarly, the expression of OCT4 also declined in 4T1-shLdha cells (**Supplementary Figure 4**). Both cellular proliferation and movement were suppressed but cellular apoptosis was rarely changed in 4T1-shLdha cancer cells (**Supplementary Figures 5A–C**). Taken together, our results highlighted a crucial role of LDHA in cancer stemness maintenance.

LDHA Promoted the Transformation of M-BCSCs From E-BCSCs in 4T1 Cells

$ALDH^+$ cells represented a population of proliferative epithelial-BCSCs (E-BCSCs) and $CD44^+CD24^-$ cells enriched the metastatic mesenchymal-BCSCs (M-BCSCs) (5). It is interesting that the proportion of $ALDH^+$ cells appeared to be increased and $CD44^+CD24^-$ population was reduced while LDHA was downregulated in 4T1 cells (**Figures 3A, B**), which suggested a regulatory effect of LDHA in the conversion between E-BCSCs and M-BCSCs. Furthermore, the co-expression analysis showed that LDHA was negatively correlated with the expression of ALDH1A1 but positively correlated with the expression of CD44 in human breast cancer samples based on the TCGA data set (**Figure 3C**). In addition, the formation of tumor spheres (enriched for proliferative BCSCs) was enhanced, but the migratory cells (enriched for metastatic BCSCs) were markedly reduced after silencing of LDHA in 4T1 cells (**Figures 3D, E**). It is important to note that pulmonary metastasis was also strongly suppressed in mice inoculated with 4T1-shLdha cells (**Figure 3F**). It was suggested that LDHA could preserve the proportion and properties of M-BCSCs; therefore, it was supposed to be a gatekeeper in BCSCs' plasticity.

Epithelial Marker E-Cadherin Was Negatively Regulated by LDHA

To investigate the mechanism of LDHA regulating BCSCs heterogeneity, we performed a bioinformatics analysis by using GSE115302 and GSE59281 data sets. CDH1 encoding E-Cadherin was an epithelial marker, and CDH2/VIM encoding N-Cadherin/vimentin, respectively, represented the mesenchymal markers. As expected, CDH1 was highly expressed in E-BCSCs and CDH2/VIM were highly expressed in M-BCSCs (**Figure 4A**), which suggested that the EMT markers could be used to distinguish those two BCSCs subgroups. It was noteworthy that the migratory cells of MCF-7 treated with oxamate were reduced, and E-Cadherin level was increased correspondingly (**Figures 4C, D**). The expression of E-Cadherin which was overexpressed in 4T1 and MCF-7 cells (**Figure 4B**) was up-regulated upon LDHA knocked down (**Figure 4E**). Immunoprecipitation assay revealed that endogenous LDHA could interact with E-cadherin in MCF7 cells (**Figure 4F**). These results suggested that E-cadherin was negatively regulated by LDHA in M-BCSCs, which perhaps contributed the E/M-BCSCs transformation.

LDHA Promoted the Tumoral Infiltration of TAMs in a 4T1 Murine Breast Cancer Model

Previous studies demonstrated that tumor immune microenvironment, especially tumor-associated macrophages (TAMs) served as the key niche cell for CSCs maintenance (21–23). Using the 4T1 orthotopic breast tumor model in mice, besides a remarkable retardation of tumor growth (**Figure 5A**), the infiltration of macrophages, marked by $CD45^+CD11b^+F4/80^+$, was significantly suppressed in 4T1-shLdha tumors (**Figure 5B**). Furthermore, the antineoplastic immune cells, including $CD8^+$ or $CD4^+$ T cells was highly increased, nevertheless the bone marrow-derived immun osuppressive cells (MDSCs) decreased sharply in 4T1-shLdha tumors (**Figure 5C**). It was also verified with immuno histochemistry that the numbers of $CD8^+$ T cells was increased and $CD206^+$ M2 macrophages was decreased infiltrated in 4T1-shLdha tumors (**Figure 5D**). Next, we explored the migratory ability and immunologic phenotype of macrophages cultured with diverse 4T1 conditional medium *in vitro*. It was notable that migratory RAW264.7 cells were dramatically decreased and the expression of M2 related genes (Arg1, CD206, IL-10, and Ccr2) in RAW264.7 or bone marrow derived macrophages (BMDMs) were markedly reduced when cultured with the conditional medium from 4T1-shLdha cells (**Figures 6A, B** and **Supplementary Figure 6**). Ccr2-expressing monocytes were effectively attracted into tumor sites by Ccl2 (24), and our results revealed that Ccl2 secreted from 4T1-shLdha cells notably decreased compared to the control cells detected by ELISA (**Figure 6C**). Intriguingly, bioinformatic analysis revealed that CCL2 and LDHA were both strongly increased in M-BCSCs population ($CD44^+$ cancer cells) compared with the bulk cancer cells according to the GSE115302 data set (**Figure 6D**). Moreover, the expression of Ccl2 was significantly increased in sorted E-BCSCs ($ALDH^+$ cancer cells) compared with the unsorted bulk 4T1 cells (**Figure 6E**). Taken together, these results suggested that tumoral infiltration of TAMs in breast cancer was intensively facilitated by Ldha/Ccl2 signaling pathway in BCSCs.

DISCUSSION

BCSCs are considered as key drivers of tumor growth, relapse, and metastasis, of which targeting the specific metabolism may

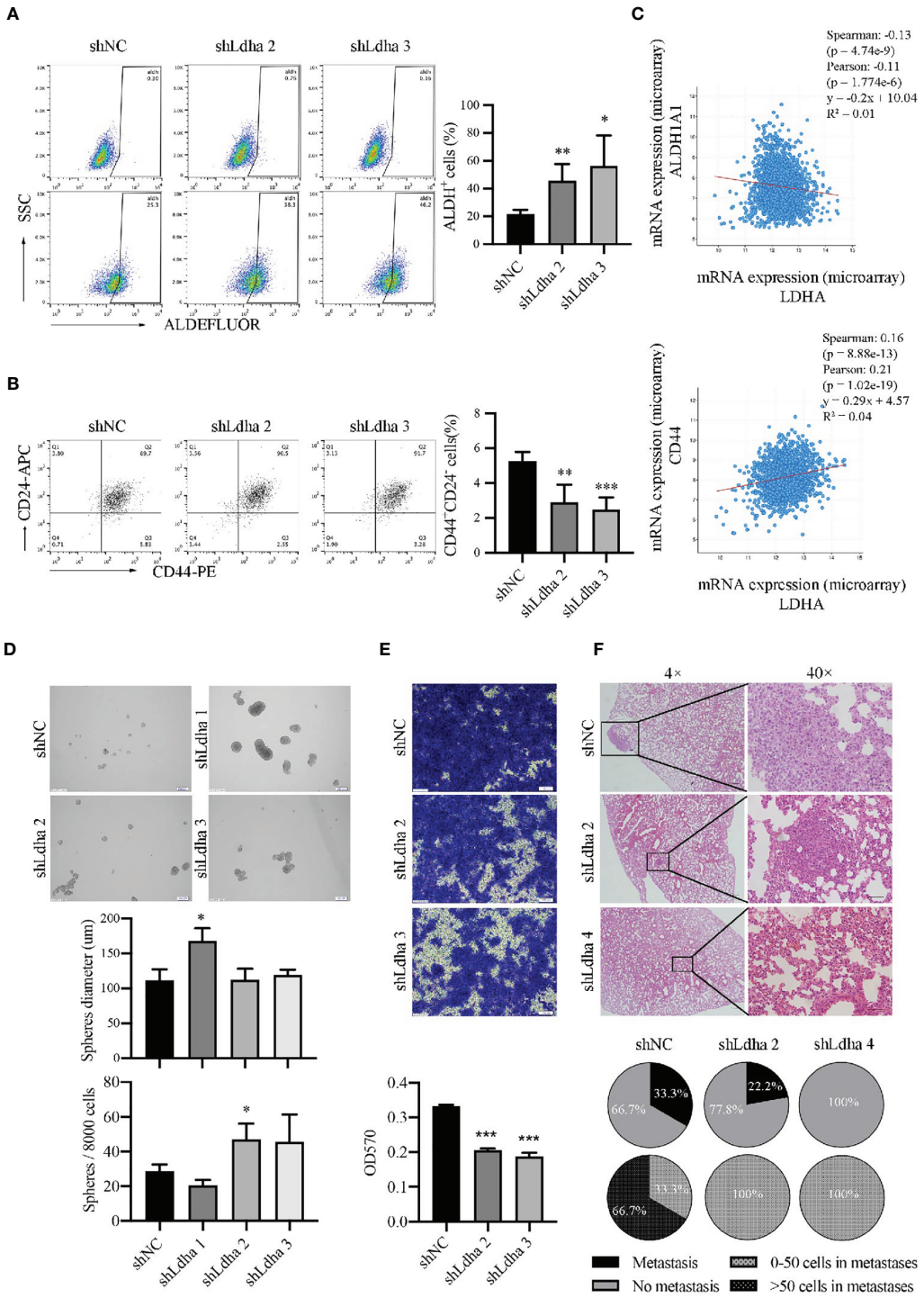

FIGURE 3 | LDHA promoted E to M transition of BCSCs in mouse 4T1 cancer cells. **(A)** Percentage of $ALDH^+$ cells in 4T1- shNC or 4T1-shLdha cells was analyzed by flow cytometry. **(B)** Percentage of $CD44^+CD24^-$ cells in 4T1- shNC or 4T1-shLdha cells was analyzed by flow cytometry. **(C)** Co-expression analysis of LDHA, ALDH1A1 and CD44 by using cBioportal for cancer genomics. **(D)** 4T1-shLDHA or control cells was cultured in sphere formation medium for 5-7 days, and the diameters and numbers of tumor-spheres were measured under a microscope. Scale bar, 200 μm (insets). **(E)** Cellular migration of 4T1-shNC or shLdha cells was measured by Transwell assay. Crystal violet was eluted by 33% acetic acid and determined at 570nm. **(F)** 4T1 shNC or shLdha cells was injected into 4^{th} breast fat pad of mice and lung was harvested on day 19 after injection. Tumor metastasis was analyzed by hematoxylin-eosin staining. Scale bar, 50 μm (insets). Number of mice with lung metastasis was 3/9(4T1 shNC), 2/9(shLdha 2) and 0/7(shLdha 4). The number of mice with more than 50 tumor cells in metastatic foci was 2/3(4T1 shNC), 0/2(shLdha 2) and 0/0(shLdha 4). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

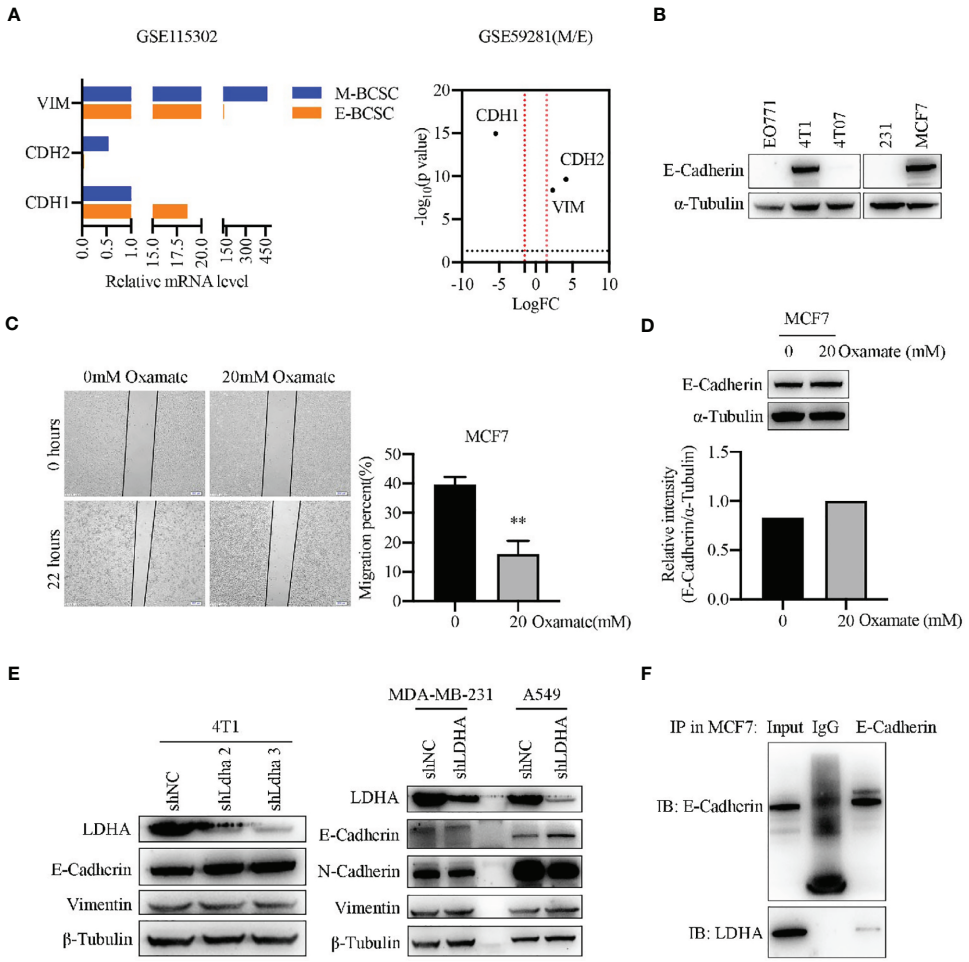

FIGURE 4 | Epithelial marker E-cadherin was negatively regulated by LDHA. **(A)** Transcripts of CDH1, CDH2, and VIM were analyzed in E-BCSCs or M-BCSCs population based on GSE115302 and GSE59281 data sets. The horizontal dotted line represents $P = 0.05$, the vertical dotted line represents logFC = 2/−2. **(B)** Western blot analysis for E-Cadherin in EO771, 4T1, 4T07, MDA-MB-231 and MCF7 cells. **(C)** Cellular migration was measured by wound healing assay in MCF7 cells treated with 0 or 20 mM oxamate for 30 h. Scale bar, 200 μm (insets). **(D)** Western blot analysis for E-Cadherin in MCF7 cells treated with 0 or 20 mM oxamate for 30 h. **(E)** Western blot analysis for LDHA, E-Cadherin, N-Cadherin and Vimentin in A549-shLDHA or MDA-MB-231-shLDHA cancer cells. **(F)** Immunoprecipitation analysis of the interaction between LDHA and E-Cadherin in MCF7 cells. $^{**}P < 0.01$.

help to eradicate this small subset of cells. The main metabolic pathway of cancer cell is aerobic glycolysis rather than oxidative phosphorylation, which is called "Warburg effect" and has been verified in many types of tumor (12, 13, 25–27). Our previous study revealed a higher glycolytic metabolism in BCSCs (20), but the mechanism of glycolysis maintaining stemness was not much clear. LDHA, a rate-limiting enzyme in glycolytic process, promotes tumor growth through variety of ways, which is verified to play an important role in stemness maintenance of BCSCs in this study. This result is in agreement with a recent report documenting that targeting LDHA inhibits lung tumor-initiating cells by using an inducible murine model (13). Recent evidence implies BCSCs are not a simplex cell population but mainly consist of two subgroups, which are termed as E-BCSCs and M-BCSCs. E-BCSCs are epithelioid, proliferative cancer stem cells, highly expressing epithelial-like marker E-Cadherin, while M-BCSCs are quiescent, metastatic cancer stem cells, highly expressing mesenchymal-like marker N-Cadherin. A combinatory approach targeting both M- and E-BCSCs illustrates a novel treatment approach targeting both BCSC states (28). For the first time, our results reveal that LDHA play a critical role in BCSCs heterogeneity, which indicated a special effect on E/M-BCSCs transition in 4T1 cells.

The transition of BCSCs from the E to the M state closely resembles the epithelial-to-mesenchymal transition (EMT), which plays an important role in the metastatic process of tumor cells (29). Our results also demonstrate a suppressive effect of LDHA on the epithelia marker E-Cadherin, which seemingly explains its essential function in E/M-BCSCs transition. Departure from cell membrane of E-Cadherin provoked by ubiquitylate degradation is an important mechanism for tumor cells to undergo EMT and enhance their

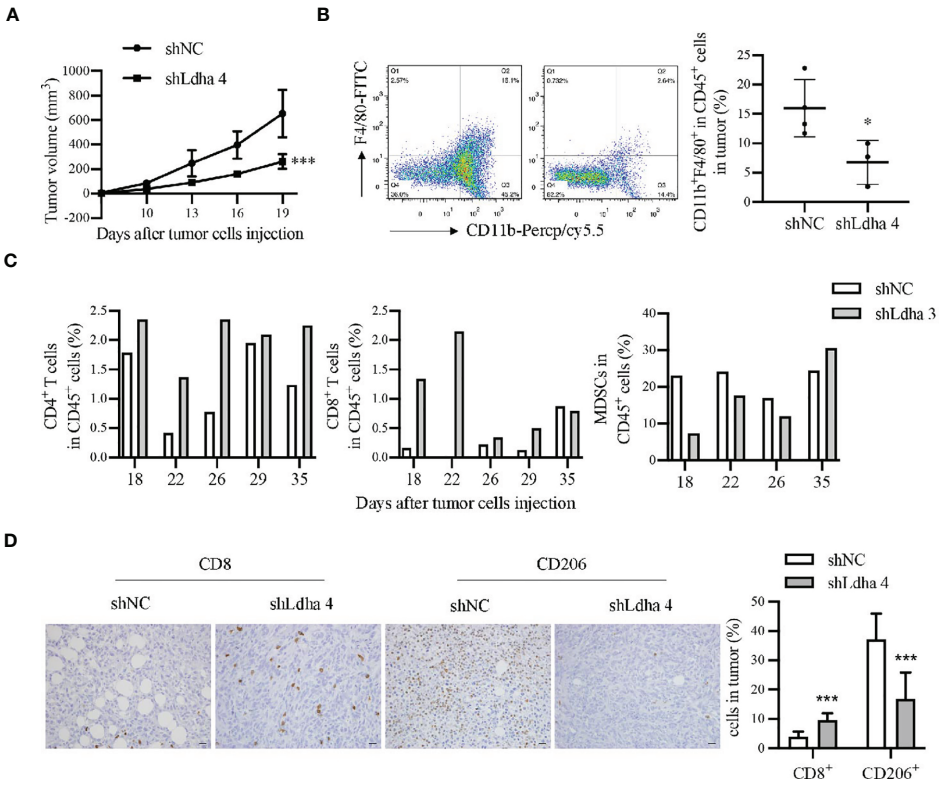

FIGURE 5 | LDHA promoted tumoral infiltration of TAMs in 4T1 murine breast cancer model. **(A)** Tumor growth curve of mice inoculated with 4T1-shNC or 4T1-shLdha cells. 1×10^5 cells per mice were injected into the fourth breast fat pad of Balb/c mice (n = 9 in 4T1-shNC group, n = 7 in 4T1 shLdha group). **(B)** Percentage of $CD11b^+F4/80^+$ macrophages in $CD45^+$ immune cells were analyzed by flow cytometry. Tumor were harvested on day 19 after the tumor cells injection. CD45-PE, CD11b-Percp/cy5.5 and F4/80-FITC were used to mark macrophages in tumor. **(C)** Percentage of $CD3^+CD8^+$ T cells, $CD3^+CD4^+$ T cells or $CD11b^+Gr1^+$ MDSCs in $CD45^+$ immune cells were analyzed by flow cytometry. Tumor were harvested on different days after the tumor cells injection (n = 1 in each group). CD45-PE, CD3ϵ-FITC, CD4-APC and CD8α-APC were used to mark T cells in tumor. CD11b-FITC and Gr1-APC were used to mark MDSCs in tumor. **(D)** The expression of CD8 and CD206 was analyzed by immunohistochemistry in 4T1 shNC or shLdha-injected tumors (n = 9 in 4T1-shNC group, n = 7 in 4T1 shLdha group). Scale bar, 20 μm (insets). $^*P < 0.05$; $^{***}P < 0.001$.

metastatic ability (30, 31). In our data, ubiquitin of E-Cadherin was also detected in LDHA pull-down sample (data not shown). Therefore, we speculate that LDHA might promote the ubiquitination and endocytosis of E-Cadherin to facilitate the transformation of CSCs from E-BCSCs to M-BCSCs ultimately and it is also suggested that solely suppression of LDHA may not be sufficient to eliminate BCSCs containing different subpopulations. Fully investigation of metabolic heterogeneity in diverse subsets of BCSCs appears urgent and therapeutic strategies by targeting BCSCs' diverse metabolism seems to have great prospect for breast cancer treatment.

The equilibrium of diverse BCSCs subgroups is regulated by the TME *via* multifaceted mechanisms including cytokine or chemokine signaling (5, 32, 33). Macrophages constitute up to 50% of the tumor infiltrating cells in human breast cancer and thus represent most non-neoplastic cells in the tumors. Our previous studies revealed that tumor associated macrophages promoted the stemness phenotypes of BCSCs by activating key inflammatory pathways (34, 35). In turn, this study demonstrated that LDHA, a cancer stemness promoter, could impede the infiltration of anti-tumor immune cells ($CD4^+$/$CD8^+$ T cells) and enhance the accumulation of the pro-tumoral immune cells (MDSCs and TAMs) in TME. Previous study revealed that the polarization of M2 phenotype was promoted by lactate in TAMs (36). Recent study revealed that lactate concentration quantified by the double quantum filtered (DQF) MRS, a non-invasive imaging method, is a sensitive marker for the prediction of tumor grades and prognosis in breast cancer. However, there has been study showing that silencing LDHA failed to alter lactic acid production in breast cancer cell line (37). Except for lactic acid, carbonic anhydrase 9 (CA-9), maintaining the acidic condition of TME, also contributed to the tumor progression (38). Interestingly, the lipid composition in TME is also an important factor for macrophages recruitment and functional remolding (39). In our study, by using the co-culturing system *in vitro*, we find that the recruitment of macrophages is strongly enhanced by LDHA. Mechanistically, this occurs at least in part through elevated secretion of macrophages-attracted chemokine CCL2 induced by LDHA. Recruitment

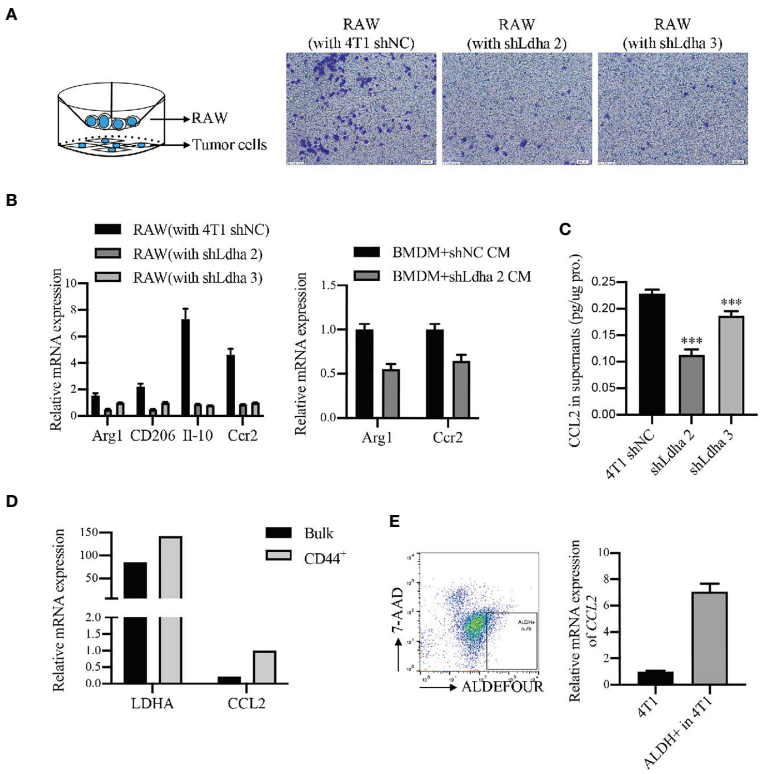

FIGURE 6 | LDHA enhanced the recruitment and M2 phenotypes of macrophages *in vitro*. **(A)** Migratory capacity of RAW264.7 cells was analyzed by Transwell assay. RAW264.7 cells were seeded in 8-μm-pore polycarbonate filters and 4T1-shNC or 4T1-shLdha cells were seeded in the bottom well. Representative images were visualized by microscope (right) and percentage of migrating cells was calculated by use of ImageJ. Scale bar, 200 μm (insets). **(B)** Relative mRNA level of M2 markers in RAW 264.7 or BMDM cultured with conditional medium from 4T1-shNC or 4T1-shLdha cancer cells were detected by RT-qPCR. **(C)** Ccl2 level in the cultural supernatant from 4T1-shNC or 4T1-shLdha cells was detected by ELISA. Results were normalized by protein content in cells. **(D)** Relative mRNA level of LDHA and CCL2 in M-BCSCs or bulk cells were analyzed according to GSE115302 data set. **(E)** The mRNA level of Ccl2 was measured by RT-qPCR in ALDH$^+$ or bulk 4T1 cells. ALDH$^+$7-AAD$^-$ cells were sorted out by flow cytometry. Error bars mean ± SD from three independent experiments. ***$P < 0.001$.

of inflammatory monocytes/macrophages responding to CCL2 is critical for tumor cell pulmonary seeding (40).These findings revealed the different mechanisms of metabolic enzyme in regulating the TME and might provide novel targets for cancer therapy. Interestingly, CCL2 expression is dramatically increased in BCSCs based on TCGA data set and we propose a hypothesis that secretion of CCL2 from BCSCs is enhanced by LDHA, which could recruit more CCR2-positive tumor associated macrophages (TAMs) to further maintained the stemness of BCSCs. Moreover, E-Cadherin, the molecular marker of E-BCSCs, was suppressed by LDHA that partially explain the phenomenon of LDHA mediated E to M transformation in BCSCs. This hypothesis needs to be further fully investigated and targeting LDHA combined with CCL2/CCR2 antagonists may provide a better therapeutic outcome for breast cancer.

In conclusion, our study demonstrates that an essential glycolytic enzyme of LDHA not only maintains the stemness and heterogeneity of BCSCs but also facilitates the infiltration of TAMs to trigger immune escape of BCSCs (**Figure 7**). The mechanisms of tumor abnormal metabolism identified here can inform translational studies by targeting heterogeneous

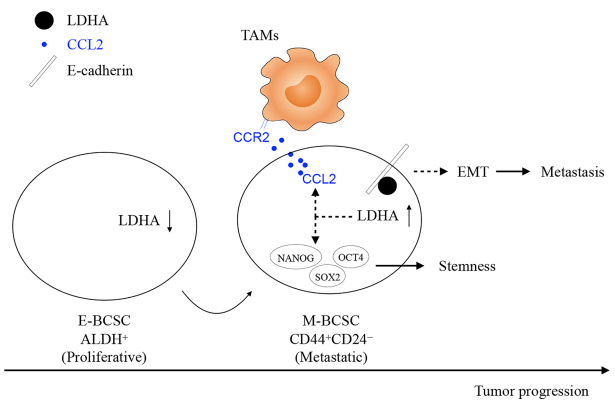

FIGURE 7 | The work model of glycolytic enzyme of LDHA not only maintains the stemness and heterogeneity of BCSCs but also facilitates the infiltration of TAMs to trigger immune escape of BCSCs. At the early stage of breast cancer, E-BCSCs promote tumor rapidly growth and recruit more CCR2-expressing monocytes/macrophages and then, LDHA reduces E-Cadherin expression and increases CCL2 secretion in M-BCSCs to facilitated tumor metastasis at the advanced stage of breast cancer.

population and supportive niche of BCSCs, to develop more efficient treatment for 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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by institutional review board of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

AUTHOR CONTRIBUTIONS

SW carried out the cellular and animal experiments. ZW carried out the bioinformatic analysis. SW and LM performed the statistical analysis. CC and SW drafted the manuscript. HH, HC, ZD, YLi, QS, and T-HC provided material support. CC and YLu conceived of the study and participated in its design. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (NSFC): grant no. 81972795 and 81672914; the Bilateral Inter-Governmental S&T Cooperation Project from Ministry of Science and Technology of China: grant no. 2018YFE0114300; the National Basic Research Program (973) of China: grant no. 2013CB967202.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.654452/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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 06 July 2021
doi: 10.3389/fonc.2021.677207

Hormone Receptor Status May Impact the Survival Benefit Between Medullary Breast Carcinoma and Atypical Medullary Carcinoma of the Breast: A Population-Based Study

Wenxing Qin[1†], *Feng Qi*[1,2†], *Mengzhou Guo*[2†], *Liangzhe Wang*[3] *and Yuan-Sheng Zang*[1*]

[1] Department of Oncology, Second Affiliated Hospital of Naval Medical University, Shanghai, China, [2] Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China, [3] Department of Pathology, Second Affiliated Hospital of Naval Medical University, Shanghai, China

Background: A rare subtype of breast cancer, atypical medullary carcinoma of the breast (AMCB), shows a highly adverse prognosis compared to medullary carcinoma of the breast (MBC). The current study aimed to establish a correlated nomogram for the identification of the prognostic factors of AMCB and MBC.

Methods: Kaplan–Meier and Cox regression analyses were applied to data acquired from the Surveillance, Epidemiology and End Results (SEER) database for 2004 to 2013 to analyse tumour characteristics and overall survival. Propensity score matching (PSM) analysis was performed to determine the overall survival (OS) among those with AMCB and MBC. A predictive nomogram was created, and the concordance index (C-index) was used to predict accuracy and discriminative ability.

Results: A total of 2,001 patients from the SEER database were diagnosed with MBC between 2004 and 2013, including 147 patients diagnosed with AMCB. The number of diagnoses gradually increased in both groups. Cox analysis of multivariate and Kaplan–Meier analysis showed that older age (HR = 3.005, 95% CI 1.906–4.739) and later stage were significantly associated with poor prognosis, while cancer-directed surgery was an independent protective factor (HR = 0.252, 95% CI 0.086–0.740). In the HR-negative stratification analysis, older age (HR = 2.476, 95% CI 1.398–4.385), later stage and histological type (HR=0.381, 95% CI 0.198-0.734) were found to be independent prognostic factors for low standard survival. The log-rank analysis demonstrated significantly worse prognostic factors for patients with AMCB than for those with MBC (P = 0.004). A nomogram (C-index for survival = 0.75; 95% CI 0.69–0.81) was established from four independent prognostic factors after complete identification.

OPEN ACCESS

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Shanghai Jiao Tong University, China

***Correspondence:**
Yuan-Sheng Zang
doctorzangys@163.com

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *07 March 2021*
Accepted: *17 June 2021*
Published: *06 July 2021*

Citation:
Qin WX, Qi F, Guo M, Wang L and Zang Y-S (2021) Hormone Receptor Status May Impact the Survival Benefit Between Medullary Breast Carcinoma and Atypical Medullary Carcinoma of the Breast: A Population-Based Study. Front. Oncol. 11:677207. doi: 10.3389/fonc.2021.677207

Conclusions: MBC is rare, and cancer-directed surgery, older age, and later stage are independently linked with prognosis. In the HR negative population, AMCB patients show a worse survival gain than those with MBC.

Keywords: hormone receptor status, breast cancer, medullary carcinoma, nomogram, atypical medullary carcinoma of the breast

INTRODUCTION

Breast cancer is a major malignant tumour in women, ranking second in incidence among female malignant tumours. Medullary carcinoma of the breast (MBC) has been declared a special type of infiltrating breast cancer, accounting for approximately 5–7% of cases (1). The boundary of the typical medullary carcinoma of the breast (TMCB) is clearer, and a large number of lymphocytes infiltrate the interstitial substance; thus, it has a slower growth and more favourable prognosis (2). However, atypical medullary carcinoma of the breast (AMCB) is characterized by no obvious histologic boundaries and a poor prognosis.

Therefore, it is important to determine and characterize the prognostic factors for AMCB patients to facilitate diagnosis and clinical treatment. Although the cellular morphology of AMCB is essentially consistent with that of MBC, it fails to conform to the diagnostic criteria of MBC and features infiltrating tumour borders (3). Several studies have reported that the overall survival (OS) and disease-free survival (DFS) of MBC patients were closely related to age, race, local metastasis, distant metastasis, tumour size, hormone receptor status, and lymph node metastasis (4–7). In addition, HR status also affected the survival of MBC patients. Some studies found that MBC patients had a higher percentage of triple-negative status, and MBC patients with PR negativity exhibited good prognosis (8, 9). However, the prognostic factors of AMCB were not mentioned. To address this, we examined patients with AMCB and MBC of the breast cancer referring to the Surveillance, Epidemiology and End Results (SEER) database and offer a related retrospective assessment. We compared the overall survival, prognostic factors, and clinical features between MBC and AMCB patients. In addition, hormone receptor status was used as a stratification analysis to analyse the survival benefit of AMCB and MBC patients.

MATERIALS AND METHODS

Patient Data

Data from patients diagnosed with breast cancer from 2004 to 2013 were downloaded using Stat version 8.2.1 of SEER. Patient data were included in this analysis if the following criteria were met: ages 18 to 79; breast cancer as the primary malignant cancer diagnosis; estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) status available; medullary carcinoma (based on ICD-O-3 8512/3) and pathological types including atypical medullary carcinoma (based on ICD-O-3 8513/3) if not specified (MBC-NOS, ICD-O-3 8510/3); and definite AJCC TNM stages; and histological grades I to IV. We excluded patients who did not have a clear tumour stage or a record of months of survival. Additionally, we also included data from patients exhibiting a history of breast cancer diagnosis prior to 2013 to ensure a sufficiently extensive follow-up period. The study was in compliance with the ethics statement of Changzheng Hospital.

Statistical Analysis

The expression status of ER and PR was combined as the hormone receptor (HR) state. The expression of HR− was further defined as ER− and PR−, while the expression of HR+ was defined as ER+ and PR+. Unclear expression of ER or PR was defined as unknown. According to the pathological type, breast cancer patients were allocated into two groups: the AMCB and MBC groups. The t-test and, where necessary, the Mann–Whitney U test were employed to compare the homogeneity of variance and continuous variables in the normal distribution. Tukey's test and one-way ANOVA were employed to compare multiple groups. The chi-square test was used to compare the clinical and demographic characteristics of the three groups. The Kaplan–Meier method was employed to plot the survival curve, and the log-rank test was used to adjust the unadjusted overall survival rates of different histological subtypes. Overall survival was defined as the period from diagnosis to the final follow-up or the complete disappearance of the tumour. The prognostic factors were computed using the Cox proportional hazards model, where HR was the 95% confidence interval. PSM analyses were performed based on age, race, sex, grade, laterality, AJCC stage, T category, N category, local treatment of the primary tumour, ER, PR and Her2 at a 1:1 ratio to adjust for the differences among the AMCB and MBC groups. All statistical analyses were computed using SPSS version 22.0 (IBM SPSS Statistics, Chicago, IL, US), and P <0.05 was considered statistically significant.

RESULTS

Demographic Analysis of Patients With AMCB and MBC

A total of 2,001 eligible patients, including 1,863 MBC patients and 147 AMCB patients, were included in our study based on the inclusion criteria. The clinical and demographic characteristics of all patients in the SEER database with Histologic Type of Breast Cancer from 2004–2013 are summarized in **Table 1**.

Abbreviations: SEER database, Surveillance, Epidemiology and End Results database; AJCC, American Joint Committee on Cancer; AMCB, atypical medullary carcinoma of the breast (MBC); MBC, medullary carcinoma of the breast; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2.

TABLE 1 | Patient characteristics with histologic type of breast cancer in the SEER database, 2004–2013.

Characteristics	Histologic type			
	AMCB	**MBC**	**Total**	***P*-value**
Number	147	1,863	2,010	
Age (years)	53.26 ± 12.25	53.68 ± 13.00		0.707
Marital status				
Married	90 (61.2%)	1,018 (54.6%)	1,180 (55.1%)	0.298
Not married[a]	52 (35.4%)	777 (41.7%)	829 (41.2%)	
Unknown	5 (3.4%)	68 (3.7%)	73 (3.6%)	
Race				
White	100 (68.0%)	1,255 (67.4%)	1,355 (67.4%)	0.548
Black	33 (22.4%)	475 (25.5%)	508 (25.3%)	
Other[b]	14 (9.5%)	129 (6.9%)	143 (7.1%)	
Unknown	0 (0%)	4 (0.2%)	4 (0.2%)	
Sex				
Female	147 (100%)	1,858 (99.7%)	2,005 (99.8%)	0.529
Male	0 (0%)	5 (0.3%)	5 (0.2%)	
Grade				
Well differentiated	1 (0.7%)	13 (0.7%)	14 (0.7%)	0.715
Moderate	8 (5.4%)	102 (5.5%)	110 (5.5%)	
Poor	122 (83.0%)	1,481 (79.5%)	1,603 (79.8%)	
Unknown	16 (10.9%)	267 (14.3%)	283 (14.1%)	
Laterality				
Right	66 (44.9%)	926 (49.7%)	992 (49.4%)	0.485
Left	81 (55.1%)	935 (50.2%)	1,016 (50.5%)	
Bilateral	0 (0%)	2 (0.1%)	2 (0.1%)	
AJCC stage				
I	52 (35.4%)	699 (37.5%)	751 (37.4%)	0.293
II	80 (54.4%)	1,027 (55.1%)	1,107 (55.1%)	
III	12 (8.2%)	124 (6.7%)	136 (6.8%)	
IV	3 (2.0%)	13 (0.7%)	16 (0.8%)	
T category				
T0	0 (0%)	2 (0.1%)	2 (0.1%)	0.780
T1	65 (44.2%)	848 (45.5%)	913 (45.4%)	
T2	70 (47.6%)	909 (48.8%)	979 (48.7%)	
T3	10 (6.8%)	84 (4.5%)	94 (4.7%)	
T4	2 (1.4%)	16 (0.9%)	18 (0.9%)	
Tx	0 (0%)	4 (0.2%)	4 (0.2%)	
N category				
N0	111 (75.5%)	1,406 (75.5%)	1,517 (75.5%)	0.544
N1	25 (17.0%)	368 (19.8%)	393 (19.6%)	
N2	8 (5.4%)	63 (3.4%)	71 (3.5%)	
N3	3 (2.0%)	23 (1.2%)	26 (1.3%)	
Nx	0 (0%)	3 (0.2%)	3 (0.1%)	
Local treatment of the primary tumour				
Surgery	145 (98.6%)	1,838 (98.7%)	1,983 (98.7%)	0.985
No surgery	2 (1.4%)	25 (1.3%)	27 (1.3%)	
ER				
Positive	34 (23.1%)	388 (20.8%)	422 (21.0%)	0.752
Negative	105 (71.4%)	1,356 (72.8%)	1,461 (72.7%)	
Unknown	8 (5.4%)	119 (6.4%)	127 (6.3%)	
PR				
Positive	20 (13.6%)	246 (13.2%)	266 (13.2%)	0.812
Negative	119 (81.0%)	1,490 (80.0%)	1,609 (80.0%)	
Unknown	8 (5.4%)	127 (6.8%)	135 (6.7%)	
Her2				
Positive	3 (2.0%)	59 (3.2%)	62 (3.1%)	0.108
Negative	26 (17.7%)	458 (24.6%)	484 (24.1%)	
Unknown	118 (80.3%)	1,346 (72.2%)	1,464 (72.8%)	

[a]*Including divorced, separated, single (never married), unmarried or domestic partner and widowed.*

[b]*Including American Indian/Alaskan Native and Asian/Pacific Islander.*

AJCC, American Joint Committee on Cancer; AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma; ER, oestrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2.

The X-tile analysis revealed that the best possible cut-off value to assess prognostic factors for age was 71 years. The comparison of MBC and AMCB showed no substantial differences in race, sex, age, degree of differentiation, tumour size, AJCC stage, lymph node metastasis, Her2 status, HR status, or surgical method. Similar to MBC, the rate of incidence of AMCB was high in triple-negative breast cancer. In molecular subtypes, three categories were defined (**Table 1**). Regarding Her2 status, the frequency of Her2-negative cases was higher in the MBC group than in the AMCB group (24.6% *vs* 17.7%, $P = 0.108$). Overall, the number of Her2-negative patients was higher than that of Her2-positive patients (24.1% *vs* 3.1%). However, 72.8% of cases were characterized as unknown, with neither Her2 negativity nor Her2 positivity. Due to limited data, the comparative analysis of the treatment method did not generate statistically significant results. Between 2004 and 2013, the overall incidence rate trends of AMCB and MBC decreased (AMBC: r = −0.88 and MBC: r = −0.90, P <0.001) (**Figure 1**).

Survival and Prognostic Factors of AMCB and MBC Patients

The overall survival of MBC and AMCB patients was estimated using the Kaplan–Meier estimator. The survival curve was stratified by race, sex, age at diagnosis, laterality, AJCC stage, primary tumour size, primary tumour differential grade, surgery for the primary tumour, lymph node status, HR status, and histological type (**Figure 2**). As illustrated, older-aged patients had poorer survival (5-year overall survival rate: 83.3% vs 94.9%). The outcomes were extremely poor for patients with advanced cancer stages; the prognostic factors were the worst (27.5% 5-year overall survival rate) for MBC and AMCB patients with stage IV cancer, while the 5-year overall survival rates for stage I, II, and III cancers were 75.3, 94.5, and 97.3%, respectively (P <0.01, **Figures 2A, E**). Similarly, the overall survival rate of MBC and AMCB patients with smaller tumour sizes was significantly higher than that of patients with larger tumour sizes (P <0.01). The

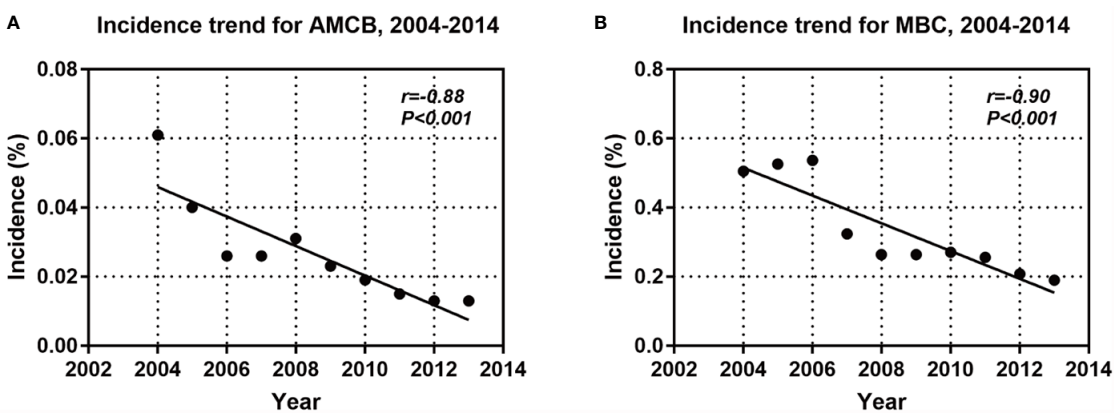

FIGURE 1 | Incidence trends for AMCB **(A)** and MBC **(B)**.

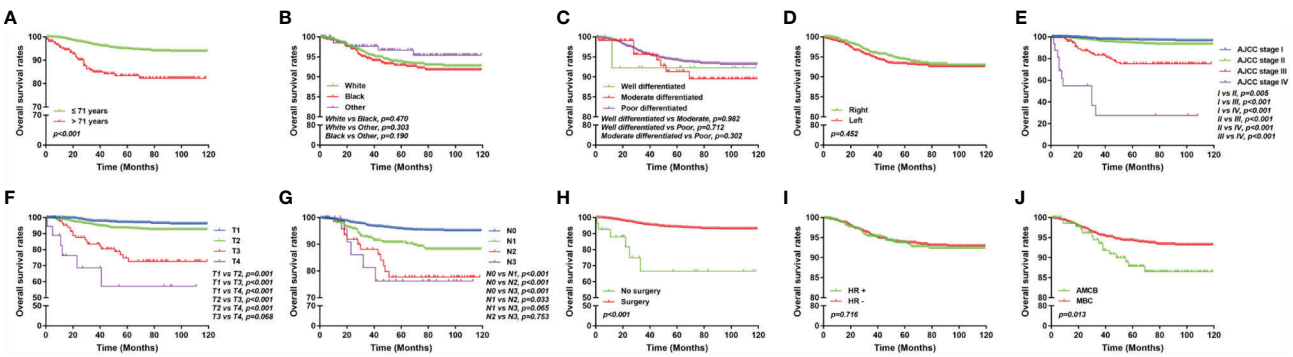

FIGURE 2 | Overall survival of MBC and AMCB patients using the Kaplan–Meier estimator stratified by **(A)** age at diagnosis; **(B)** race; **(C)** grade; **(D)** laterality; **(E)** AJCC stage; **(F)** primary tumour size; **(G)** lymph node status; **(H)** surgery for primary tumour; **(I)** HR status; and **(J)** histological type.

prognostic factors for MBC and AMCB patients were much worse when breast cancer cells were detected in lymph nodes (P <0.01) (**Figures 2F, G**). **Figure 2H** shows that the median overall survival for the MBC and AMCB patients significantly improved after cancer-directed surgery (P <0.01, mOS: 16.5 m *vs* 28.0 m). In the histological type analysis for OS, patients with MBC had better survival than those with AMCB (P = 0.013). The 5-year overall survival rates of MBC and AMCB patients were 94.3 and 87.8%, respectively.

The Cox regression models in both forms, i.e., univariate and multivariate analyses, were applied to the overall survival results to further analyse the prognostic factors. According to univariate factor analysis, as shown in **Table 2**, older age, larger tumour size, later stage, lymph node metastasis, and histological type of AMCB were significantly related to worse prognosis (P <0.01). Cancer-directed surgery was found to be significantly related to extended overall survival (P <0.01). After adjusting the multivariate analysis, as shown in **Table 2**, the independent prognostic factors of poorer survival in MBC and AMCB patients were older age and later stage only. However, cancer-directed surgery was found to be an independent protective factor that reduced the probability of death by 74.8% (HR = 0.252, 95% CI 0.086–0.740) in MBC and AMCB patients.

PSM analysis was performed to adjust for the unmatching cohort, and a total of 147 AMCB patients were matched with 147 MBC patients (1:1) (**Table 3**). According to univariate and multivariate analyses, as shown in **Table 4**, compared with MBC, histological type of AMCB was identified as an independent risk factor (HR = 4.767, 95% CI 3.408–6.669).

TABLE 2 | Univariate and multivariate Cox proportional hazard analyses of the association of clinical characteristics with overall survival rates in patients with AMCB and MBC.

Variance	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age				
≤71 years	1		1	
>71 years	3.603 (2.360–5.500)	<0.001	3.005 (1.906–4.739)	<0.001
Race				
White	1		1	
Black	1.163 (0.772–1.753)	0.470	1.076 (0.704–1.645)	0.734
Other	0.625 (0.253–1.546)	0.309	0.707 (0.283–1.768)	0.458
Grade				
Well differentiated	1		1	
Moderate	1.009 (0.126–8.068)	0.993	0.970 (0.110–8.546)	0.978
Poor	0.691 (0.096–4.965)	0.713	0.597 (0.076–4.713)	0.625
Laterality				
Right			1	
Left	1.154 (0.794–1.676)	0.453	0.987 (0.666–1.461)	0.947
Bilateral	17.641 (2.423–128.446)	0.005	0.258 (0.005–14.380)	0.509
AJCC stage				
I	1		1	
II	2.066 (1.229–3.472)	0.006	0.674 (0.255–1.783)	0.426
III	9.083 (5.025–16.416)	<0.001	0.813 (0.187–3.541)	0.783
IV	53.150 (24.633–114.681)	<0.001	13.065 (3.639–46.904)	<0.001
Stage T				
T0	1		1	
T1	0.027 (0.006–0.114)	<0.001	0.132 (0.015–1.174)	0.069
T2	0.058 (0.014–0.237)	<0.001	0.340 (0.042–2.751)	0.312
T3	0.232 (0.054–0.996)	0.049	0.839(0.094–7.454)	0.875
T4	0.542 (0.109–2.691)	0.454	1.511 (0.144–15.875)	0.731
Stage N				
N0	1		1	
N1	2.467 (1.626–3.745)	<0.001	1.951 (1.155–3.294)	0.012
N2	4.924 (2.642–9.177)	<0.001	2.918 (0.927–9.184)	0.067
N3	5.851 (2.345–14.600)	<0.001	3.459 (0.968–12.355)	0.056
Local treatment of the primary tumour				
None	1		1	
Surgery	0.135 (0.059–0.307)	<0.001	0.252 (0.086–0.740)	0.012
HR				
Positive	1		1	
Negative	0.928 (0.620–1.388)	0.716	1.055 (0.685–1.626)	0.808
Histologic type				
AMCB	1		1	
MBC	0.508 (0.295–0.874)	0.015	0.684 (0.371–1.260)	0.223

AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma; HR, hormone receptor.

TABLE 3 | Patient characteristics with histologic type of breast cancer after propensity score matching in the SEER database, 2004–2013.

Characteristics	Histologic type			
	AMCB	MBC	Total	*P* value
Number	147	147	294	
Age (years)	53.26 ± 12.25	62.10± 14.46		<0.001
Marital status				
Married	90 (61.2%)	31 (21.1%)	121 (41.2%)	<0.001
Not married[a]	52 (35.4%)	92 (62.6%)	144 (49.0%)	
Unknown	5 (3.4%)	24 (16.3%)	29 (9.9%)	
Race				
White	100 (68.0%)	106 (72.1%)	206 (70.1%)	0.180
Black	33 (22.4%)	35 (23.8%)	68 (23.1%)	
Other[b]	14 (9.5%)	6 (4.1%)	20 (6.8%)	
Sex				
Female	147 (100%)	142 (96.6%)	289 (98.3%)	0.024
Male	0 (0%)	5 (3.4%)	5 (1.7%)	
Grade				
Well differentiated	1 (0.7%)	0 (0.7%)	1 (0.3%)	0.194
Moderate	8 (5.4%)	5 (3.4%)	13 (4.4%)	
Poor	122 (83.0%)	115 (78.2%)	237 (80.6%)	
Unknown	16 (10.9%)	27 (18.4%)	43 (14.6%)	
Laterality				
Right	66 (44.9%)	99 (67.3%)	165 (56.1%)	<0.001
Left	81 (55.1%)	47 (32.0%)	128 (43.5%)	
Bilateral	0 (0%)	1 (0.7%)	1 (0.3%)	
AJCC stage				
I	52 (35.4%)	82 (55.8%)	134 (45.6%)	0.003
II	80 (54.4%)	59 (40.1%)	139 (47.3%)	
III	12 (8.2%)	5 (3.4%)	17 (5.8%)	
IV	3 (2.0%)	1 (0.7%)	4 (1.4%)	
T category				
T1	65 (44.2%)	92 (62.6%)	157 (53.4%)	0.009
T2	70 (47.6%)	46 (31.3%)	116 (39.5%)	
T3	10 (6.8%)	9 (6.1%)	19 (6.5%)	
T4	2 (1.4%)	0 (0.0%)	2 (0.7%)	
N category				
N0	111 (75.5%)	115 (78.2%)	226 (76.9%)	0.293
N1	25 (17.0%)	27 (18.4%)	52 (17.7%)	
N2	8 (5.4%)	2 (1.4%)	10 (3.4%)	
N3	3 (2.0%)	2 (1.4%)	5 (1.7%)	
Nx	0 (0%)	1 (0.7%)	1 (0.3%)	
Local treatment of the primary tumour				
Surgery	145 (98.6%)	143 (97.3%)	288 (98.0%)	0.409
No surgery	2 (1.4%)	4 (2.7%)	6 (2.0%)	
ER				
Positive	34 (23.1%)	19 (12.9%)	53 (18.0%)	0.068
Negative	105 (71.4%)	117 (79.6%)	222 (75.5%)	
Unknown	8 (5.4%)	11 (7.5%)	19 (6.5%)	
PR				
Positive	20 (13.6%)	21 (14.3%)	41 (13.9%)	0.867
Negative	119 (81.0%)	116 (78.9%)	235 (79.9%)	
Unknown	8 (5.4%)	10 (6.8%)	18 (6.1%)	
Her2				
Positive	3 (2.0%)	50 (34.0%)	53 (18.0%)	<0.001
Negative	26 (17.7%)	75 (51.0%)	101 (34.4%)	
Unknown	118 (80.3%)	22 (15.0%)	140 (47.6%)	

[a]*Including divorced, separated, single (never married), unmarried or domestic partner and widowed.*

[b]*Including American Indian/Alaskan Native and Asian/Pacific Islander.*

AJCC, American Joint Committee on Cancer; AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

TABLE 4 | Univariate and multivariate Cox proportional hazard analyses of the association of clinical characteristics with overall survival rates in patients with AMCB and MBC after propensity score matching.

Variance	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age				
≤71 years	1		1	
>71 years	1.169 (0.872–1.568)	0.296	0.549 (0.389–0.774)	<0.001
Race				
White	1		1	
Black	0.825 (0.615–1.107)	0.199	0.856 (0.619–1.184)	0.349
Other	0.751 (0.456–1.237)	0.261	1.006 (0.576–1.757)	0.984
Grade				
Well differentiated	1		1	
Moderate	1.763 (0.225–13.805)	0.589	1.625 (0.199–13.285)	0.650
Poor	2.275 (0.318–16.264)	0.413	1.678 (0.213–13.209)	0.623
Laterality				
Right			1	
Left	0.869 (0.681–1.109)	0.260	1.159 (0.889–1.511)	0.275
Bilateral	3.420 (0.474–24.684)	0.223	3.910 (0.379–40.316)	0.252
AJCC stage				
I	1		1	
II	0.893 (0.696–1.145)	0.372	1.535 (0.808–2.916)	0.191
III	0.886 (0.500–1.569)	0.678	1.978 (0.320–12.220)	0.463
Stage T				
T1	1		1	
T2	0.907 (0.706–1.165)	0.445	0.794 (0.449–1.405)	0.428
T3	0.902 (0.488–1.668)	0.743	0.730 (0.298–1.788)	0.491
T4	0.727 (0.102–5.207)	0.751	1.032 (0.123–8.691)	0.977
Stage N				
N0	1		1	
N1	1.013 (0.740–1.386)	0.938	0.826 (0.527–1.295)	0.405
N2	0.932 (0.459–1.893)	0.846	0.851 (0.140–5.182)	0.861
N3	0.659 (0.211–2.063)	0.474	0.604 (0.080–4.537)	0.624
Local treatment of the primary tumour				
Surgery	1		1	
None	1.197 (0.445–3.218)	0.721	1.105 (0.338–3.609)	0.868
HR				
Positive	1		1	
Negative	1.142 (0.874–1.492)	0.329	1.025 (0.765–1.372)	0.870
Histologic type				
MBC	1		1	
AMCB	3.025 (2.340–3.911)	<0.001	4.767 (3.408–6.669)	<0.001

AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma; HR, hormone receptor.

Baseline Characteristics and Survival Benefits in the Hormone Receptor Subgroups

We analyzed the characteristics of the patients belonging to the HR-negative subgroup, which included 1,291 MBC and 102 AMCB patients (**Table 5**). The X-tile analysis revealed that the best possible cut-off value to assess prognostic factors for age was 71 years. The results were consistent with the entire population, and the comparison of MBC and AMCB showed no significant differences in sex, race, age, AJCC stage, degree of differentiation, tumour size, lymph node metastasis, or surgical method. The results of the Kaplan–Meier estimator and the Cox regression including univariate and multivariate analyses showed that later stage, older age, and histological type of AMCB were independently related to shortened OS, while surgery was independently related to prolonged overall survival (P <0.01, **Figure 3** and **Table 6**). The MBC group showed a better survival benefit than the AMCB group, with a lower hazard ratio of 0.38 (95% CI, 0.198–0.734, P = 0.004).

Prognostic Nomogram for AMCB and MBC Patients With Hormone Receptor Negativity

A nomogram based on the prognostic factors that combined all significant independent variables for overall survival in the MBC and AMCB groups with HR negativity is shown in **Figure 4**. All patients were divided into three groups based on their age using the X-tile program, and the probabilities of 1-, 3-, or 5-year overall survival were determined. The optimal cut-points were 54 and 70 years. To determine the survival of MBC and AMCB patients more accurately with HR negativity, a nomogram based on the prognostic factors that included all significant independent variables in a multivariate Cox analysis was created (**Table 4**). Univariate and multivariate Cox analyses were used to calculate the association of the overall survival rate with the clinical characteristics of HR− patients with MBC and AMCB. The C-index for overall survival was found to be 0.75 (95% CI 0.69–0.81). As shown in **Figures 4B–D**, the actual

TABLE 5 | HR (–) patient characteristics with AMCB and MBC.

Characteristics	Histologic type			
	AMCB	**MBC**	**Total**	***P*-value**
Number	102	1,291	1,393	
Age (years)	53.31 ± 11.76	53.60 ± 12.93		0.830
Marital status				
Married	65 (63.7%)	716 (55.5%)	781 (56.1%)	0.269
Not married[a]	34 (33.3%)	527 (40.8%)	561 (40.3%)	
Unknown	3 (2.9%)	48 (3.7%)	51 (3.7%)	
Race				
White	73 (71.6%)	856 (66.3%)	929 (66.7%)	0.357
Black	20 (19.6%)	350 (27.1%)	370 (26.6%)	
Other[b]	9 (8.8%)	84 (6.5%)	93 (6.7%)	
Unknown	0 (0%)	1 (0.1%)	1 (0.1%)	
Sex				
Female	102 (100%)	1,290 (99.9%)	1,392 (99.9%)	0.779
Male	0 (0%)	1 (0.1%)	1 (0.1%)	
Grade				
Well differentiated	0 (0.0%)	9 (0.7%)	9 (0.6%)	0.715
Moderate	6 (5.9%)	54 (4.2%)	60 (4.3%)	
Poor	82 (80.4%)	1,052 (81.5%)	1,134 (81.4%)	
Unknown	14 (13.7%)	176 (13.6%)	190 (13.6%)	
Laterality				
Right	49 (48.0%)	645 (50.0%)	694 (49.8%)	0.894
Left	53 (52.0%)	645 (50.0%)	698 (50.1%)	
Bilateral	0 (0%)	1 (0.1%)	1 (0.1%)	
AJCC stage				
I	35 (34.3%)	479 (37.1%)	514 (36.9%)	0.490
I	59 (57.8%)	712 (55.2%)	771 (55.3%)	
III	6 (5.9%)	91 (7.0%)	97 (7.0%)	
IV	2 (2.0%)	9 (0.7%)	11 (0.8%)	
T category				
T0	0 (0%)	1 (0.1%)	1 (0.1%)	0.727
T1	45 (44.1%)	596 (46.2%)	641 (46.0%)	
T2	50 (49.0%)	626 (48.5%)	676 (48.5%)	
T3	7 (6.9%)	54 (4.2%)	61 (4.4%)	
T4	0 (0%)	12 (0.9%)	12 (0.9%)	
Tx	0 (0%)	2 (0.2%)	2 (0.1%)	
N category				
N0	81 (79.4%)	967 (74.9%)	1,048 (75.2%)	0.569
N1	15 (14.7%)	259 (20.1%)	274 (19.7%)	
N2	4 (3.9%)	49 (3.8%)	53 (3.8%)	
N3	2 (2.0%)	16 (1.2%)	18 (1.3%)	
Local treatment of the primary tumour				
No surgery	0 (0%)	17 (1.3%)	17 (1.2%)	0.487
Surgery	102 (100%)	1,273 (98.6%)	1,375 (98.7%)	
Unknown	0 (0%)	1 (0.1%)	1 (0.1%)	

[a]*Including divorced, separated, single (never married), unmarried or domestic partner and widowed.*

[b]*Including American Indian/Alaskan Native and Asian/Pacific Islander.*

AJCC, American Joint Committee on Cancer; AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma.

observation and the prediction by the nomogram displayed optimal agreement based on the calibration plot for the probability of overall survival at 1, 3, or 5 years.

DISCUSSION

Medullary carcinoma is a distinct subgroup of breast cancers that makes up less than 5% of all advanced breast cancers. It has been considered that medullary breast carcinoma has a better prognosis than other common subtypes of histological breast cancer (10). In 1977, Ridolfi clarified the definition of MBC and proposed clear diagnostic criteria (six basic criteria) for the identification and diagnosis of medullary carcinoma (11). AMCB was defined to satisfy the first criterion of Ridolfi but not the remaining criteria. Through these precise diagnostic criteria, some institutions have reclassified breast medullary carcinoma (12) according to histopathological measures. Hormone receptors such as ER and PR are predictive prognostic factors and can serve as the foundations of a patient's treatment for breast cancer. In previous studies, MBC was observed to have the lowest frequencies of ER, PR, and Her/neu-2 expression (13). ER positivity is infrequent in MBC, which is why previous studies reported only 547 patients (16.3%) with ER+ tumours. A positive ER in MBC patients is accompanied by the

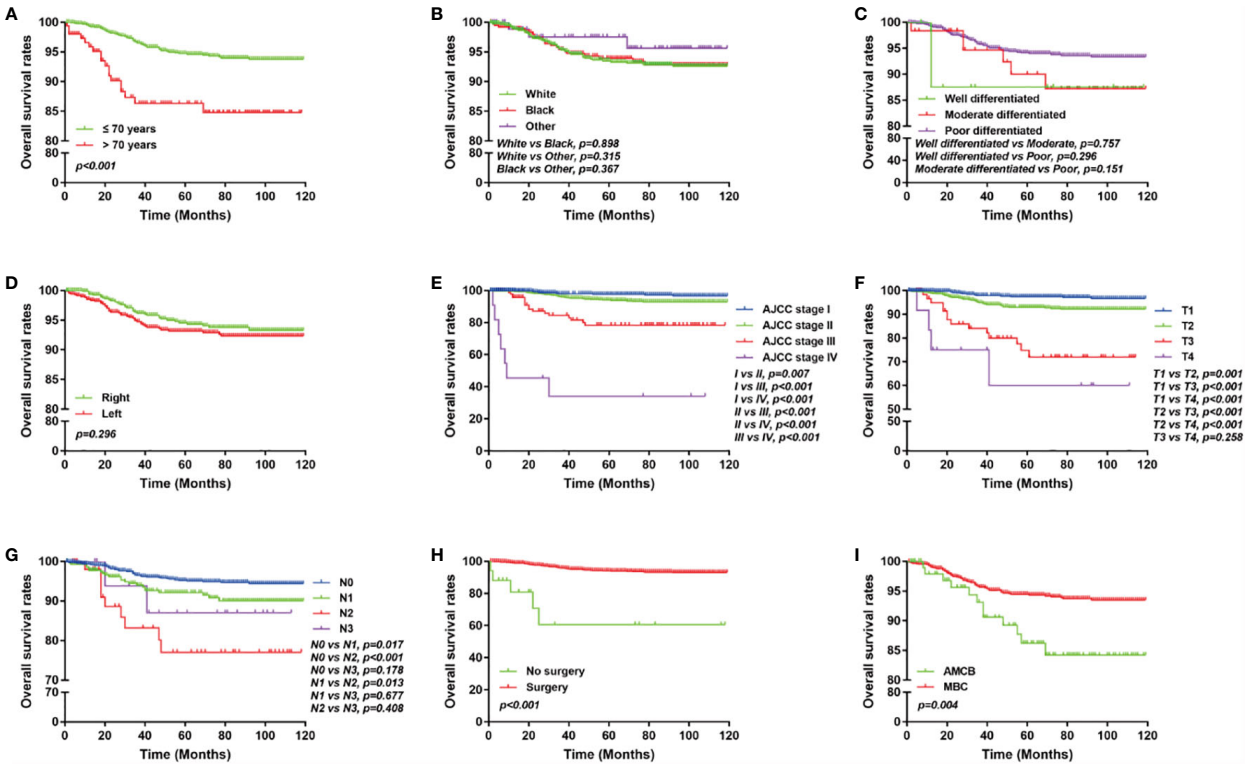

FIGURE 3 | Overall survival of MCB and AMCB patients using the Kaplan–Meier estimator with HR negativity stratified by **(A)** age at diagnosis; **(B)** race; **(C)** grade; **(D)** laterality; **(E)** AJCC stage; **(F)** primary tumour size; **(G)** lymph node status; **(H)** surgery for primary tumour; **(I)** and histological type.

worst overall survival (5). Pinto et al. found no survival difference in patients with ER+/Her2/neu+ carcinomas and ER−/Her2/neu− carcinomas, indicating resistance to hormone therapy (14). Identification and characterization of prognostic factors for MBC patients will help in dictating diagnosis and suggesting more applicable treatments. In the current study, we tried to assess the clinicopathological features and prognostic factors of MBC and AMCB using data from patients in the SEER database from 2004 to 2013. MBC has many prognostic variables in common with ductal carcinoma infiltration. They both involve increasing age, the growing size of the tumour, metastasis at the lymph node, and the existence of cancer cells in distant lymph nodes, all of which shorten overall survival. Previous studies have identified numerous distinctive factors that are exclusively prognostic of survival in MBC (7, 15). Several factors, such as age, marital status, tumour size, stage, lymph node status, subtype of breast cancer, and radiation therapy, were substantially linked to overall survival in MBC (5). Our findings suggest that AMCB has a worse prognosis than MBC. Importantly, according to multivariate regression analysis, the prognosis of AMCB has close associations with age, stage, tumour size, surgical type, and hormone receptor positivity. In our research, the ratio of high-grade (grade III/IV) AMCB was 83.0%. Therefore, AMCB was considered a more aggressive tumour and was predominantly determined to be a high-grade tumour, similar to other studies (16). Moreover, the prevalence rate of HR-negative AMCB was 81.0%, which was more prevalent than HR-positive AMCB (13.6%), similar to other studies (17). According to further stratification studies, patients with hormone receptor-positive AMCB and MBC showed similar OS. In hormone receptor-negative patients, the prognosis of AMCB was significantly worse than that of MBC (data not shown). It was shown that the hormone receptor can be a clear independent prognostic factor for AMCB. It is important to realize that the clinicopathological features and prognostic factors of AMCB can serve as a reference for patients to provide more accurate clinical treatment.

Reported studies have indicated that the prognosis of MBC is better than that of IDC. Huober et al. found that the overall survival rates and 14-year distant recurrence-free intervals for invasive ductal tumours and medullary tumours of the full cohort were 57, 66, 64 and 76%, respectively (16). Similar findings were also found in the report of Dongjun Dai et al. (18). However, the results of the AMCB comparison between MBC and IDC were different. MBC occurs in patients of younger age; however, there was variation in the results of previous studies (5, 6, 19). Ethnic variations may play a role in such differences. Compared with patients who had an atypical characteristic, Rakha et al. (20) achieved better survival rates in patients with the typical characteristics of MBC. However, they found that the difference was not statistically significant. Conversely, Aksoy et al. (8) found that patients with atypical MBC

TABLE 6 | Univariate and multivariate Cox proportional hazard analyses of the association of clinical characteristics with overall survival rates in HR (–) patients with AMCB and MBC.

Variance	Univariate HR (95% CI)	*P* value	Multivariate HR (95% CI)	*P* value
Age				
≤70 years	1		1	
>70 years	3.087 (1.822–5.232)	<0.001	2.476 (1.398–4.385)	0.002
Race				
White	1		1	
Black	0.968 (0.585–1.602)	0.899	0.981 (0.585–1.645)	0.943
Other	0.556 (0.174–1.779)	0.323	0.588 (0.179–1.925)	0.380
Grade				
Well differentiated	1		1	
Moderate	0.675 (0.081–5.608)	0.716	3.482 (0.337–36.016)	0.295
Poor	0.368 (0.051–2.655)	0.321	1.486 (0.161–13.749)	0.727
Laterality				
Right			1	
Left	1.268 (0.812–1.981)	0.297	1.134 (0.705–1.822)	0.605
AJCC stage				
I	1		1	
II	2.368 (1.247–4.498)	0.008	0.477 (0.131–1.743)	0.263
III	8.844 (4.222–18.524)	<0.001	0.376 (0.056–2.542)	0.316
IV	56.164 (22.067–142.943)	<0.001	13.166 (2.922–59.331)	0.001
Stage T				
T0	1		1	
T1	0.029 (0.004–0.219)	0.001	0.043 (0.004–0.468)	0.010
T2	0.076 (0.010–0.555)	0.011	0.178 (0.020–1.606)	0.124
T3	0.298 (0.039–2.272)	0.243	0.747 (0.076–7.356)	0.802
T4	0.567 (0.063–5.079)	0.612	1.016 (0.080–12.941)	0.990
Stage N				
N0	1		1	
N1	1.842 (1.106–3.067)	0.019	1.472 (0.751–2.882)	0.260
N2	4.780 (2.336–9.781)	<0.001	4.377 (1.116–17.166)	0.034
N3	2.541 (0.617–10.477)	0.197	1.992 (0.329–12.077)	0.454
Local treatment of the primary tumour				
None	1		1	
Surgery	0.099 (0.040–0.246)	<0.001	0.066 (0.022–0.195)	<0.001
Histologic type				
AMCB	1		1	
MBC	0.416 (0.225–0.770)	0.005	0.381 (0.198–0.734)	0.004

AMCB, atypical medullary carcinoma; MBC, medullary breast carcinoma.

presented significantly superior rates of recurrence and RFS. This may be associated with a smaller number of selected cases, and these patients have less tumour growth and angiogenesis with fewer lymphocytes and mononuclear infiltration. Some studies of immunohistochemical staining and gene expression analysis revealed that MBCs expressed a substantially higher fraction of triple-negative subtypes (ER, PR, and Her2) (21, 22). Considering local invasion, IDC has a more aggressive manner than MBC, and a previous study showed that MBC expressed a higher negative rate of lymph nodes than IDC (75.0% *vs* 47.9%, $P = 0.0014$) (23). In our study, the clinical features of 2,001 patients with MBC were analysed, and hormone receptor-negative cases comprised the majority. Her2 expression status was not included in this study as a prognostic factor. This was related to the number of cases that did not report Her2 expression before the 2010 SEER database. In our research, the prognosis of hormone receptor-negative AMCB patients was significantly worse than that of MBC patients. This was similar to the study of Shokouh et al. (24) and referred to the relationship of Ki67, Her2, p53, ER, and PR status and breast carcinoma subtypes. These previous studies have confirmed the possibility that hormone receptors will be used as prognostic indicators for AMCB.

The SEER database provides extensive data on breast cancer patients, which makes our study more convenient. However, there are limits to what we are able to do using this information. It is well known that there are no Her2 expression data in the SEER database prior to 2010. Thus, we are not able to group patients according to the 2015 St. Gallen consensus breast cancer classification; we can only separate the hormone receptor status into a single stratified study of the prognosis. In addition, different treatments for breast cancer have different effects on prognosis. The SEER database lacks data about patients who have received targeted therapy, endocrine therapy, and chemotherapy. Previous studies also used the SEER database and demonstrated that MBCs had distinctive clinicopathological characteristics, such as higher grade, larger tumour size, advanced stage, younger age at diagnosis, and a higher proportion of triple-negative breast cancer (7). The SEER database is more convenient for a specific pathologic review of specimens than for histological diagnosis.

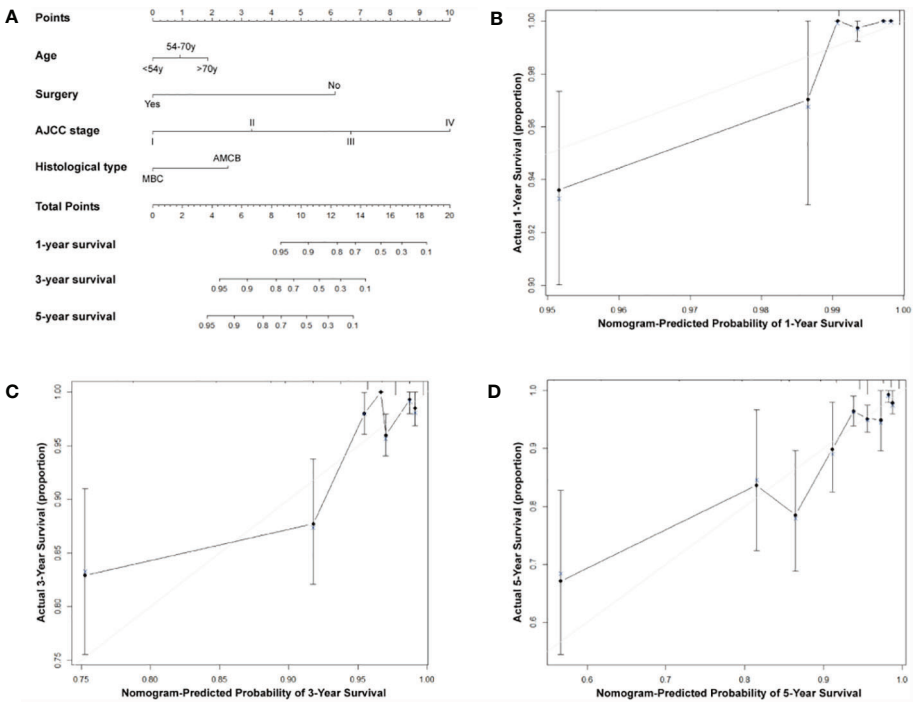

FIGURE 4 | Predictive nomogram assessed by clinical characteristics for 1-, 3-, and 5-year overall survival in MBC and AMCB patients who were HR-negative. **(A)** The probability of 1-, 3-, or 5-year overall survival was estimated by connecting the probability scale to the total point scale through a vertical line. The calibration curves for overall survival at 1 year **(B)**, 3 years **(C)**, and 5 years **(D)** are shown.

In conclusion, the Cox analysis of multivariate and Kaplan–Meier analyses revealed that the prognostic factors of AMCB were worse than those of MBC in the hormone receptor-negative cohort. The prognostic factors were related to age, stage, and cancer-directed surgery. Finally, a novel nomogram based on the prognostic factors that combined all significant independent variables was established for the overall survival of AMCB patients and could be used to suggest a more applicable treatment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://seer.cancer.gov/data/.

ETHICS STATEMENT

Our study was approved by the Ethics Committee of Changzheng Hospital. All patients provided written informed consent prior to enrolment in the study.

AUTHOR CONTRIBUTIONS

WQ, FQ, and MG participated in the conception and design of the study. FQ, LZW, and Y-SZ performed the statistical and bioinformatics analyses. WQ, FQ, MG, and Y-SZ coordinated, drafted, revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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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.

frontiers in Oncology

REVIEW
published: 26 July 2021
doi: 10.3389/fonc.2021.654428

Anticancer Mechanisms of Salinomycin in Breast Cancer and Its Clinical Applications

Hui Wang[1†], *Hongyi Zhang*[2†], *Yihao Zhu*[1], *Zhonghang Wu*[3,4], *Chunhong Cui*[1,3*‡] *and Fengfeng Cai*[2*‡]

[1] Laboratory of Tumor Molecular Biology, School of Basic Medical Sciences, Shanghai University of Medicine and Health Sciences, Shanghai, China, [2] Department of Breast Surgery, Yangpu Hospital, School of Medicine, Tongji University, Shanghai, China, [3] Department of Scientific Research, Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital, Shanghai, China, [4] Shanghai Key Laboratory of Molecular Imaging, Shanghai University of Medicine and Health Sciences, Shanghai, China

Breast cancer (BC) is the most frequent cancer among women worldwide and is the leading cause of cancer-related deaths in women. Cancer cells with stem cell-like features and tumor-initiating potential contribute to drug resistance, tumor recurrence, and metastasis. To achieve better clinical outcomes, it is crucial to eradicate both bulk BC cells and breast cancer stem cells (BCSCs). Salinomycin, a monocarboxylic polyether antibiotic isolated from *Streptomyces albus*, can precisely kill cancer stem cells (CSCs), particularly BCSCs, by various mechanisms, including apoptosis, autophagy, and necrosis. There is increasing evidence that salinomycin can inhibit cell proliferation, invasion, and migration in BC and reverse the immune-inhibitory microenvironment to prevent tumor growth and metastasis. Therefore, salinomycin is a promising therapeutic drug for BC. In this review, we summarize established mechanisms by which salinomycin protects against BC and discuss its future clinical applications.

Keywords: salinomycin, breast cancer, mechanism, clinical application, cancer stem cells

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***Correspondence:**
Fengfeng Cai
caifengfeng@tongji.edu.cn
Chunhong Cui
cuich@sumhs.edu.cn

[†]These authors have contributed equally to this work and share first authorship

[‡]These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *16 January 2021*
Accepted: *28 June 2021*
Published: *26 July 2021*

Citation:
Wang H, Zhang H, Zhu Y, Wu Z, Cui C and Cai F (2021) Anticancer Mechanisms of Salinomycin in Breast Cancer and Its Clinical Applications. Front. Oncol. 11:654428. doi: 10.3389/fonc.2021.654428

INTRODUCTION

According to the International Agency for Research on Cancer of the World Health Organization, the number of new breast cancer (BC) cases increased to 2.26 million in 2020, exceeding the number of lung cancer cases (2.21 million in 2020) for the first time; thus, BC has officially replaced lung cancer as the most common cancer worldwide. BC accounts for 1/4 of female cancer cases and 1/6 of female cancer-related deaths. In most countries, it is now the greatest threat to the health of women.

Owing to the limitations of current therapies, many patients die from metastasis, recurrence, and drug resistance (1, 2). In the last two decades, studies have revealed that breast cancer stem cells (BCSCs), a small subpopulation of cells, have been shown to have the ability to self-renew and differentiate into tumor cells. The clinical relevance of BCSCs has been a focus of many studies (3, 4), which have demonstrated that these cells are resistant to conventional chemotherapy and radiation

treatment and are very likely to be the origin of cancer relapse and metastasis (5–9). Hence, to improve clinical outcomes, it is crucial to eradicate both bulk BC cells and BCSCs.

Salinomycin is a monocarboxylic polyether antibiotic derived from *Streptomyces albus* (10). Its chemical structure is shown in **Figure 1**. It functions on different biological membranes and shows high affinity for positive ions, especially potassium, interfering with the balance of ion concentrations between the inside and outside of cells, thereby affecting osmotic pressure and eventually leading to germ cell disruption (12). At the end of the last decade, the preferential toxicity towards cancer stem cells (CSCs) has been reported by Gupta and colleagues (13). In addition to BC, salinomycin can also selectively kill CSCs in many other types of cancers, such as ovarian, lung, prostate, and colorectal cancers (14–19). The multiple function of salinomycin against CSCs and its molecular mechanism have been intensively studied in many types of cancer cells, with these studies extensively reviewed in several publications (11, 20–22). These reviews have also described the chemical properties of salinomycin in detail and summarized its role in cancer cells and CSCs. Extensive studies on BC have established that salinomycin inhibits cell proliferation, invasion, and migration, modulates cell death, and reverses the immune-inhibitory microenvironment to prevent tumor growth and metastasis (13, 16, 23–26). Moreover, salinomycin can be used to kill BC cells with drug resistance (27, 28). Therefore, salinomycin is expected to be a potent therapeutic drug for BC.

In this review, we summarize our current understanding of the mechanisms by which salinomycin inhibits BC, including its precise effects on BCSC proliferation, invasion, migration, and apoptosis. Furthermore, we discuss its potential applications in BC therapy, including the potential utility of nanocarrier-based delivery systems and combination therapies.

EFFECTS OF SALINOMYCIN ON BCSCS

CSCs are tumor cells with stem cell characteristics and initiate cancer formation. They form and maintain heterogeneous tumor entities through self-renewal and asymmetric division (29). Compared with highly differentiated tumor cells, CSCs have a lower degree of differentiation, stronger drug metabolism, and the ability to repair drug-induced damage (30). They can renew themselves through epigenetic changes, acquire new mutations, and quickly adapt to the environment, thereby forming a new tumor entity with molecular characteristics very differently from those of the initial tumor. Therefore, CSCs are also considered to be the source of tumor metastasis and drug resistance (31). If therapies only target differentiated tumor cells and cannot effectively eliminate CSCs, tumor recurrence will be inevitable. Hence, effective inhibition of differentiated tumor cells and simultaneous and specific elimination of CSCs are the focus of current research in the field of tumor therapy.

Salinomycin is over 100 times more effective against BCSCs than paclitaxel, the traditional chemotherapy drug for the treatment of BC (26). Salinomycin increases the apoptosis of BC mammosphere cells, which is accompanied by downregulation of Bcl-2 expression, and decreases their migration capacity, which is accompanied by downregulation of c-Myc and Snail expression (23). Furthermore, extensive studies on BCSCs have established that salinomycin decreases CSC population (32–34). Global gene expression analyses have shown that salinomycin inhibits the expression of BCSC genes (13). In addition, consistent with previous findings in prostate cancer, salinomycin reduces aldehyde dehydrogenase activity and the expression of MYC, AR, and ERG; it induces oxidative stress and inhibits nuclear factor (NF)-κB activity (35). In BC cells, salinomycin influences stem cell signaling, such as Wnt and Hedgehog signaling, or ALDH1 activity, to suppress mammosphere formation, induce cell apoptosis, or inhibit cell proliferation (34, 36–39). Further, salinomycin effectively inhibits mammary tumor growth *in vivo*. In BC xenograft tumors, it significantly reduces tumor growth, which is accompanied by decreased PTCH, SMO, Gli1, and Gli2 expression (23).

Although the exact mechanisms underlying the effects of salinomycin on BCSCs are not fully understood, recent studies have provided insights into its molecular mechanisms and modes of action. The effect of salinomycin on BCSCs and BC cells through various mechanisms of action and its molecular targets have been summarized in this review (see **Tables 1**, **2** and **Figure 2**).

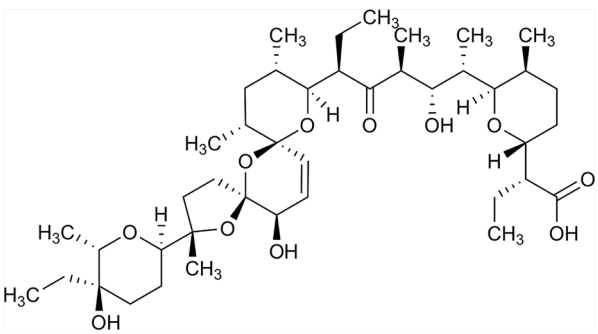

FIGURE 1 | Chemical structure of Salinomycin, edit from (11).

MULTIPLE FUNCTIONS OF SALINOMYCIN IN BC

Salinomycin and its derivatives have multiple functions in BC cells. We review evidence for its precise effects on various cellular processes in BC and the mechanisms underlying these effects.

Three major cell death pathways have been described: apoptosis, autophagy, and necrosis (48). Cancer cell death induced by salinomycin is achieved by multiple mechanisms (13, 16, 24). In fact, accumulating evidence shows that apoptosis and autophagic cell death can both occur, with crosstalk between the two pathways. Furthermore, both pathways may eventually lead to necrosis (secondary necrosis), depending on the stimulus and cell type (49).

TABLE 1 | Anticancer mechanisms of salinomycin in BCSCs.

Mechanisms	BCSCs	Molecular targets of salinomycin	Activity *in vitro/ in vivo*	Ref.
Affect iron homeostasis	HMLER $CD44^{high/}$ $CD24^{low}$ cells	Salinomycin accumulates and sequesters iron in lysosomes and triggers ferritin degradation in response to iron depletion, further activating a cell death pathway consistent with ferroptosis.	*in vitro* and *in vivo*	(40)
Affect apoptosis, cell growth and migration	MCF7 mammosphere cells	Salinomycin increases apoptosis by a decreased expression of Bcl-2; decreases the migration capacity accompanied by a decreased expression of c-Myc and Snail in MCF7 MS cells, and significantly reduces the tumor growth accompanied by decreased expression of the critical components of the Hedgehog pathway (PTCH, SMO, Gli1 and Gli2) in xenograft tumors.	*in vitro* and *in vivo*	(23)
Affect autophagic flux	ALDH(+) HMLER cells	Salinomycin has a relatively greater suppressant effect on autophagic flux, which may be affected by ATG7 and correlates with an increase in apoptosis.	*in vitro*	(41)
	$CD44+/CD24^{low}$ HMLER cells	Acidic conditions improve the ability of salinomycin to inhibit the autophagic flux and kill CSCs.	*in vitro*	(32)
Induce cell differentiation	MDA-MB-435 mammosphere cells	Salinomycin blocks the PKCa signaling pathway in the CSCs, resulting in the occurrence of plastic differentiation of CSCs	*in vitro* and *in vivo*	(33)
	$ALDH^{br}$ cell population of Br-Ca-MZ01, MDA-MB-436, S68, SUM149, and SUM15	Salinomycin or salinomycin/JQ1 drug combination reduces the BCSC pool by inducing cell differentiation	*in vitro* and *in vivo*	(42)

Effect of Salinomycin on the Apoptosis Pathway

Apoptosis is the major cell death pathway contributing to the removal of unnecessary and harmful cells during embryonic development, tissue homeostasis, and immune regulation. Apoptosis is mainly executed by intracellular cysteine proteases called caspases, which may be activated by two principal pathways: death receptor-dependent (extrinsic) and -independent (intrinsic or mitochondrial) pathways (50).

Salinomycin induces apoptosis in CSCs of different origins (18, 51, 52). However, the precise mechanisms vary substantially and are tightly correlated with the origin of the CSCs (26, 53–55).

Salinomycin increases DNA breaks in BC cells as well as the expression of phosphorylated p53 and γH2AX in Hs578T cells.

TABLE 2 | Anticancer mechanisms of salinomycin in BC cells.

Mechanisms	Cell lines	Molecular targets of salinomycin	Activity *in vitro/in vivo*	Ref.
Apoptosis	MCF7	Salinomycin inhibits the antiapoptotic genes BCL-2, BCL-XL, and BIRC5.	*in vitro*	(43)
	MCF7, T47D and MDA-MB-231	Salinomycin induces apoptosis through DNA damage, by which it activates γH2AX, a marker of double strand breaks, and through downregulation of survivin expression.	*in vitro*	(44)
	MCF7, MDA-MB-231, Hs578T	Salinomycin increases DNA damage, by which the phosphorylated levels of p53 and γH2AX was increased. Salinomycin also decreases the p21 protein level, which by the increased proteasome activity.	*in vitro*	(26)
	JIMT-1, MCF-7, and HCC1937	Salinomycin-induced ER Ca^{2+} depletion upregulates C/EBP homologous protein, which inhibits Wnt signaling by downregulating β-catenin. The increased cytosolic Ca2+ also activates protein kinase C, which has been shown to inhibit Wnt signaling.	*in vitro*	(37)
ROS induced autophagy and apoptosis	MCF-7, T47D, MDA-MB-453	Salinomycin leads to the formation of ROS eliciting JNK activation and induction of the transcription factor JUN. Moreover, salinomycin induces autophagy through the JNK pathway.	*in vitro*	(45)
	MCF-7, MDA-MB-231	Salinomycin-mediated ROS production leads to mitochondrial dysfunction and induces apoptosis and autophagy. Moreover, autophagy inhibition is involved in acceleration of apoptosis induced by salinomycin.	*in vitro*	(46)
Mitophagy and mitoptosis	SKBR3, MDAMB468	Salinomycin increases mitochondrial membrane potential (ΔΨ), decreases cellular ATP level, and induces mitophagy, mitoptosis	*in vitro*	(16)
Angiogenesis	MCF-7, T47D, MDA-MB-231, MDA-MB-468, 4T1	Salinomycin inhibits HIF-1α transcription factor activity and inhibits hypoxia-induced HIF-1α/VEGF signaling axis.	*in vitro* and *in vivo*	(47)
Cell proliferation	MCF-7, HS578T and MDA-MB-231 cells	Salinomycin inhibits LRP6, activates GSK3β, and suppresses the expression of cyclin D1 and survivin, two targets of both Wnt/β-catenin and mTORC1 signaling, displaying remarkable anticancer activity.	*in vitro*	(36)
	MCF7, T47D and MDA-MB-231	Salinomycin induces a p53-independent upregulation of $p21^{waf/cip}$ and leads to growth arrest.	*in vitro*	(44)
	MCF7	Salinomycin inhibits cell proliferation *via* downregulation of Smo and Gli1 in Hedgehog–Gli1 signaling.	*in vitro*	(39)
	MDA-MB-231	Salinomycin inhibits cell proliferation by downregulating the expression of key elements Shh, Smo, and Gli1 in the Hedgehog pathway.	*in vitro*	(38)
	MCF7, T47D and MDA-MB-231	Slinomycin suppresses mammosphere formation and tumor growth *in vivo via* the inhibition of ALDH1 activity and downregulates the transcription factors Nanog, Oct4 and Sox2.	*in vitro* and *in vivo*	(34)

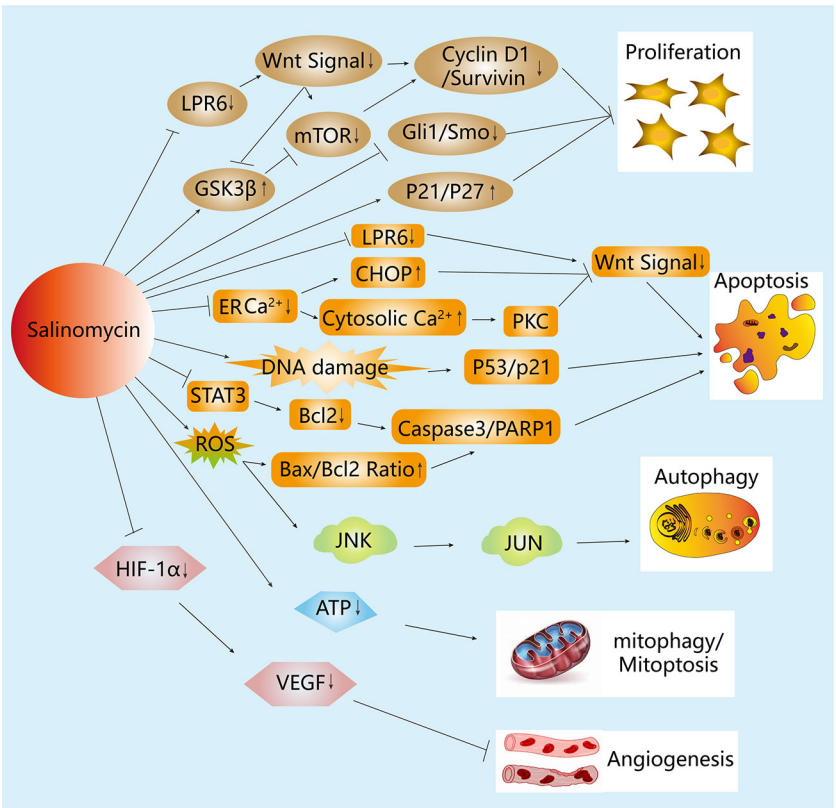

FIGURE 2 | Mechanisms of the anticancer activity of salinomycin in breast cancer (BC) cells. Salinomycin inhibits cell proliferation in BC cells through the Hedgehog and Wnt signaling pathways. Additionally, salinomycin is capable of inhibiting Wnt signaling by blocking the phosphorylation of LRP6 or by ER stress, which leads to apoptosis in BC cells. Moreover, salinomycin induces the apoptosis of BC cells by increasing DNA damage or intracellular ROS level, thereby influencing the p53 and caspase cascade separately. Salinomycin also induces autophagy by increasing intracellular ROS level, which is accompanied by MAPK signaling pathway activation. Furthermore, salinomycin can affect the cell membrane potential and reduce the level of ATP to induce mitophagy and mitoptosis. Moreover, salinomycin inhibits angiogenesis through the VEGF signaling pathway.

It was indicated that salinomycin increases DNA damage, and this effect plays an important role in increasing apoptosis (26). Furthermore, salinomycin induces the expression of the pro-apoptotic protein Bax but inhibits the expression of the anti-apoptotic protein Bcl-2 in MDA-MB231 cells, suggesting that an increase in the Bax/Bcl-2 ratio may be involved in salinomycin-induced apoptosis (46). The same result was shown in cisplatin-resistant $MCF7^{DDP}$ cells; in these cells, salinomycin abrogated nuclear translocation of NF-κB and caused a concurrent reduction in NF-κB-regulated expression of pro-survival proteins (27). Moreover, salinomycin reportedly activates a distinct apoptotic pathway that is not accompanied by cell cycle arrest and is independent of the tumor suppressor protein p53, caspase activation, the CD95/CD95L system, and proteasome (54). Interestingly, Yusra Al Dhaheri et al. reported that combination of salinomycin with paclitaxel or docetaxel can synergistically increase apoptosis in the BC cell line MDA-MB-231, which is not sensitive enough to salinomycin, providing a new insight for the clinical application of salinomycin (53).

These results suggest that the induction of apoptotic or non-apoptotic cell death by salinomycin in cancer cells depends on the particular cell type and that the detailed mechanisms underlying salinomycin-induced cell death in cancer cells remain to be fully elucidated.

Effect of Salinomycin on the Autophagy Pathway

Autophagy is the catabolic process that regulates the degradation of a cell's own components *via* the lysosomal machinery, characterized by the appearance of large autophagic vacuoles in the cytoplasm. It can be considered a physiological process used for the recycling of damaged organelles and energy supply during "lean times," leading to cell death if extensively activated. At low levels, autophagy promotes cell survival by removing damaged proteins and organelles while supplying extra energy; however, the excessive and long-term upregulation of autophagy eventually results in the destruction of essential proteins and organelles beyond a certain threshold, resulting in cell death. Salinomycin reportedly induces and inhibits autophagy (56).

On the one hand, a substantial autophagic response to salinomycin (substantially stronger than the response to the

commonly used autophagy inducer rapamycin) has been detected in BC cells (SKBR3 and MDA-MB-468 cells) and to a lesser degree in normal human dermal fibroblasts (16). Salinomycin strongly decreases ATP levels in BC cells in a time-dependent manner. Conversely, following treatment with salinomycin, human normal dermal fibroblasts exhibit decreased mitochondrial mass, although they are largely resistant to salinomycin-induced ATP depletion. Autophagy caused by salinomycin has a protective effect on various cancer cell lines, but experiments have shown that by blocking the expression of ATG7 with siRNA (41), this protective effect can be reversed. Therefore, the combined use of autophagy blockers in the clinic will be more effective.

On the other hand, the inhibitory effect of salinomycin on autophagy may explain its ability to block the degradation of LC3 and long-lived proteins. The suppressive effect of salinomycin on autophagic flux is relatively higher in the ALDH(+) population in HMLER cells than in the ALDH(−) population, and this differential effect is correlated with an increase in apoptosis in the ALDH(+) population. ATG7 depletion accelerates the proapoptotic capacity of salinomycin in the ALDH(+) population (41). Similarly, Pellegrini et al. found that CSC-like cells have greater sensitivity to autophagy inhibition than that of cells not expressing CSC markers (32).

Effect of Salinomycin on Necrosis

Different from apoptosis, necrosis is another type of cell death that is typically not associated with the activation of caspases. It is characterized by the swelling of the endoplasmic reticulum (ER), mitochondria, and cytoplasm, with the subsequent rupture of the plasma membrane and cell lysis. Necrotic cell death is the result of interplay between several signaling cascades. The determinants of necrosis are mainly RIP3, calcium, and mitochondria. RIP3 interacts with RIP1 and binds to several enzymes involved in carbohydrate and glutamine metabolism. Calcium controls the activation of PLA, calpains, and NOS, inducing a series of events leading to necrotic cell death.

According to Xipell et al., in glioblastoma cells, salinomycin induces substantial ER stress, triggering the unfolded protein response and an aberrant autophagic flux that culminates in necrosis due to mitochondria and lysosomal alterations (57). However, the mechanism underlying necrosis in BC has not been revealed. It may occur as a consequence of massive cell injury or in response to extreme changes in physiological conditions.

Effects of Salinomycin on Cell Cycle Arrest

Treatment with salinomycin hampers the proliferation of BC cells, and this effect is mediated by different mechanisms. For example, salinomycin inhibits proliferation in MDA-MB-231 cells in concentration- and time-dependent manners; in particular, it blocks the G1-to-S phase transition by the downregulation of genes downstream of the Hedgehog signaling pathway (38). In addition, salinomycin blocks the proliferation of BC cells by suppressing cyclin D1 expression and the GSK3β-mediated inhibition of the Wnt signaling pathway (36). It also suppresses BCSC proliferation, concomitant with the downregulation of cyclin D1 and increased p27(kip1) nuclear accumulation (34). Furthermore, an interesting study has shown that at low concentrations, salinomycin could induce transient G1 arrest at early time points and G2 arrest at late time points as well as senescence, in addition to inducing an enlarged cell morphology, the upregulation of p21 protein, and increases in histone H3 and H4 hyperacetylation and SA-β-Gal activity (44).

Salinomycin and Reactive Oxygen Species (ROS)

ROS, which are products of normal metabolism and xenobiotic exposure, can be beneficial or harmful to cells and tissues depending on their concentration. ER stress can be an initiator of apoptosis and occurs when unfolded proteins accumulate; ER Golgi transport is inhibited, or the Ca^{2+} equilibrium is disrupted in cells. Excessive ROS production triggers oxidative modification of cellular macromolecules, inhibits protein function, and promotes cell death.

The elevated oxidative stress and mitochondrial membrane depolarization in response to salinomycin-mediated apoptosis were first reported in prostate cancer cells (55). Salinomycin can induce an increase in intracellular ROS levels, accompanied by decreased mitochondrial membrane potential, causing ER damage, with increased levels of Ca^{2+} being released into the cytoplasm; this process is regulated by Bcl-2 and Bax/Bak, further activating caspase-3 and the cleavage of PARP-1, ultimately leading to mitochondrial apoptosis. Moreover, Bcl-2, Bax, and Bak are also localized in the ER; thus, the ER serves as an important organelle for apoptotic control that is further enhanced by the mitochondria ER connection.

In BC cell lines (MCF-7, T47D, and MDA-MB-453), salinomycin induces ROS formation, causing JNK activation and induction of the transcription factor JUN. Salinomycin-mediated cell death was partially inhibited by *N*-acetyl-cysteine (NAC), a free radical scavenger, suggesting ROS formation contributes to the toxicity of salinomycin (45). This has been observed in MDA-MB-231 cells, in which salinomycin-mediated ROS production leads to mitochondrial dysfunction, and NAC attenuates salinomycin-induced apoptosis and autophagy. This result seems to conflict with the fact that the acceleration of apoptosis induced by salinomycin is related to autophagy inhibition. NAC thereby makes the function of salinomycin more complex. This indicates that the crosstalk between two different physiological responses (autophagy and apoptosis) induced by salinomycin might play a pivotal role in the determination of the fate of cancer cells (46).

Furthermore, ionomycin (AM5), a synthetic derivative of salinomycin, exhibits potent and selective activity against BCSCs *in vitro* and *in vivo* by accumulating and sequestering iron in lysosomes. Iron-mediated ROS production reportedly promotes lysosomal membrane permeabilization, thereby activating a cell death pathway consistent with ferroptosis (40). These findings highlight the importance of iron homeostasis in BCSCs, suggesting both iron- and iron-mediated processes to be potential therapeutic targets in BC.

Effects on Tumor Cell Migration

Epithelial-to-mesenchymal transition (EMT) is the major cause of BC invasion and metastasis. In addition to substantial inhibitory effects on invasion and metastasis in BC, as determined by single-cell tracking, salinomycin significantly reduces the metastatic tumor burden in mice (58). Furthermore, various migration-related parameters in MDA-MB-231 cells are significantly lower after salinomycin treatment than in control cells, and the effects of salinomycin are concentration-dependent (59). Further, salinomycin inhibits the TGF-β1-induced EMT phenotypic transition and activation of Smad (p-Smad2/3 and Snail1) and non-Smad (β-catenin, p-p38, and MAPK) signaling molecules, which cooperatively regulate EMT induction. Importantly, these findings were confirmed in a series of BC specimens, in which there were strong correlations among levels of E-cadherin and β-catenin and the lymph node metastatic potential of BC (60).

Effect on Neovascularization in BC

The characteristics of CSCs have been associated with angiogenesis. It has been reported that the expression of several CSC biomarkers correlates with that of angiogenesis markers, and some stem cell signaling pathways (*e.g.*, Notch) are used by both CSCs and by pro-angiogenic factors (61). This indicates that treatment linked to anti-angiogenic therapy and targeted at CSCs may be effective in the treatment of many malignant tumors, including BC. The antiangiogenic and anticancer efficacies of salinomycin in BC have been explored. In particular, salinomycin interrupts HIF-1α/VEGF signaling to inhibit VEGF-induced angiogenesis and BC growth. Moreover, it inhibits the expression of the pro-angiogenic cell surface marker CD31, thereby disrupting endothelial tubulogenesis, thereby interrupting endothelial tubulogenesis, decreases the binding of HIF-1α to the HRE sequence in human BC cells and suppresses neovascularization in a chick chorioallantoic membrane and a Matrigel plug-implanted mouse model (47). Salinomycin inhibits BC growth and tumor angiogenesis in mice based on bioluminescence and immunofluorescence imaging analyses. It also suppresses serum VEGFA levels in tumor-bearing mice and induces caspase-dependent apoptosis in BC cells (47).

Effects on the Immune Microenvironment

Salinomycin could play a role in modulating the immune microenvironment in BC. It may exert these modulatory effects by two different mechanisms. First, at insufficient concentrations for direct antitumor activity, it could effectively stimulate M1 type macrophages and limit tumor growth and metastasis. In a previous study, the intratumoral injection of salinomycin increased the proportion of CD86 cells and decreased CD206 cells in transplant 4T1 tumors, thereby preventing tumor growth and pulmonary metastasis (62). Second, salinomycin could potently reverse tumor immune tolerance *via* the repression of IDO1 enzymatic activity. Moreover, it suppresses and inhibits IFN-γ-induced activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the nuclear factor NF-κB pathway, respectively, by inhibiting IκB degradation and NF-κB phosphorylation. Furthermore, it restores the proliferation of T cells co-cultured with IFN-γ-treated BC cells and potentiates the antitumor activity of cisplatin *in vivo* (63).

THE POSSIBLE MOLECULAR MECHANISMS OF SALINOMYCIN

Salinomycin is a membrane ionophore that facilitates ion flux through the cytoplasmic and mitochondrial membranes. It works as a mobile carrier and discharges K^+ rapidly (64–66). However, recent studies showed that salinomycin physically targets the lysosomal compartment, and not the ER, mitochondria, or the Golgi apparatus in CSCs (40, 67). It then accumulates in the lysosomal compartment in an endocytosis-independent manner (40). In the lysosomal compartment, salinomycin can interact with iron(II) and inhibit the effective translocation of the metal into the cytosol, thereby blocking the release of iron from lysosomes and resulting in lysosomal iron accumulation. The elevated iron level in lysosomes promotes enhanced ROS reaction *via* Fenton chemistry (68), possibly through lysosomal degradation of ferritin and the release of additional soluble redox-active iron, subsequently inducing lysosomal membrane permeabilization (LMP) (69). Compared with normal cells, cancer cells are sensitive to LMP through a variety of mechanisms, such as altered lysosomal localization, decreased LAMP-1 and LAMP-2 level (70), increased lysosomal size, altered heat shock protein 70 localization, and elevated sphingosine level (71–74). Particularly, CSCs contain significantly higher levels of iron, which either directly alter their differentiation or cause them to be selected for proliferation in favor of a subpopulation exhibiting a pronounced CSC phenotype.

Therefore, in CSCs, salinomycin can trigger a much stronger ROS reaction and severe LMP. Salinomycin has been shown to activate distinct cell responses, including autophagy, apoptosis, and necrosis. This is likely linked to the extent of LMP in various cancer cells in response to salinomycin. Lysosomes are intrinsically heterogeneous, and not all lysosomes are permeabilized simultaneously in response to lysosomal stress (75, 76). Thus, damage to a small proportion of lysosomes can be fixed by activating lysophagy and other endolysosomal damage response mechanisms, thereby ensuring cell survival. However, when majority of lysosomes are damaged or autophagy is inhibited, the damaged lysosomes can no longer be eliminated by lysophagy, causing cell death. This point has not been addressed by previous investigations on salinomycin, and thus requires clarification. In some cancer cells, salinomycin triggered LMP and resulted in the translocation of cathepsins from lysosomes to the cytosol, where they induced the proteolytic activation of substrates, such as Bid and Bax, which in turn, promoted mitochondrial outer membrane permeabilization and caspase activation (77–80). This cell death can be inhibited by blocking cathepsin activity with protease inhibitors or by increasing the activity of iron

chelators and endogenous cathepsin inhibitors, such as serpins and cystatins (81). However, under some circumstances, complete lysosomal rupture can also lead to uncontrolled necrosis. Ferroptosis is a regulated cell death pathway. A recent publication showed that salinomycin can kill CSCs by sequestering iron in lysosomes *via* a process triggered by severe lipid peroxidation related to ROS production and iron availability, and such cell death could be partially prevented by the ferroptosis inhibitor, ferrostatin-1, whereas the apoptosis and necrosis inhibitors Z-VAD-FMK and necrostatin-1, respectively, could not influence the cell death profiles (67). The possible molecular mechanisms underlying the effect of salinomycin through the lysosomal compartment on BC cells have been summarized in this review (see **Figure 3**).

APPLICATION OF SALINOMYCIN IN BC THERAPY

As a potent therapeutic agent against cancer, salinomycin is still in the initial phase of the preclinical stage. Up to date, there is still no registered clinical trial about salinomycin. Two individual cases have been described in the literature as follows: one describing a 40-year-old female patient with metastatic (bone and subcutaneous) invasive ductal BC and another describing an 82-year-old female patient with advanced and metastatic (pelvic lymphatic metastasis) squamous cell carcinoma of the vulva. Both cases were treated systemically with intravenous administration of salinomycin (200–250 μg/kg salinomycin every second day), which partially regressed metastasis for 3 weeks and showed only minor adverse effects, as opposed to the severe adverse effects commonly observed with conventional chemotherapy (24). These findings confirm the safety and efficacy of salinomycin for selective clinical use. However, the potential toxicity of salinomycin may need to be studied in larger clinical trials.

POTENTIAL COMBINATION THERAPY WITH SALINOMYCIN

Tumors are composed of heterogenous cell populations with various mutations and/or phenotypes. Therefore, it is generally ineffective to use a single drug to prevent cancer progression. For the complete eradication of cancers, several drugs with distinct mechanisms but complementary anticancer activities (*i.e.*, combination therapies) are often used to enhance the antitumor efficacy and minimize the risk of developing drug resistance. The recent development of innovative delivery methods using biocompatible nanocarriers has enabled studies of various combinations. In particular, combination therapy with salinomycin can achieve promising results, and selective delivery *via* biocompatible nanocarriers provides a novel therapeutic approach (82–85). The effects of various combination strategies with salinomycin on BC cells have been summarized in **Table 3**.

Salinomycin and dasatinib (a Src kinase inhibitor) have a synergistic effect in BC cells *via* the horizontal suppression of multiple pathways (96). This effect possibly involves the promotion of cell cycle arrest at the G1/S phase through both the estrogen-mediated S phase entry and BRCA1 and DNA damage response pathways.

Additionally, salinomycin increases cancer cell sensitivity to the apoptotic effects of doxorubicin (DOX) or etoposide (ETO). pH2AX, pBRCA1, p53BP1, and pChk1 levels substantially increase in response to co-treatment with salinomycin and either DOX or ETO. The level of the anti-apoptotic p21

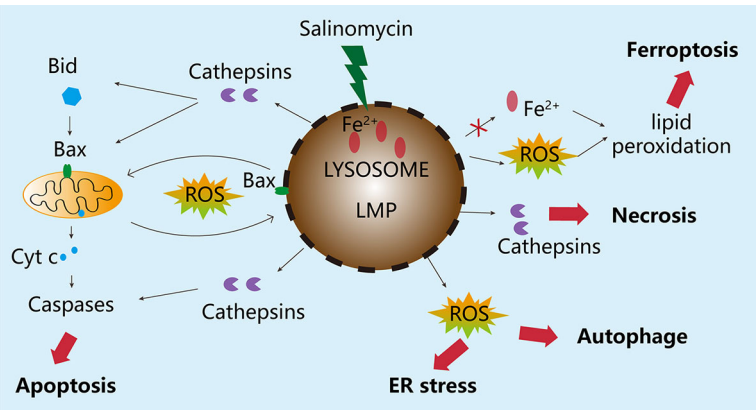

FIGURE 3 | Molecular mechanisms underlying the effects of salinomycin through the lysosomal compartment. Salinomycin is enriched in the lysosome of breast cancer tumor cells, leading to imbalance in Fe+ metabolism, excessive accumulation of Fe ions, and degradation of ferritin, which causes the release of a large amount of free radicals and ultimately induces LMP. Depending on the degree of damage to the lysosome, different responses will be triggered. If the damage to the lysosome is minor, the stress sensor and response mechanisms, including autophagy, will be activated to ensure cell survival. However, when the degree of LMP is very high, the damage cannot be repaired. As a result, special cathepsins will be released from the lysosome into the cytoplasm, causing the disruption of mitochondrial membrane integrity, leading to apoptosis. In the case of severe damage to the lysosome, the release of enzymes from the lysosome can also cause uncontrolled cell death, such as necrosis edit from (69).

TABLE 3 | Synergistic anticancer co-action of salinomycin with other agents in BC.

Synergy	Cell lines	Effect against cancer cells	Activity *in vitro*/ *in vivo*	Ref.
Conventional chemotherapy	Hs578T	Salinomycin sensitizes paclitaxcel-, docetaxcel-, vinblastin-, or colchicine-treated cancer cell lines; on one side salinomycin increases pH2AX level and reduces p21 level; this leads to mitotic catastrophe; on the other side, salinomycin reduces cyclin D1 level to prevent G2 arrest.	*in vitro*	(53)
	MDA-MB-231 or MCF7 mammospheres	Combination therapies of salinomycin with paclitaxel or lipodox show a potential to improve tumor cell killing	*in vitro*	(86)
	MDA-MB-231, MCF7 and Hs578T	Salinomycin increases the sensitivity of cancer cells to the apoptotic effects of doxorubicin or etoposide	*in vitro*	(26)
	MCF7 cells and MCF7-MS	Nanoparticles loaded with salinomycin and docetaxel at molar ratio of 1:1 is synergistic.	*in vitro* and *in vivo*	(87)
	MCF7 side population cells	Octreotide-modified paclitaxel-loaded PEG-b-PCL polymeric micelles and salinomycin-loaded PEG-b-PCL polymeric micelles combination has the strongest antitumor efficacy.	*in vitro* and *in vivo*	(88)
	MDA-MB-231, MCF7	Combination of hyaluronic acid-coated salinomycin nanoparticles and paclitaxel nanoparticles shows the highest cytotoxicity against CD44(+) cells	*in vitro*	(89)
	MDA-MB-231 $CD44^{+}$/ $CD24^{-/low}$ CSCs and no CSCs	Single-walled carbon nanotubes conjugated to CD44 antibodies with salinomycin and paclitaxel could target and eradicate both whole tumor cells and CSC populations.	*in vitro* and *in vivo*	(85)
	MCF7	Hyaluronic acid-coated vitamin E-based redox-sensitive salinomycin prodrug nanoparticles were fabricated to deliver paclitaxel for cancer-targeted and combined chemotherapy, and this led to maximize the chemotherapeutic effect	*in vitro*	(90)
HER2	BT-474 and MDA-MB-361 and breast cancer xenografts	Sali-NP-HER2 nanoparticles efficiently bound to HER2-positive BCSCs and BC cells, resulting in enhanced cytotoxic effects compared with non-targeted nanoparticles or salinomycin	*in vitro* and *in vivo*	(91)
	MCF7	Herceptin-immobilized salinomycin-encapsulated poly (lactic-co-glycolic acid) nanoparticles could successfully be uptaken by MCF7 cells	*in vitro*	(92)
	MCF7 mammospheres	Combinatorial treatment of mammospheres with trastuzumab and salinomycin is superior to single treatment with each drug	*in vitro*	(93)
Estrogen receptor	MCF7, T47D	Salinomycin induces an additional cytotoxic effect when treated combinational with tamoxifen by inhibiting the ligand independent activation of Erα.	*in vitro*	(94)
	MCF7/LCC2 and MCF7/LCC9 cell lines	Salinomycin has synergistic effect with tamoxifen and enhanced tamoxifen sensitivity by decreasing AIB1 expression	*in vitro*	(95)
Small molecule inhibitor	MDA-MB-468, MDA-MB-231, MCF7	The combination of salinomycin and dasatinib (a Src kinase inhibitor) shows enhanced potency against human BC cell lines and tumor spheroids.	*in vitro*	(96)
	HCC1937 cells	Combination of LBH589(a histone deacetylase inhibitor) and salinomycin has a synergistic inhibitory effect on TNBC BCSCs by inducing apoptosis, arresting the cell cycle, and regulating EMT.	*in vitro* and *in vivo*	(82)
	Hs578T	Co-treatment of salinomycin sensitizes AZD5363 (an inhibitor of protein kinase B)-treated cancer cells through increased apoptosis	*in vitro*	(97)
Phytoalexin	MDA-MB-231, MDA-MB-468 and T47D	Resveratrol combination with salinomycin exerts synergistic anti-proliferative activity on BC cells.	*in vitro*	(98)
	MDA-MB-231	Resveratrol and salinomycin inhibit EMT (fibronectin, vimentin, N-cadherin, and slug); chronic inflammation (Cox2, NF-kB, p53), autophagy (Beclin and LC3) and apoptotis (Bax, Bcl-2) markers.	*in vitro* and *in vivo*	(99)

protein increases in response to DOX or ETO but decreases in response to salinomycin, which increases proteasome activity. These results indicate that the ability of salinomycin to sensitize cancer cells to DOX or ETO is associated with an increase in DNA damage and a decrease in p21 levels. Accordingly, salinomycin-based chemotherapy may be beneficial for patients with cancer receiving DOX or ETO (26).

The development of innovative delivery systems has significantly improved the efficacy of combination therapy (100). For example, Gao et al. (87) found that the synergistic effects of salinomycin and docetaxel could be effectively maintained *in vivo* by the co-encapsulation of these agents in PLGA/TPGS nanoparticles, providing a promising strategy for BC treatment.

Moreover, combining therapies targeting CSCs with conventional chemotherapy may be a useful strategy for cancer treatment (83). A single-walled carbon nanotube (SWCNT) drug delivery system combining paclitaxel, salinomycin, and biocompatible CD44 antibody-conjugated SWCNTs *via* a hydrazone linker had better effects than did individual drug-conjugated nanocarriers or free drug suspensions in both *in vitro* and *in vivo* assays (85). Moreover, another study has shown that an elastin-like polypeptide-salinomycin nanoparticle has a long release half-life *in vitro* and results in a lower CSC frequency in 4T1 orthotopic tumors than in untreated tumors or free salinomycin-treated tumors (84). Furthermore, HER2 is overexpressed in both breast CSCs and cancer cells and can also be utilized for targeted delivery systems. Salinomycin-loaded

polymer-lipid hybrid anti-HER2 nanoparticles (Sali-NP-HER2) were developed to target both HER2-positive breast CSCs and cancer cells, with a high delivery efficacy and low untargeted toxicity (91).

Simultaneous eradication of both CSCs and cancer cells is necessary for optimizing therapeutic efficacy (101). The combination of salinomycin and traditional therapies has proven to be effective in BC; however, the proximal mechanisms underlying the effects of salinomycin on CSCs remain unclear and should be a focus of future studies.

CONCLUSION

The potency of tumor initiation and drug resistance of BCSCs are key factors contributing to the metastasis and recurrence of BC and thus increasing mortality. Salinomycin in combination with traditional chemotherapy and targeted therapy can kill both common cancer cells and CSCs, providing a very promising clinical strategy to treat BC. Salinomycin has multiple functions in the regulation of cellular processes in BC cells. Importantly, it functions in the regulation of cell death by a highly complex mechanism involving multiple pathways and interactions among several cell death-related biological processes. Its effects may be mediated by Bax/Bak, the activation of mitoptosis, irreversible deterioration of mitochondrial structure, or other apoptogenic factors associated with apoptosis released from mitochondria. Owing to the limited research focused on the mechanisms of action of salinomycin, further studies of its precise effects on apoptosis and autophagy, as well as the interactions among mitoptosis, mitophagy, ferroptosis, and necrosis are needed. In addition, suitable methods for the identification and isolation of CSCs are lacking despite their key importance for studies of the effects of salinomycin and the development of CSC-targeted therapy. Furthermore, a better understanding of the different characteristics of somatic stem cells and CSCs, such as the signaling pathways that regulate self-renewal and cell fate, would be important for the design of new strategies targeting CSCs.

AUTHOR CONTRIBUTIONS

HW, HZ, and FC: conceptualization. HW, HZ, and CC wrote the manuscript. YZ, ZW, and FC: manuscript review and critical comments. All authors contributed to the article and approved the submitted version.

FUNDING

This review article was supported by the National Natural Science Foundation of China (no. 81802961), the Shanghai Yangpu District Health and Family Planning Commission Fund for Hao Yi Shi Training Project (2020-2023), and the Natural Science Foundation of Shanghai (grant no. 18ZR1436000).

ACKNOWLEDGMENTS

We thank all of the participants for their participation.

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GLOSSARY

AIB1	amplified in breast cancer 1
ALDH	acetaldehyde dehydrogenase
ATG7	autophagy related protein 7
ATP	adenosine triphosphate
BCSCs	breast cancer stem cells
BIRC5	baculoviral IAP repeat containing 5
C/EBP	CCAAT/enhancer binding protein
CHOP	C/EBP homologous protein
Cox2	cyclooxygenase-2
CSCs	cancer stem cells
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
Erα	estrogen receptor α
GSK3β	glycogen synthase kinase-3 β
H2AX	H2A histone family member X
HIF-1	hypoxia inducible factor 1
JNK	Jun N-terminal kinase
LC3	the microtubule-associated protein 1 light chain 3
LMP	lysosomal membrane permeabilization
LRP6	LDL-receptor-related protein 6
mTORC1	mechanistic target of rapamycin complex 1
NF-kB	nuclear factor κ-light chain-enhancer of activated B cells
PARP1	poly(ADP-ribose) polymerase 1
PEG-b-PCL	poly (ethylene glycol)-block-poly (ε-caprolactone)
PKCα	protein kinase C α
PTCH	patched homolog
ROS	reactive oxygen species
Sali-NP-HER2	salinomycin-loaded polymer-lipid hybrid anti-HER2 nanoparticles
SMO	smoothened
STAT3	signal transducer and activator of transcription 3
TNBC	triple-negative breast cancer
VEGF	vascular endothelial growth factor
Wnt	wingless/integrated

frontiers in Oncology

ORIGINAL RESEARCH
published: 09 September 2021
doi: 10.3389/fonc.2021.667843

Mammary Tumorigenesis and Metabolome in Male Adipose Specific Monocyte Chemotactic Protein-1 Deficient MMTV-PyMT Mice Fed a High-Fat Diet

Lin Yan, Sneha Sundaram, Bret M. Rust, Matthew J. Picklo and Michael R. Bukowski*

U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND, United States

Male breast cancer, while uncommon, is a highly malignant disease. Monocyte chemotactic protein-1 (MCP-1) is an adipokine; its concentration in adipose tissue is elevated in obesity. This study tested the hypothesis that adipose-derived MCP-1 contributes to male breast cancer. In a 2x2 design, male MMTV-PyMT mice with or without adipose-specific *Mcp-1* knockout [designated as *Mcp-1*$^{-/-}$ or wild-type (WT)] were fed the AIN93G standard diet or a high-fat diet (HFD) for 25 weeks. *Mcp-1*$^{-/-}$ mice had lower adipose *Mcp-1* expression than WT mice. Adipose *Mcp-1* deficiency reduced plasma concentrations of MCP-1 in mice fed the HFD compared to their WT counterparts. *Mcp-1*$^{-/-}$ mice had a longer tumor latency (25.2 weeks *vs.* 18.0 weeks) and lower tumor incidence (19% *vs.* 56%), tumor progression (2317% *vs.* 4792%), and tumor weight (0.23 g *vs.* 0.64 g) than WT mice. Plasma metabolomics analysis identified 56 metabolites that differed among the four dietary groups, including 22 differed between *Mcp-1*$^{-/-}$ and WT mice. Pathway and network analyses along with discriminant analysis showed that pathways of amino acid and carbohydrate metabolisms are the most disturbed in MMTV-PyMT mice. In conclusion, adipose-derived MCP-1 contributes to mammary tumorigenesis in male MMTV-PyMT. The potential involvement of adipose-derived MCP-1 in metabolomics warrants further investigation on its role in causal relationships between cancer metabolism and mammary tumorigenesis in this male MMTV-PyMT model.

Keywords: adipose MCP-1, MMTV-PyMT, plasma metabolome, diet, male, mice

OPEN ACCESS

Edited by:
Pu Li,
Shanghai Jiao Tong University, China

Reviewed by:
Luca Gelsomino,
University of Calabria, Italy
Syn Yeo,
University of Cincinnati, United States

****Correspondence:***
Lin Yan
Lin.Yan@usda.gov

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *14 February 2021*
Accepted: *13 August 2021*
Published: *09 September 2021*

Citation:
Yan L, Sundaram S, Rust BM, Picklo MJ and Bukowski MR (2021) Mammary Tumorigenesis and Metabolome in Male Adipose Specific Monocyte Chemotactic Protein-1 Deficient MMTV-PyMT Mice Fed a High-Fat Diet. Front. Oncol. 11:667843. doi: 10.3389/fonc.2021.667843

INTRODUCTION

Breast cancer in men accounts for roughly 1% of all breast cancer cases (1). However, breast cancer is an aggressive disease in men. Approximately, 90% of all breast cancer diagnosed in men are invasive carcinoma (2), and 25% of male breast cancer patients have distant metastasis at the time of clinical presentation (3). Additionally, results from the Surveillance, Epidemiology, and End Results

(SEER) program show that improvements in breast cancer survival for men lag behind that for women (4).

Similar to female breast cancer, male breast cancer is classified into various subtypes including luminal b (5), an aggressive subtype of a higher grade with poorer prognosis (6) that accounts for 21% of breast cancer in men (5). The mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) model is a commonly used model in research of luminal b breast cancer (7). The MMTV-PyMT model conserves many of the defining characteristics of human subtypes (7). Compared to females, male MMTV-PyMT mice exhibit a delayed onset of palpable mammary tumors with a lower penetrance of metastasis (8). This delayed onset mimics clinical observations that breast cancer in men occurs approximately five to 10 years later than the average age of breast cancer occurrence in women (9).

Obesity is a major risk factor for breast cancer. Obese breast cancer patients often present high-grade lesions, elevated risk of recurrence, and increased incidence of lymph node involvement and metastasis (10, 11). Body fat accumulation, a hallmark of obesity (12, 13), may account for this association. Adipose tissue produces proinflammatory adipokines, including monocyte chemotactic protein-1 (MCP-1), that are elevated by obesity and contribute to obesity-related diseases.

MCP-1 is a major member of the adipokine family (14). In response to obesity, adipocytes increase the production of MCP-1 leading to obesity-induced inflammation (14–16). Clinical studies show that an elevation in MCP-1 occurs with cancer progression and has prognostic value for breast cancer. Poor outcomes and short disease-free intervals are related to high levels of MCP-1 in breast cancer patients (10, 17, 18). Silencing the expression of *Mcp-1* or its receptor protects mice against obesity-mediated inflammation in visceral adipose tissue (19, 20) and inhibits mammary tumor growth and metastasis in MDA-MB-231 mice (21). Depletion of MCP-1 reduces mammary tumorigenesis in C3(1)/SV40Tag mice (22) and spontaneous metastasis of Lewis lung carcinoma in C57BL/6 mice (23).

We have reported that adipose specific *Mcp-1* knockout reduces high-fat diet-enhanced mammary tumorigenesis in female mice (24) and metastasis of Lewis lung carcinoma (25) in male mice. However, the role of adipose-derived MCP-1 in male breast cancer remains unelucidated. We hypothesized that adipose-derived MCP-1 contributes to mammary tumorigenesis in male mice. The present study tested this hypothesis by investigating the effects of adipose specific MCP-1 deficiency on mammary tumorigenesis in male MMTV-PyMT mice fed a high-fat diet.

MATERIALS AND METHODS

Animals and Diets

The Grand Forks Human Nutrition Research Center vivarium provided mice for this study. The breeders were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Hemizygous male MMTV-PyMT mice on an FVB background were bred to female C57BL/6 mice with both alleles of the MCP-1 exons 2-3 flanked by loxP sites (MCP-$1^{fl/fl}$). Adipose tissue-specific knockout of MCP-1 was achieved by breeding male mice homozygous for MCP-$1^{fl/fl}$ and heterozygous for the PyMT oncogene (MMTV-$PyMT^{+}$/MCP-$1^{fl/fl}$) with female MCP-$1^{fl/fl}$ mice expressing Cre recombinase driven by the adiponectin promoter (MCP-$1^{fl/fl}$/Adipoq-Cre^{+}). Male mice heterozygous for the PyMT oncogene carrying two floxed MCP-1 alleles and positive for Cre expression (MMTV-$PyMT^{+}$/MCP-$1^{fl/fl}$/Adipoq-Cre^{+}) were designated as adipose *Mcp-1* knockout (*Mcp-1*$^{-/-}$) mice. Male littermates that were negative for Cre expression (MMTV-$PyMT^{+}$/MCP-$1^{fl/fl}$/Adipoq-Cre^{-}) served as wild-type (WT) controls. All mice used in this study were on a combination of the FVB and C57BL/6 backgrounds. Mice were maintained in a pathogen-free room on a 12:12-hour light/dark cycle with a temperature of 22 ± 1°C. The standard AIN93G diet (26) and a high-fat diet (HFD) providing 16% and 45% of energy from soybean oil, respectively, were used in this study (**Table 1**). Both diets were powder diets and were stored at −20°C until feeding. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (27).

Experimental Design

Mice were weaned onto the AIN93G diet at three weeks of age. Following one week of acclimation with the AIN93G diet, WT and *Mcp-1*$^{-/-}$ mice each were randomly assigned into one of four groups (32 per group for WT and 34 per group for *Mcp-1*$^{-/-}$ mice fed the AIN93G and HFD, respectively). Mice were housed two per cage to avoid stress related to single housing and weighed weekly. Food intake (12 mice per group) was recorded daily for three consecutive weeks one week after the initiation of the HFD. Body composition of conscious, immobilized mice was assessed one week prior to the end of the study by using the Echo Whole Body Composition Analyzer (Model 100, Echo Medical Systems,

TABLE 1 | Composition of diets.

Ingredient	AIN93G	High-fat
	g/kg	g/kg
Corn Starch	397.5	42.5
Casein	200	239
Dextrin	132	239
Sucrose	100	120
Soybean oil	70	239
Cellulose	50	60
AIN93 mineral mix	35	42
AIN93 vitamin mix	10	12
L-Cystine	3	3.6
Choline bitartrate	2.5	3
t-Butylhydroquinone	0.014	0.017
Total	1000	1000
Energy	%	%
Protein	20	20
Fat	16	45
Carbohydrate	64	35
Analyzed gross energy kcal/g[a]	4.3 ± 0.1	5.2 ± 0.1

[a]*Values are means ± SEM of three samples analyzed from each diet using oxygen bomb calorimeter (Model 6200; Oxygen Bomb Calorimeter, Parr Instrument, Moline, IL, USA).*

Houston, TX, USA). The percent body fat mass was calculated by using the formula: (fat mass/body mass) x 100; the percent body lean mass was obtained by using the formula: (lean mass/body mass) x 100.

Measurement of Mammary Tumors

Mice were palpated for mammary tumors twice weekly. Tumor latency was defined as the age at which the first mammary tumor was detected (28). Palpable tumors were measured weekly by using a digital caliper (Fred V Fowler Company, Newton, MA, USA). Tumor volume was calculated by using the formula: length x width^2 x 0.5 (28). Tumor progression was defined as the percentage change in tumor volume over time and calculated by using the formula: [(end volume – start volume)/start volume] x 100 (29). End volume was the tumor volume measured at the end of the study; start volume was the volume of the palpable tumor when it was first detected.

Tissue Harvest

At termination, mice were fasted for six hours before they were euthanized with an intraperitoneal injection of a mixture of ketamine and xylazine followed by exsanguination. Mammary tumors were collected and weighed. Epididymal adipose tissue and plasma were harvested and stored at -80°C. Lungs were collected and fixed in Bouin's solution for assessing the extent of metastasis (30).

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from epididymal adipose tissue by using the RNeasy Lipid Tissue Mini Kit following the manufacturer's protocol (Qiagen, Germantown, MD, USA). The quality and quantity of RNA were analyzed by using Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's protocol. Real-time qPCR of *Mcp-1* was analyzed and normalized to the 18s rRNA by using the TaqMan Assay of Demand primers on the ABI QuantStudio 12K-Flex Real-time PCR system (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative changes in gene expression (31).

Quantification of MCP-1 in Plasma

Sandwich enzyme-linked immunosorbent assay (ELISA) kit was used to quantify MCP-1 (R&D Systems, Minneapolis, MN, USA) in plasma following the manufacturer's protocol. Samples were read within the linear range of the assay. The accuracy of the analysis was confirmed by using the controls provided in the kit.

Metabolomics Analyses

Metabolomics analysis was conducted on plasma samples from mice without palpable mammary tumors (n = 10 per group) (32, 33). This was because few mice developed palpable tumors, particularly *Mcp-1*$^{-/-}$ mice. Samples were extracted and derivatized by silylation methyloximation and analyzed by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) for untargeted metabolomics of primary metabolism. The analysis was performed and obtained data were processed by using the BinBase database (34) at the West Coast Metabolomics Center (University of California-Davis, Davis, CA, USA). Unidentified peaks were removed from the dataset and excluded from the subsequent analysis. For the remaining identified compounds, quantifier ion peak heights were normalized to the sum intensities of all known compounds. Compounds representing less than 0.02% of total signal intensity for identified compounds were excluded from statistical analysis. Additional compounds were excluded if they could not be identified as either an intermediate species or metabolic endpoint common to mammalian metabolism based upon the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database or the Human Metabolome Database (35–37).

Statistical Analyses

Two-way analysis of variance (ANOVA) and Tukey contrasts were performed to examine the effects of diet (AIN93G or HFD), genotype (WT or *Mcp-1*$^{-/-}$), and their interactions on *Mcp-1* expression in adipose tissue, MCP-1 concentration in plasma, body weight, body composition, and energy intake among the four dietary groups. The LIFEREG procedure was used to fit the Lognormal model to latency data. The LIFETEST procedure was used to produce the Kaplan-Meier plots. Results are reported as means ± standard error of the mean (SEM); tumor latency is reported as medians and 95% confidence intervals (95% CI). Data were analyzed by using SAS 9.4 (SAS Institute, Cary, NC, USA). Differences with a $p \leq 0.05$ are considered significant.

Metabolomics analyses were performed by using MetaboAnalyst 5.0 (McGill University, Sainte Anne de Bellevue, Quebec, Canada). Data were scaled by Pareto scaling method and analyzed by sparse partial least square-discriminant analysis (sPLSDA) (38, 39). Hierarchical clustering heatmap was constructed by using the normalized peak intensity with Euclidean distance for distance measurement and the Ward error sum of squares hierarchical clustering methods for Cluster algorithm. Group averages were reported for the top 25 metabolites identified. The MACRO procedure (SAS 9.4) was used to examine effects of diet, genotype, and their interactions on changes in plasma metabolites with the false discovery rate-corrected *p*-values reported. Results of metabolomics analyses from treatment groups are presented as fold changes in comparison to the WT control group fed the AIN93G diet.

Pathway and network analyses were performed by using MetaboAnalyst 5.0 (40). Pathway analysis of alterations in metabolic pathways in MMTV-PyMT mice was performed by using the pathway library for *Mus musculus* according to the KEGG database (41). Pathway enrichment analysis coupled with pathway topology analysis was performed to identify the altered metabolic pathways. Obtained *p* values from the pathway enrichment analysis were adjusted by the Holm method (42). Network analysis was performed to map the functional relationships of identified metabolites between the AIN93G and HFD, between WT and *Mcp-1*$^{-/-}$ mice, and between WT and *Mcp-1*$^{-/-}$ mice fed the HFD by using the KEGG Global Metabolic Network and the Metabolite-Metabolite Interaction Network. Differences with a $p \leq 0.05$ are considered significant.

RESULTS

Body Weight, Adipose *Mcp-1* Expression, and Plasma MCP-1 Concentrations

Mice fed the HFD were heavier than mice fed the AIN93G diet, regardless of genotype (**Figure 1A**). The difference was significant after three weeks on the HFD and remained for the remainder of the study ($p < 0.05$) (**Figure 1A**). *Mcp-1*$^{-/-}$ mice fed the AIN93G diet were smaller than their WT counterparts; the difference was significant in the last four weeks of the study ($p < 0.05$) (**Figure 1A**).

The HFD elevated *Mcp-1* expression in adipose tissue by 166% compared to the AIN93G diet, regardless of genotype (**Figure 1B**). Adipose *Mcp-1* knockout diminished *Mcp-1* elevation by 61% compared to WT mice, regardless of diet (**Figure 1B**). Plasma concentrations of MCP-1 from WT mice fed the HFD were 95% higher than that from WT mice fed the AIN93G diet (**Figure 1C**). Adipose *Mcp-1* deficiency prevented plasma MCP-1 elevation in HFD-fed *Mcp-1*$^{-/-}$ mice, which did not differ from that of AIN93G-fed *Mcp-1*$^{-/-}$ mice (**Figure 1C**).

Body Composition and Energy Intake

Regardless of genotype, the percent body fat mass of mice fed the HFD was 37% greater than that of mice fed the AIN93G diet (28.8 ± 0.9% *vs.* 21.1 ± 0.9%) (**Figure 2A**). The percent body lean mass of the HFD-fed mice was 11% less than that of the AIN93G-fed mice (69.7 ± 0.9% *vs.* 77.9 ± 0.9%) (**Figure 2B**). The absolute lean mass of mice fed the HFD was slightly higher than that of mice fed the AIN93G diet (28.5 ± 0.4 g *vs.* 26.6 ± 0.4 g) (**Figure 2C**). Energy intake of the HFD-fed mice was 7% higher than that of the AIN93G-fed mice (39.4 ± 0.7 kcal per day *vs.* 37.0 ± 0.7 kcal per day) (**Figure 2D**). There were no significant differences in percent body fat mass, percent body lean mass, absolute lean mass, and energy intake between WT and *Mcp-1*$^{-/-}$ mice, regardless of diet (**Figures 2A–D**).

Mammary Tumorigenesis and Lung Metastasis

Fewer *Mcp-1*$^{-/-}$ mice developed palpable mammary tumors than WT mice. The tumor incidence was 19% for *Mcp-1*$^{-/-}$ mice (13 out of 68 mice) and 56% for WT mice (36 out of 64 mice) ($p < 0.01$), regardless of diet. There was no difference in tumor

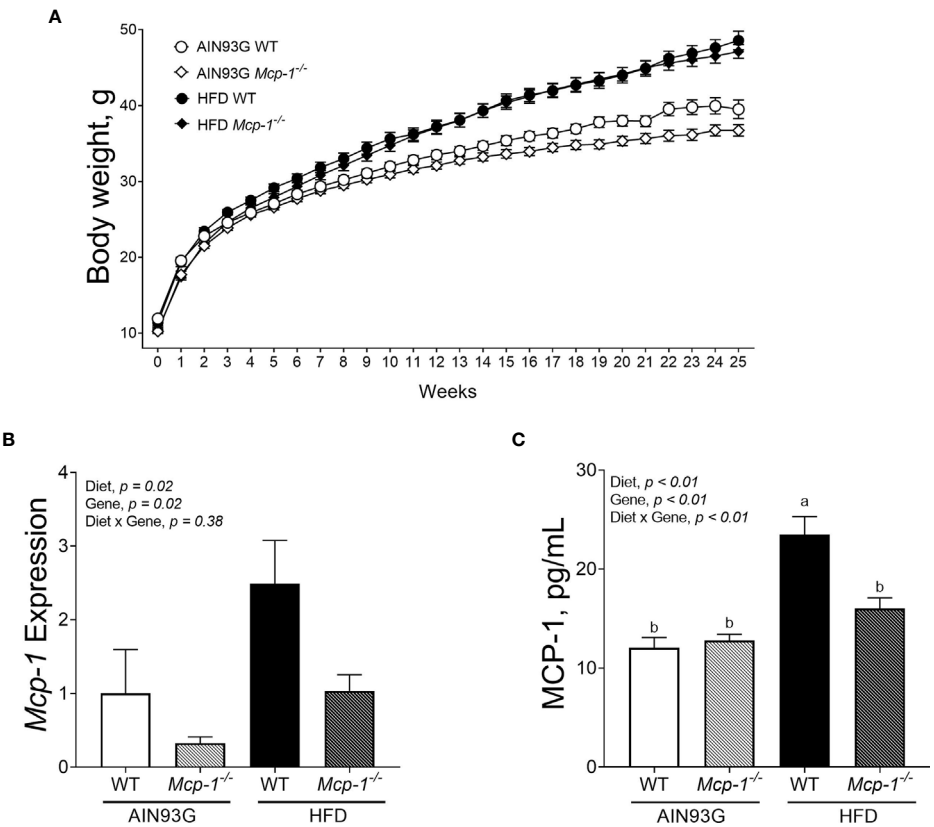

FIGURE 1 | Body weight **(A)**, adipose *Mcp-1* expression **(B)**, and plasma concentrations of MCP-1 **(C)** in MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD). Mice fed the HFD were heavier than mice fed the AIN93G diet; the difference was significant three weeks after the HFD ($p < 0.05$). *Mcp-1*$^{-/-}$ mice fed the AIN93G diet were smaller than their WT counterparts; the difference was significant for the last four weeks of the study ($p < 0.05$). Values are means ± SEM [n = 32 per group for WT mice, n = 34 per group for *Mcp-1*$^{-/-}$ mice for **(A)**; n = 10 per group for **(B)**; n = 16 per group for **(C)**]. Values with different letters are significant at $p \leq 0.05$ for **(C)**.

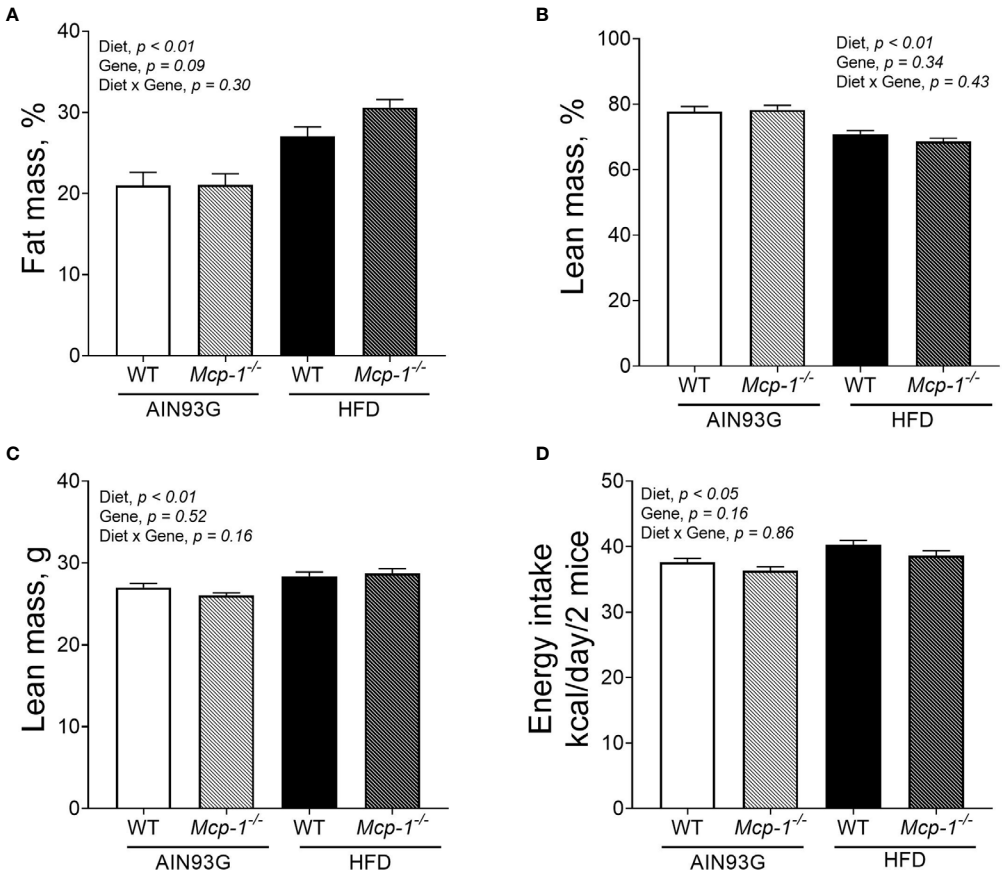

FIGURE 2 | The percent body fat mass **(A)**, percent body lean mass **(B)**, absolute lean mass **(C)**, and energy intake **(D)** of MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD). Values are means ± SEM [n = 32 per group for WT mice, n = 34 per group for *Mcp-1*$^{-/-}$ mice for **(A–C)**; n = 12 mice per group for **(D)**].

incidence between the HFD (26 out of 66 mice) and the AIN93G diet (23 out of 66 mice), regardless of genotype.

Palpable mammary tumors were detected later in *Mcp-1*$^{-/-}$ mice than in WT mice. Tumor latency of *Mcp-1*$^{-/-}$ mice fed the AIN93G and HFD was 25.2 weeks and 25.1 weeks, respectively (**Figure 3A**). Tumor latency of WT mice fed the AIN93G and HFD was 17.4 weeks and 18.5 weeks, respectively (**Figure 3A**). The difference between *Mcp-1*$^{-/-}$ and WT mice was significant ($p < 0.01$), regardless of diet (**Figure 3A**).

Mice fed the HFD had greater tumor progression than mice fed the AIN93G diet (4679 ± 786% *vs.* 2430 ± 700%), regardless of genotype (**Figure 3B**). The tumor progression of *Mcp-1*$^{-/-}$ mice was lower than that of WT mice (2317 ± 901% *vs.* 4792 ± 545%), regardless of diet (**Figure 3B**). At the end of the study, mammary tumors from *Mcp-1*$^{-/-}$ mice weighed 64% less than that from WT mice (0.23 ± 0.15 g *vs.* 0.64 ± 0.09 g), regardless of diet (**Figure 3C**). There was no significant difference in tumor weight between the two diets, regardless of genotype (**Figure 3C**).

Few tumor-bearing mice in each group had detectable metastases in the lungs. The incidence of metastasis was 38% for *Mcp-1*$^{-/-}$ mice (5 out of 13 mice) and 39% for WT mice (14 out of 36 mice), regardless of diet. The incidence of metastasis was 50% for the HFD (13 out of 26 mice) and 26% for the AIN93G diet (6 out of 23 mice) regardless of genotype. There were no significant differences in these comparisons.

Plasma Metabolomics Analysis

Plasma metabolomics analysis was performed to investigate effects of adipose MCP-1 deficiency and HFD on metabolome. Because few mice developed palpable mammary tumors, particularly *Mcp-1*$^{-/-}$ mice, the analysis was performed by using plasma samples from mice that did not develop palpable tumors. We identified 127 compounds from 467 discrete signals detected in plasma by the GC-TOF-MS. Eighty-seven of the 127 compounds met the criteria for statistical analysis (**Supplementary Table 1**). Fifty-six of these 87 compounds differed significantly among the four groups, including 22 differed by diet, 22 by genotype, and 34 by diet and genotype interactions (**Tables 2–4**).

The heatmap analysis of plasma metabolites provided intuitive visualization of the results. It produced five responsive clusters, 1) those that elevated in WT mice fed the HFD, 2) those

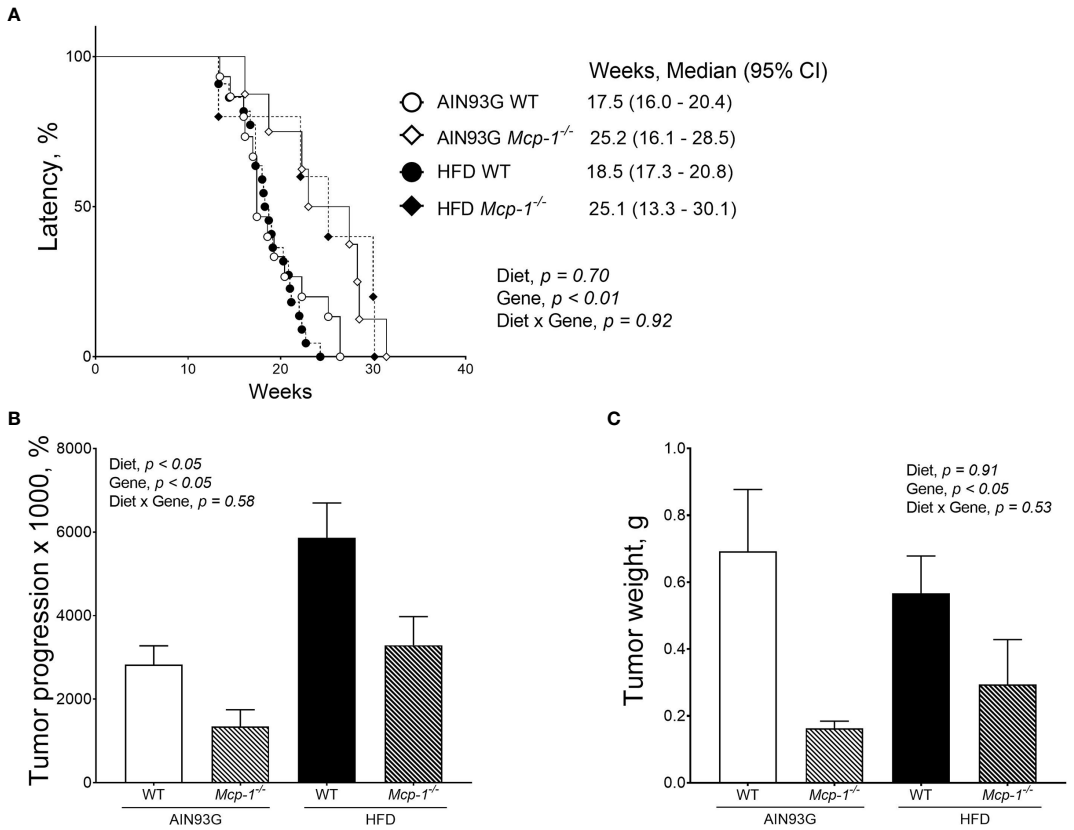

FIGURE 3 | Mammary tumor latency **(A)**, tumor progression **(B)**, and tumor weight **(C)** of MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD). Values are means ± SEM [(n = 5-22 per group) for **(B, C)**]. N = 32-34 per group for **(A)**.

that elevated in *Mcp-1*$^{-/-}$ mice regardless of diet, 3) those that remained relatively high in mice fed the AIN93G diet regardless of genotype, 4) those that elevated in *Mcp-1*$^{-/-}$ mice fed the AIN93G diet and WT mice fed the HFD, and 5) those that were higher in *Mcp-1*$^{-/-}$ mice fed the AIN93G diet compared to other groups (**Figure 4**).

The sPLSDA scores plot showed separations by diet and genotype (**Figures 5**, **6**). Along the x-axis, component 1 showed that WT mice fed the HFD and *Mcp-1*$^{-/-}$ mice fed the AIN93G diet were separated from WT mice fed the AIN93G diet whereas *Mcp-1*$^{-/-}$ mice fed the HFD remained similar to WT mice fed the AIN93G diet (**Figure 5A**). The loadings plot for component 1 identified the amino acids (alanine, isoleucine, leucine, phenylalanine, threonine, and valine) and carbohydrate metabolites (fumaric acid, glucuronic acid, hexuronic acid, and malic acid) as major determinants of separation (**Figure 6A**).

Along the y-axis, component 2 separated the groups into two categories based upon the diet, mice fed the AIN93G diet and mice fed the HFD, regardless of genotype (**Figure 5A**). The loadings plot for component 2 identified carbohydrate metabolites (1,5-anhydroglucitol, glucose, and phosphate), cholesterol, fatty acids (myristic acid, oleic acid, and palmitoleic acid), and amino acid derivatives (2-aminobutyric acid, aminomalonate, and 2-ketoisocapronic acid) as major determinants of separation (**Figure 6B**).

Along the x-axis, component 3 separated HFD-fed mice, but not AIN93G-fed mice, by genotype (**Figure 5B**). The loadings plot for component 3 identified amino acids (lysine and tyrosine), carbohydrate metabolites (phosphate and sorbitol), cholesterol, linoleic acid, lipid metabolites (glycerol and 1-monoolein), vitamin α-tocopherol and uric acid as major determinants of separation (**Figure 6C**).

Pathway Analysis

Pathway analysis was conducted to determine metabolic pathways that were altered in MMTV-PyMT mice. The identified metabolites (**Supplementary Table 2**) were mapped into 55 metabolic pathways according to the KEGG database (35, 41). Six pathways were altered the most (**Table 5** and **Figure 7**). These included aminoacyl-tRNA biosynthesis, arginine biosynthesis, valine, leucine and isoleucine biosynthesis, alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, and citrate cycle (**Table 5**).

TABLE 2 | Identified plasma metabolites related to amino acid metabolism that differed in MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD).

	AIN93G WT	AIN93G *Mcp-1*$^{-/-}$	HFD WT	HFD *Mcp-1*$^{-/-}$	Diet, *p*	Gene, *p*	Diet x Gene, *p*
Proteinogenic amino acids							
Alanine	1.00 ± 0.15^{b}	1.74 ± 0.15^{a}	1.24 ± 0.12^{b}	1.19 ± 0.07^{b}	0.21	0.01	<0.01
Glycine	1.00 ± 0.08	1.18 ± 0.07	0.97 ± 0.06	0.90 ± 0.08	0.04	0.45	0.09
Isoleucine	1.00 ± 0.07^{b}	1.38 ± 0.09^{a}	1.29 ± 0.06^{a}	0.87 ± 0.04^{b}	0.09	0.80	<0.01
Leucine	1.00 ± 0.07^{b}	1.28 ± 0.08^{a}	1.34 ± 0.08^{a}	0.84 ± 0.05^{b}	0.48	0.13	<0.01
Lysine	1.00 ± 0.12	1.40 ± 0.11	0.86 ± 0.06	1.25 ± 0.15	0.22	<0.01	0.99
Phenylalanine	1.00 ± 0.08^{b}	1.37 ± 0.08^{a}	1.25 ± 0.06^{ab}	0.98 ± 0.09^{b}	0.34	0.52	<0.01
Methionine	1.00 ± 0.06^{b}	1.33 ± 0.08^{a}	0.99 ± 0.07^{b}	0.91 ± 0.10^{b}	0.01	0.12	0.01
Proline	1.00 ± 0.20^{b}	1.87 ± 0.27^{a}	1.19 ± 0.20^{ab}	0.92 ± 0.15^{b}	0.08	0.16	0.01
Serine	1.00 ± 0.06	1.28 ± 0.08	0.96 ± 0.04	1.07 ± 0.10	0.09	0.01	0.24
Threonine	1.00 ± 0.09^{b}	1.65 ± 0.16^{a}	1.02 ± 0.07^{b}	1.00 ± 0.08^{b}	<0.01	<0.01	<0.01
Tyrosine	1.00 ± 0.06	1.30 ± 0.08	1.00 ± 0.04	1.35 ± 0.12	0.74	<0.01	0.77
Valine	1.00 ± 0.06^{b}	1.43 ± 0.08^{a}	1.28 ± 0.05^{a}	0.92 ± 0.06^{b}	0.09	0.59	<0.01
Nonproteinogenic amino acids and derivatives							
2-Aminobutyric acid	1.00 ± 0.24	1.61 ± 0.22	0.67 ± 0.06	0.69 ± 0.07	<0.01	0.07	0.08
Aminomalonate	1.00 ± 0.09	0.91 ± 0.11	0.66 ± 0.07	0.84 ± 0.10	0.04	0.66	0.15
2-Hydroxybutanoic acid	1.00 ± 0.17^{a}	0.41 ± 0.05^{b}	0.60 ± 0.15^{ab}	0.70 ± 0.15^{ab}	0.70	0.84	0.02
Indole-3-propionic acid	1.00 ± 0.37	1.81 ± 0.47	0.59 ± 0.15	0.52 ± 0.12	0.01	0.25	0.17
2-Ketoisocaproic acid	1.00 ± 0.14	1.13 ± 0.13	0.86 ± 0.06	0.65 ± 0.05	<0.01	0.71	0.11
Oxoproline	1.00 ± 0.07	1.17 ± 0.07	1.23 ± 0.06	1.01 ± 0.06	0.59	0.74	0.01
Taurine	1.00 ± 0.16	1.82 ± 0.32	1.78 ± 0.23	1.41 ± 0.19	0.44	0.33	0.01
Urea cycle metabolites							
Citrulline	1.00 ± 0.07	1.25 ± 0.09	0.89 ± 0.09	0.92 ± 0.06	0.01	0.08	0.17
Urea	1.00 ± 0.04^{a}	0.42 ± 0.17^{b}	0.47 ± 0.16^{b}	0.79 ± 0.11^{ab}	0.52	0.32	<0.01

Values of treatment groups are standardized to that of the AIN93G WT group. Values (means ± SEM) in the same row with different letters are significant at p ≤ 0.05 (false discovery rate-adjusted p values) (n = 10 per group).

TABLE 3 | Identified plasma metabolites related to carbohydrate metabolism that differed in MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD).

	AIN93G WT	AIN93G *Mcp-1*$^{-/-}$	HFD WT	HFD *Mcp-1*$^{-/-}$	Diet, *p*	Gene, *p*	Diet x Gene, *p*
1,5-Anhydroglucitol	1.00 ± 0.07^{a}	0.79 ± 0.06^{a}	0.34 ± 0.06^{b}	0.40 ± 0.05^{b}	<0.01	0.22	0.03
Erythritol	1.00 ± 0.09	1.32 ± 0.10	1.31 ± 0.11	1.09 ± 0.04	0.67	0.58	<0.01
Fumaric acid	1.00 ± 0.17^{b}	2.48 ± 0.36^{a}	1.35 ± 0.19^{b}	1.11 ± 0.14^{b}	0.03	0.01	<0.01
Glucose	1.00 ± 0.07^{b}	0.98 ± 0.03^{b}	1.05 ± 0.03^{b}	1.24 ± 0.05^{a}	<0.01	0.09	0.03
Glucose-1-phosphate	1.00 ± 0.14	0.79 ± 0.05	0.81 ± 0.07	1.23 ± 0.18	0.31	0.39	0.01
Glucuronic acid	1.00 ± 0.21^{b}	3.59 ± 0.86^{a}	1.13 ± 0.13^{b}	1.09 ± 0.14^{b}	0.01	0.01	0.01
Glycerol-α-phosphate	1.00 ± 0.11	1.09 ± 0.15	1.45 ± 0.12	1.06 ± 0.10	0.09	0.22	0.05
Glyceric acid	1.00 ± 0.18	1.74 ± 0.22	1.62 ± 0.23	1.28 ± 0.16	0.69	0.32	0.01
Hexuronic acid 1	1.00 ± 0.26^{b}	3.63 ± 0.90^{a}	1.11 ± 0.17^{b}	1.04 ± 0.18^{b}	0.02	0.01	0.01
Hexuronic acid 2	1.00 ± 0.10^{b}	1.88 ± 0.33^{a}	1.05 ± 0.04^{b}	1.17 ± 0.08^{ab}	0.07	0.01	0.04
3-Hydroxybutyric acid	1.00 ± 0.27	0.87 ± 0.13	1.03 ± 0.11	0.43 ± 0.06	0.22	0.03	0.16
Isocitric acid	1.00 ± 0.09^{ab}	1.30 ± 0.13^{a}	1.03 ± 0.12^{ab}	0.83 ± 0.07^{b}	0.04	0.62	0.03
Lactic acid	1.00 ± 0.28	1.65 ± 0.37	0.62 ± 0.06	0.57 ± 0.09	<0.01	0.21	0.16
Malic acid	1.00 ± 0.22^{b}	2.52 ± 0.40^{a}	1.07 ± 0.23^{b}	1.00 ± 0.23^{b}	0.01	0.01	0.01
Mannose	1.00 ± 0.07	1.08 ± 0.06	1.16 ± 0.07	0.94 ± 0.03	0.87	0.28	0.02
Myoinositol	1.00 ± 0.07^{ab}	1.32 ± 0.09^{a}	1.05 ± 0.11^{ab}	0.95 ± 0.05^{b}	0.07	0.21	0.02
Phosphate	1.00 ± 0.06^{b}	1.07 ± 0.07^{b}	1.49 ± 0.10^{a}	0.95 ± 0.05^{b}	0.02	<0.01	<0.01
Pyruvic acid	1.00 ± 0.20	1.42 ± 0.16	0.69 ± 0.10	0.88 ± 0.13	0.01	0.05	0.46
Sorbitol	1.00 ± 0.16	1.74 ± 0.21	1.07 ± 0.16	1.49 ± 0.09	0.57	<0.01	0.33
Succinic acid	1.00 ± 0.20	1.35 ± 0.21	1.54 ± 0.34	0.76 ± 0.14	0.91	0.37	0.02
Xylose	1.00 ± 0.04^{b}	1.11 ± 0.07^{ab}	1.26 ± 0.08^{a}	1.03 ± 0.05^{ab}	0.18	0.36	0.01

Values of treatment groups are standardized to that of the AIN93G WT group. Values (means ± SEM) in the same row with different letters are significant at p ≤ 0.05 (false discovery rate-adjusted p values) (n = 10 per group).

Network Analysis

Network analysis of plasma metabolites by using the KEGG Global Metabolic Network identified 9 metabolic pathways that differed between the AIN93G diet and HFD, regardless of genotype (**Table 6**). These pathways were glycine, serine, and threonine metabolism, valine, leucine, and isoleucine biosynthesis, arginine biosynthesis, citrate cycle, pyruvate metabolism, glycolysis/gluconeogenesis, alanine, aspartate, and glutamine metabolism, glyoxylate and dicarboxylate metabolism, and cysteine and methionine metabolism (**Table 6**). Nine pathways differed

TABLE 4 | Identified plasma metabolites related to lipid, nucleotide, and vitamin metabolisms that differed in MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout [*Mcp-1*$^{-/-}$ or wild-type (WT)] and fed the AIN93G or high-fat diet (HFD).

	AIN93G WT	AIN93G *Mcp-1*$^{-/-}$	HFD WT	HFD *Mcp-1*$^{-/-}$	Diet, *p*	Gene, *p*	Diet x Gene, *p*
Lipid metabolism							
Cholesterol	1.00 ± 0.12	0.74 ± 0.08	1.32 ± 0.10	1.05 ± 0.08	<0.01	0.01	0.98
Diglycerol	1.00 ± 0.15	1.28 ± 0.11	1.42 ± 0.14	1.21 ± 0.08	0.16	0.80	0.05
Glycerol	1.00 ± 0.06	0.77 ± 0.05	1.05 ± 0.11	0.84 ± 0.03	0.38	<0.01	0.92
Heptadecanoic acid	1.00 ± 0.09	1.20 ± 0.04	1.27 ± 0.12	1.12 ± 0.05	0.24	0.79	0.04
Linoleic acid	1.00 ± 0.18	0.77 ± 0.09	1.24 ± 0.04	0.78 ± 0.08	0.29	<0.01	0.31
1-Monoolein	1.00 ± 0.13	0.76 ± 0.06	1.40 ± 0.18	0.99 ± 0.07	0.01	0.01	0.51
Myristic acid	1.00 ± 0.08	0.95 ± 0.07	0.88 ± 0.04	0.63 ± 0.08	<0.01	0.03	0.14
Oleic acid	1.00 ± 0.36^{b}	2.37 ± 0.95^{ab}	4.21 ± 0.67^{a}	2.41 ± 0.64^{ab}	0.02	0.75	0.02
Palmitoleic acid	1.00 ± 0.18	0.78 ± 0.10	0.30 ± 0.02	0.16 ± 0.02	<0.01	0.11	0.68
Nucleotides							
Pseudo uridine	1.00 ± 0.11	1.25 ± 0.08	1.33 ± 0.13	1.01 ± 0.07	0.66	0.72	0.01
Thymidine	1.00 ± 0.11	1.09 ± 0.10	0.67 ± 0.10	1.01 ± 0.11	0.06	0.05	0.25
Uric acid	1.00 ± 0.17	0.65 ± 0.12	1.06 ± 0.12	0.65 ± 0.08	0.82	0.01	0.82
Vitamins							
Threonic acid	1.00 ± 0.15	1.69 ± 0.22	1.76 ± 0.28	1.29 ± 0.12	0.37	0.59	<0.01
α-Tocopherol	1.00 ± 0.19	0.52 ± 0.11	1.50 ± 0.15	0.98 ± 0.15	<0.01	<0.01	0.91

Values of treatment groups are standardized to that of the AIN93G WT group. Values (means ± SEM) in the same row with different letters are significant at p ≤ 0.05 (false discovery rate-adjusted p values) (n = 10 per group).

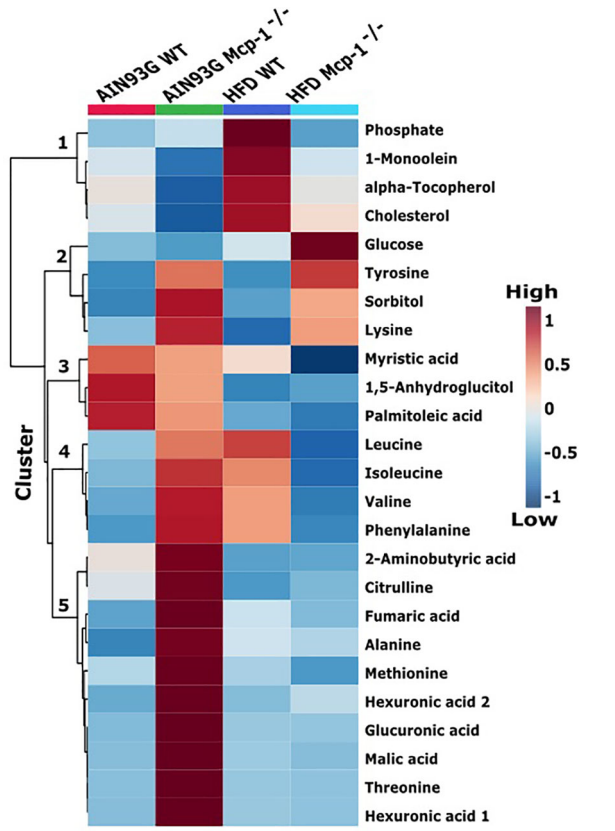

FIGURE 4 | Hierarchical clustering heatmap of the top 25 plasma metabolites that differ among the four dietary groups. AIN WT (red): wild-type mice fed the AIN93G diet; AIN *Mcp-1*$^{-/-}$ (green): *Mcp-1*$^{-/-}$ mice fed the AIN93G diet; HFD WT (blue): wild-type mice fed the high-fat diet; HFD *Mcp-1*$^{-/-}$ (cyan): *Mcp-1*$^{-/-}$ mice fed the high-fat diet. Each cell on the map represents the group average of a metabolite (n = 10 per group).

between WT and *Mcp-1*$^{-/-}$ mice, regardless of diet (**Table 6**). These included alanine, aspartate, and glutamine metabolism, glycine, serine, and threonine metabolism, tyrosine metabolism, citrate cycle, pyruvate metabolism, galactose metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, linoleic metabolism, and biotin metabolism (**Table 6**). Three pathways differed when HFD-fed *Mcp-1*$^{-/-}$ mice were compared to HFD-fed WT mice (**Table 6**). They were valine, leucine, and isoleucine biosynthesis, valine, leucine, and isoleucine degradation, and pantothenate and CoA biosynthesis (**Table 6**). Networks of the metabolites mapped by the Metabolite-Metabolite Interaction Network between AIN93G diet and HFD, between WT and *Mcp-1*$^{-/-}$ mice, and between the HFD-fed *Mcp-1*$^{-/-}$ and the HFD-fed WT mice are in **Figures 8A, B**, and **9**, respectively; their network statistics are in **Supplementary Table 3**.

DISCUSSION

The present study showed that adipose-derived MCP-1 contributed to mammary tumorigenesis in male MMTV-PyMT mice. We have three important findings with this male breast cancer model. First, mammary tumorigenesis is slow and less aggressive in male MMTV-PyMT mice. This is evidenced by a longer latency before detection of palpable mammary tumors (17.4 weeks) and a lower tumor incidence (56%) compared to female MMTV-PyMT mice reported to have a shorter latency (6 to 8 weeks) and 100% tumor incidence (24, 28, 43). The observed delay in onset of mammary tumorigenesis is consistent with a previous report showing a 14-week tumor latency with a 70% tumor incidence in male MMTV-PyMT mice (44). Findings from this study support clinical observations that breast cancer occurs late in life in men than that in women (9).

Second, mammary tumorigenesis in male MMTV-PyMT mice responds to dietary changes, evidenced by the increased tumor progression in mice fed the HFD. Mice fed the HFD

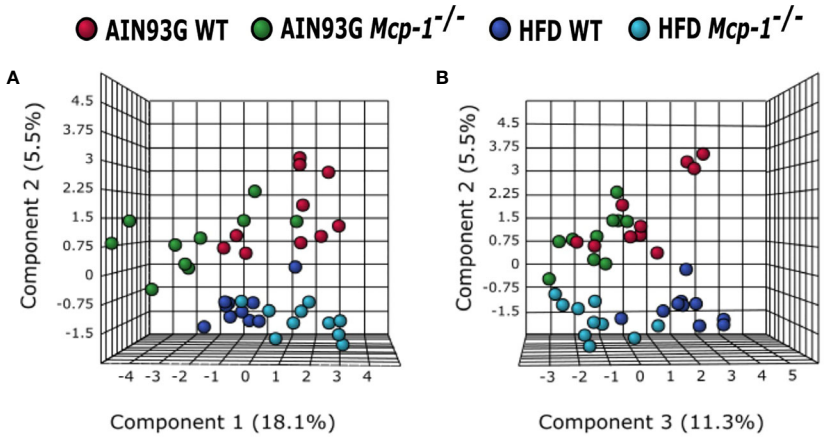

FIGURE 5 | Sparse partial least square discriminant analysis of the plasma metabolome among the four dietary groups. **(A)** Components 1 *vs* 2; **(B)** Components 2 *vs* 3. AIN WT (red): wild-type mice fed the AIN93G diet; AIN *Mcp-1*$^{-/-}$ (green): *Mcp-1*$^{-/-}$ mice fed the AIN93G diet; HFD WT (blue): wild-type mice fed the high-fat diet; HFD *Mcp-1*$^{-/-}$ (cyan): *Mcp-1*$^{-/-}$ mice fed the high-fat diet (n = 10 per group).

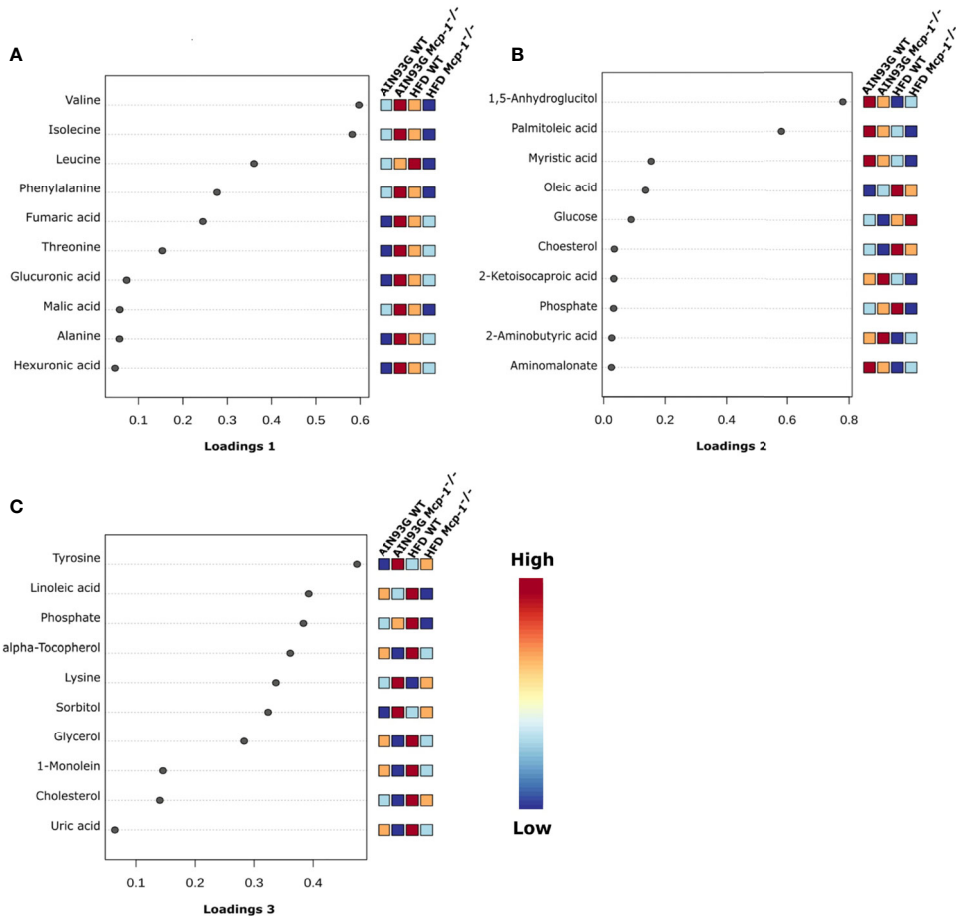

FIGURE 6 | Loadings plots of the 10 metabolites that are the most influential in treatment separation among the four groups for components 1 **(A)**, 2 **(B)**, and 3 **(C)** (n = 10 per group).

TABLE 5 | Metabolic pathways identified by the pathway analysis that are significantly altered in MMTV-PyMT mice.

KEGG pathway	Number of metabolites identified	p^a	Impact[b]
Aminoacyl-tRNA biosynthesis	15	<0.01	0.17
Arginine biosynthesis	7	<0.01	0.41
Valine, leucine and isoleucine biosynthesis	5	<0.01	0
Alanine, aspartate and glutamate metabolism	8	<0.01	0.36
Glyoxylate and dicarboxylate metabolism	8	0.01	0.26
Citrate cycle	6	0.03	0.30

[a]*p-values are obtained by the over-representation analysis and adjusted by the Holm method.*
[b]*Impact is the pathway impact score obtained by the pathway topology analysis.*

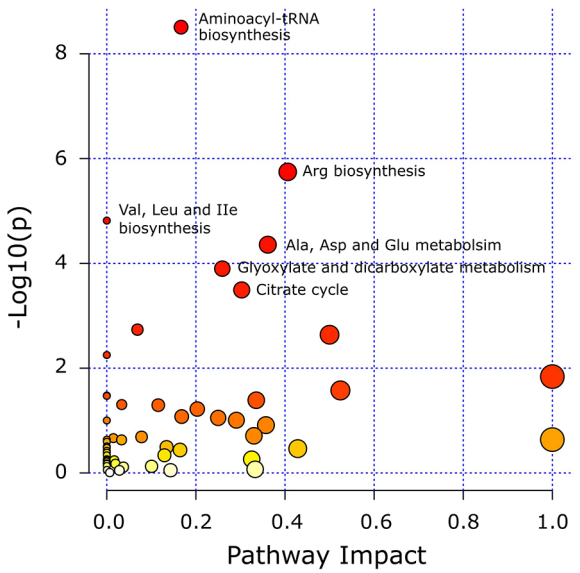

FIGURE 7 | Matched metabolic pathway plot by using identified metabolites in MMTV-PyMT mice. The x-axis marks the pathway impact. The y-axis marks the pathway enrichment. Each node represents a pathway. The nodes with larger sizes and darker colors (from yellow to red) positioning towards top right region represent higher pathway impact values and higher pathway enrichment. Pathways that are significantly altered are presented with their names next to their nodes and are in **Table 5**. All detected metabolic pathways are presented in **Supplementary Table 2**.

gained body fat mass. Diet-induced obesity enhances mammary tumorigenesis in female MMTV-PyMT mice (24, 28, 43). Adipose tissue, considered an endocrine organ, produces proinflammatory cytokines that can be tumorigenic in rodent models of cancer, including the MMTV-PyMT model (24, 28, 43). Results from this study suggest that adipose-derived cytokines may be responsible, at least partly, for the HFD-enhanced mammary tumorigenesis in male mice.

Third, adipose-derived MCP-1 contributes to mammary tumorigenesis in male MMTV-PyMT mice. This is demonstrated by longer latency and lower tumor incidence, tumor progression, and tumor weight in *Mcp-1*$^{-/-}$ mice. These

TABLE 6 | Metabolic pathways identified by the KEGG Global Metabolic Network that are significantly altered by the diet (AIN93G *vs.* high-fat diet) and genotype (wild-type *vs. Mcp-1*$^{-/-}$) in MMTV-PyMT mice with or without adipose monocyte chemotactic protein-1 knockout (*Mcp-1*$^{-/-}$ or wild-type) and fed the AIN93G or high-fat diet.

Metabolic pathways	*p*
AIN93G diet *vs.* high-fat diet, regardless of genotype	
Glycine, serine, and threonine metabolism	<0.01
Valine, leucine, and isoleucine metabolism	<0.01
Arginine biosynthesis	<0.01
Citrate cycle	0.01
Pyruvate metabolism	0.01
Glycolysis/gluconeogenesis	0.02
Alanine, aspartate, and glutamate metabolism	0.03
Glyoxylate and dicarboxylate metabolism	0.03
Cysteine and methionine metabolism	0.03
Wild-type mice *vs. Mcp-1*$^{-/-}$ mice, regardless of diet	
Alanine, aspartate, and glutamine metabolism	<0.01
Glycine, serine, and threonine metabolism	<0.01
Tyrosine metabolism	0.02
Citrate cycle	0.02
Pyruvate metabolism	0.02
Galactose metabolism	0.05
Phenylalanine, tyrosine, and tryptophan biosynthesis	0.05
Linoleic acid metabolism	0.05
Biotin metabolism	0.05
Wild-type mice *vs. Mcp-1*$^{-/-}$ mice, high-fat diet	
Valine, leucine, and isoleucine biosynthesis	<0.01
Valine, leucine, and isoleucine degradation	<0.01
Pantothenate and CoA biosynthesis	0.05

observations are MCP-1 specific and independent on body fat, because there were no differences in body fat mass between *Mcp-1*$^{-/-}$ and WT mice, particularly those fed the HFD. These findings are consistent with a previous report that adipose MCP-1 deficiency mitigates mammary tumorigenesis in female MMTV-PyMT mice (24).

In this study, we analyzed plasma metabolome in male MMTV-PyMT mice. Pathway analysis showed disturbances in metabolic pathways in MMTV-PyMT mice. The two most significant alterations are pathways related to amino acid and carbohydrate metabolisms. The aminoacyl-tRNA biosynthesis pathway is important in protein synthesis (45). It is a group of aminoacyl-tRNA synthetases that catalyze aminoacylations by covalently linking an amino acid to its cognate tRNA in the first step of protein translation. This includes glycine, serine, and threonine metabolism, valine, leucine and isoleucine biosynthesis, arginine biosynthesis, alanine, aspartate and glutamate metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis identified by the network analysis in MMTV-PyMT mice.

Alteration in the citrate cycle and pyruvate metabolism is an evidence of disturbed carbohydrate metabolism. However, it is interest to find the glyoxylate and dicarboxylate metabolism pathway in MMTV-PyMT mice. The glyoxylate cycle, a modification of the citrate cycle, was found in plants and some microorganisms but not in animals because animals lack two key enzymes of the cycle (isocitrate lyase and malate synthase). However, available studies have showed that the glyoxylate cycle occurs in animals (46, 47). The detection of isocitric acid

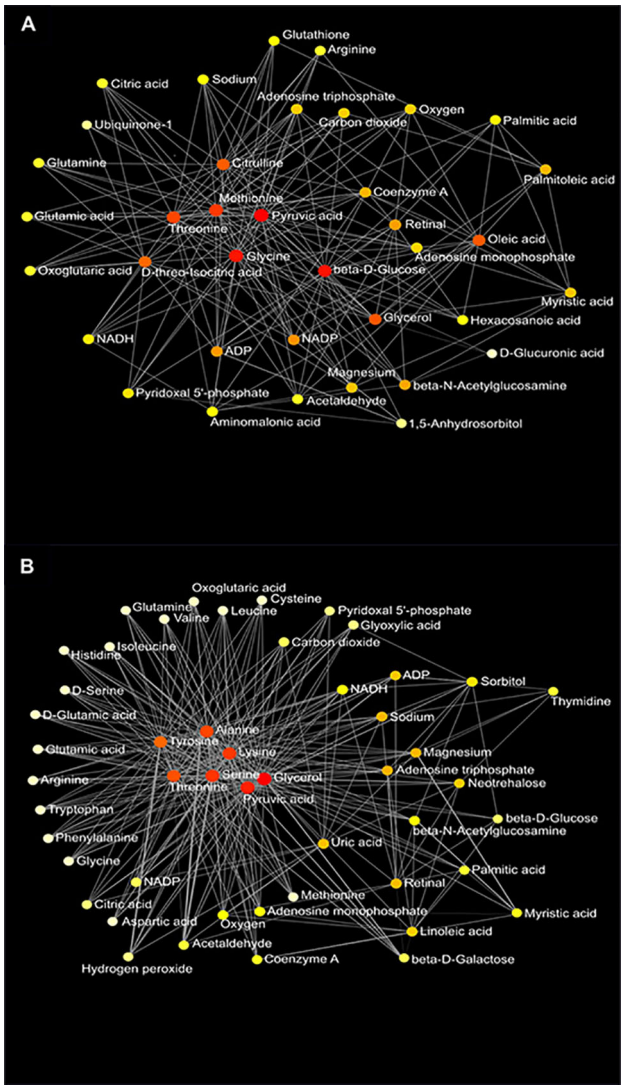

FIGURE 8 | Metabolic network of plasma metabolites between the AIN93G and high-fat diet **(A)** and between wild-type and *Mcp-1*$^{-/-}$ mice **(B)**. Colors, from white-yellow to red, indicate levels of impact the metabolites have on the network in an ascending order (the number of connections a node has to other nodes and the number of shortest paths going through the node). Network statistics for **(A, B)** are presented in **Supplementary Table 3**.

and malic acid in plasma of MMTV-PyMT mice support the existence of the glyoxylate cycle in these mice. The potential involvement of glyoxylate and dicarboxylate metabolism in altered carbohydrate metabolism in MMTV-PyMT mice and its impact on mammary tumorigenesis certainly warrant further investigation.

Results from the network analysis support findings from the pathway analysis. It further demonstrates that both the HFD and adipose MCP-1 deficiency alter amino acid and carbohydrate metabolisms in male MMTV-PyMT mice. It is particularly interesting that both branched-chain amino acid (BCAA) biosynthesis and degradation pathways were altered when HFD-fed *Mcp-1*$^{-/-}$ mice were compared to their WT counterparts. Our findings of accelerated BCAA metabolism support the clinical observations of disturbed BCAA metabolism in human breast cancer patients (48, 49).

Heatmap analysis illustrates the top 25 metabolites that are major determinants in separation of the four dietary groups. The expression of 17 of them was higher in AIN93G-fed *Mcp-1*$^{-/-}$ mice than in their WT counterparts. These include essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine), nonessential amino acids (alanine and tyrosine), amino acid metabolites (2-aminobutyric acid and citrulline), metabolites of carbohydrate metabolism (fumaric acid, glucuronic acid, malic acid, and sorbitol), and fatty acid metabolite hexuronic acids. These findings indicate that MCP-1 deficiency may have altered metabolism in AIN93G-fed mice, particularly amino acid metabolism, and that adipose-derived MCP-1 may contribute to metabolic homeostasis in mice fed a non-obesogenic diet. It is interest to note that the expression of 14 of these 17 metabolites (except lysine, sorbitol, and tyrosine) were lower in HFD-fed *Mcp-1*$^{-/-}$ mice. It suggests that a high-energy intake may attenuate, at least partly, the metabolic alterations caused by adipose MCP-1 deficiency.

Multivariate and clustered heatmap analyses showed the separation of plasma metabolome among the four groups. Component 1 of the sPLSDA scores plot showed that clusters of WT mice differed by diet. However, the cluster of HFD-fed *Mcp-1*$^{-/-}$ mice remained similar to that of AIN93G-fed WT controls. This is the case for the major determinant metabolites shown in the loadings plot for component 1, in which BCAAs and phenylalanine are major determinants that separate the dietary groups. BCAAs account for one-third dietary essential amino acids and make up 20% of total protein content (50). BCAAs, specifically leucine, activate mammalian target of rapamycin complex (mTORC) signal pathway that is essential for initiation of protein synthesis (51, 52). Elevated expression of BCAAs in HFD-fed WT mice, compared to that in AIN93G-fed WT mice, are consistent with findings from both human and rodent studies (53, 54) and support the concept that the impaired BCAA catabolic pathway in obesity leads to BCAA buildup in the blood (53, 54). Significantly lower expression of BCAAs in HFD-fed *Mcp-1*$^{-/-}$ mice, compared to that in HFD-fed WT mice, indicates that adipose MCP-1 deficiency may attenuate the impaired BCAA metabolism in mice fed an obesogenic diet.

The elevated expression of four metabolites (cholesterol, phosphate, 1-monoolein, and α-tocopherol) identified by the heatmap in HFD-fed WT mice suggests accelerated metabolism in mice fed an obesogenic diet. Phosphate is involved in many anabolic and catabolic metabolisms. Cholesterol is essential for membrane biogenesis and a precursor for steroid hormone synthesis. Cholesterol is elevated in mammary tumors in MMTV-PyMT mice fed an HFD (55). Hypercholesterolemia promotes mammary tumorigenesis in MMTV-PyMT mice (56, 57). Elevations in α-tocopherol (an essential nutrient) and 1-

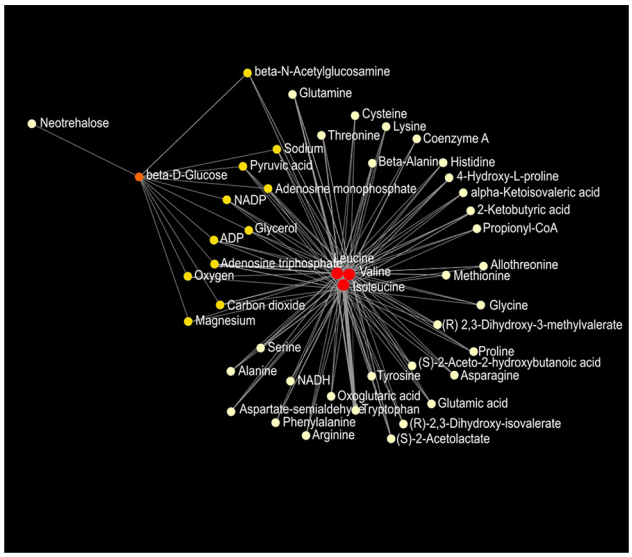

FIGURE 9 | Metabolic network of plasma metabolites between wild-type and *Mcp-1*$^{-/-}$ mice fed the high-fat diet. Colors, from white-yellow to red, indicate levels of impact the metabolites have to the network in an ascending order (the number of connections a node has to other nodes and the number of shortest paths going through the node). Network statistics are presented in **Supplementary Table 3**.

monoolein (a major hydrolysis product of dietary triacylglycerol) (58) indicate an increase in nutrient uptake from the diet and an up-regulation in lipolysis of triacylglycerol in these mice. The attenuated expression of these metabolites in HFD-fed *Mcp-1*$^{-/-}$ mice suggests that adipose-derived MCP-1 may contribute to the diet-induced metabolic dysregulation in WT mice.

The significant elevation in glucose in HFD-fed *Mcp-1*$^{-/-}$ mice suggests impaired glucose metabolism under the MCP-1 deficiency in mice consuming an obesogenic diet. This elevation is not solely due to MCP-1 deficiency nor HFD alone, because such elevation was not observed in *Mcp-1*$^{-/-}$ mice fed the AIN93G diet nor WT mice fed the HFD. Rather, it is likely an interaction between MCP-1 deficiency and the HFD. The impairment is supported by the lower expression of 1,5-anhydroglucitol in these HFD-fed *Mcp-1*$^{-/-}$ mice. 1,5-Anhydroglucitol is a marker of glycemic control; its blood level is inversely correlated with blood glucose (59). Cancer cells demand high glucose uptake for their rapid, uncontrolled proliferation (60). The roles of adipose-derived MCP-1 in glucose metabolism under the obesogenic condition and its impact on mammary tumorigenesis warrant further investigation.

We found there were no differences in plasma concentrations of MCP-1 between *Mcp-1*$^{-/-}$ and WT mice fed the AIN93G diet. In this study, the expression of *Mcp-1* in adipose tissue was low in *Mcp-1*$^{-/-}$ mice. However, it does not exclude the possibility that MCP-1 from non-adipose tissue may contribute to plasma concentrations in mice consuming the AIN93G diet. Nevertheless, significant decreases in plasma MCP-1 in HFD-fed *Mcp-1*$^{-/-}$ mice, compared to their WT counterparts, demonstrates the validity of the model and that decreases in plasma MCP-1 are a result of MCP-1 deficiency in adipose tissue.

A limitation of this study is that we were not able to analyze plasma metabolome from tumor-bearing mice because few mice, particularly *Mcp-1*$^{-/-}$ mice, developed palpable mammary tumors. This made us unable to examine the metabolic profile in the presence of mammary tumor. Furthermore, cautions should be taken in data interpretation, as the observed plasma alterations could be results from undetected nonpalpable tumors, stromal or systematic changes, or their combinations. Nevertheless, this study showed plasma metabolome in mice carrying PyMT oncogene and its changes resulted from adipose *Mcp-1* knockout and high-fat consumption. To our knowledge, this is the first study providing an assessment of plasma metabolic profile in this male MMTV-PyMT breast cancer model. Metabolomics differences between mammary tumors and mammary glands and the resulting systematic changes by adipose MCP-1 deficiency certainly warrant further investigation.

In summary, the present study showed that adipose-specific MCP-1 deficient mice had longer tumor latency and lower tumor incidence, tumor progression, and tumor burden compared to WT mice. It indicates that adipose-derived MCP-1 may contribute to mammary tumorigenesis in male MMTV-PyMT mice. Plasma metabolomics analysis identified 56 metabolites that differed significantly among the four dietary groups. Pathway and network analyses of the identified metabolites showed that amino acid and carbohydrate metabolisms are the most disturbed pathways in MMTV-PyMT mice. These metabolomics findings warrant further investigation on the role of adipose-derived MCP-1 in causal relationships between cancer metabolism and mammary tumorigenesis with this MMTV-PyMT model and through which to build strategies for prevention and treatment of male breast cancer.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee, Grand Forks Human Nutrition Research Center, Grand Forks, ND, USA.

AUTHOR CONTRIBUTIONS

LY and SS designed the study, conducted experiments, and collected and analyzed data. All authors contributed to data interpretation, wrote and revised the manuscript, and agreed to be accountable for the content of the work.

FUNDING

This work was funded by the USDA Agricultural Research Service Projects #3062-51000-050-00D and #3062-51000-056-00D.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance the following staff of the Grand Forks Human Nutrition Research Center: Lana DeMars, Shawn Krueger, and Jack He for technical support, Dr. Susan Komanetsky for suggestions to manuscript revision, and vivarium staff for preparing experimental diets and providing high-quality animal care.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.667843/full#supplementary-material

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frontiers in Oncology

ORIGINAL RESEARCH
published: 17 January 2022
doi: 10.3389/fonc.2021.670897

Lipid Changes During Endocrine Therapy in Breast Cancer Patients: The Results of a 5-Year Real-World Retrospective Analysis

Tao He[1†], Xu Li[2†], Jiayuan Li[3], Zhu Wang[4], Yuan Fan[1], Xiusong Li[5], Zhoukai Fu[6], Yunhao Wu[7], Qing Lv[6], Ting Luo[8,9], Xiaorong Zhong[8,9] and Jie Chen[6]*

[1] Department of Breast Surgery, West China School of Medicine/West China Hospital, Sichuan University, Chengdu, China, [2] Center of Biostatistics, Design, Measurement and Evaluation (CBDME), Department of Clinical Research Management, West China Hospital, Sichuan University, Chengdu, China, [3] West China School of Public Health and West China Fourth Hospital, Sichuan University, Chengdu, China, [4] Laboratory of Molecular Diagnosis of Cancer, West China Hospital, Sichuan University, Chengdu, China, [5] Department of Clinical Medicine, Fujian Medical University, Fuzhou, China, [6] Department of Breast Surgery, Clinical Research Center for Breast Diseases, West China Hospital, Sichuan University, Chengdu, China, [7] West China School of Medicine/West China Hospital, Sichuan University, Chengdu, China, [8] Department of Head and Neck and Mammary Gland Oncology, Cancer Center, West China Hospital, Sichuan University, Chengdu, China, [9] Department of Medical Oncology, Cancer Center, West China Hospital, Sichuan University, Chengdu, China

OPEN ACCESS

Edited by:
Pu Li,
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Reviewed by:
Helena Jernström,
Lund University, Sweden
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Baylor College of Medicine, United States

***Correspondence:**
Jie Chen
chenjiewestchina@163.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Breast Cancer, a section of the journal Frontiers in Oncology

Received: *22 February 2021*
Accepted: *23 December 2021*
Published: *17 January 2022*

Citation:
He T, Li X, Li J, Wang Z, Fan Y, Li X, Fu Z, Wu Y, Lv Q, Luo T, Zhong X and Chen J (2022) Lipid Changes During Endocrine Therapy in Breast Cancer Patients: The Results of a 5-Year Real-World Retrospective Analysis. Front. Oncol. 11:670897. doi: 10.3389/fonc.2021.670897

Background: The aim of this study was to investigate the status of serum lipids during endocrine therapy.

Methods: We retrospectively analysed lipid profiles during the 5-year treatment of 1487 consecutive postoperative BC patients. Lipid parameters included triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL-C) and high-density lipoprotein (HDL-C). Those biomarkers were measured at baseline and 1, 2, 3, 4 and 5 years following the initiation of endocrine therapy.

Results: For premenopausal BC patients, LDL levels rapidly decreased at 1 year in the tamoxifen (TAM) group compared with baseline levels ($p<0.05$), and this decline remained for the following 4 years. Additionally, LDL levels were significantly lower in the TAM group than in the nonendocrine group at all assessment time points ($p<0.05$). Similarly, TC levels also decreased in the TAM group compared with baseline levels at all assessment time points ($p<0.05$), and compared with the levels in the nonendocrine group, TC levels were also lower for the first 4 years. For postmenopausal BC patients, there was no significant difference in the lipid profiles (TG, TC, LDL and HDL) in the letrozole (LET), anastrozole (ANA) or exemestane (EXE) groups compared with the nonendocrine group. For patients who received TAM, compared with the nonendocrine group, TC levels decreased at 1 year, and LDL levels decreased at 1 and 2 years.

Conclusions: TAM may improve LDL and TC levels in premenopausal BC patients. In postmenopausal BC patients, aromatase inhibitors (AIs) may have no adverse effects on lipid profiles, and TAM may have limited beneficial effects on serum lipids.

Keywords: breast cancer, endocrine therapy, lipids, tamoxifen, letrozol, anastrozole, exemesatne

BACKGROUND

Breast cancer (BC) is one of the most common malignancies, with an incidence of approximately 2100000 new cases and a mortality of approximately 630000 worldwide in 2018 (1, 2). Different BC subtypes have different biological behaviours and clinical prognoses. Among these, hormone receptor (HR)-positive BC, accounting for 65%-75% of BC cases, is the most common type deserving urgent exploration (3–5). Extensive studies have shown that the better prognosis of HR-positive BC patients may be due to adjuvant endocrine therapy, which substantially reduces recurrence rates and improves overall survival (6).

Prior studies have demonstrated that oestrogen plays a vital role in the development and progression of HR-positive BC (7, 8), and thus, deregulation of oestrogen signalling may be the basic therapeutic strategy for endocrine therapy. Endocrine drugs include selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) (9, 10). As a first-generation SERM, tamoxifen, which was applied in the 1970s, blocks oestrogenic effects by antagonizing oestrogen binding to oestrogen receptors (ERs) and interfering with receptor-mediated transcriptional events (11). AIs (including letrozole, anastrozole and exemestane) inhibit the aromatase enzyme, which is the rate-limiting enzyme in the conversion of androgens to oestrogens, thus leading to the reduction of plasma and intratumoural oestrogen levels (12, 13).

For BC patients receiving endocrine therapy, the oestrogen level might be reduced due to the ovarian function decrease and the side effects of endocrine drugs. While, oestrogen possesses several physiological functions, including involvement in bone and lipid metabolism as well as cardiovascular, cognitive and sexual functions (14). Thus, the decrease in oestrogen may cause some side effects during the endocrine therapy period. In particular, endocrine therapy lasts for 5-10 years (15), and the long-term potential adverse effects of endocrine therapy deserve exploration. Beneficial effects of oestrogen on lipid metabolism have been widely reported. Additionally, previous studies have revealed that the decrease in oestrogen is associated with an increased rate of hyperlipidaemia and cardiovascular disease (CVD), including myocardial infarction and stroke (16, 17). Based on the latest available statistics, with the increasing survival rate of BC patients, the proportion of non-cancer-related death has increased gradually. CVD, accounting for 35% of non-cancer-related deaths in BC patients, is the most common cause of non-cancer-related death and even competes with BC as the leading cause of death in older patients with early BC (18). Thus, possible long-term consequences of endocrine treatment-related changes in lipid metabolism are gradually drawing increasing attention in BC patients receiving endocrine therapy.

Abbreviations: BC, breast cancer; TG, triglycerides; TC, total cholesterols; LDL-C, low-density lipoprotein; HDL-C, high-density lipoprotein; CVD, cardiovascular diseases; ECOG, Eastern Cooperative Oncology Group; BMI, body mass index; DV, difference value; SERMs, selective estrogen receptor modulators; TAM, tamoxifen; AI, aromatase inhibitor; LET, letrozole; ANA, anastrozole; EXE, exemestane; TOR, toremifene.

To date, there has been little agreement about the role of endocrine drugs on lipid metabolism (19–21). Thus, in the present study, we retrospectively investigated the changing trend of lipid profiles during the entirety of endocrine treatment and explored the effects of different endocrine drugs on lipid metabolism in HR-positive BC patients.

MATERIALS AND METHODS

Patient Selection

From February 2009 to January 2015, the medical records of all consecutive BC patients were retrospectively collected from the Department of Breast Surgery, West China Hospital, Sichuan University. The Institutional Review Board and Ethics Committee of West China Hospital approved this retrospective study (IRB No. 2019-1023). The inclusion criteria were as follows: (1) female patients aged ≥18 years; (2) patients pathologically diagnosed with BC; (3) postoperative BC patients and the method of operation were according to the NCCN guidelines (4) stage I-III BC patients; (5) postoperative systemic therapies including endocrine therapy, chemotherapy, targeted therapy, and radiotherapy were recommended according to the NCCN guidelines, (6)patients who did not receive endocrine therapy or patients who received one of the following endocrine drugs: tamoxifen (TAM), letrozole (LET), anastrozole (ANA), or exemestane (EXE); and (7) patients who had adequate organ function before surgery and Eastern Cooperative Oncology Group (ECOG) ≤2. The exclusion criteria were as follows: (1) patients with bilateral BC; (2) patients with inflammatory BC; (3) patients with tumors in other systems; (4) patients who received endocrine therapy before being diagnosed with BC; (5) patients who had not completed the established endocrine regimens or who had changed endocrine drugs for various reasons; (6) patients diagnosed with dyslipidemia and taking lipid-lowering drugs before endocrine therapy; (7) patients with heart disease and liver dysfunction before endocrine therapy; and (8) pregnant and lactating women.

Data Collection and Evaluated Parameters

All clinicopathological data were reviewed from medical records by two clinicians who signed confidentiality agreements. The variables of interest included height, weight, body mass index (BMI), age at diagnosis, menopausal status, tumour location, surgical procedures, TNM (Tumour, Node, Metastasis) stage (22), ER, progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), Ki-67 status and lipid parameters at baseline (within one week prior to drug administration) and at 1, 2, 3, 4 and 5 years following the initiation of endocrine therapy. Height and weight were assessed for the determination of BMI, which was calculated as the weight divided by the height squared (kg/m^2). Lipid parameters includes triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL). All patients fasted overnight before blood sampling, and blood samples were collected the next

morning on an empty stomach. To avoid lipid changes associated with freezing and storage, all lipid analyses were carried out from unfrozen samples within 2 hours after sampling. All measurements were performed on a Cobas8000 system (Roche Diagnostics GmbH, Germany) in the Department of Laboratory Medicine, West China Hospital, Sichuan University, which is a College of American Pathologists (CAP)-accredited laboratory. According to the Chinese guidelines for the management of dyslipidaemia (23), dyslipidaemia was considered if patients met at least one of the following criteria: TG≥ 1.7 mmol/L, TC ≥ 5.2 mmol/L, LDL-C ≥ 3.4 mmol/L or HDL-C ≤ 1.0 mmol/L.

Endocrine Drugs

For premenopausal patients, the nonendocrine group (ER and PR negative) did not receive any endocrine therapeutic agents, while the TAM treatment group (ER or PR positive) received 5-year continuous oral administration of 10 mg TAM twice daily.

Similarly, for postmenopausal patients, the nonendocrine group (ER and PR negative) did not receive any endocrine therapeutic agents, while ER or PR positive patients received continuous oral administration of one of the following endocrine agents: TAM (20mg/d), LET (2.5 mg/d), ANA (1 mg/d) or EXE (25 mg/d). Medication was begun within 2 weeks of the end of chemotherapy treatment and planned to continue for 5 years.

Statistical Analysis

According to the menopausal status, the participants were stratified into premenopausal and postmenopausal groups. The differences in baseline lipid profiles and the baseline covariates in several endocrine therapy groups were evaluated by ANOVA and the chi-square test.

The generalized linear mixed model was used to evaluate the changing rules of lipid profiles in different endocrine therapy groups during the treatment period. First, the time of administration was taken as a continuous variable to evaluate the overall trend of blood lipid changes with time and the influence of different endocrine agents on blood lipids in both premenopausal and postmenopausal patients. In both premenopausal and postmenopausal subgroups, BMI, smoking, hypertension, age, and chemotherapy, endocrine therapy medication type, medication time (classified or continuous), and medication type * time were taken as the fixed effects, the intercept was taken as the random effect, and the UN covariance matrix (unstructured covariance) was used. Since the distribution of postmenopausal baseline LDL in the different endocrine groups was significantly different, in the analysis of postmenopausal LDL, the difference between LDL levels at the assessment time points and baseline LDL levels were used as the response variables of the generalized linear mixed model. Then, the time of administration was taken as a classified variable to explore the changes in lipid profiles in different medication groups and the differences among different medication groups at 1, 2, 3, 4, and 5 years. The trend of serum lipid levels in premenopausal and postmenopausal groups and the blood lipid levels of different AI groups were described by model adjusted least-square means.

All the analyses in this study were carried out with SAS 9.4 software (SAS Institute, Cary, NC, USA). Statistical tests were all 2-sided and $p < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

A total of 1487 eligible and consecutive postoperative BC patients were identified for this study, with 756 premenopausal patients and 731 postmenopausal patients (**Figure 1**). The clinicopathological characteristics are summarized in **Tables 1**, **2**. For the premenopausal women, 486 patients received TAM treatment, and 270 patients did not receive endocrine drugs. For the postmenopausal patients, 198 patients received TAM, 151 patients received LET, 118 patients received ANA, 82 patients received EXE, and 182 patients did not receive endocrine therapy.

The Variations in Lipid Profiles in Premenopausal Patients

The time-dependent changes in lipid profiles and the results of intergroup tests using least-square means are shown in **Figure 2**. The means and standard error are presented for lipid parameters measured at baseline and 1, 2, 3, 4 and 5 years.

TC rapidly decreased at 1 year (p<0.05) and then showed a slow increasing trend that was still below the baseline values (p<0.05) in the TAM group. Compared with the nonendocrine group, TC was significantly lower in the TAM group in the first 1-4 years (p<0.05). At 5 years, TC values were still lower in the TAM group than in the nonendocrine group, but there was no statistically significant difference.

TG was stable in the TAM group. Compared with the nonendocrine groups, it was found that TG levels were higher in the TAM group than in the nonendocrine group at 2 years (p<0.05); nevertheless, no difference was found at other measurement time points.

LDL rapidly decreased in the TAM group compared with baseline levels at 1 year (p<0.05), and the decline remained for the following 4 years (p<0.05). Notably, LDL levels were significantly lower in the TAM group than in the nonendocrine group at all assessment time points (p<0.05).

HDL trends witch increased at 1 year and remained at high levels at the following assessment time points were similar in both groups. Only at 5 years, HDL levels were lower in the TAM group than in the nonendocrine group (p<0.05).

Over all, TAM had beneficial effects on LDL and TC levels during the 5-year follow-up period (**Table 3**). Compared with the nonendocrine group, patients who received TAM had lower LDL and TC levels, and with the increase in medication time, the effects of TAM on reducing LDL increased (β=-0.05, 95% CI -0.07, -0.03). In addition, we found that TC and LDL levels were positively correlated with age (β=0.03, 95% CI 0.02, 0.04 and β=0.02, 95% CI 0.01, 0.03, respectively). Similarly, the levels of TC, TG, and LDL were also positively correlated with BMI (β=0.02, 95% CI 0.001, 0.04; β=0.05, 95% CI 0.02, 0.07; β=0.03, 95% CI 0.02, 0.04, respectively), while the HDL levels

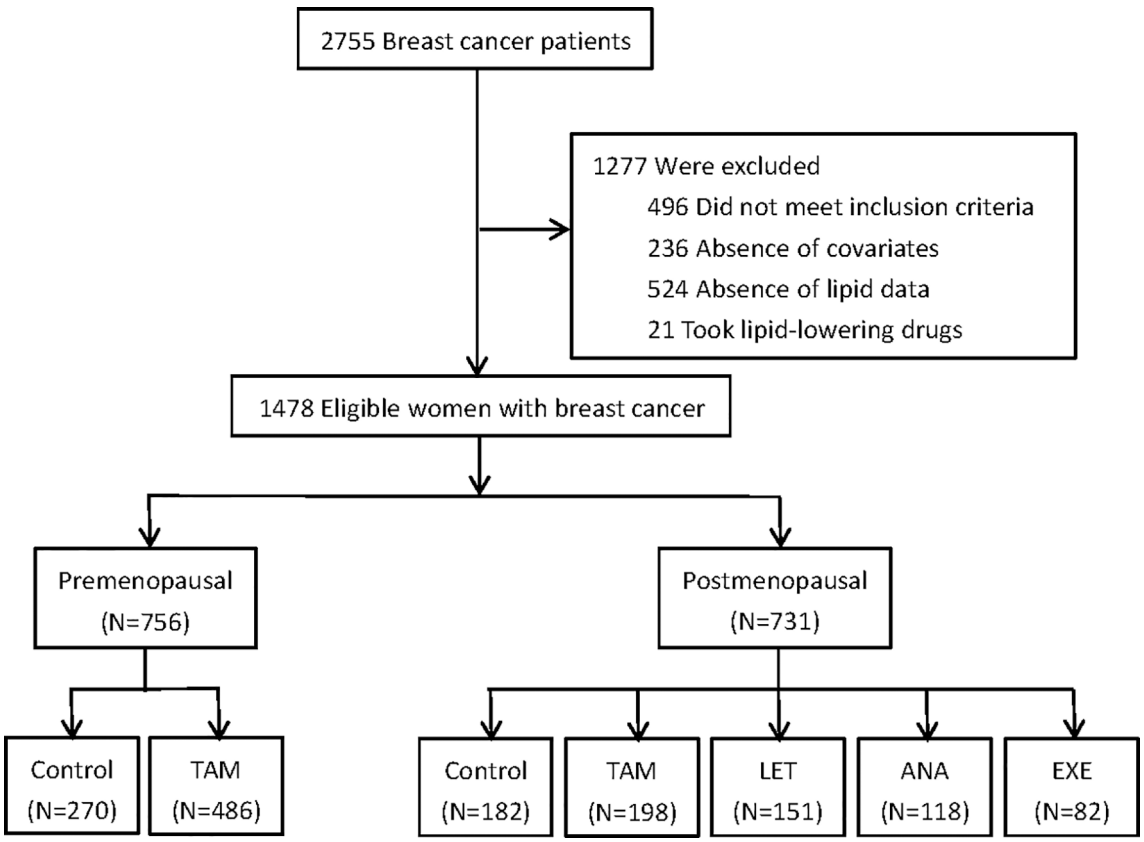

FIGURE 1 | Flow chart of included and excluded patients.

TABLE 1 | Baseline characteristics of premenopausal breast cancer patients.

VARIABLE	Control (n = 270)	TAM (n = 486)	F/X^2	P
Age, mean (SD)	42.83 (5.90)	42.36 (5.22)	1.35	0.25
Smoking, n (%)			1.00	0.30
Yes	1 (0.37%)	5 (1.03%)		
No	269 (99.63%)	481 (98.97%)		
Hypertension, n (%)			0.04	0.83
Yes	5 (1.85%)	8 (1.65%)		
No	265 (98.15%)	478 (98.35%)		
BMI, mean (SD)	22.78 (2.83)	22.95 (3.34)	0.50	0.48
Surgery, n (%)			5.72	0.06
Mastectomy	25 (9.26%)	59 (12.14%)		
Breast conserving operation	235 (87.04%)	392 (80.66%)		
Breast reconstruction	10 (3.70%)	35 (7.20%)		
Chemotherapy, n (%)			5.02	0.08
anthracycline-plus-taxane based regimens	124 (45.93%)	208 (42.80%)		
anthracycline-based regimens	34 (12.59%)	92 (18.93%)		
taxane-based regimens	112 (41.48%)	186 (38.27%)		
TNM stage, n (%)			0.68	0.71
Stage I	87 (32.22%)	144 (29.63%)		
Stage II	176 (65.19%)	331 (68.11%)		
Stage III	7 (2.59%)	11 (2.26%)		
Baseline TC level, mean (SD)	4.97 (0.86)	4.89 (0.96)	1.41	0.24
Baseline TG level, mean (SD)	1.73 (0.98)	1.71 (1.12)	0.09	0.77
Baseline LDL level, mean (SD)	3.00 (0.70)	2.93 (0.74)	1.62	0.20
Baseline HDL level, mean (SD)	1.43 (0.38)	1.45 (0.36)	0.43	0.51

TABLE 2 | Baseline characteristics of postmenopausal breast cancer patients.

VARIABLE	Control (n = 182)	TAM (n = 198)	LET (n = 151)	ANA (n = 118)	EXE (n = 82)	F/X^2	P
Age, mean (SD)	57.12 (6.53)	53.35 (6.44)	58.99 (6.48)	59.18 (6.81)	61.07 (8.51)	95.09	<0.01
Smoking, n (%)						8.47	0.076
Yes	0 (0.00%)	5 (2.53%)	1 (0.66%)	1 (0.85%)	1 (1.22%)		
No	182 (100.00%)	193 (97.47%)	150 (99.34%)	117 (99.15%)	81 (98.78%)		
hypertension, n (%)						9.98	0.041
Yes	7 (3.85%)	9 (4.55%)	13 (8.61%)	14 (11.86%)	7 (8.54%)		
No	175 (96.15%)	189 (95.45%)	138 (91.39%)	104 (88.14%)	75 (91.46%)		
BMI, mean (SD)	23.15 (3.13)	23.66 (3.00)	23.85 (3.15)	24.68 (3.58)	23.52 (2.90)	4.39	<0.01
Surgery, n (%)						15.10	0.06
Mastectomy	15 (8.24%)	3 (1.52%)	8 (5.30%)	11 (9.32%)	8 (9.76%)		
Breast conserving operation	164 (90.11%)	193 (97.47%)	142 (94.04%)	107 (90.68%)	73 (89.02%)		
Breast reconstruction	3 (1.65%)	2 (1.01%)	1 (0.66%)	0 (0.00%)	1 (1.22%)		
chemotherapy, n (%)						113.81	<0.01
anthracycline-plus-taxane based regimens	113 (62.09%)	144 (72.73%)	90 (59.60%)	71 (60.17%)	42 (51.22%)		
Anthracycline based regimens	2 (0.01%)	9 (0.05%)	1 (0.01%)	0 (0.00%)	20 (24.39%)		
Taxane based regimens	67 (36.81%)	45 (22.73%)	60 (39.74%)	47 (39.83%)	20 (24.39%)		
TNM stage, n (%)						14.03	0.08
Stage I	54 (29.67%)	54 (27.27%)	39 (25.83%)	37 (31.36%)	31 (37.80%)		
Stage II	127 (69.78%)	136 (68.69%)	111 (73.51%)	80 (67.80%)	50 (60.98%)		
Stage III	1 (0.55%)	8 (4.04%)	1 (0.66%)	1 (0.85%)	1 (1.22%)		
Baseline TC level, mean (SD)	5.22 (0.97)	5.07 (0.95)	5.24 (1.03)	5.28 (1.07)	5.19 (0.94)	1.14	0.34
Baseline TG level, mean (SD)	1.82 (1.14)	1.77 (1.05)	1.81 (0.79)	1.87 (0.87)	1.66 (0.83)	0.62	0.65
Baseline LDL level, mean (SD)	3.16 (0.85)	3.00 (0.76)	3.27 (0.79)	3.35 (0.79)	3.12 (0.83)	4.42	<0.01
Baseline HDL level, mean (SD)	1.45 (0.40)	1.39 (0.42)	1.39 (0.32)	1.42 (0.36)	1.51 (0.46)	1.96	0.10

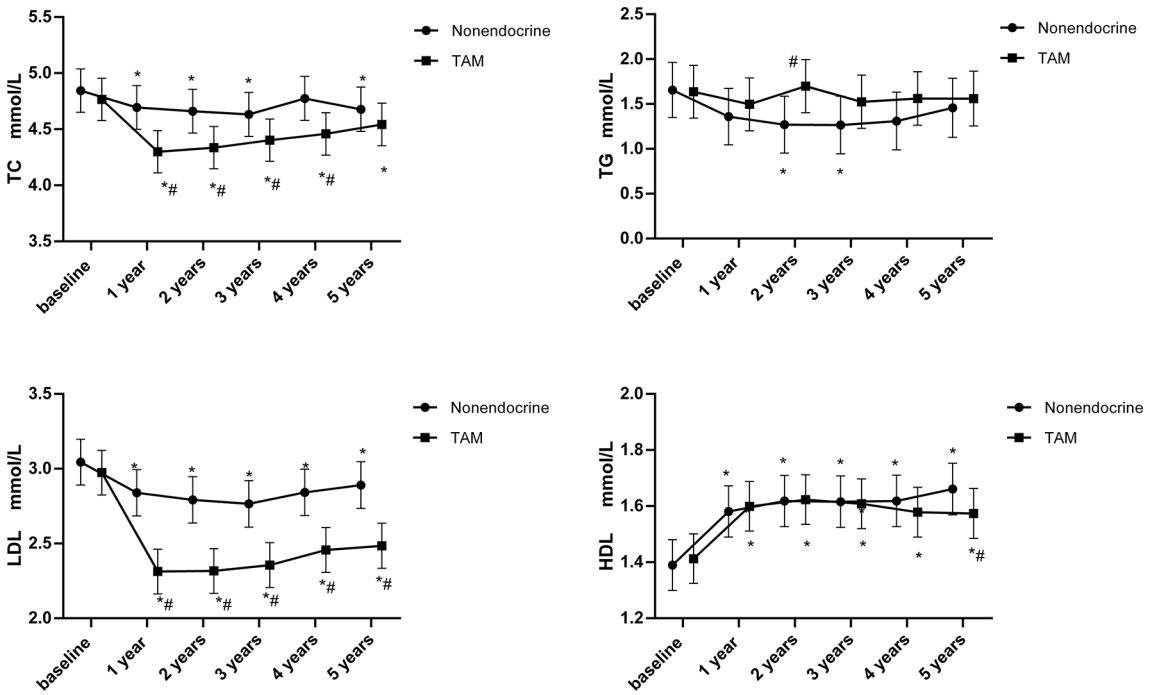

FIGURE 2 | Line chart of lipid profiles during 5-years endocrine therapy in premenopausal BC patients. *$p < 0.05$ for comparison with baseline. #$p < 0.05$ for comparison with the nonendocrine group.

were negatively correlated with BMI (β=-0.03, 95% CI -0.04, -0.02).

In addition, among the 756 premenopausal patients, 445 patients were diagnosed with dyslipidaemia (58.86%) before endocrine therapy. Moreover, the proportion of newly diagnosed dyslipidaemia during endocrine therapy in premenopausal BC was also assessed (**Figure 3**). At all assessment time points during the whole endocrine therapy

TABLE 3 | Comparison of lipid profiles in premenopausal BC patients.

	TC β (95%CI)	TG β (95%CI)	LDL β (95%CI)	HDL β (95%CI)
ET				
control	0.00	0.00	0.00	0.00
TAM	-0.24 (-0.37,-0.12)	0.09 (-0.15,0.32)	-0.29 (-0.39,-0.19)	0.05 (-0.01,0.11)
Time	-0.02 (-0.04,-0.002)	-0.05 (-0.11,0.01)	-0.03 (-0.05,-0.02)	0.05 (0.04,0.06)
ET*Time				
control*time	0.00	0.00	0.00	0.00
TAM*time	-0.01 (-0.04,0.02)	0.04 (-0.04,0.12)	-0.05 (-0.07,-0.03)	-0.02 (-0.03,-0.01)
Chemotherapy				
anthracycline-plus-taxane-based regimens	0.00	0.00	0.00	0.00
anthracycline-based regimens	0.05 (-0.10,0.21)	-0.10 (-0.34,0.14)	0.04 (-0.09,0.16)	0.02 (-0.05,0.09)
taxane-based regimens	-0.05 (-0.17,0.07)	0.01 (-0.18,0.19)	-0.04 (-0.14,0.05)	0.00 (-0.06,0.07)
Smoking				
No	0	0	0	0
Yes	0.03 (-0.57,0.63)	0.22 (-0.68,1.12)	-0.11 (-0.59,0.37)	0.04 (-0.24,0.32)
Hypertension				
No	0	0	0	0
Yes	0.26 (-0.16,0.68)	-0.20 (-0.79,0.56)	0.03 (-0.30,0.37)	0.05 (-0.14,0.25)
Age	0.03 (0.02,0.04)	0.01 (-0.01,0.02)	0.02 (0.01,0.03)	0.00 (-0.003,0.01)
BMI	0.02 (0.001,0.04)	0.05 (0.02,0.07)	0.03 (0.02,0.04)	-0.03 (-0.04,-0.02)

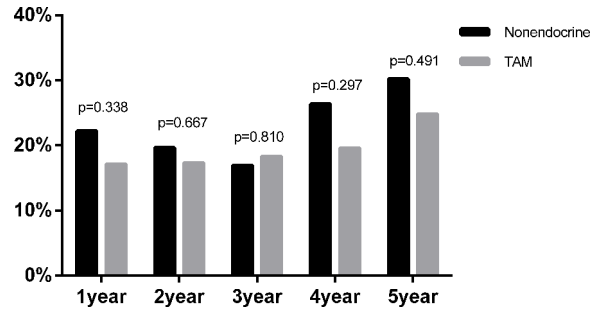

FIGURE 3 | The proportion of newly diagnosed dyslipidaemia after endocrine therapy in premenopausal BC.

period, there was no statistically significant difference in the proportion of newly diagnosed dyslipidaemia between the TAM and nonendocrine groups.

The Variations in Lipid Profiles in Postmenopausal Patients

Additionally, we assessed the time-dependent variations in lipid profiles in postmenopausal patients, and the results are shown in **Figure 4**. The means and standard deviations for lipid parameters were still measured at baseline and at 1, 2, 3, 4 and 5 years.

TC decreased in the TAM group at 1, 2, 3 and 4 years compared with baseline levels (p<0.05). Additionally, TC also decreased in the EXE group at 1 and 2 years compared with baseline levels (p<0.05). While, TC levels remained basically stable in the LET, ANA and nonendocrine groups. We found that only at 1 year, TC levels in the TAM group were lower than those in the nonendocrine group (p<0.05).

TG decreased in the nonendocrine group at 2, 3, 4 and 5 years compared with baseline levels (p<0.05). Similarly, TG decreased in the ANA group at 1 and 4 years compared with baseline levels (p<0.05). Also, TG decreased in the EXE group at 1, 2 and 4 years compared with baseline levels (p<0.05). In the TAM and LET groups, TG levels did not change significantly. The difference between the four endocrine drugs and the nonendocrine group was not significant.

LDL decreased in both TAM and ANA groups at all assessment time points compared with baseline levels (p<0.05). In the LET group, LDL decreased at 4 years compared with baseline levels (p<0.05). Additionally, LDL levels were significantly lower at 1 and 2 years in the TAM group than in the nonendocrine group (p<0.05).

HDL levels increased in the TAM group at 1 year compared with baseline levels (p<0.05), while HDL levels did not change significantly in the LET, ANA, EXE or nonendocrine groups. Additionally, the difference between the four endocrine drugs and the nonendocrine group was not significant.

Additionally, we performed further analysis to assess the effects of endocrine drugs on serum lipids in postmenopausal patients (**Table 4**). The present study showed that there was no statistically significant alteration in the lipid profiles (TG, TC, LDL and HDL) in the LET, ANA or EXE groups compared with the nonendocrine group. For patients who received TAM, we found that LDL levels decreased from the first year, and with the increase in medication time, the effects of TAM on reducing LDL were weakened (β=0.06, 95% CI 0.02, 0.11). Additionally, BMI was positively correlated with TG (β=0.05, 95% CI 0.02, 0.07), and age was positively correlated with TC (β=0.01, 95% CI 0.00, 0.02).

Additionally, among the 731 postmenopausal patients, 521 patients were diagnosed with dyslipidaemia (71.23%) before endocrine therapy. Furthermore, we compared the proportion of newly diagnosed dyslipidaemia in patients received different endocrine drugs (**Figure 5**). Similar to the results of

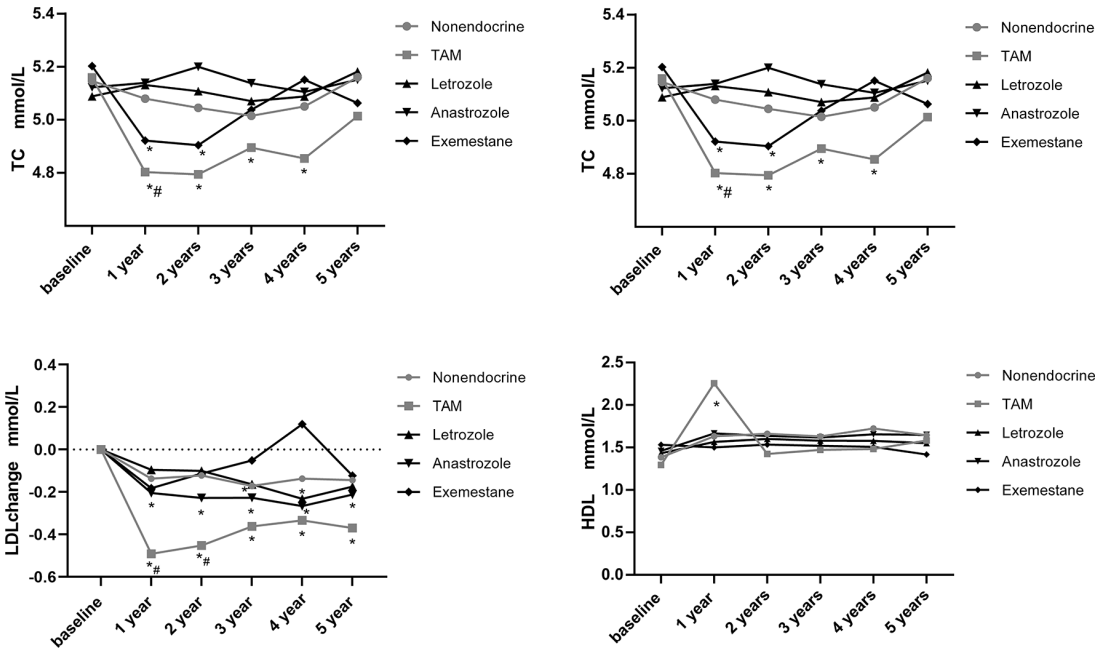

FIGURE 4 | Line chart of lipid profiles during 5-years endocrine therapy in postmenopausal BC patients. *$p < 0.05$ for comparison with baseline. $^{\#}p < 0.05$ for comparison with the nonendocrine group.

premenopausal patients, the proportion of newly diagnosed dyslipidaemia were also no statistical difference among nonendocrine, TAM, LET, ANA and EXE groups at all assessment time points during the whole endocrine therapy period.

Comparison of Variations in Lipid Profiles Among Letrozole, Anastrozole and Exemestane in Postmenopausal BC Patients

To assess the effects of LET, ANA and EXE on lipid profiles, intergroup comparisons were performed. The mean absolute values for lipid parameters and the P value of pairwise comparisons across the three endocrine drugs during the study period are shown in **Table 5**. Overall, there was almost no difference in the effects of LET, ANA and EXE on lipid profiles. We found that TC levels were lower in the EXE than in the LET or ANA groups at 1 year (p=0.04; p=0.047, respectively). The LDL levels increased in the EXE group but decreased in the LET group and ANA group at 4 years (p=0.01; p=0.01, respectively).

DISCUSSION

This was a real-world retrospective study with a relatively large sample size in a single institution in China to investigate the potential effects of endocrine therapy on serum lipids. In this study, we found that for premenopausal BC patients, TAM had beneficial effects on LDL and TC levels. For postmenopausal BC patients, the effects of AIs (including LET, ANA and EXE) on lipid metabolism might be insignificant, while TAM has a modest beneficial impact on LDL.

The majority of previous studies which showed beneficial effects of TAM on LDL and TC levels were usually prospective randomized controlled trials, although the variables affecting serum lipids were relatively strictly controlled, the sample size was usually small, and the follow-up time was generally short, mostly from 3 months to 2 years (24–26). This real-world retrospective study complemented and further confirmed previous results with a relatively large sample size and longer follow-up. The beneficial effects of TAM on lower LDL levels may be explained by the fact that TAM may inhibit enzymes, including sterol-Δ8,7-isomerase and acetyl-coenzyme A acetyltransferase, which are involved in the cholesterol metabolism pathway to decrease LDL levels (27). Additionally, other research have reported that the TAM-induced decrease in LDL levels might be associated with upregulating apo B-100 receptors (28).

Regarding the effects of TAM on HDL levels, previous studies have shown different results. Wasan KM et al. reported that after 2 years of TAM administration, a moderate increase in HDL levels was observed in postmenopausal BC women (29). On the other hand, Vehmanen et al. reported that the changes in HDL cholesterol levels after 3 and 6 months of tamoxifen therapy were insignificant both in the TAM group and in the nonendocrine group (30). Similarly, another report published by Sawada also showed that TAM did not change HDL levels (24). Similarly, the present study showed that except for the lower HDL levels in the

TABLE 4 | Comparison of lipid profiles in postmenopausal BC patients.

	TC β (95%CI)	TG β (95%CI)	LDL change β (95%CI)	HDL β (95%CI)
ET				
control	0.00	0.00	0.00	0.00
TAM	-0.14 (-0.43,0.14)	-0.01 (-0.31,0.30)	-0.50 (-0.77,-0.24)	0.14 (-0.39,0.67)
LET	-0.05 (-0.28,0.19)	-0.14 (-0.39,0.12)	-0.02 (-0.25,0.22)	0.01 (-0.48,0.51)
ANA	0.02 (-0.23,0.28)	-0.12 (-0.40,0.15)	0.03 (-0.22,0.28)	0.06 (-0.47,0.59)
EXE	-0.05 (-0.35,0.25)	-0.26 (-0.57,0.06)	-0.19 (-0.49,0.12)	0.06 (-0.54,0.66)
Time	-0.01 (-0.04,0.03)	-0.06 (-0.10,-0.03)	-0.02 (-0.05,0.01)	0.05 (-0.07,0.17)
ET*Time				
control*time	0.00	0.00	0.00	0.00
TAM*time	-0.03 (-0.08,0.01)	0.05 (-0.001,0.10)	0.06 (0.02,0.11)	-0.07 (-0.24,0.09)
LET*time	0.01 (-0.04,0.06)	0.06 (0.00,0.11)	-0.01 (-0.06,0.04)	-0.03 (-0.21,0.15)
ANA*time	0.00 (-0.05,0.06)	0.01 (-0.05,0.06)	-0.01 (-0.06,0.04)	-0.02 (-0.21,0.16)
EXE*time	0.00 (-0.06,0.06)	0.04 (-0.03,0.10)	0.05 (-0.005,0.11)	-0.07 (-0.27,0.14)
Chemotherapy				
anthracycline-plus-taxane-based regimens	0.00	0.00	0.00	0.00
anthracycline-based regimens	-0.20 (-0.53,0.13)	-0.10 (-0.45,0.25)	0.31 (0.02,0.61)	-0.05 (-0.57,0.46)
taxane-based regimens	0.16 (-0.27,0.59)	-0.07 (-0.52,0.38)	-0.32 (-0.70,0.05)	-0.13 (-0.77,0.50)
Smoking				
No	0.00	0.00	0.00	0.00
Yes	0.28 (0.00,0.55)	0.10 (-0.19,0.39)	-0.12 (-0.36,0.13)	-0.01 (-0.42,0.41)
Hypertension				
No	0.00	0.00	0.00	0.00
Yes	-0.03 (-0.25,0.19)	-0.05 (-0.29,0.18)	0.12 (-0.07,0.32)	-0.08 (-0.42,0.25)
Age	0.01 (0.00,0.02)	0.00 (-0.01,0.01)	-0.001 (-0.01,0.01)	0.00 (-0.02,0.01)
BMI	-0.00 (-0.03,0.02)	0.05 (0.02,0.07)	0.01 (-0.01,0.03)	-0.03 (-0.06,0.01)

TAM group at 5 years, there was no significant difference between the TAM and nonendocrine groups.

In terms of the TG levels, different studies have different results. Tominaga et al. demonstrated that TAM did not affect TG levels (31), whereas Sawada reported that TAM increased TG by 21.7% (24). Our results showed that TAM seemed more adverse than the nonendocrine at 2 years, but the adverse effects only lasted for a short time, and there was no significant difference at other assessment time points between the TAM and nonendocrine groups. Due to the lack of long-term prospective randomized controlled trials to confirm the effects of TAM on lipid profiles, further research is urgently needed.

Regarding the effects of LET on lipid metabolism, previous studies have shown different results. Elisaf et al. have reported an increase in TC and LDL levels after the administration of LET (32). Similarlly, in the sub-study of the MA.17 trial, significant increases in TC, LDL and HDL levels were observed in the LET group compared with baseline levels, although the placebo arm was not available (33). Moreover,the first results of the BIG 1-98 trial comparing LET with TAM showed that 43.6% of LET-treated patients developed mild to moderate hypercholesterolaemia vs 19.2% of TAM-treated patients (34). While, Harper-Wynne et al. (35) and Heshmati et al. (36) demonstrated that LET had no significant effects on TC, LDL or HDL levels following 3 and 6 months of therapy. Similarly, a multicentre open randomized study containing 27 patients in the LET and TOR groups, respectively reported that after 2 years of follow-up, lipid profiles were essentially unchanged in the LET group (37). Additionally, a sub-study of the MA.17 trial (183 in the LET group and 164 in the placebo group with a 3-year follow-up) demonstrated that after 5 years of TAM treatment, LET therapy did not significantly alter TG, HDL, LDL, or TC levels, and the frequency of hypercholesterolemia was comparable between patients treated with LET and those treated with placebo (38). Overall, the current information is conflicting and insufficient to fully determine the longer-term effects of LET on lipid metabolism.

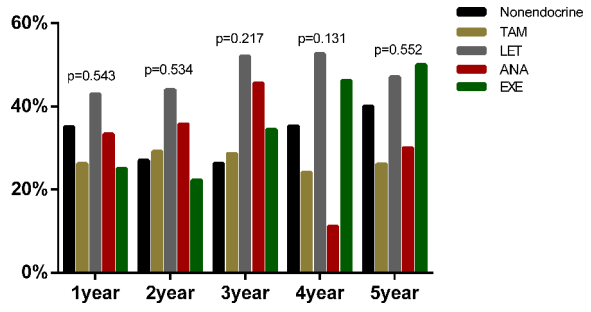

FIGURE 5 | The proportion of newly diagnosed dyslipidaemia after endocrine therapy in postmenopausal BC.

In regard to the changes in lipid profiles during the ANA treatment period, there has been much controversy. In the Italian TAM Arimidex (ITA) trial, patients switching to ANA after 2 or more years of TAM were found to have higher levels of lipid metabolism disorders than those continuing on TAM (9.3% vs 4%, respectively) (39). In addition, another study containing 38 postmenopausal BC patients showed significant increases in TC, LDL, and HDL levels (40). However, there have also been studies that showed beneficial effects on lipid profiles during the ANA treatment period. For instance, Banerjee et al. reported a

TABLE 5 | Comparision of lipid profiles among LET, ANA and EXE in postmenopausal BC patients.

	LET Lsmean (SE)	N	ANA Lsmean (SE)	N	EXE Lsmean (SE)	N	LET vs. ANA t (*P*)	LET vs. EXE t (*P*)	ANA vs. EXE t (*P*)
TC									
baseline	5.11 (0.10)	151	5.16 (0.10)	118	5.20 (0.11)	82	-0.5 (0.62)	-0.71 (0.48)	-0.28 (0.78)
1 year	5.16 (0.10)	122	5.16 (0.11)	100	4.85 (0.12)	58	0 (0.99)	2.04 (0.04)	1.98 (0.047)
2 years	5.12 (0.10)	103	5.18 (0.11)	92	4.90 (0.13)	51	-0.46 (0.64)	1.46 (0.15)	1.8 (0.07)
3 years	5.09 (0.10)	94	5.18 (0.11)	82	5.02 (0.13)	43	-0.72 (0.47)	0.46 (0.64)	1.01 (0.31)
4 years	5.15 (0.11)	80	5.16 (0.11)	75	5.20 (0.14)	34	-0.1 (0.92)	-0.28 (0.78)	-0.19 (0.85)
5 years	5.21 (0.11)	62	5.17 (0.12)	59	5.06 (0.15)	24	0.32 (0.75)	0.88 (0.38)	0.62 (0.54)
TG									
baseline	1.71 (0.10)	151	1.74 (0.11)	118	1.71 (0.12)	82	-0.21 (0.84)	0.01 (0.99)	0.18 (0.86)
1 year	1.69 (0.11)	122	1.46 (0.12)	100	1.38 (0.13)	58	1.74 (0.08)	1.91 (0.06)	0.49 (0.63)
2 years	1.57 (0.11)	103	1.63 (0.12)	92	1.38 (0.14)	51	-0.48 (0.63)	1.09 (0.28)	1.46 (0.14)
3 years	1.60 (0.11)	94	1.63 (0.12)	82	1.46 (0.14)	43	-0.24 (0.81)	0.79 (0.43)	0.96 (0.34)
4 years	1.71 (0.12)	80	1.50 (0.12)	75	1.41 (0.15)	34	1.49 (0.14)	1.62 (0.10)	0.46 (0.65)
5 years	1.77 (0.12)	62	1.53 (0.13)	59	1.59 (0.16)	24	1.52 (0.13)	0.89 (0.37)	-0.31 (0.76)
HDL									
baseline	1.44 (0.17)	151	1.47 (0.19)	118	1.52 (0.22)	82	-0.15 (0.88)	-0.28 (0.78)	-0.14 (0.89)
1 year	1.60 (0.19)	122	1.68 (0.21)	100	1.50 (0.26)	58	-0.3 (0.77)	0.32 (0.75)	0.55 (0.58)
2 years	1.63 (0.21)	103	1.64 (0.21)	92	1.55 (0.27)	51	-0.03 (0.97)	0.24 (0.81)	0.26 (0.79)
3 years	1.62 (0.21)	94	1.63 (0.22)	82	1.54 (0.30)	43	-0.04 (0.97)	0.23 (0.82)	0.26 (0.80)
4 years	1.59 (0.22)	80	1.65 (0.23)	75	1.51 (0.33)	34	-0.23 (0.82)	0.19 (0.85)	0.36 (0.72)
5 years	1.59 (0.25)	62	1.64 (0.26)	59	1.43 (0.36)	24	-0.13 (0.90)	0.39 (0.70)	0.49 (0.63)
LDLchange									
1 year	-0.10 (0.09)	122	-0.21 (0.09)	100	-0.18 (0.11)	58	1.06 (0.29)	0.66 (0.51)	-0.17 (0.86)
2 years	-0.10 (0.09)	103	-0.23 (0.09)	92	-0.11 (0.11)	51	1.21 (0.23)	0.09 (0.93)	-0.85 (0.40)
3 years	-0.16 (0.09)	94	-0.23 (0.09)	82	-0.05 (0.11)	43	0.59 (0.55)	-0.81 (0.42)	-1.25 (0.21)
4 years	-0.23 (0.09)	80	-0.27 (0.10)	75	0.12 (0.12)	34	0.31 (0.76)	-2.44 (0.01)	-2.64 (0.01)
5 years	-0.17 (0.09)	62	-0.21 (0.10)	59	-0.12 (0.12)	24	0.32 (0.75)	-0.34 (0.73)	-0.59 (0.56)

significant increase in HDL levels for 176 BC patients who received 3 months of ANA treatment (41). Additionally, after 3 months of ANA treatment, a significant decrease in TG and an increase in HDL levels were observed by Sawada S (24). While, there were also studies which showed that ANA did not alter lipid profiles. Anan et al. reported a 2-year multicentre open randomized study containing 36 patients in the TOR group and 33 patients in the ANA group, and the results showed that ANA did not significantly alter lipid profiles (19). Considering the controversial results, further studies are needed to confirm the effects of ANA on lipid metabolism.

In terms of the effects of EXE on lipid metabolism, different studies have shown different results. A study showed that after 2 years of follow-up, TC, LDL, and TG levels decreased in both the EXE and placebo groups, and the only difference was a small decrease in HDL observed in the EXE group only (42). A companion sub-study of the EORTC trial (72 patients included in the statistical analysis) indicated that EXE decreased TG levels and had no adverse effects on TC or HDL levels 8, 24 and 48 weeks after treatment (43). In the Greek sub-study of the TEAM trial, baseline lipid levels were compared with levels at 3 and 6 months of treatment. At 6 months, EXE appeared to stabilize TC and HDL levels. In terms of TG levels, EXE reduced TG at both time points and by approximately 10% at 6 months (44). In brief, these studies showed that EXE seemed to have little adverse effects on lipid profiles.

Overall, few studies have explored the effects of endocrine drugs on lipid metabolism, and the results are largely controversial. Additionally, there has been no study that directly compares the effects of TAM, LET, ANA and EXE on lipid profiles in postmenopausal HR-positive BC patients. More importantly, no consensus has been reached so far. To complement previous results, we conducted a retrospective real-world analysis, and our findings indicated that there was no significant difference in the lipid profiles (TG, TC, LDL and HDL) in the LET, ANA and EXE groups compared with the nonendocrine group. For patients who received TAM, we found that LDL levels decreased from the first year, and with the increase in medication time, the effects of TAM on reducing LDL levels were weakened. In conclusion, endocrine therapy had almost no adverse effects on serum lipids in postmenopausal HR-positive BC patients. Due to the lack of long-term prospective randomized controlled trials to confirm the effects of endocrine drugs on lipid profiles, further research is urgently needed.

To the best of our knowledge, the present study was one of the first real-world retrospective studies with a 5-year follow-up and a relatively large sample size to provide comprehensive information on lipid alterations and to explore the effects of different endocrine agents on lipid profiles in both premenopausal and postmenopausal BC patients.There are still several limitations to this study that should be highlighted. First, as a retrospective study, some data were lost, and some confounding factors could not be completely excluded. Second, the present study lacks data on calorie intake and consumption, energy variation and body composition, such as dietary intake, physical activity and basic metabolic rates. Third, although the portion of patients who take

hyperlipidemia drugs was less than 1%, the data was collected only from West China Hospital. It is not clear whether patients were prescribed lipid-lowering drugs in other hospitals or took drugs themselves. However, based on clinical experience, few patients took lipid-lowering drugs and thus, the results of this study were mildly affected. Additionally, many factors may have intertwining effects on lipid profiles; thus, the specific mechanism of lipid profile alterations throughout endocrine therapy was not explained clearly. Last but not least, we lacked the prognostic value of follow-up in later stages, and we did not further explore the relationship between endocrine therapy and the incidence of CVD and the long-term prognosis of BC patients. Thus, further multicenter RCT studies are urgently warranted to supplement and confirm lipid metabolism during endocrine treatment period, and the sustained effects after the end of endocrine treatment deserve increasing attention.

CONCLUSION

In conclusion, favourable changes in lipid profiles, especially LDL and TC levels, were observed in premenopausal BC patients treated with TAM. Additionally, for postmenopausal BC patients, aromatase inhibitors (AIs) may have no adverse effects on lipid profiles, and TAM may have limited beneficial effects on serum lipids.

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 Institutional Review Board and Ethics Committee of West China Hospital. 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

All authors contributed to the conception, design and interpretation of the data; the preparation of the manuscript; and the final editing and approval of the manuscript. All authors read and approved the final manuscript.

FUNDING

This study was supported by Sichuan Science and Technology Program (2019YFS0338) and National Natural Science Foundation of China (32071284).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the whole staff of the Department of Breast Surgery, West China Hospital, who generously provided assistance in the collection of data throughout the duration of the study.

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