NON-CODING RNAs IN BREAST CANCER

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PUBLISHED IN: Frontiers in Oncology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-974-7
DOI 10.3389/978-2-88971-974-7

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Citation: Zhang, W., Kataoka, N., Guan, X., eds. (2021). Non-coding RNAs in Breast Cancer. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-974-7

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frontiers in Oncology

EDITORIAL
published: 11 November 2021
doi: 10.3389/fonc.2021.789798

Editorial: Non-Coding RNAs in Breast Cancer

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Keywords: non-coding RNAs, breast cancer, ceRNA, therapeutic targets, biomarkers

Editorial on the Research Topic

Non-Coding RNAs in Breast Cancer

Breast cancer (BC) is a highly heterogeneous disease and the most common malignancy in women worldwide. Despite early diagnosis and comprehensive treatment, including endocrine therapy, molecular targeted therapy and emerging immunotherapy, breast cancer mortality remains relatively high (1). Thus, identification of novel biomarkers for therapeutic targets and efficacy prediction in breast cancer is urgently required. Apart from about 2% protein-coding genes, the vast majority of the human genome is made up of non-coding RNA (ncRNA), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNA (circRNA), small nucleolar RNA (snoRNA), and PIWI-interacting RNAs (piRNA). To date, a large number of ncRNAs have been identified and found to be dysregulated in various types of cancers including breast cancer. The biological functions of ncRNAs have been extensively studied, and several ncRNAs have been reported to play important roles in various biological processes of breast cancer, including cell proliferation, apoptosis, migration, invasion, angiogenesis, and drug resistance. In addition, the potential of ncRNAs as diagnostic, prognostic biomarkers, and therapeutic targets has been extensively explored in breast cancer (2–4). This Research Topic collected 10 scientific studies (six original research articles, and four reviews), which focused on the new findings or reviewed recent advances of ncRNAs in breast cancer.

miRNAs and lncRNAs are the two most widely studied ncRNAs in breast cancer. miRNAs are small noncoding RNAs of approximately 19 to 25 nucleotides, that can modulate gene expression by targeting selective mRNAs sequences, inducing translational repression or mRNA degradation. Tommasi et al. summarized the mechanisms by which miRNAs interact with BRCA genes, and the role of miRNAs in influencing the risk and diagnosis of BRCA-related breast cancer. They also discussed the biological and clinical significance of the link between nutritional and lifestyle interventions, miRNA expression and germline BRCA mutations. Pedroza et al. performed a whole human miRNome profiling to identify altered miRNAs and miRNA-mRNA network hubs, after AG-205 treatment and PGRMC1 silencing in the TNBC cell line MDA-MB-468. Enrichment analysis showed that the target genes of PGRMC1-altered miRNAs were uniquely involved in signaling pathways, including pathways in cancer, cell cycle and p53 signaling pathway.

LncRNAs are untranslated transcripts with a length of 200 nucleotides or more. Recently, lncRNAs have been reported to have multiple regulatory functions, including acting as regulators of transcription and chromatin remodeling, splicing factors, regulators of mRNA stability, protein

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Edited and reviewed by:
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Specialty section:
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

Received: *05 October 2021*
Accepted: *27 October 2021*
Published: *11 November 2021*

Citation:
Zhang W, Kataoka N and Guan X (2021) Editorial: Non-Coding RNAs in Breast Cancer. Front. Oncol. 11:789798. doi: 10.3389/fonc.2021.789798

decoys and miRNA sponges (5, 6). Among them, lncRNAs are widely studied to act as molecular sponges of miRNAs that compete for miRNA-targeted mRNAs, thereby forming a complex post-transcriptional regulatory network, called the competitive endogenous RNAs (ceRNA) network. In this research topic, several reports have shown that sponge lncRNAs play a critical role in regulating the cancer initiation, progression and drug resistance of breast cancer. Li et al. identified LINC01977 as a key oncogenic driver, that promotes breast cancer progression and chemoresistance to doxorubicin by targeting the miR-212-3p/GOLM1 axis. Cisneros-Villanueva et al. observed that the LINC00460 expression is significantly enriched in the basal-like 2 (BL2) triple negative breast cancer (TNBC) subtype. LINC00460 potentially binds to miR-103-a-1, and acts as a potential regulator of WNT7A expression, resulting in activating the WNT differentiation pathway. Moreover, they also found that the LINC00460:WNT7A ratio could serve as a composite marker to predict favorable overall survival (OS) and distant metastasis-free survival (DMFS) in TNBC, and that their combined expression could predict anthracycline therapy response in ER-positive breast cancer patients.

Other two studies constructed ceRNA regulatory network using differentially expressed lncRNAs, miRNAs, and mRNAs from Gene Expression Omnibus (GEO) or The Cancer Genome Atlas (TCGA) databases. Qin et al. established a new ceRNA regulatory network in TNBC, based on six lncRNAs, 295 miRNAs, and 573 mRNAs. They developed a predictive model for recurrence and pathological stage of TNBC patients, on the basis of a prognostic scoring model of eight differentially expressed genes. Besides, they also constructed a network of small-molecule drugs targeting these eight differentially expressed genes to predict potential therapeutic agents. Another study established a novel strategy to construct a cell-specific ceRNA network to explore the function of hub lncRNAs in the regulation of estrogen in breast cancer. Chen et al. built a cell-specific RNA-RNA co-expression network, based on single-cell expression profiles of predefined reference cells. Next, they constructed a cell-specific ceRNA network to specify breast cancer cell subtypes, by integrating the cell-specific RNA-RNA co-expression network with the existing ceRNA network. They found that NEAT1 is a hub lncRNA of the early estrogen response subtype, and lncRNA DLEU2 is potentially involved in GPCR signaling.

Several authors focused on other ncRNAs in this Research Topic. Circular RNAs (circRNAs), structurally stable non-coding RNAs with a covalently closed circular structure, have been identified and shown to play an important role in the development and progression of breast cancer. Xu et al. summarized recent advances in the regulatory network of circRNA biogenesis, degradation and distribution, as well as the functions, mechanisms and clinical significances of circRNA in breast cancer. PIWI-interacting RNAs (piRNAs) were reported to bind with PIWI family proteins to form PIWI–piRNA complexes to regulate gene expression at the epigenetic and post-transcriptional levels. Qian et al. reviewed the advances, challenges and perspectives of oncogenic or tumor suppressor piRNAs and their regulatory mechanisms in breast cancer.

In addition, two articles provide new research approaches to explore the role of ncRNAs in breast cancer. Mathias et al. proposed a novel bioinformatic approach to incorporate lncRNAs complexity into breast cancer molecular and immune subtypes, by using signal-to-noise ratio metrics to build these subtype-specific signatures. They obtained five immune-related signatures from approximately ten specific lncRNAs, which act as regulators of the immune response, and are associated with different breast cancer specific molecular subtypes. Such as, MEG3, EBLN3P, XXYLT1-AS2, LINC01871, and LINC02613 were associated with immune response activation (or suppression) in Luminal A, Luminal B, HER2-enriched, basal-like and normal-like subtypes, respectively. Animal xenotransplantation means the implantation of human tumor cells into animal hosts for *in vivo* monitoring of tumor development, elucidating pathogenesis and designing new therapeutic strategies. Mouse xenotransplantation is the most commonly used animal model, but the cost and complexity of raising mice are problems that have to be considered. The zebrafish xenograft model offers the accessibility of a xenograft assay as well as economic and experimental advantages. Zampedri et al. summarized the advantages of zebrafish xenotransplants compared to other models, and the use of zebrafish xenotransplants to study the role of lncRNAs in breast cancer development, including proliferation, differentiation, migration, metastasis, angiogenesis and response to drugs.

Taken together, all these studies in the present Research Topic provide new insights into the role of ncRNAs during the development and progression of breast cancer. However, the potential of ncRNAs as therapeutic targets for breast cancer should be more extensively explored in the future. We hope that ncRNAs-based therapeutics would soon evolve into viable options for the treatment of breast cancer patients, either alone or in combination with existing therapeutic agents.

AUTHOR CONTRIBUTIONS

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

FUNDING

This research was supported by National Natural Science Foundation of China (No. 81773102 to XG, 81802667 to WZ), Natural Science Foundation of Jiangsu Province (BK20180133 to WZ), Nanjing Outstanding Youth Fund (No. JQX20009 to WZ), Key International Cooperation of the National Natural Science Foundation of China (No. 81920108029 to XG), Key Foundation for Social Development Project of the Jiangsu Province, China (BE2021741 to XG), and Grants-in-Aid for Scientific Research (18K06012 to NK).

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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: 31 March 2021
doi: 10.3389/fonc.2021.657094

LINC01977 Promotes Breast Cancer Progression and Chemoresistance to Doxorubicin by Targeting miR-212-3p/GOLM1 Axis

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

Received: 22 January 2021
Accepted: 15 March 2021
Published: 31 March 2021

Citation:
Li Z, Li Y, Wang X, Liang Y, Luo D, Han D, Li C, Chen T, Zhang H, Liu Y, Wang Z, Chen B, Wang L, Zhao W and Yang Q (2021) LINC01977 Promotes Breast Cancer Progression and Chemoresistance to Doxorubicin by Targeting miR-212-3p/GOLM1 Axis. Front. Oncol. 11:657094. doi: 10.3389/fonc.2021.657094

Long non-coding RNAs(lncRNAs) play an important role in cancer initiation and progression. However, hub lncRNAs involved in breast cancer still remain underexplored. In this study, integrated bioinformatics analysis was used to define LINC01977 as a key oncogenic driver in breast cancer. Subsequently, *in vitro* assays showed that LINC01977 could significantly promote breast cancer progression and chemoresistance to doxorubicin. To further investigate its biological mechanism, we performed dual-luciferase reporter assay, real-time PCR, RNA immunoprecipitation (RIP), and rescue assay. Our results indicated that LINC01977 may function as ceRNA to prevent GOLM1 gene from miRNA-mediated repression by sponging miR-212-3p. Overall, LINC01977 can serve as a novel prognostic indicator, and help develop more effective therapeutic approaches for breast cancer patients.

Keywords: long non-coding RNAs, breast cancer, bioinformatics, chemoresistance, miR-212-3p, GOLM1

INTRODUCTION

Breast cancer was reported as the leading cause of cancer-related death among women worldwide (1). Although conventional cancer therapies including surgery, radiotherapy and chemotherapy have vastly improved, many patients with breast cancer still have poor clinical outcomes (2–4). Consequently, we should continue to investigate the mechanisms of breast cancer malignant progression to develop more effective therapeutic strategies for breast cancer patients.

Long non-coding RNAs (lncRNAs) are classified as a subtype of non-protein coding RNAs longer than 200 nucleotides in length, which have been found to regulate various biological processes including immunity, apoptosis, autophagy, and cell proliferation (5–7). Moreover, emerging evidence showed that lncRNAs dysregulation contributed to cancer malignant progression by regulating gene expression at epigenetic, transcriptional, and post-transcriptional levels (8–10). For instance, Sun Z et al. reported that lncRNA MALAT1 could promote angiogenesis and epithelial–mesenchymal transition *via* regulating YAP1-MALAT1-miR-126-5p axis in colorectal cancer (11). Xiao G et al. revealed that lncRNA TTTY15 upregulation was associated

with the malignant progression of prostate cancer by sponging the microRNA let-7 to suppress miRNA-mediate CDK6 and FN1 degradation (12). Wang J et al. demonstrated that H19 lncRNA(H19), a highly abundant and conserved imprinted gene, contributed to tamoxifen resistance in breast cancer patients *via* inducing autophagy activation (7). Additionally, Tsai KW et al. identified that LINC00659 promoted cell proliferation and suppress cell apoptosis by activating PI3K-AKT signaling pathway in colon cancer (13).

MicroRNAs(miRNA) are small (19–25 nucleotides) non-protein coding RNAs that commonly affect the process of gene expression *via* gene translation silencing or mRNA degradation (14, 15). Since their discovery, miRNA have been proved to involve in many pathological processes, such as carcinogenesis (16). To our knowledge, altered miRNA expression have been found in most tumor types, including breast cancer. Mechanically, miRNA can load onto the RNA-induced silencing complex (RISC) where they are directly associated with the Argonaute (AGO) family proteins (17). Once they capture a target mRNA, the RISC Argonaute RNase may initiate the process of mRNA degradation. Intriguingly, lncRNAs are able to inhibit miRNA-mediate mRNA degradation *via* binding to miRNA in RISC, namely competing endogenous RNAs (ceRNAs) mechanism (18). Thereby, it is essential to further explore their interactive mechanism for yielding a promising therapeutic avenue for breast cancer patients.

Doxorubicin (DOX), an anthracyclic antitumor antibiotic, is one mainstream chemotherapeutic drug, either in single or combined regimen (19). DOX kills cancer cells by causing DNA damage that typically lead to cell cycle arrest or cell death (20). During tumor evolution, some tumor cells would escape from DNA damage response network to acquire chemoresistance (21). Previous studies reported that the drug transporter ABCB1 can export DOX from cells and then contribute to resistance to DOX (22). However, ABCB1 inhibitor failed to reverse unresponsiveness to DOX in cancer treatment. Currently, accumulating evidence indicated that the aberrant expression of lncRNAs was associated with the development of drug resistance (23), which further exploration can provide a novel means to reduce tumor recurrence after treatment with DOX.

In this study, we performed integrated bioinformatics analysis to select LINC01977, which was significantly upregulated in breast cancer samples and DOX resistant cell lines. Subsequently, the biological function of LINC01977 was evaluated by *in vitro* experiments. Finally, our findings indicated that LINC01977 could promote breast cancer progression and chemoresistance to DOX by targeting miR-212-3p/GOLM1 axis. Overall, LINC01977 may act as a novel prognostic indictor and potential therapeutic target for breast cancer patients.

METHODS

Gene Expression Dataset and Data Processing

Breast cancer RNA-seq count data (level3) and corresponding clinical information were obtained from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/). These data were normalized by edgeR package (24) and then transformed by voom function in limma package (25). Different analysis for 112 tumors and paired normal samples was performed using limma algorithm (25). LncRNAs with FDR <0.05 and absolute log2(fold changes) >1 were considered as significant difference. TCGA pancancer and GTEx datasets were downloaded from UCSC Xena (http://xena.ucsc.edu/). GSE155478 microarray data containing three DOX resistant cell lines and three DOX sensitive cell lines were gained from the GENE EXPRESSION OMNIBUS (GEO, https://www.ncbi.nlm.nih.gov/geo/). Here, we re-annotated probe sets of GPL22755 by mapping all probes to the human genome (GRCh38) with SeqMap method (26). All probes were mapped to the genome without mismatch, and the probes mapped to protein-coding and pseudogene transcripts were removed. The drug response data were sourced from the Genomics of Drug Sensitivity in Cancer (GDSC, https://www.cancerrxgene.org/).

Cell Lines and Cell Culture

In this study, all cell lines were obtained from American Type Culture Collection (ATCC). MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) containing 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin. MCF-10A, MCF-10AT, MCF-10CA1A, and MCF-10CA1H cell lines were cultured in DMEM/F12 (Invitrogen, Carlsbad, California) containing 5% horse serum (Invitrogen), 500 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, Missouri), 100 ng/ml cholera toxin (Sigma-Aldrich), 10μg/ml insulin (Invitrogen), and 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich). All cells used for the experiments were cultured in a humidified incubator with 5% CO2 at 37°C.

Plasmid Establishment and Transfection

All plasmids were sourced from Vigene Biosciences (Rockville, Maryland). The LINC01977 sequence was cloned into PCDH vector, and then transfected into MDA-MB-231 or MCF-7 using Lipofectamine 2000 (Invitrogen, MA, USA). Empty PCDH vector was used as negative control. PLKO.1 lentiviral plasmids for control or carrying shRNA sequences for LINC01977 were used to infect MDA-MB-231 or MCF-7 cells according to the provided protocol. The short hairpin RNAs (shRNA) sequence targeted to LINC01977 are listed as follows:

Sh-1, 5'-TGTTCCTAATTTGGACACTGGTTTA-3';
Sh-2, 5'-AATGAGAAACCAGATACCATGGAAT-3'

Cell Proliferation and Cytotoxicity Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used to assess cell viability. In 96-well plates, the transfected cells were plated at a density of 2×10^3 cells per well and then incubated overnight. Subsequently, the medium was replaced by the solutions containing indicated concentrations of

DOX. After incubation for the indicated time, 20μl MTT (5mg/ml) was added into each well and incubated for another 4-6h. The supernatants were aspirated and 100 μl of dimethyl sulfoxide (DMSO) was add into each wells. Microplate Reader (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance values at 490nm.

Colony Formation Assay

The transfected cells were seeded in 6-well plates at a density of 800 cells/well and incubated for two weeks. Then they were washed with PBS, fixed with methanol, and stained with 0.1% crystal violet. After excess staining was washed with PBS, images were obtained with a microscope.

Ethynyl Deoxyuridine Incorporation Assay

For cell proliferation analysis, the EDU incorporation assay was performed using an EDU assay kit (Ribobio, Guangzhou, China) following the manufacturer's protocols. Breast cancer cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After incubation in DMEM with EDU labeling for 2h, the cells were fixed, permeated, and stained with Apollo Dye Solution. Lastly, the nucleic acid was labeled with Hoechst33342. Laser scanning microscope was applied to observe the treated cells.

Apoptosis Assay

Cell apoptosis assay was performed using Annexin V Apoptosis Detection Kit (BD Biosciences, NJ, USA) according to the manufacturer's instruction. Briefly, the transfected cells were stained with 5μL Annexin V-FITC and 5μL PI, followed by collecting and washing with ice cold PBS. Then cells were incubated in the dark for 15 min. Lastly, apoptosis cells were measured *via* flow cytometry. Further data analysis was evaluated using FlowJo 7.6.1 software.

Transwell Assay

In vitro migration ability was assessed using a migration assay, which was performed using transwell inserts (8-μm pore size, Corning Costar, USA) in 24-well plates (Corning Costar, USA). 1×10^5 MDA-MB-231 or 2×10^5 MCF-7 were suspended in 200μL serum-free medium and seeded into the inside of each insert, while 700μL medium containing 20% FBS was placed in the lower well. After incubation for 24-72h, the infiltrating cells, on the lower surface, were fixed with methanol and stained with 0.1% crystal violet. Cell invasion assay was conducted using the same procedure as in the cell migration assay, except that the inside of each insert was coated with Matrigel. After the infiltrating cells were photographed, ImageJ software was used to count the number of cells.

Wound Healing Assay

For the wound-healing assay, transfected cells were seeded in 24-well plates and cultured in full DMEM medium supplemented with 10% FBS until a confluent monolayer was achieved. Then cells were scratched with a sterile 10 μl sterile micropipette tip, washed with PBS, and replenished with serum-free DMEM. The images were captured by phase-contrast microscope at both 0 and 24h and the wound area was measured by the Image J software.

RNA Immunoprecipitation Assay

For RNA immunoprecipitation (RIP) assay, the procedure was carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, Massachusetts) following the manufacturer' protocol. Antibodies against Ago2 and IgG were purchased from Millipore. The coprecipitated RNAs (total RNA) was extracted for the detection of miRNA and lncRNA expressions by real-time PCR.

Dual-Luciferase Reporter Assay

Full length LINC01977 sequence with wild-type miR-212-3p binding sites were synthesized and fused to the luciferase reporter vector pmirGLO (Promega, Madison, Wisconsin). Wild-type LINC01977 constructs and miR-212-3p mimic cotransfected into breast cancer cells. After incubation for 24h, transfected cells were plated in 96-well plates. According to the manufacturer's instructions, the dual-luciferase reporter assay system (Promega) was used to measure the firefly and Renilla luciferase activities at 48h after transfection. Renilla luciferase activity was employed to normalize against firefly luciferase activity.

RNA Extraction, Reverse Transcription, and Real-time PCR Analysis

Total RNA from breast cancer cells was extracted using The TRIzol reagent (Invitrogen). Complementary DNA (cDNA) of lncRNA and mRNA was reversely transcribed using PrimeScript reverse transcriptase (RT) reagent kit (TaKaRa, Shiga, Japan), while cDNA of miRNA was reversely transcribed using the Prime-Script miRNA cDNA Synthesis Kit (TaKaRa). Biosystems StepOne plus System was employed to perform real-time PCR. Primers used for real-time PCR are listed in **Supplementary Table 1**.

Protein Isolation and Western Blot

Western blot assay was conducted as previously described (27). Briefly, equivalent amounts of total cellular protein from each sample were separated on 10% SDS-PAGE gels and transferred onto a PVDF membrane (Millipore). After 5% non-fat milk was used to block non-specific binding sites, membranes were incubated with specific primary antibodies overnight at 4°C and appropriate secondary antibodies for 2 hours at room temperature. The protein bands were detected using enhanced chemiluminescence (ECL; Bio-Rad, USA). The antibodies used in the experiments are available in **Supplementary Table 2**.

WGCNA Network Construction and Module Preservation Analysis

Here, DESeq2 (28) and preprocessCore packages were used to normalize RNA-seq count data from TCGA. Genes with zero variance between high- and low- groups were removed, and the first 25% genes with median absolute deviation (MAD) value at

least greater than 0.01 were retained. Subsequently, WGCNA was performed according to the previous description (6). Briefly, two weighted gene co-expression networks were established according to GOLM1's median expression value, the low-expression group serving as the reference network and the high-expression group serving as the test network. After defining 5 as an optimum soft threshold power, a scale-free network and topological overlap matrix (TOM) were constructed. In this study, we identified 12 modules in the group with high GOLM1 expression and 13 modules in the group with low GOLM1 expression by hierarchical clustering analysis (**Supplementary Figures1D, E**). Module preservation analysis in WGCNA package was used to evaluate the preservation of gene pairs between two networks. In order to make more accurate result, permutation testing with 1000 times was performed. Z-summary score was applied to evaluate which modules were the lowly preserved module. Z-summary values less than 10 indicated that this module had weak preservation. Finally, clusterprofiler package (29) was employed to perform the gene ontology(GO) analysis for top100 eigengene-based connectivity (kME) genes associated with the lowly preserved module.

Statistical Analysis

All the experiments in the study were repeated at least thrice. Experiment data were presented as the mean ± SD (standard deviation). Continuous data between two groups were assessed using Student's t-test and multiple groups comparison were performed using Kruskal-Wallis test. Comparison between survival curves was carried out using the Kaplan–Meier method followed by the log-rank test. Statistical analysis was performed by GraphPad Prism 8 and R software (Version 3.6.1). Differences with p value < 0.05 were considered statistically significant.

RESULTS

LINC01977 Functioned as an Oncogenic Driver and Predicted a Poor Prognosis in Breast Cancer Patients

We performed different analysis based on limma package in TCGA and GSE155478 datasets, respectively (**Figures 1A, B; Supplementary Figure 1A**). Of upregulated genes, 18 lncRNAs were overlapped (**Supplementary Figure 1B**). Our results indicated that LINC01977 expression was significantly upregulated in breast cancer samples and DOX resistant cell lines (**Figures 1A, B**). LINC01977 is located at 17q25.3 in humans consisting of three exons with 1799bp in length (**Supplementary Figure 1C**). Based on the GDSC drug response data, the high expression group of LINC01977 represented a higher IC50 value for DOX than other subgroups (**Figure 1C**). Kaplan-Meier survival analysis showed that patients with high LINC01977 expression had shorter survival time than those with low LINC01977 expression (**Figure 1D**). Subsequently, breast cancer progression cell line model was enrolled into this study. Real-time PCR result suggested that LINC01977 upregulation may play an important role in the onset and progression of breast cancer (**Figure 1E**). The Pancancer Analysis of transcriptional level revealed that LINC01977 expression was commonly upregulated in most cancer samples compared to their normal samples, including breast cancer (**Figure 1F**).

LINC01977 Knockdown Reduced Breast Cancer Cell Proliferation, Metastasis, and Chemoresistance to DOX

To further identify the biological role of LINC01977 in breast cancer, MDA-MB-231 and MCF-7 cell lines were selected and stably transfected with control or knockdown vectors. The efficiency of shRNA knockdown was evaluated by real-time PCR (**Figure 2A**). MTT and colony formation assays demonstrated that LINC01977 knockdown resulted in a decreased cell growth rate and less colony formation numbers (**Figures 2B, C**). The cytotoxicity assay showed that LINC01977 downregulation made breast cancer cells more sensitive to DOX and represented a decreased IC50 value compared to the control group (**Figures 2D, E**). Besides, decreased metastasis capability was observed in the LINC01977 knockdown group *via* transwell and wound healing assays (**Figures 2F, G**). Taken together, our results revealed that LINC01977 knockdown can inhibit cell proliferation, metastasis, and resistance to DOX in breast cancer cells.

LINC01977 Overexpression Promoted Breast Cancer Cell Proliferation, Metastasis, and Chemoresistance to DOX

Here, we transfected MDA-MB-231 and MCF-7 cell lines with control or overexpression vectors. The efficacy of LINC01977 overexpression was confirmed by real-time PCR in two cell lines (**Figure 3A**). MTT, colony formation, and EDU assays revealed that LINC01977 had a significant positive effect on cell proliferation of MDA-MB-231 and MCF-7 cells (**Figures 3B–D**). Accordant with the knockdown results, LINC01977 overexpression increased IC50 value for DOX in MDA-MB-231 and MCF-7 cells (**Figure 3E**). Meanwhile, the group with LINC01977 overexpression displayed a stronger resistance to DOX than the control group in a time dependent manner (**Figure 3F**). Furthermore, LINC01977 impeded DOX-induced cell apoptosis, which was confirmed by flow cytometry (**Figure 3G**). In the transwell assay, we observed increased tumor cell migration and invasion in the group with LINC01977 overexpression (**Figure 3H**). To sum up, our findings demonstrated that LINC01977 upregulation could promote cell proliferation, metastasis, and resistance to DOX in breast cancer cells.

LINC01977 Served as a Molecular Sponge *via* Binding to miR-212-3p

Cellular fractionation assay revealed that LINC01977 was mainly localized in the cytoplasm rather than nucleus (**Figures 4A, B**). This result suggested that LINC01977 may exert its downstream

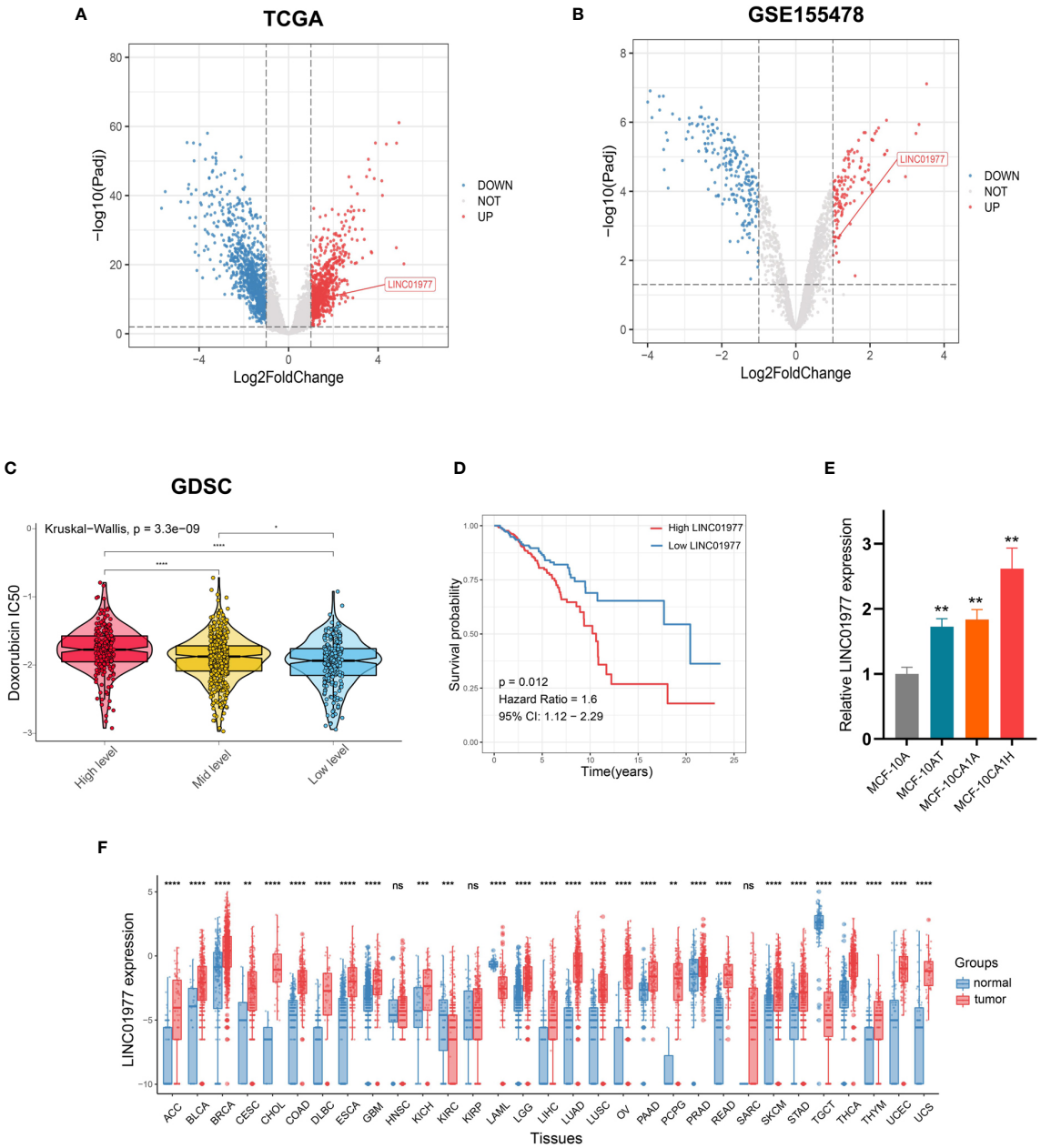

FIGURE 1 | LINC01977 functioned as an oncogenic driver and predicted a poor prognosis in breast cancer patients. **(A)** Different analysis between 112 tumors and paired normal samples from TCGA breast cancer data. **(B)** Different analysis between DOX sensitive and resistant cell lines from GSE155478. **(C)** IC50 value for DOX of TCGA breast cancer patients was estimated on the basis of GDSC drug response data. Three subgroups represented the expression level of LINC01977. **(D)** Kaplan-Meier survival analysis in TCGA breast cancer patients. **(E)** Expression profiles of LINC01977 in breast cancer progression cell line model. **(F)** Expression profiles of LINC01977 in pancancer dataset. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Student's t-test).

function by post-transcriptional regulation. RegRNA2.0 speculated that LINC01977 had potential binding site with the seed sequence of miR-212-3p. RNA-binding protein immunoprecipitation (RIP) assay was performed to investigate whether LINC01977 and miR-212-3p existed in the RNA-induced silencing complex (RISC), which main component was Argonaute2 (AGO2) (30). As presented in **Figure 4C**, the anti-AGO2 group pulled down more amounts of LINC01977 and miR-212-3p than the IgG group, indicating that LINC01977 might sponge miR-212-3p existed in RISC to inhibit downstream mRNA degradation. Subsequent real-time PCR confirmed that LINC01977 overexpression was reversed by transfection of miR-212-3p mimics, whereas miR-212-3p expression was repressed by transfection of LINC01977 overexpression vectors (**Figures 4D, E**).

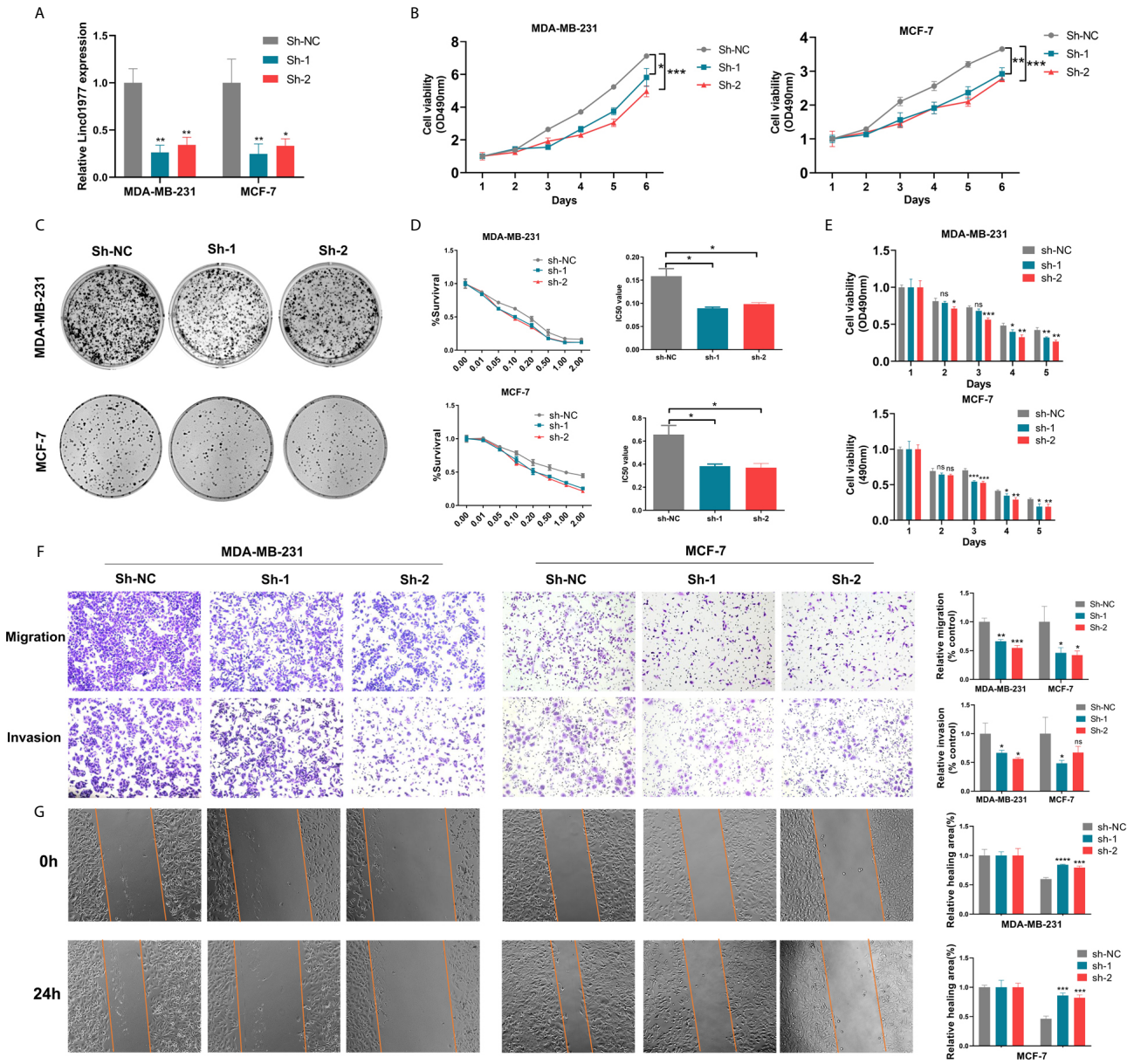

FIGURE 2 | LINC01977 knockdown reduced breast cancer cell proliferation, metastasis, and chemoresistance to DOX. **(A)** The efficiency of shRNA knockdown was evaluated by real-time PCR in MDA-MB-231 and MCF-7 cells. **(B)** MTT assay was used to evaluate cell viability of MDA-MB-231 and MCF-7 transfected by control or knockdown plasmids. **(C)** Colony formation assay displayed the influence of LINC01977 knockdown on breast cancer cell proliferation. **(D)** IC50 value for DOX in MDA-MB-231 and MCF-7 transfected by control or knockdown plasmids. **(E)** Cytotoxicity assay was used to test the suppression of DOX resistance caused by LINC01977 knockdown in MDA-MB-231 and MCF-7 cells. DOX concentration: 0.1μM in MDA-MB-231 and 0.5μM in MCF-7 cells. **(F)** Cell migration and invasion assays. **(G)** Wound healing assay. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Student's t-test).

To further investigate the relationship between LINC01977 and miR-212-3p, the luciferase reporter plasmids containing their putative binding site were constructed (**Figure 4F**). Dual luciferase reporter assay showed that luciferase activity was evidently decreased with the raising amount of transfected miR-212-3p mimics (**Figure 4G**).

LINC01977 Rescued GOLM1 Expression *via* ceRNA Mechanism

To identify the biological function of miR-212-3p, miR-212-3p mimics was transfected into MDA-MB-231 and MCF-7 cells. As shown in **Figures 5A-C**, we found that miR-212-3p was able to attenuate LINC01977-induced cell proliferation, migration, and

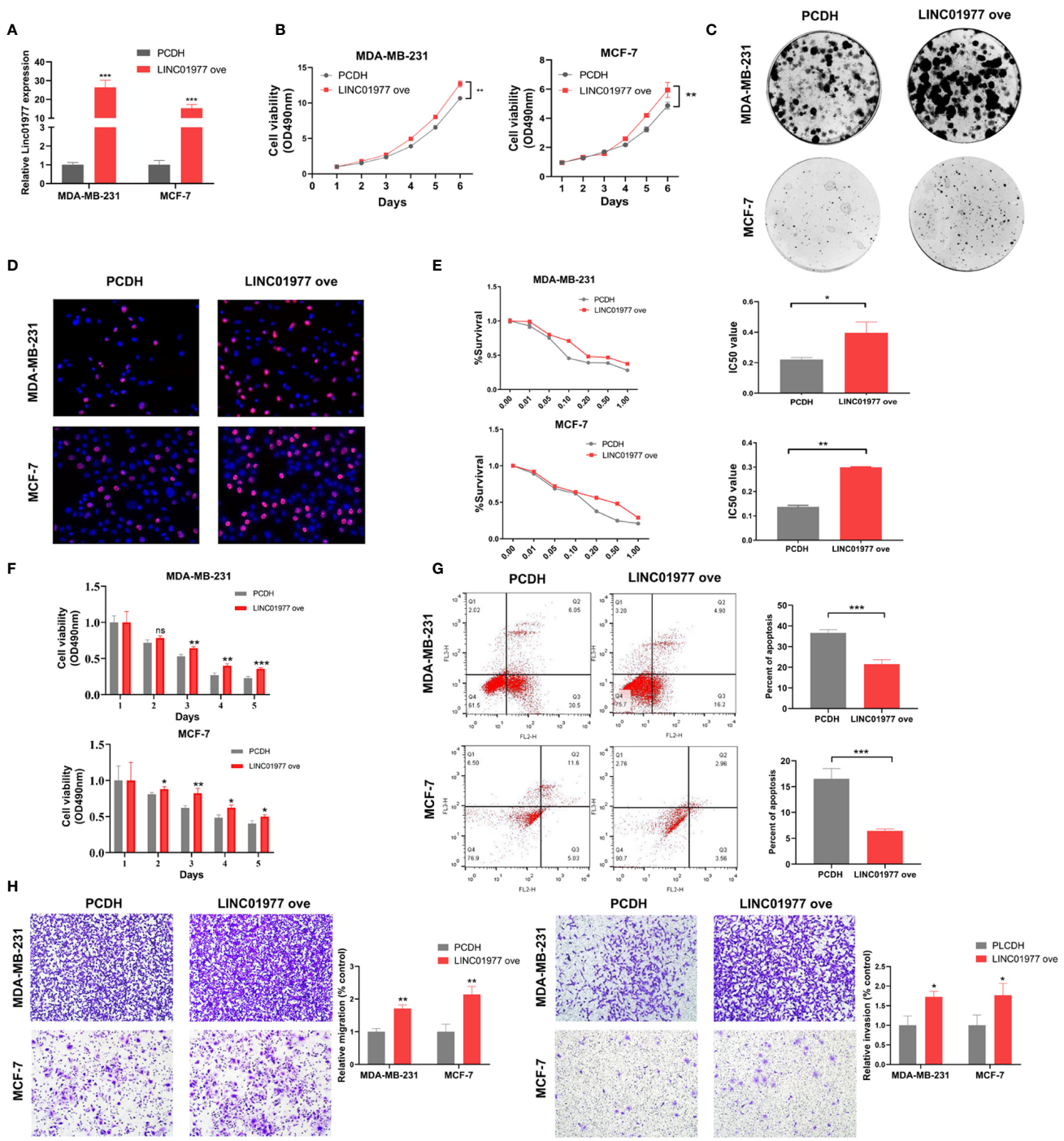

FIGURE 3 | LINC01977 overexpression promoted breast cancer cell proliferation, metastasis, and chemoresistance to DOX. **(A)** The efficiency of LINC01977 expression was evaluated by real-time PCR in MDA-MB-231 and MCF-7 cells. **(B)** MTT assay. **(C)** Colony formation assay. **(D)** EDU assay. **(E)** IC50. **(F)** Cytotoxicity assay. **(G)** Apoptosis assays. As shown in right panel, total apoptosis cells were quantified. **(H)** Transwell assay. Left panel: Cell migration assay. Right panel: Cell invasion assay. PCDH: empty PCDH vector using as negative control. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Student's t-test).

chemoresistance to DOX in MDA-MB-231 and MCF-7 cells. TargetScanHuman 7.2 was used to conjecture that miR-212-3p could combine with the 3' untranslated region (UTR) of GOLM1 (**Figure 5D**). Subsequently, real-time PCR showed that miR-212-3p suppressed GOLM1 expression, whereas this effect can be partly rescued by LINC01977 expression (**Figure 5E**).

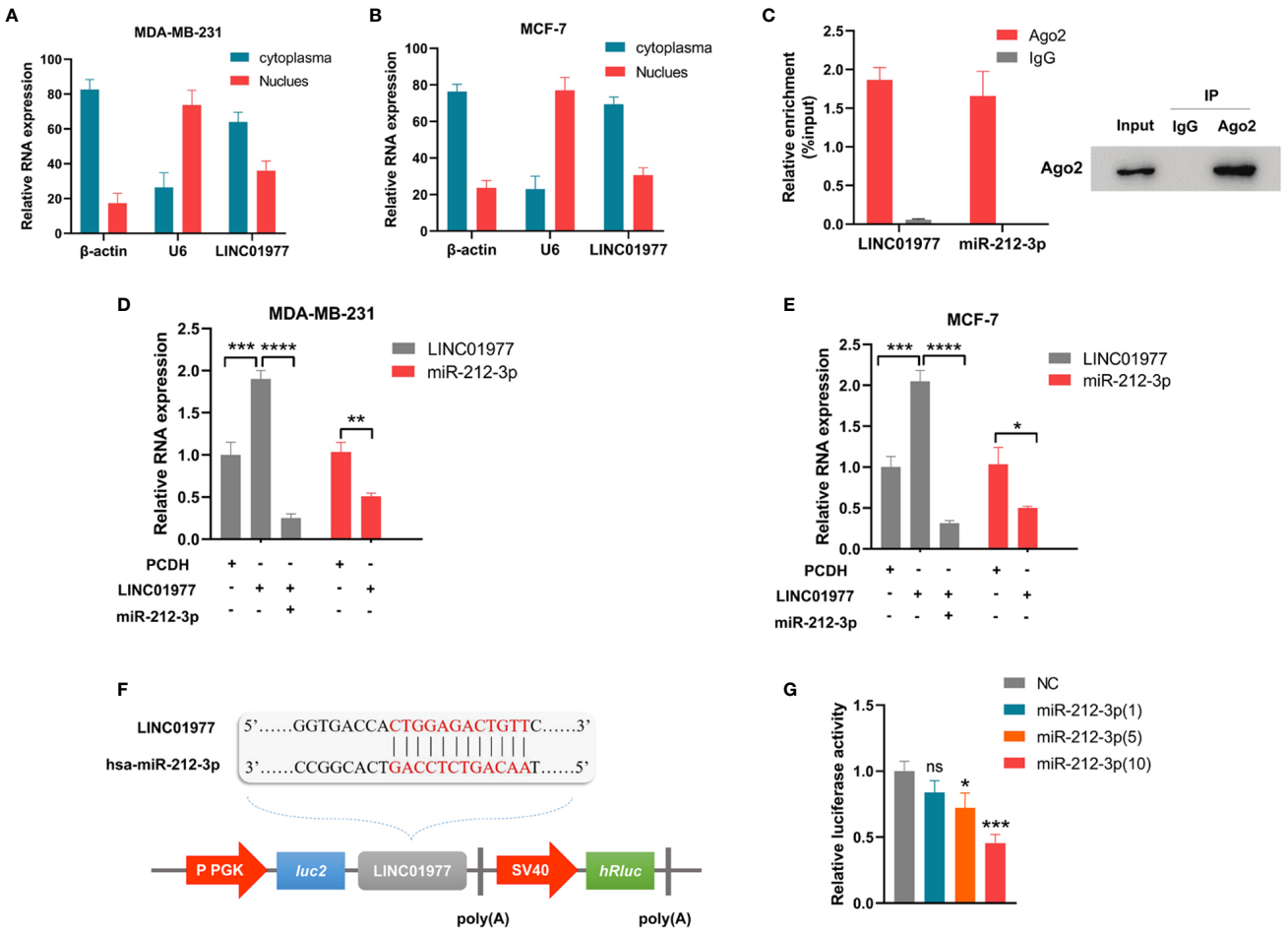

FIGURE 4 | LINC01977 served as a molecular sponge *via* binding to miR-212-3p. **(A, B)** The subcellular location of LINC01977. β-actin: positive control for cytoplasm; U6: positive control for nuclear. **(C)** Left panel: Real-time PCR analysis of LINC01977 and miR-212-3p enriched by Ago2 proteins in MCF-7 cells. Right panel: Western blot was used to confirm the specific immunoprecipitation of Ago2. **(D, E)** Real-time PCR confirmed the interaction between LINC01977 and miR-212-3p in MDA-MB-231 and MCF-7 cells. **(F)** A schematic of wild-type LINC01977 luciferase reporter vectors. **(G)** Dual-luciferase reporter assay. NC: negative control of miR-212-3p mimics. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; Student's t-test).

LINC01977 Promoted Breast Cancer Progression and Chemoresistance to DOX by Targeting miR-212-3P/GOLM1 Axis

After module preservation analysis was performed, purple module (Zsummary =9.2) was defined as the lowly preserved module, which represented the special properties of the group with high GOLM1 expression (**Figure 6A**). We selected top100 kME genes associated with purple module and then performed the GO analysis (**Figure 6B**). In the purple module, several GO terms associated with GOLM1 biological processes were detected, mainly including epithelial cell proliferation, epidermal cell differentiation, cell-cell adhesion *via* plasma-membrane adhesion molecules, stem cell differentiation, and cell junction organization.

To further identify GOLM1's biological function, vectors for control or GOLM1 overexpression were transfected into MDA-MB-231 and MCF-7 cells. The efficacy of GOLM1 overexpression was validated by western blot assay in two cell lines (**Figure 6C**). Next, cell proliferation, chemoresistance, and migration were evaluated using MTT, cytotoxicity, and transwell assays, respectively (**Figures 6D–F**). Our results demonstrated that GOLM1 could promote cell proliferation, metastasis, and resistance to DOX.

DISCUSSION

With the development of RNA sequencing, thousands of lncRNAs have been demonstrated to participate in various biological processes, including tumor malignant progression (11). However, breast cancer-related lncRNAs are still underexplored. In this study, integrated bioinformatics analysis was performed to identify LIN01977 as a key lncRNA in malignant progression of breast cancer. Consistent with a previous report in papillary thyroid

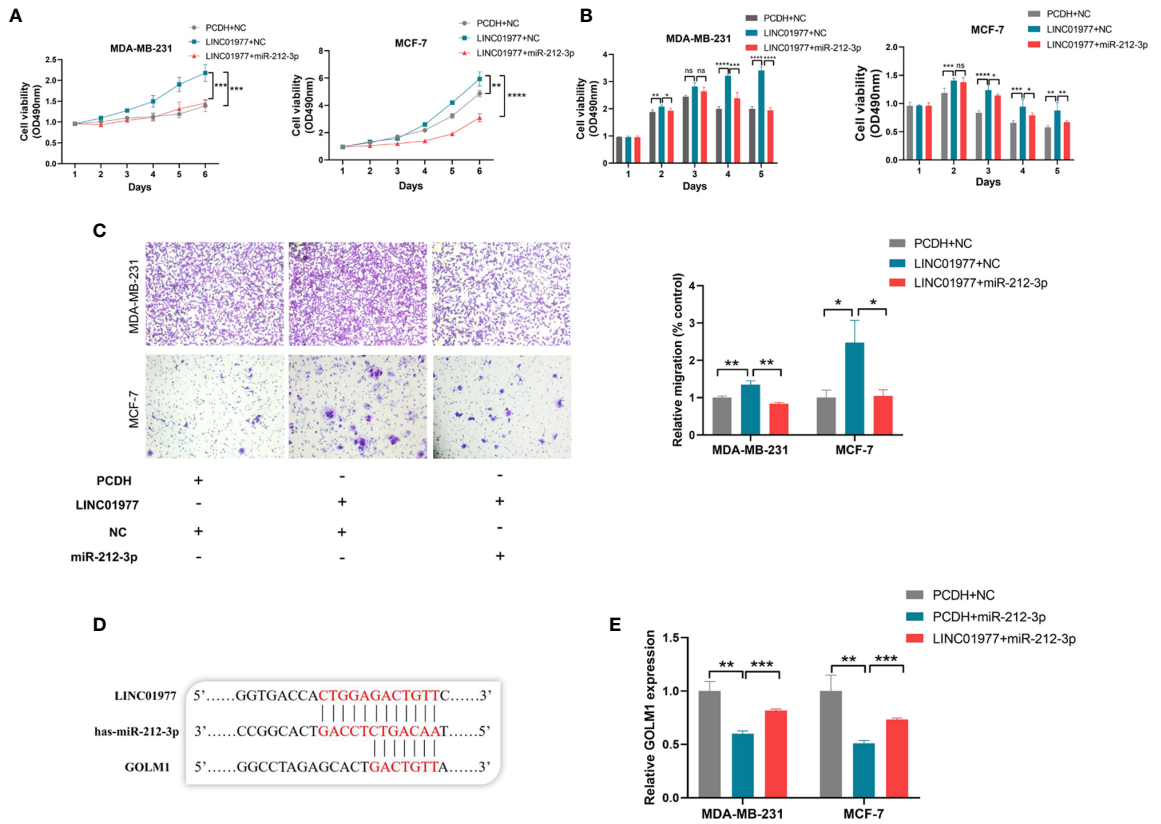

FIGURE 5 | LINC01977 rescued GOLM1 expression *via* ceRNA mechanism. **(A)** Promotion of cell proliferation by LINC01977 was rescued by miR-212-3p expression. **(B)** Promotion of DOX resistance by LINC01977 was rescued by miR-212-3p expression. **(C)** Promotion of cell migration by LINC01977 was rescued by miR-212-3p expression. **(D)** The predicted binding site for GOLM1. **(E)** Real-time PCR was used to identify that GOLM1 expression could be suppressed by miR-212-3p expression and promoted by LINC01977 expression. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Student's t-test).

carcinoma (31), LINC01977 is also remarkably upregulated in breast cancer tissues compared to their adjacent normal tissues. Moreover, GEO and GDSC datasets were employed to show that high LINC01977 expression was significantly associated with resistance to DOX. Survival analysis indicated that high LINC01977 expression predicted shorter survival time in patients with breast cancer. Thus, the biological role of LINC01977 is worth more investigation in the development of breast cancer.

To continue to explore the biological function of LINC01977, we performed *in vitro* experiments using breast cancer cell lines, which further demonstrated that LINC01977 could promote breast cancer cell proliferation, metastasis, and resistance to DOX. Subsequently, cellular fractionation assay was conducted to determine the subcellular localization of LINC01977. Our result indicated that LINC01977 was predominantly located in cytoplasm. Besides, bioinformatics analysis showed that LINC01977 was able to act as a molecular sponge *via* binding to miR-212-3p. Hence, we speculated that LINC01977 may exert its biological function *via* ceRNA mechanism.

miR-212-3p was previously reported as a tumor suppressor in multiple cancer types. Liu H et al. demonstrated that miR-212-3p suppressed tumor cell growth in glioblastoma by miRNA-mediated gene degradation (32). Additionally, Wada R et al. revealed that miR-212 suppressed the development of gastric cancer *via* MECP2 silencing (33). To validate whether LINC01977 could bind to miR-231-3p as a molecular sponge, we performed RIP, real-time PCR, dual-luciferase report assay, and rescue assay. Our findings indicated that miR-231-3p can sponge LINC01977 and reverse the oncogenic phenotype of LINC01977.

To further identify a target gene of LINC01977/miR212-3p axis in breast cancer, bioinformatics analysis was performed to define that GOLM1 was a potential target of miR-212-3p. Real-time PCR was used to validate the relationship among the three players. GOLM1, known as GP73 and GOLPH2, can encode a Golgi associated protein, which is a highly-phosphorylated protein located in the cis and medial-Golgi apparatus (34). Many studies have suggested that GOLM1 can function as a promoter of oncogenic phonotype in several cancer types (35). Ye QH et al. proved that GOLM1 played a key role in cell cycle and metastasis of hepatocellular carcinoma (HCC) cells, and may serve as a prognostic indicator and therapeutic target in HCC patients (36). In our study, WGCNA was carried out to evaluate the biological role of GOLM1 in breast cancer. Purple module in the group with high GOLM1 expression was defined as

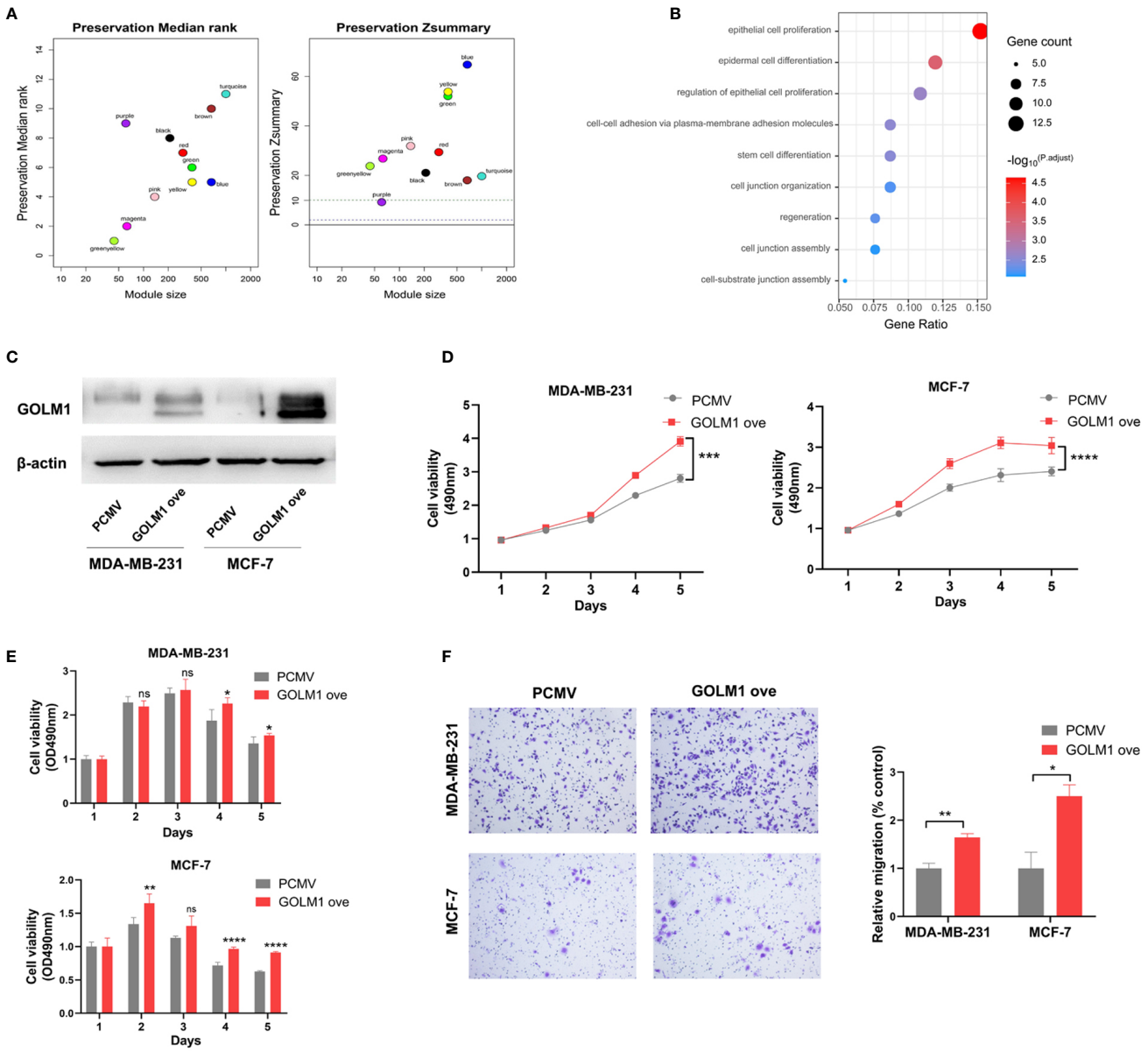

FIGURE 6 | LINC01977 promoted breast cancer progression and chemoresistance to DOX by targeting miR-212-3p/GOLM1 axis. **(A)** Module preservation analysis in WGCNA. Each module is represented by its color-code and name. Left plot shows the preservation median rank. Right plot shows the preservation Zsummary value. Zsummary value <10 represented lowly preserved modules. **(B)** Gene ontology analysis. **(C)** Expression efficiency of GOLM1 was validated by western blotting assay. **(D)** MTT assay. **(E)** Cytotoxicity assay. **(F)** Cell migration array. (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Student's t-test).

non-preservation module. Subsequent GO analysis indicated that the biological processes related to GOLM1 were mainly enriched in epithelial cell proliferation, epidermal cell differentiation, cell-cell adhesion *via* plasma-membrane adhesion molecules, stem cell differentiation, and cell junction organization. Next, we further confirmed that GOLM1 overexpression could evidently promoted breast cancer cell proliferation, metastasis, and resistance to DOX by *in vitro* experiments.

Our data presented here suggested that LINC01977 was an attracting therapeutic target for breast cancer treatment. However, this study was mainly based on human breast cancer cell lines, and lack of the xenograft tumor model and other non-clinical test. Addressing this issue will be a critical direction for our future work.

In summary, LINC01977 was identified as a key oncogenic driver in breast cancer, and significantly promoted breast cancer cell proliferation, metastasis, and chemoresistance to DOX. Moreover, our results demonstrated that LINC01977 may exert its biological effect by targeting miR-212-3p/GOLM1 axis, which broaden our insights into the post-transcriptional regulation

mechanism and help provide a novel prognostic indicator and therapeutic target for patients with breast cancer.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. These can be found in TCGA (http://cancergenome.nih.gov/), GDSC (https://www.cancerrxgene.org/), GTEX (http://xena.ucsc.edu/), and GEO (https://www.ncbi.nlm.nih.gov/geo/) (GPL22755 platform: GSE155478).

AUTHOR CONTRIBUTIONS

QY conceived the project. ZL and YL designed the study and performed the experiments. ZL wrote the manuscript and analyzed the data. ZL, YL, and QY reviewed the data and proofread the manuscript. XW, YL, DL, DH, CL, TC, HZ, YL, ZW, BC, LW and WZ assisted the experiments. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Key Research and Development Program (No. 2020YFA0712400), Special Foundation for Taishan Scholars (No. ts20190971), National Natural Science Foundation of China (No. 81874119; No. 82072912), Special Support Plan for National High Level Talents (Ten Thousand Talents Program W01020103), National Key Research and Development Program (No. 2018YFC0114705), Foundation from Clinical Research Center of Shandong University (No.2020SDUCRCA015), Qilu Hospital Clinical New Technology Developing Foundation (No. 2018-7; No. 2019-3).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.657094/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: 12 April 2021
doi: 10.3389/fonc.2021.628027

LINC00460 Is a Dual Biomarker That Acts as a Predictor for Increased Prognosis in Basal-Like Breast Cancer and Potentially Regulates Immunogenic and Differentiation-Related Genes

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Breast cancer (BRCA) is a serious public health problem, as it is the most frequent malignant tumor in women worldwide. BRCA is a molecularly heterogeneous disease, particularly at gene expression (mRNAs) level. Recent evidence shows that coding RNAs represent only 34% of the total transcriptome in a human cell. The rest of the 66% of RNAs are non–coding, so we might be missing relevant biological, clinical or regulatory information. In this report, we identified two novel tumor types from TCGA with LINC00460 deregulation. We used survival analysis to demonstrate that LINC00460 expression is a marker for poor overall (OS), relapse-free (RFS) and distant metastasis-free survival (DMFS) in basal-like BRCA patients. LINC00460 expression is a potential marker for aggressive phenotypes in distinct tumors, including HPV-negative HNSC, stage IV KIRC, locally advanced lung cancer and basal-like BRCA. We show that the LINC00460 prognostic expression effect is tissue-specific, since its upregulation can predict poor OS in some tumors, but also predicts an improved clinical course in BRCA patients. We found that the LINC00460 expression is significantly enriched in the Basal-like 2 (BL2) TNBC subtype and potentially regulates the WNT differentiation pathway. LINC00460 can also modulate a plethora of immunogenic related genes in BRCA, such as *SFRP5, FOSL1, IFNK, CSF2, DUSP7* and *IL1A* and interacts with miR-103-a-1, *in-silico*, which, in turn, can no longer target WNT7A. Finally, LINC00460:WNT7A ratio constitutes a composite

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

Received: *10 November 2020*
Accepted: *10 March 2021*
Published: *12 April 2021*

Citation:
Cisneros-Villanueva M, Hidalgo-Pérez L, Cedro-Tanda A, Peña-Luna M, Mancera-Rodríguez MA, Hurtado-Cordova E, Rivera-Salgado I, Martínez-Aguirre A, Jiménez-Morales S, Alfaro-Ruiz LA, Arellano-Llamas R, Tenorio-Torres A, Domínguez-Reyes C, Villegas-Carlos F, Ríos-Romero M and Hidalgo-Miranda A (2021) LINC00460 Is a Dual Biomarker That Acts as a Predictor for Increased Prognosis in Basal-Like BreastCancer and Potentially Regulates Immunogenic and Differentiation-Related Genes. Front. Oncol. 11:628027. doi: 10.3389/fonc.2021.628027

marker for decreased OS and DMFS in Basal-like BRCA, and can predict anthracycline therapy response in ER-BRCA patients. This evidence confirms that LINC00460 is a master regulator in BRCA molecular circuits and influences clinical outcome.

Keywords: LINC00460, breast cancer, basal-like, biomarker, increased prognosis, mir-103a, sponge, WNT7A

INTRODUCTION

Breast cancer (BRCA) is a major public health problem, as it is the most frequent malignant tumor in women worldwide. According to GLOBOCAN (2018), at least 2.088 million new cases and a total of 626,679 deaths were reported globally (1). Breast cancer is a phenotypically heterogeneous disease, with well-defined histological types. In 2000, Perou et al. (2), suggested that this physical heterogeneity is also reflected on molecular level, particularly on transcriptome, where same histological types can display a variety of differentially expressed genes. Four BRCA subgroups are differentiated by the expression of 50 genes (the PAM50 signature): Luminal A, Luminal B, HER2-enriched and Basal-like tumors. This molecular classification has been used to discern between aggressive and non-aggressive tumors, evaluate metastatic potential, establish clinical prognosis and estimate survival, among other relevant cancer-related features. Furthermore, patients´ therapy response-associated expression profiles can be subjected to the same classification, allowing us to predict a type of therapy a patient can benefit from, e.g. chemotherapy or anti-hormonal therapy (3). All these clinical advances are only focused on coding RNAs profiles; however, more recent reports have shown that coding (messenger) RNAs represent only 2% of the total transcriptome in a human cell (4). The remaining 98% of transcriptome are non-coding RNAs, that might nevertheless carry relevant biological and clinical information.

Long non-coding RNAs (lncRNAs) are non-translated transcripts with a length of 200 nucleotides or superior. In recent years, several papers have focused on their role in different types of cancer showing their contribution to critical biological processes including carcinogenesis, apoptosis, differentiation, proliferation, invasion and metastasis among others (5–8). LncRNAs display multiple regulatory functions, as they can act as modulators of transcription and chromatin remodeling (9, 10), as splicing factors (11, 12), as regulators of mRNA decay (13), mRNA stability (14), as protein decoys (15) and microRNA (miRNA) sponges (16, 17). In this sponging effect, a lncRNA competes with a miRNA to release the inhibition of other genes (18, 19). Several reports have shown that sponge lncRNAs play a pivotal role in various cancer types (20–24), including BRCA (25, 26) and abnormal expression of lncRNAs can significantly contribute to BRCA initiation and progression (25, 27, 28).

The long intergenic non-coding RNA 460 (LINC00460) is a human lncRNA gene, transcribed from chromosome 13q33.2 and measuring 913 bp (29). The LINC00460 is constituted by three exons and it has been shown that it lacks coding capability (30, 31). According to the ENSEMBL database, the human LINC00460 gene has seven splice variant transcripts reported to date (32). It has been observed that LINC00460 functions as an oncogene, acting as a miRNA sponge (17, 33–43). In these models, LINC00460 sponging activity and its association with cancer-related processes such as increase in proliferation, epithelial to mesenchymal transition (EMT), migration, invasion and metastasis (44–46) is well described. For example, LINC00460 promotes hepatocellular carcinoma progression by sponging miR-342-3p and hence increasing AGR2 expression level (47). Similar LINC00460 sponging and cancer-related mechanisms across distinct tumors are described in several reports (38–43, 48).

The LINC00460 role in clinical cancer features has also been studied. LINC00460 over-expression is strongly associated with poor survival rates in different tumors, such as lung, ovary, larynx, nasopharynx, head and neck, meningioma, kidney, thyroid, colorectal, glioma, osteosarcoma, bladder and cervix (31, 35, 48–56). Thus, LINC00460 is a well-defined sponge lncRNA with significant prognostic potential in several tumors, however its clinical and biological relevance in BRCA is poorly understood (33), and thorough studies are needed.

In this study, we aimed to elucidate the potential role of LINC00460 in BRCA. For this purpose, we performed a series of analysis to infer its biological relevance and verify the underlying role of LINC00460 in BRCA. We identified two novel (not previously reported) tumor types from the TCGA cohort with LINC00460 deregulation. In addition, we used a multivariate Cox regression analysis to demonstrate that LINC00460 expression is related to poor prognosis in three different tumors. We show here that LINC00460 upregulation is significantly associated with improved survival in BRCA in three independent cohorts. This increased survival effect is replicated in Basal-like BRCA and furthermore, we demonstrate that LINC00460 is significantly enriched in the Basal-like 2 (BL2) Triple Negative Breast Cancer (TNBC) subtype. Using differential expression and correlation analysis, we show that LINC00460 overexpression impairs several breast cancer-related pathways: WNT signaling pathway, cytokine inflammatory response and DNA damage response. Among the most relevant potential LINC00460 gene targets, we found *WNT7A, SFRP5, FOSL1* and *IFNK* to be deregulated in the LINC00460-high condition. Using *in-silico* prediction analysis, we show that LINC00460 could interact with miR-103-a, which in turn, potentially regulates *WNT7A*. Finally, we demonstrate that LINC00460: WNT7A ratio is a composite marker for increased OS and DMFS in Basal-like BRCA, and their combined expression can predict anthracycline therapy response in ER-BRCA patients, which further pinpoints the biological and clinical role of these transcripts in TNBC.

MATERIALS AND METHODS

The Cancer Genome Atlas (TCGA) Datasets

LINC00460 expression levels were screened in 31 TCGA tumor datasets and their corresponding GTEx normal tissues using the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) platform (http://gepia2.cancer-pku.cn/#index) (57). The 31 tumors included in the analysis are summarized in **Supplementary Table 1** and enlisted as follows: Acute myeloid leukemia (LAML); Adrenocortical carcinoma (ACC); Bladder urothelial carcinoma (BLCA); Brain lower grade glioma (LGG); Breast invasive carcinoma (BRCA); Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC); Cholangiocarcinoma (CHOL); Colon adenocarcinoma (COAD); Esophageal carcinoma (ESCA); Glioblastoma multiforme (GBM); Head and neck squamous cell carcinoma (HNSC); Kidney chromophobe (KICH); Kidney renal clear cell carcinoma (KIRC); Kidney renal papillary cell carcinoma (KIRP); Liver hepatocellular carcinoma (LIHC); Lung adenocarcinoma (LUAD); Lung squamous cell carcinoma (LUSC); Lymphoid neoplasm diffuse large B-cell lymphoma (DLBC); Ovarian serous cystadenocarcinoma (OV); Pancreatic adenocarcinoma (PAAD); Pheochromocytoma and Paraganglioma (PCPG); Prostate adenocarcinoma (PRAD); Rectum adenocarcinoma (READ); Sarcoma (SARC); Skin cutaneous melanoma (SKCM); Stomach adenocarcinoma (STAD); Testicular germ cell tumors (TGCT); Thymoma (THYM); Thyroid carcinoma (THCA); Uterine carcinosarcoma (UCS); Uterine corpus endometrial carcinoma (UCEC). Mesothelioma (MESO) and Uveal melanoma (UVM) were removed from the analysis, since the correspondent normal tissues were not available in the GEPIA2 platform.

Additional analysis with TCGA breast cancer, normal tissues and HNSC HPV status, was performed using data retrieved from the TANRIC platform (https://ibl.mdanderson.org/tanric/_design/basic/main.html) (58). Violin plots, box plots and notch plots were constructed using the ggplot2 R package. Validation of LINC00460 over-expression in BRCA versus normal tissues was performed using data retrieved from the GSE29431 dataset (https://www.ncbi.nlm.nih.gov/geo/) and analyzed using the lnCAR platform (https://lncar.renlab.org/) (59).

LUAD, LUSC and KIRC tumor stage plots were generated in the GEPIA2 platform. LINC00460 expression levels were considered significantly correlated with tumors when log2 fold change (Log2FC)>1 and p-value<0.01.

Breast Cancer Samples Differential Expression Analysis

Breast cancer RNA seq counts were obtained from The Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov). After dataset preparation, we identified the LINC00460 ID (ENSG00000233532), which is located in chromosome 13: 106,376,563-106,378,217. We then downloaded the LINC00460 expression counts and performed filtering of transcripts with 10 counts or less. In order to generate the high and low LINC00460 expression groups, we calculated two percentiles from the count expression data. The first percentile (25) contains the lowest LINC00460 expression counts, and the upper percentile (60), contains the highest expression levels for this transcript. We then performed differential expression analysis with the DESeq2 tool from the Gene Pattern platform, using the default parameters (http://software.broadinstitute.org/cancer/software/genepattern/) (61).

Genes were considered differentially expressed when Log2FC was >1.5 and -<1.5 and p- adjusted value <0.05. Volcano plots were generated with the ggplot2 and ggrepel R packages.

Breast Cancer Patients and Biological Samples

A total of 74 biological samples (frozen tissues) were collected from breast cancer patients attending Fundación de Cáncer de Mama A.C. (FUCAM) in Mexico City, Mexico. Formalin fixed paraffin embedded (FFPE) breast cancer samples (n=19) were collected from the Hospital Central Sur de Alta Especialidad, Petróleos Mexicanos (PEMEX). None of the patients received neoadjuvant therapy. All patients signed a written informed consent. The studies involving human participants were reviewed and approved by the Research Ethics Committee (INMEGEN) and the FUCAM Ethics Committee (Registration number: CE2009/11).

Biological samples were bisected; one portion was fixed in formaldehyde (10%), paraffin embedded (FFPE) (Paraplast Plus®; Sigma Aldrich ®, St Louis, Missouri, USA) and then submitted to Hematoxylin and Eosin (H&E) staining for histopathological examination by a certified pathologist. Tumor stage was assessed, according to international standards. The second portion of the sample was used for RNA extraction and functional downstream analysis. All tissues were liquid nitrogen-frozen and stored at -80°C. Samples with more than 80% tumor cells were included in the analysis, otherwise were discarded.

An expert pathologist performed the standardized evaluation of stromal tumor-infiltrating lymphocytes (TILs) based on H&E -stained slides of breast tumoral tissue. Briefly, TILs assessment was performed as follows: a) only TILs within the borders of the invasive tumors were evaluated; b) the invasive edge was included in the evaluation; c) only mononuclear infiltrates were included; d) immune infiltrates in adjacent normal tissue or areas of central necrosis or fibrosis were not included; e) the average TILs of the stromal area were reported. For the purposes of this research, the cut-off points of TILs were defined in less than 50% and more than 50% in the stromal area.

In all cases, demographic (age, sex), clinical (date of diagnosis, therapy received), pathologic (stage, grade, histological type) and prognostic data (recurrence, progression and overall survival) were available and correlated with LINC00460 expression status.

RNA Extraction

Total RNA was extracted using the commercial kit AllPrep® DNA/RNA FFPE (Qiagen® Inc, Valencia, CA) following manufacturer's instructions. Briefly, the tissues were deparaffinized, disrupted

and lysed. RNA was then precipitated, washed, purified and suspended in RNAse free water. RNA concentration was evaluated by spectrophotometry (NanoDrop Technologies, Wilmington, Delaware, USA). RNA integrity was analyzed using the BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA), only high-quality samples were used. Samples were stored at -80°C until processing.

Microarray Re-Analysis

Using the BRCA cohort previously reported by our group in (62) (n = 74; Luminal A = 24, Luminal B = 23, HER2 = 14, Basal = 13) in which samples were analyzed using Human Transcriptome Array 2.0 (Affymetrix, Inc, Santa Clara, CA), we were able to identify LINC00460 expression levels across BRCA subtypes. We used the Robust Multi-chip Analysis (RMA) algorithm to minimize the effect of probe-specific affinity differences and to normalize samples (63). Log 2 relative fluorescent signal intensities were computed using the Transcriptome Analysis Console (Affymetrix, Inc, Santa Clara, CA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

cDNA was synthetized using SuperScript III RT-PCR (Invitrogen, ThermoFisher™ Scientific, Waltham, Massachusetts, USA) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Foster City, California, USA), following the manufacturer's instructions. Briefly, 100 ng of total RNA from cell lines or breast cancer samples were used to synthesize cDNA in a final reaction volume of 20μL. The PCR reaction contained 1μL of cDNA, 5 μL 2X TaqMan Universal Master Mix (Applied Biosystems, ThermoFisher™ Scientific, Waltham, Massachusetts, USA), 0.5 μL TaqMan probes (custom-made for LINC00460) and 3.5 μL of nuclease-free water. Both primers and the reporter were designed to target LINC00460, exon 2, transcript variant 1 (NCBI Reference Sequence: NR_034119.2). Forward primer: CCTGGATGAACCACCATTGC; reverse primer: ATGAGAACGAAGGTTACGACCATT; reporter: ATGTTGCAGCTTTCCCA). GAPDH (Hs99999905) and SCARNA5 (Hs03391742_cn) transcripts were used as endogenous controls.

Pathway Enrichment Analysis

Pathway enrichment analysis was performed using Ingenuity Pathway Analysis® (IPA) software. Z-scores and p-values were also computed using this platform. Only differential statistically significant genes were included in this analysis (see criteria above).

Overrepresentation Enrichment Analysis (ORA) was performed with the web-based Gene SeT Analysis Toolkit (WebGestalt) platform (www.webgestalt.org) (64). LINC00460 significantly correlated genes were included in this analysis.

BRCA and HNSC Datasets for Validation

Survival analyses from independent BRCA Gene Expression Omnibus (GEO) cohorts were performed using the Kaplan-Meier plotter (KM plotter) site (http://kmplot.com/analysis/) (65). These GEO datasets contain gene expression data from 6,234 BRCA samples analyzed with multiple microarray platforms, including Human Genome U133 and Human Genome U133 Plus 2.0 Arrays (Affymetrix). We used GEO-derived cohorts, the GSE16446 and GSE21653 datasets, or a combination of several datasets. The GSE16446 dataset was selected, since it contains 120 microarray experiments from primary ER-negative breast tumors of anthracycline-treated patients (66). The GSE21653 dataset contains data of 266 invasive breast adenocarcinomas, with all BRCA subtypes included (67, 68). We used the Affymetrix probe ID 1558930_at, that targets LINC00460, and the Affymetrix probe ID 210248_at, which targets WNT7A. In addition, we performed survival analysis of hsa-miR-103a expression levels, using the mirPower tool (69), included in the KM plotter website.

LINC00460:WNT7A ratio for TNBC survival analyses was also performed in the KM plotter tool. LINC00460 and WNT7A signature expression validation as anthracycline predictive markers were analyzed with the ROC Plotter platform (http://www.rocplot.org/site/index) (70).

For HNSC validation, we downloaded the GEO dataset GSE3292. This cohort has 36 freshly frozen HNSC samples, stratified by HPV status (negative or positive) and analyzed by gene expression microarrays (Affymetrix Human Genome U133 Plus 2.0 Array).

For further TNBC validation, we downloaded the GEO dataset GSE76250, which includes 165 samples. We then analyzed the expression profile of these samples, and classified them with the TNBCtype tool to obtain the Lehman subtypes (http://cbc.mc.vanderbilt.edu/tnbc) (71). We then re-classified these samples into the TNBCtype4 re-assigning IM and MSL subtypes to the second highest correlated centroid, as described in (72). For LINC00460 expression in TNBC cell lines validation, we screened the Cancer Cell Line Encyclopedia (CCLE) online tool (https://portals.broadinstitute.org/ccle) (73).

The validation datasets analyzed for this study can be found in the Gene Expression Omnibus (GEO) web site (https://www.ncbi.nlm.nih.gov/geo/).

miRNA Interaction Prediction Analysis

We used the miRcode prediction tool (http://www.mircode.org/) (74) in order to identify potential LINC00460 miRNA targets. We further corroborated these potential interactions using the lncTAR tool (http://www.cuilab.cn/lnctar) (75). Interaction prediction was considered valid when normalized delta G (ndG) values reached the -0.1 cutoff.

We then performed a third *in-silico* prediction analysis, using the miRPathDB tool (https://mpd.bioinf.uni-sb.de/) (60). We searched the top 15 correlated mRNAs with LINC00460 expression levels (see **Table 4**) and downloaded the miRNAs interaction list. We then compared these new miRNAs list with the previous one described above, and identified potential miRNA-mRNA-linc00460 interactions.

Statistical Analysis

It has been previously shown that cancer gene expression profiles are not normally-distributed, either on the complete-experiment or on the individual-gene level (76). Thus, LINC00460 expression distributions from the TCGA and validation BRCA, KIRC, LUAD and HSNC datasets were first tested for normality

distribution using the Kolmogorov-Smirnov and the Shapiro-Wilk tests. After results computation, we selected comparison statistical tests accordingly.

Overall Survival (OS) using all patients from CESC, GBM, HNSC, LGG, LUAD, PAAD and SARC and the Relapse Free Survival (RFS) analysis using all patients from KIRC, LUAD, READ and SARC was performed in the GEPIA2 platform. Distant Metastasis Free Survival (DMFS) was computed using the KM plotter tool described above.

OS of the BRCA TCGA patients and our independent cohort was analyzed with the Kaplan-Meier model and the multivariable Cox regression model. Relative Risk was also calculated. This analysis was performed with the PASW statistics software (SPSS, IBM®, Quarry Bay, Hong Kong). Chi-square tests were calculated in order to correlate clinical variables status with LINC00460 expression level (high or low expression).

For all statistical tests, the level of significance was <0.05.

RESULTS

LINC00460 Expression Is Deregulated in Multiple Tumors

LINC00460 expression was evaluated in 31 tumor types and normal tissues included in TCGA database. We found that LINC00460 is overexpressed in five different epithelial cancers, namely: Breast cancer (BRCA) (**Figure 1B**); Colon adenocarcinoma (COAD); Head and Neck Squamous cell carcinoma (HNSC), Pancreatic adenocarcinoma (PAAD) and Rectum adenocarcinoma (READ) (Log2FC>1; p<0.05) (**Figure 1A**). Interestingly, we have also detected two central nervous system cancers with LINC00460 low expression, comparing with its normal tissue counterparts: Glioblastoma Multiforme (GBM) and Low-Grade Glioma (LGG) (Log2FC<1; p<0.01) (**Supplementary Figure S1**). These observations potentially suggest a different role for LINC00460 in central nervous system tumors, which can further be analyzed in future studies.

Among the seven LINC00460-deregulated tumors from the TCGA cohort, we identified two novel (not previously reported) tumors, namely LGG and GBM (**Supplementary Figure S1**). We have also confirmed previous observations regarding LINC00460 overexpression in BRCA (33), comparing with normal tissue, using two independent cohorts, namely TCGA (Tanric data, T test; p=1.6845E-14) and the GEO cohort GSE29431 (**Figure 1** and **Supplementary Figure S2**). These observations suggest a ubiquitous role for LINC00460 in cancer biology.

LINC00460 Expression Is Associated With Advanced Clinical Stages and Aggressive Phenotypes in Different Cancers

LINC00460 over-expression was significantly associated with advanced and locally advanced tumor clinical stages in three

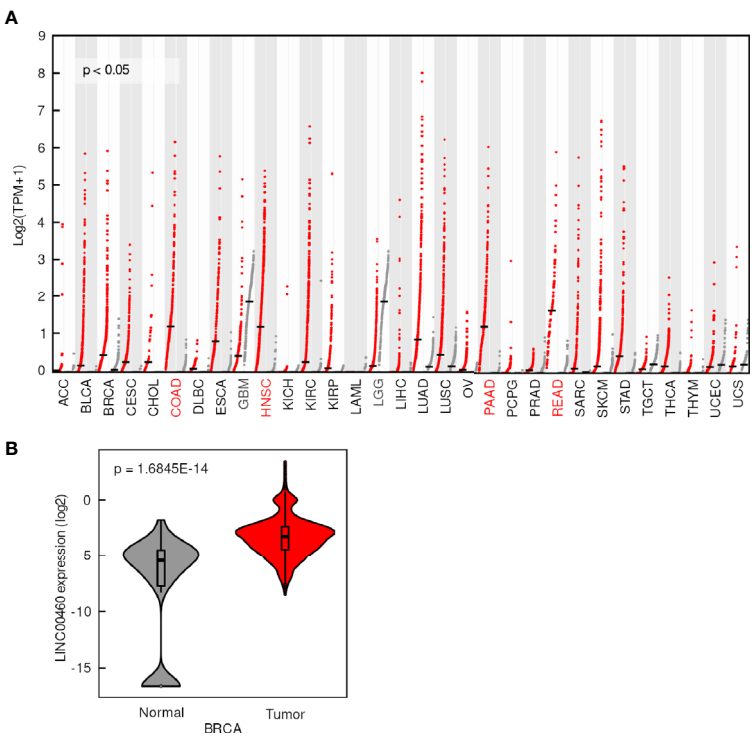

FIGURE 1 | LINC00460 is aberrantly expressed in different tumors. **(A)** LINC00460 expression levels in 33 tumors (red dots) and its correspondent normal tissues (grey dots). Tumors with overexpression and down expression of LINC00460 have red and grey color abbreviations, respectively. Data was obtained from GEPIA2 and modified. **(B)** LINC00460 is over-expressed in breast cancer compared with normal tissues. Data was retrieved from TANRIC and then re-plotted.

distinct cancers: LUSC, LUAD and KIRC (ANOVA; p<0.05; **Supplementary Figures S3A–C**). In the KIRC model, LINC00460 over-expression is significantly associated with high histological grade (Mann Whitney U-test; p=0.0001; **Supplementary Figure S3D**). In addition, LINC00460 overexpression is correlated with further aggressive tumor phenotypes, such as HPV negative HNSC (TCGA: Mann-Whitney U, p< 0.05. GSE3292: one-way ANOVA, p= 0.02) and basal-like BRCA (TCGA: Kruskal-Wallis test, p=3.14E-13. Mexican cohort: Kruskal-Wallis test, p=0.012), using two independent cohorts for each cancer type (**Supplementary Figure S4** and **Figures 2A and B**). This last observation was further validated in a panel of BRCA cell lines, where we detected that LINC00460 is mainly expressed in basal TNBC cells (**Figure 2C**). CCLE screening confirmed TNBC LINC00460 enrichment (**Supplementary Figure S6**). As LINC00460 expression is significantly related to Basal-like BRCA, we reasoned that LINC00460 over-expression might also be associated with Immunohistochemistry (IHC)-detected Estrogen Receptor (ER) and Progesterone Receptor (PR) status in BRCA patients. As expected, LINC00460 expression is enriched in IHC-detected negative Estrogen receptor (ER) (Mann-Whitney U; p=0.000079) and Progesterone Receptor (PR) (Mann-Whitney U; p= 0.024) TCGA BRCA patients (**Figure 2D**). These observations were validated in an independent mexican cohort (Chi squared test ER p-value= 0.028; Chi squared test PR p-value=0.001) (**Figure 2E**).

LINC00460 Is Related With Poor Prognosis in Eight Different Tumors, but Increased Survival Rate in BRCA

After demonstrating that LINC00460 is significantly related to aggressiveness markers in different tumors, we then aimed to know if its deregulation is also related to overall survival (OS) and relapse free survival (RFS) in these cancers, using the TCGA cohorts. As shown in **Figure 3**, LINC00460 overexpression is significantly related to high risk of death in eight different tumors, analyzed in a single model (CESC, GBM, HNSC, KIRC, LGG, LUAD, PAAD and SARC) (log2HR>1; p<0.05) (**Figure 3A**), and with high risk of relapse in four cancers (KIRC, LUAD, READ and SARC) (log2HR>1; p<0.05) (**Figure 3B**). Taken together with previous findings reported in the field (30, 34, 47, 48, 50–52, 54, 55, 57), this data suggests a relevant role for LINC00460 in clinical cancer biology.

An unexpected observation in this regard is the effect of LINC00460 over-expression in BRCA, which is associated with improved OS (**Figure 4A**), compared with the LINC00460 low expression group in two independent cohorts, namely TCGA (through Tanric tool, n=743; logrank p=0.01) and mexican (n=93; HR=1.655; 95% CI [1.038-26.41]; logrank p=0.045) (**Figure 4B**). The clinical and pathological characteristics of the TCGA and mexican BRCA patients are described in **Table 1**.

In addition, we observed that LINC00460 expression level statistically interacts with PR, HER2 status, patient age, and

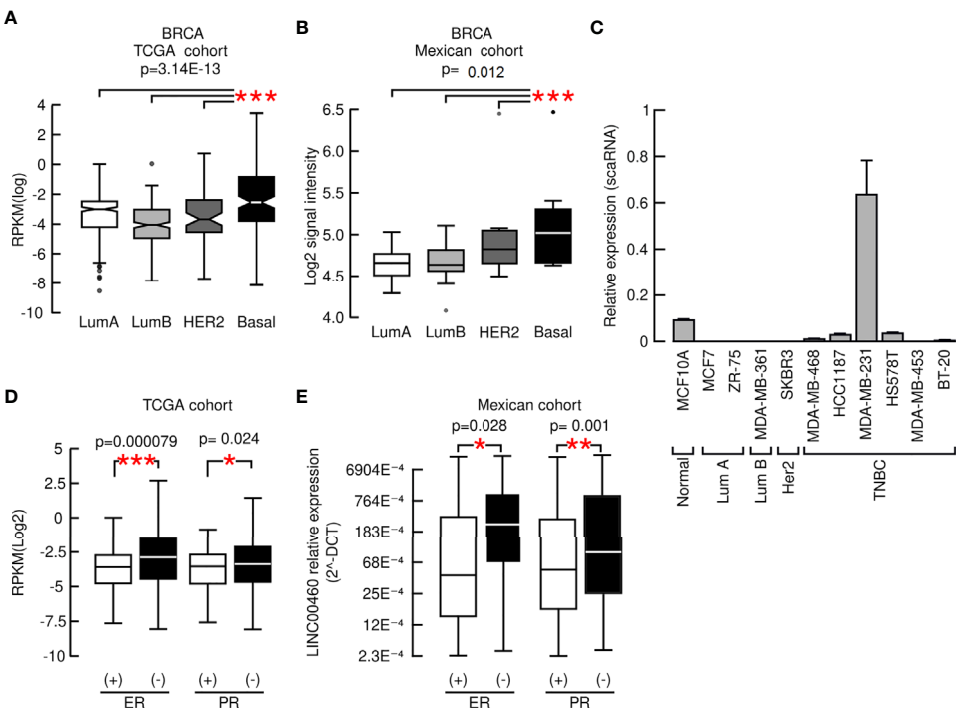

FIGURE 2 | LINC00460 high expression is associated with aggressive phenotypes in BRCA. **(A)** LINC00460 expression is significantly related with Basal-like BRCA in the TCGA cohort, **(B)** in a Mexican independent cohort and **(C)** enriched in TNBC cell lines, **(D)** LINC00460 is significantly enriched in ER- and PR- BRCA in the TCGA and **(E)** an independent Mexican BRCA cohort. *p<0.05, **p<0.005, ***p<0.0005.

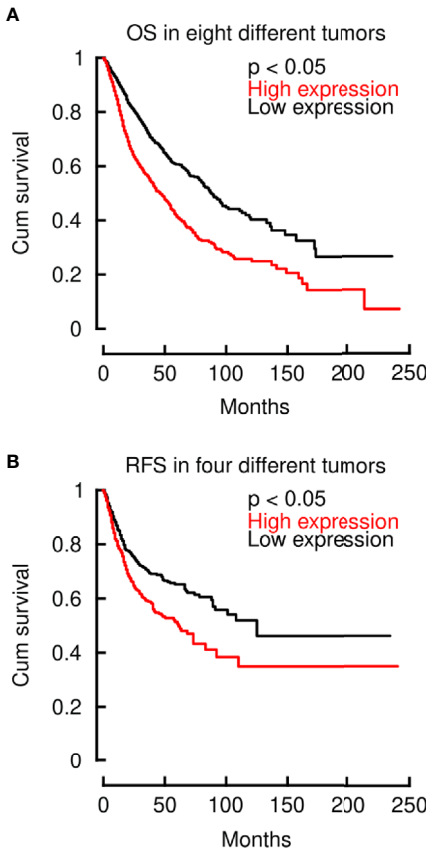

FIGURE 3 | LINC00460 deregulation is related to high risk of decease and relapse across multiple cancers. **(A)** LINC00460 overexpression is significantly associated with elevated HR of decease in eight different tumors (CESC, GBM, HNSC, KIRC, LGG, LUAD, PAAD and SARC). **(B)** High LINC00460 expression is associated with increased risk of relapse in four different tumors (KIRC, LUAD, READ and SARC).

TABLE 1 | Clinicopathological characteristics of the mexican BRCA cohort (n = 107).

Variable	Stratification	Frequency	n		
Age	<50	>50	NA		
	31	69	7		
ER	Negative	Positive	NA		
	35	68	4		
PR	Negative	Positive	NA		
	47	56	4		
HER2	Negative	Positive	NA		
	79	23	5		
Clinical Stage	I	II	III	IV	
	27	64	9	7	
Grade	I	II	III	NA	
	14	64	20	9	
Defunction	Positive	Negative	NA		
	11	94	2		
Metastasis	Positive	Negative	NA		
	9	26	72		
Survival (months)	< 60	>60	NA		
	16	91	0		
Tumor size	<20mm	>21 a 49 mm	>50 mm	NA	
	33	49	9	16	
Molecular subtype (IHC)	Luminal A	Luminal B	Her2	Basal	NA
	44	25	11	17	10
Lymphocyte infiltration	≤50	≥50	NA		
	61	1	22		

tumor grade in the survival Cox regression analysis of the Mexican cohort (**Supplementary Table S2**). These interactions suggest that LINC00460 is involved in important cancer-related processes like tumor differentiation, hormonal status and HER2 expression in mexican BRCA patients, although the exact related mechanisms remain unclear.

We have also assessed RFS in the GSE21653 dataset (n=240; HR = 0.59; 95% CI [0.38 – 0.94]; logrank p = 0.024) and DMFS (n=120; HR=0.78; 95% CI [0.6-1.02]; logrank p= 0.062) in BRCA patients in the GEO-derived cohorts (**Figure 4C** and **Supplementary Figure S5**). We found a corresponding significant association with improved RFS for LINC00460-high patients. Although non-significant, we observed a solid tendency to a higher DMFS in the BRCA LINC00460-overexpressed group (**Supplementary Figure S5**).

These data strongly suggest that LINC00460 might play a dual prognostic role across different tumors, as high LINC00460 expression predicts an increased OS, RFS and DMFS in the BRCA model, but it is also a marker for poor prognosis in at least

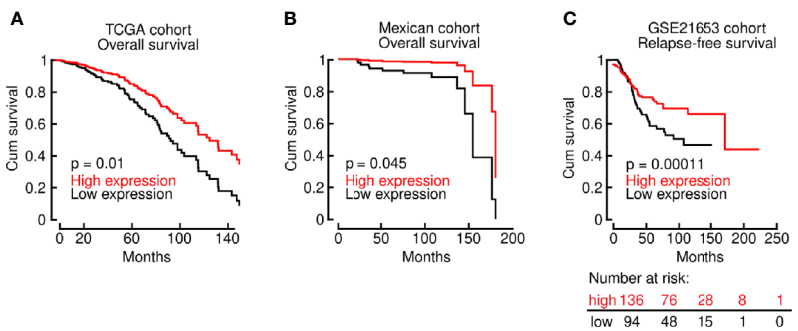

FIGURE 4 | LINC00460 up-regulation is associated with improved OS and RFS in multiple BRCA cohorts. **(A)** LINC00460 over-expression is significantly associated with improved OS in the BRCA TCGA cohort; **(B)** in an independent mexican patient cohort and **(C)** the GSE21653 GEO dataset.

eight distinct solid tumors (see **Figure 3C**). These intriguing findings aimed us to further investigate the role of LINC00460 in BRCA.

LINC00460 Is Significantly Enriched in Basal-Like 2 TNBC and Its Overexpression Predicts a Favorable Clinical Course

We then directed our efforts to elucidate the role of LINC00460 in OS prediction of the aggressive Basal-like BRCA model, a subtype which comprises the majority of TNBC cases (77). Interestingly, the improved OS effect shown in all BRCA patients (**Figures 4A, B**), is reproduced when we analyzed two independent cohorts of Basal-like BRCA tumors only, namely the TCGA cohort (TCGA *via* Tanric tool, n=139; logrank p=0.042) and GEO derived cohort GSE16446 (using basal-like samples only; n= 76; HR=0.23[0.08 –0.63]; logrank p = 0.0019) (**Figures 5A, B**). RFS/DMFS (GSE16446; n= 76; HR = 0.27; [0.11 – 0.68]; logrank p = 0.0027) were also significantly improved in LINC00460 over-expressed BRCA basal-like samples (**Figure 5C**). Analysis with LINC00460 predicted an increased OS in the TNBC GEO derived cohort GSE16446 (using all TNBC samples; n= 107; HR = 0.26; 95% CI [0.09 – 0.72]; logrank p = 0.0053) (**Figure 5D**).

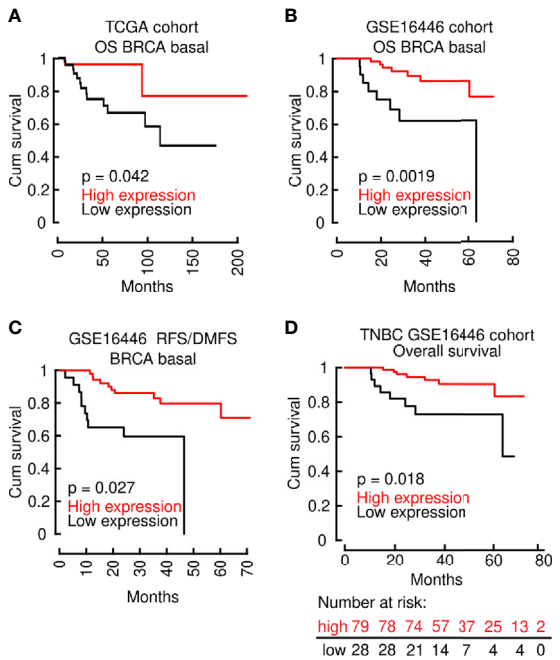

FIGURE 5 | The effect of improved OS, RFS and DMFS in the LINC00460-high group is maintained in Basal type BRCA. **(A)** OS in the TCGA BRCA Basal cohort; **(B)** OS in the GSE16446 BRCA Basal cohort; **(C)** RFS/DMFS in the GSE16446 BRCA Basal cohort; **(D)** OS in the GEO TNBC cohort.

To further characterize LINC00460 association with aggressive BRCA, we analyzed its expression in Lehman refined triple negative breast cancer classification (TNBCtype4) (**Figure 6A**). We observed a significant LINC00460 enrichment in the Basal-like 2 (BL2) subtype (n= 135, Jonckheere-Terpstra test for ordered variables; p=0.026) in a Chinese BRCA cohort (GSE76250). LINC00460 high expression level is also able to predict a favorable clinical response in BL2 triple negative BRCA GEO cohorts, both in OS (n=28; logrank, p=0.0062; **Figure 6B**) and RFS (n= 52; logrank, p=0.04; **Figure 6C**).

Altogether, these observations suggest that LINC00460 expression is generally related to intrinsically aggressive tumor phenotypes, as shown for HNSC, LUSC, LUAD, KIRC and basal-like BRCA (**Figure 2** and **Supplementary Figures S3, 4**). These findings are further corroborated when we showed that LINC00460 is enriched in the TNBCtype4 BL2 subtype in an independent cohort (**Figure 6A**). Interestingly, in aggressive BRCA subtypes, high LINC00460 expression is able to predict a favorable clinical course, further strengthening the dual role for this lncRNA in OS and RFS prediction in cancer.

LINC00460 Potentially Regulate a Plethora of Cancer-Related Genes in BRCA Involved in Proliferation, Cell Cycle and Migration

In order to elucidate the intriguing role of LINC00460 in BRCA, we aimed to identify potential LINC00460 expression targets. Differential expression analysis was performed in TCGA BRCA

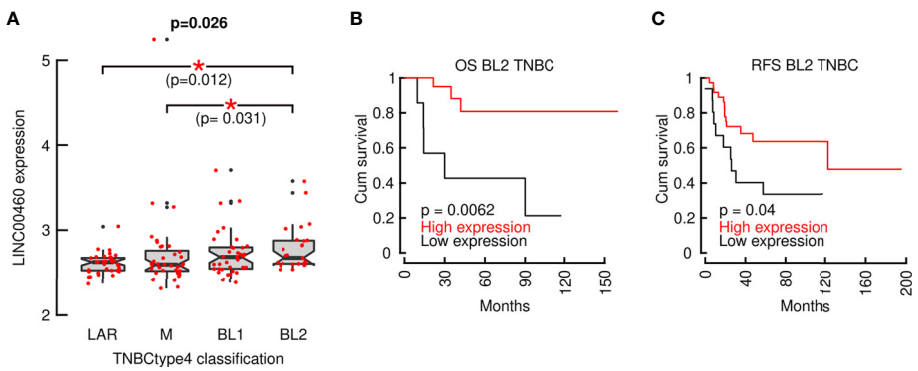

FIGURE 6 | LINC00460 is enriched in Basal-like 2 TNBC (TNBCtype4) and its expression can predict improved OS and RFS. **(A)** LINC00460 is significantly enriched in BL2 TNBC; **(B)** LINC00460 over-expression in associated with increased OS and **(C)** RFS in BL2 TNBC. *p<0.05.

samples between the LINC00460 high expression group and the LINC00460 low-group (see methods for details). This approach revealed 874 significantly deregulated transcripts (FC<1.5 and <-1.5, p.adj value<0.01; **Figure 7A**). Of those, 73% (638 RNAs) were up-regulated, and the remaining were found down-regulated (27%; 236 transcripts) between groups. Among the up-regulated RNAs, we identified several cancer-related transcripts, such as *HOXD13, CXCL1, CXCL5, FOXG1, SERPINB4, CLDN6* and *CLDN10* (see **Table 2**). Down-regulated transcripts list includes *EMX1, CYP2G1P, AMELX* and *SOX5-AS1* (**Table 3**). Furthermore, Ingenuity Pathway Analysis revealed that the proliferation process was negatively enriched in the high LINC00460 group (z score= -0.513). Cellular migration, adhesion, cell cycle progression and proliferation were also enriched in these BRCA samples, although no z-score enrichment was detected in these pathways (see **Figure 7B**).

This data pinpoints the role of LINC00460 as a potential regulator of transcripts and cellular cancer-related processes like proliferation, migration and cell proliferation in BRCA.

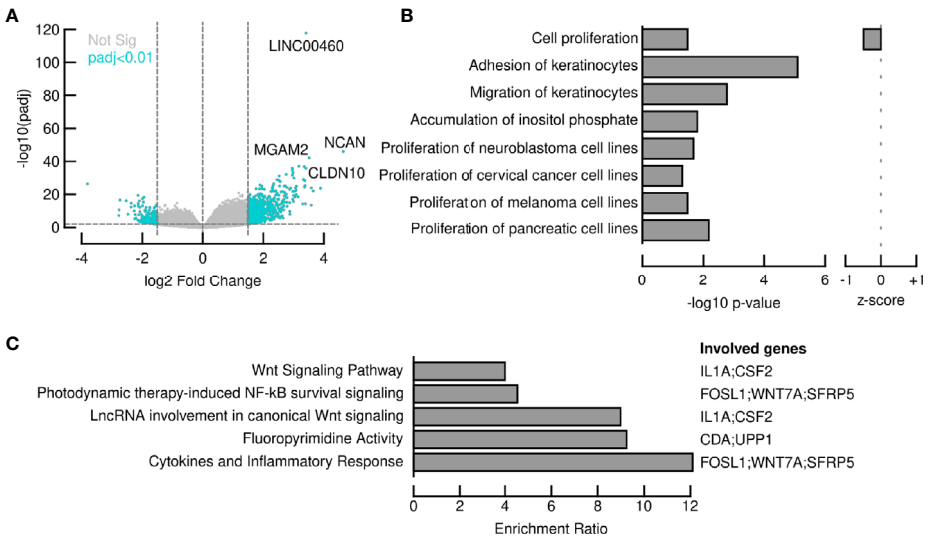

FIGURE 7 | LINC00460 potentially regulates WNT signaling pathway and the cytokine inflammatory response in BRCA. **(A)** 874 transcripts were significantly deregulated after comparing LINC00460-high samples vs LINC00460-low samples; **(B)** Several cancer-related pathways, such as cell proliferation, cellular migration, adhesion, cell cycle progression and carbohydrate metabolism are potentially altered in LINC00460-high BRCA samples; **(C)** ORA revealed LINC0460 involvement in WNT signaling and cytokine inflammatory response among other pathways in BRCA samples.

TABLE 2 | LINC00460 high vs low up-regulated genes.

	Gene symbol	Gene name	log2FC	padj-value
ENSG00000130287	NCAN	Neurocan	4.7	5.41E-50
ENSG00000103355	PRSS33	Serine Protease 33	3.7	1.22E-24
ENSG00000140522	RLBP1	Retinaldehyde binding protein 1	3.7	4.03E-27
ENSG00000275216	AL161431.1		3.7	8.28E-15
ENSG00000112238	PRDM13	PR Domain-containing protein 13	3.5	9.31E-17
ENSG00000177359	AC024940.2		3.4	2.06E-38
ENSG00000233532	LINC00460		3.4	1.00E-126
ENSG00000134760	DSG1	Desmoglein 1	3.4	4.23E-31
ENSG00000224887	AL513318.1		3.4	4.13E-16
ENSG00000166535	A2ML1	Alpha-2-Macroglobulin-Like 1	3.3	4.23E-31
ENSG00000206073	SERPINB4	Serpin Family B member 4	3.3	7.15E-15
ENSG00000181617	FDCSP	Follicular dendritic cell secreted protein	3.2	3.44E-21
ENSG00000229921	KIF25-AS1	KIF25 antisense RNA 1	3.2	2.85E-24
ENSG00000163735	CXCL5	C-X-C motif chemokine ligand 5	3.2	8.62E-34
ENSG00000047936	ROS1	ROS proto-oncogene 1	3.2	8.15E-28
ENSG00000134873	CLDN10	Claudin 10	3.2	2.36E-39
ENSG00000128714	HOXD13	Homebox D13	3.0	7.62E-27
ENSG00000163739	CXCL1	C-X-C motif chemokine ligand 1	3.0	1.66E-36
ENSG00000184697	CLDN6	Claudin 6	2.9	1.62E-25
ENSG00000176165	FOXG1	Forkhead box G1	2.7	2.22E-13

TABLE 3 | LINC00460 high vs low down-regulated genes.

	Gene symbol	Gene name	log2FC	padj-value
ENSG00000234398	AC134915.1		-1,5	0,00659454174
ENSG00000236532	LINC01695		-1,5	6,95E-09
ENSG00000137948	BRDT	Bromodomain Testis associated	-1,5	1,66E-07
ENSG00000238391	RNA5SP233	5S ribosomal pseudogene 233	-1,5	0,003359924242
ENSG00000275251			-1,5	0,004665490892
ENSG00000249691	AC026117.1		-1,5	0,003145246539
ENSG00000130612	CYP2G1P	Cytochrome P450 family 2 subfamily G member 1, pseudogene	-1,5	7,07E-07
ENSG00000125363	AMELX	Amelogenin X-Linkes	-1,5	0,005986652631
ENSG00000135638	EMX1	Empty spiracles homebox 1	-1,5	2,26E-07
ENSG00000256120	SOX5-AS1	GRIK1 antisense-RNA1	-1,5	0,009555483978
ENSG00000255155	AP004371.1		-1,6	1,77E-06
ENSG00000225795	AC006463.1		-1,6	0,004581123508
ENSG00000256612	CYP2B7P	Cytochrome P450 family 2 subfamily B member 7	-1,6	2,64E-06
ENSG00000265595	MIR4756		-1,6	0,001510008292
ENSG00000186732	MPPED1	Metallophosphoesterase domain containing 1	-1,6	1,43E-08
ENSG00000248350	AC010265.1		-1,6	0,004980797457
ENSG00000139352	ASCL1	Achaete-scute family bHLH transcription factor 1	-1,6	3,46E-05
ENSG00000233420	AC002127.2		-1,6	0,001874852522
ENSG00000250223	LINC01216		-1,6	0,007490194855

LINC00460 Expression Is Significantly Correlated With the WNT Pathway and Cytokine Inflammatory Response Genes

LINC00460 expression level is correlated with at least 100 coding transcripts in BRCA (PCC>0.45, p<0.05; shown in **Table 4**). Some of these transcripts are classic cancer-related, such as *WNT7A, SFRP5, FOSL1, IFNK, CSF2, DUSP7* and *IL1A*. Overrepresentation Enrichment Analysis (ORA) showed that WNT signaling pathway, cytokine inflammatory response, fluoropyrimidine activity pathway and photodynamic therapy-induced NF-kB survival signaling are enriched in LINC00460 over-expressed BRCA samples (**Figure 7C**). In addition, we observed that 73% (61/84) of the BRCA Mexican cohort samples displayed high levels of stromal TILs (see **Table 1**).

TABLE 4 | LINC00460 correlated genes.

	Gene symbol	Gene name	PCC*
ENSG00000179046.8	TRIML2	Tipartite motif family like 2	0.61
ENSG00000175592.8	FOSL1	FOS like 1, AP-1 transcription factor subunit	0.6
ENSG00000115008.5	IL1A	Interleukin 1 alpha	0.57
ENSG00000163915.7	IGF2BP2-AS1		0.56
ENSG00000164400.5	CSF2	Colony stimulating factor 2	0.55
ENSG00000240476.1	LINC00973		0.55
ENSG00000147896.3	IFNK	Interferon kappa	0.54
ENSG00000154764.5	WNT7A	Wnt family member 7A	0.54
ENSG00000261780.2	CTD-2354A18.1		0.53
ENSG00000254403.1	OR10Y1P	Olfactory receptor family 10 subfamily Y member 1 pseudogene	0.53
ENSG00000158825.5	CDA	Cytidine deaminase	0.53
ENSG00000104327.7	CALB1	Calbindin 1	0.51
ENSG00000203783.4	PRR9	Proline rich 9	0.51
ENSG00000176797.3	DEFB103A	Defensin beta 103A	0.48
ENSG00000182591.5	KRTAP11-1	Keratin associated protein 11-1	0.48

**PCC, Pearson correlation coefficient.*

Analyzing the LINC00460 co-expressed genes list, we observed that similar genes had been previously identified as enriched in Lehman's TNBC subtypes (77) (**Supplementary Table S3**). Altogether, these enrichment data suggests that LINC00460 is potentially related with inflammatory pathways and might partially explain its effect in good prognosis prediction in BRCA.

LINC00460 Is an In-Silico Predicted miR-103-a-1 Sponge

LINC00460 has been described as a miRNA sponge lncRNA in different tumors (38, 44, 60, 75). With this evidence in mind, we aimed to further characterized its biological role in BRCA. We used an *in-silico* approach to predict LINC00460 interaction with novel miRNAs, using the miRcode tool (**Table 4**).

In the former section, we found that a group of mRNAs are significantly correlated with LINC00460 levels (see **Table 4**). We then reasoned that, if LINC00460 is acting as a miRNA sponge as previously reported, then some of these correlated mRNAs may be miRNAs targets as well. We used two *in-silico* tools to predict mRNA-miRNA binding, namely miRcode and mirPATHDB. As shown in **Table 5**, the 10 LINC00460 most correlated mRNAs have potential miRNA interactants. Furthermore, we observed that some of these mRNAs, such as WNT7A and KRTAP11-1, can potentially bind to the same miRNA: miR-103-a-1 (**Tables 5** and **6**). These findings suggest a role for LINC00460 as a miR-103-a-1 interactant and as a potential regulator of WNT7A and KRTAP11-1 expression.

The LINC00460: WNT7A Ratio Is a Composite Marker for Increased OS and DMFS in Basal-Like BRCA and Can Predict Anthracycline Response in ER- BRCA Patients

To further demonstrate the role of LINC00460 in potential regulation of WNT7A and its combined role in BRCA prognosis, we computed the LINC00460:WNT7A ratio to construct an OS and

TABLE 5 | Matching sites and predicted miRNAs interacting with LINC00460.

microRNA family	Seed position	Seed type
miR-503	chr13:107029280	7-mer-m8
miR-143/1721/4770	chr13:107028970	7-mer-m8
miR-150/5127	chr13:107029727	7-mer-m8
miR-1ab/206/613	chr13:107030346	7-mer-A1
miR-200bc/429/548a	chr13:107029706	7-mer-A1
miR-221/222/222ab/1928	chr13:107029417	7-mer-A1
miR-23abc/23b-3p	chr13:107029820	7-mer-A1
miR-24/24ab/24-3p	chr13:107029217	7-mer-A1
miR-24/24ab/24-3p	chr13:107029855	7-mer-A1
miR-24/24ab/24-3p	chr13:107029953	8-mer
miR-103a/107/107ab	chr13:107030479	8-mer
miR-338/338-3p	chr13:107029282	7-mer-A1
miR-338/338-3p	chr13:107029426	7-mer-A1
miR-425/425-5p/489	chr13:107029977	7-mer-A1
miR-129-5p/129ab-5p	chr13:107030286	7-mer-A1

TABLE 6 | Predicted miRNAs that are potentially interacting with LINC00460 and some coexpressed mRNAs.

miRNA	mRNA	PCC (mRNA vs LINC00460)
miR-133a-3p	TRIML2	0.61
miR-130a	FOSL1	0.6
miR-544b	CSF2	0.55
miR-216b	IFNK	0.54
miR-103a-1	WNT7A	0.54
miR-34a	CDA	0.53
miR-140-5p	CALB1	0.51
miR-124-1	DEFB103A	0.48
miR-103a-1	KRTAP11-1	0.48
miR-455-5p	FERMT1	0.47

DMFS models for Basal-like BRCA. As shown in **Figure 8**, the LINC00460:WNT7A ratio is able to predict an increased OS (n=153, logrank p=0.028) and DMFS (n=145, logrank p=0.0057) in Basal-like BRCA, using GEO cohorts. In contrast, analysis with LINC00460:KRTAP11-1 ratio did not retrieve any significant survival effect (data not shown). Survival analysis using the mature sequence of miR-103-a-1 (hsa-miR-103a), showed a marginal, non-significant association between overexpression of the of miR-103-a-1 and decreased survival in TCGA TNBC cohort (n=97, logrank p=0.059; **Supplementary Figure S7**).

Taken together, these data show that the expression ratio of two genes, LINC00460:WNT7A is a composite marker that accurately predicts Basal-like BRCA OS and DMFS. Furthermore, we identified that the combination of LINC00460 and WNT7A over-expression is significantly associated with pathological complete response (pCR) after anthracycline therapy in ER- BRCA patients (n= 665, Mann-Whitney U test p= 0.0047) (**Figure 8C**). These evidences clearly indicate that both transcripts exert a central and beneficial role to basal-like, ER- BRCA patients.

DISCUSSION

Long non-coding RNAs exert numerous roles in human cancers, as their biological activities involve regulation of cell proliferation, cell death, differentiation, migration, invasion and metastasis. Deregulation in lncRNAs expression has also been associated with clinical outcome. LncRNAs can affect expression of thousands of genes, so they are regarded as key master regulators (78).

In this work, our aim was to investigate if LINC00460 expression was deregulated in different tumors, and if it was associated with clinical and pathological characteristics in these tumors. We then focused on its clinical role in aggressive (basal-like) breast cancer and the identification of potential LINC00460 targets in this model. We aimed to know if LINC00460 can target miRNAS *in-silico*, which can in turn bind to relevant mRNAs. Finally, we sought to investigate if some of the potential candidate genes would have a combinatorial role for OS and therapy response in basal-like BRCA.

We found that LINC00460 was deregulated in seven different tumor types in the TCGA database, including two not previously reported tumors, namely LGG and GBM. We have also confirmed LINC00460 deregulation in BRCA using two independent cohorts. Interestingly, LINC00460 expression is associated with clinically aggressive tumors, such advanced stage LUAD, LUSC and KIRC, high histology grade in KIRC, HPV-negative HNSC and basal-like BRCA, suggesting an important role of LINC00460 in the progression or intrinsically aggressiveness of these tumors. Indeed, it has been previously shown that LINC00460 expression can promote cancer progression (34, 35, 38, 50), metastasis (36) and influences therapy response (79).

Several reports show that LINC00460 is a marker for poor OS prognosis across different tumors, such as CESC (48), HNSC (80), KIRC (81), LUAD (49) and PAAD (82). In addition, we describe here that LINC00460 high expression is significantly associated with poor survival in three different tumors (GBM, LGG and SARC) but related with a favorable survival rate in BRCA, *i.e.*, its association to clinical outcome varies between tumors. This data suggests that the expression levels and its impact on OS, RFS or DMFS may be tissue-specific. Indeed, it has previously been shown that the same lncRNA may exert dual prognostic roles in distinct tumors. For example, high MALAT-1 expression has been reported as a marker for poor prognosis in various tumors, including COAD, NSCLC, STAD, PAAD, ESCA, among others (83), but also as a good prognosis factor for BRCA, acting as a metastasis suppressor (84). Another interesting example of this dual phenomenon is the expression of XIST. It has been shown that high expression of this lncRNA is related to poor clinical outcome in different cancers (85), but in another study, authors demonstrate that high XIST expression is related to an increased brain metastasis–free survival in BRCA patients (6). We suggest that LINC00460 can perform as a dual tissue-specific prognostic marker, similar to MALAT-1 and XIST; although we cannot discard alternative mechanisms, such as differences in the splicing variants measured between studies. These variant transcripts expression patterns should be taken into account to evaluate lncRNA-based predictive biomarkers (86). This latter possibility must be addressed in future studies.

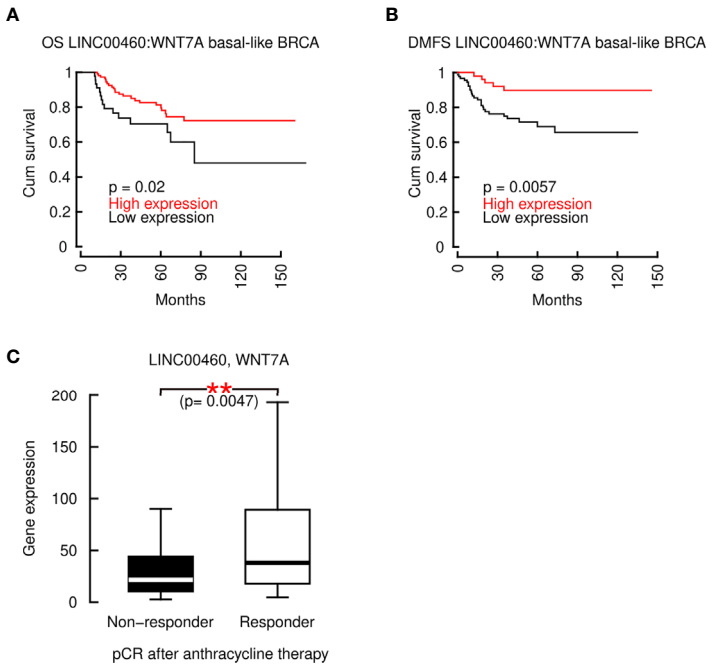

FIGURE 8 | The composite marker LINC00460:WNT7A is predicts an increased OS and RFS in basal-like BRCA and its median is associated with a complete pathological response to chemotherapy in ER- BRCA patients. **(A)** LINC00460:WNT7A ratio predicts a significantly improved OS and **(B)** RFS in basal-like BRCA; **(C)** LINC00460 and WNT7A combined expression is significantly associated with pCR after anthracycline-based therapy in ER- patients. **p<0.005.

In the BRCA model, LINC00460 expression is associated with the phenotypic makeup of the tumor, where overexpression of LINC00460 is associated with a negative result to hormone receptors (ER and PR) when subject to IHC. It was also observed that in the most aggressive phenotype, basal-like BRCA, it actually favored the clinical outcome (**Figure 6**), even when being subject to analysis in the different TNBC subtypes. Its similar behavior through different BRCA subtypes and BRCA different cohorts, led us to propose LINC00460 as a potential biomarker for improved OS, RFS and conceivably DMFS prediction in basal-like BRCA (**Figures 4–6**). This behavior can potentially be explained through the candidate co-expressed genes found in the differential expression analyses and ORA (**Figure 7**). We propose five main mechanisms that potentially explain the role of LINC00460 in the increased prognosis of BRCA patients: (1) regulation/co-expression of good prognosis-related genes, (2) co-expression/modulation of genes that promote an immunogenic niche, (3) decrease of tumor cell proliferation, (4) regulation of WNT7A through sponging of miR-103-a-1 and (5) promoting chemotherapy (anthracycline) complete pathologic response. As the majority of these interactions are *in-silico* predicted, or associated with clinical features in patient-based cohorts, experimental validation is needed to support these hypotheses.

In this regard, we observed that the LINC00460 expression is significantly enriched in the BL2 TNBC subtype. This finding is of particular interest, since the BL2 subtype displays a variety of gene ontologies enriched in components and pathways involved in cell proliferation, growth, survival and cell differentiation pathways, such as the WNT pathway (77). In accordance, we identified important members of the WNT pathway, such as WNT7A, as being potentially regulated by LINC00460 (see **Figure 7C** and the discussion below). These data strengthen the role of LINC00460 in BL2 TNBC, and further clarify its mechanism of action in these tumors.

We detected that some of the LINC00460 co-expressed genes, such as *SFRP5, HOXD13*, are also related to increased survival rate in different tumors. High expression of *SFRP5* is significantly associated with a better prognosis in PAAD (87) and BRCA (88). HOXD13 protein levels are related to an increased OS in BRCA (89). Thus, this LINC00460 candidate target genes potentially contribute to the increased survival effect observed in BRCA patients.

On the other hand, we found that several LINC00460 co-expressed genes (*TRIML2, SFRP5, FOSL1, IFNK, CSF2, DUSP7, DEFB103A* and *IL1AA)* are immunogenic-related. Immune response pathways are clinically relevant, as it has been previously described that a highly immunogenic niche in a tumor may improve the outcome of the disease (90). These genes and pathways are frequently enriched in TNBC (77, 91). Furthermore, TNBC also display enrichment of tumor-infiltrating lymphocytes (TILs) (92). We suggest that LINC00460 is related with good prognosis in TNBC/basal-like due to its potential relation with these genes and

with the presence of tumor-infiltrating lymphocytes (TILs), as shown in our samples (see **Table 1**). It has been previously shown that the presence of TILs improves prognosis as it modulates cancer progression and enhances chemotherapy response in TNBC, conferring a protective immunity in these patients (93).

Therefore, we suggest that the correlation between these immunogenic genes and LINC00460 can partially explain the clinical behavior in the breast cancer cohorts, as the overexpression of LINC00460 is associated with upregulation of immunogenic factors that in turn, permit the migration of components of the immune system. These mechanisms can promote an immunogenic tumor environment and thus favor tumor cell death. Future studies are needed to experimentally validate these data, as it is relevant for clinical outcome in aggressive TNBC.

In addition to promoting the immunogenic niche, LINC00460 could decrease proliferation of the tumor cells, as we observed that the proliferation pathway is negatively enriched in the high LINC00460 group in BRCA samples. This could also explain the increased survival rates of the BRCA patients. Indeed, multiple lines of evidence suggest that LINC00460 can modulate cell proliferation, cell death, migration and invasion and EMT, through its sponging activity and targeting various key transcripts in several cancer types (31, 37, 43, 94–96).

We predicted that LINC00460 potentially binds to miR-103-a-1 *in-silico*, which, in turn, can target WNT7A. In this regard, although there have been reports that suggest that WNT7A is an oncoprotein (97), it has also been shown that loss of WNT7A expression is significantly associated with poor RFS in BRCA (98) and it is also involved in tumor cell differentiation (99). Thus, the exact role of WNT7A in BRCA is currently unclear. Our results might suggest that WNT7A potentially play an important clinical role in the BRCA, as LINC00460 could sponge miR-103-a-1 and henceforth, liberate WNT7A. Further research must validate these predictions experimentally.

We observed that the LINC00460:WNT7A ratio is a composite marker that can predict a favorable OS and DMFS in TNBC. These results highlight the clinical and biological role of LINC00460 and WNT7A transcripts in TNBC and constitute valuable data, as simple ratios of gene expression levels can be used to accurately diagnose (100) and predict cancer outcomes (101, 102), while circumventing many of the limitations that preclude the use of microarray techniques in extensive clinical applications (103, 104). In accordance with our observations regarding immunogenic factor potential upregulation and previous findings in the field (92, 93, 105), we have also identified a significant association between the expression enrichment of LINC00460 and WNT7A with anthracycline responsive ER- patients. This finding further strengthens the beneficial role of both transcripts in patient's prediction and prognosis as, it has been demonstrated that ER-negative breast cancers with high levels of TILs have heightened sensitivity to anthracycline-based chemotherapy (106), and that TILs are an independent predictor of good response to anthracycline/taxane neoadjuvant chemotherapy (105). All these observations, however, are limited by the size of GEO patient cohorts and will require validation in a larger independent cohort.

Regarding the role of miR-103-a-1, we identified a marginal association with poor OS in BRCA. This is in accordance with previous findings, as it has been shown that miR-103-a-1 acts as an oncogene to promote TNBC cells migration and invasion (107). In another report, authors show that serum miR-103 over-expression was significantly correlated with worse clinical factors, as well as poorer recurrence-free survival or overall survival in colorectal cancer (108). MiR-103/107 expression displays stemness-promoting functions, and a signature of miR-103/107 high and Axin2 low expression profile correlates with poor prognosis in colorectal cancer patients (109). In gastric cancer patients, high expression of miR-103 was significantly associated with poor overall survival and disease-free survival and is a key factor that contributes to tumor progression (110). Altogether, these data suggest that of miR-103-a-1 is a marker for poor prognosis in several tumors, including BRCA. To the best of our knowledge, this is the first report that suggests a potential connection between LINC00460, WNT7A and miR-103-a-1. Future research must elucidate the exact mechanisms involved in this potential 3-gene network, and its impact in basal-like BRCA biology.

In conclusion, LINC00460 expression is a dual potential marker for aggressive phenotypes and poor clinical outcome in distinct tumors, including HNSC, KIRC LUSC and LUAD, that is also associated with increased prognosis in basal-like BRCA. LINC00460 is enriched in BL2 TNBC, and potentially regulates the WNT differentiation pathway. LINC00460 can also modulate a plethora of immunogenic related genes in BRCA, such as *SFRP5, FOSL1, IFNK, CSF2, DUSP7* and *IL1A* and interacts with miR-103-a-1, *in-silico*, which, in turn, can no longer target WNT7A. LINC00460:WNT7A ratio is a composite marker that can predict a favorable OS and DMFS in TNBC, and combination of LINC00460 and WNT7A over-expression is associated with complete pathological response (pCR) after anthracycline therapy in ER- BRCA patients. This data confirms that LINC00460 is a master regulator in BRCA molecular circuits and influences clinical outcome.

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 Research and Ethics Committee of National Institute of Genomic Medicine and the Institute of Breast

Diseases, FUCAM (Registration number: CE2009/11). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH-M, MR-R, LH-P and MC-V conceived and designed the study. MM-R and LH-P handled the samples, constructed the clinical database and tested the expression levels in biological samples. SJ-M, RA-L, LA-R, and LH-P handled the samples and clinical database. AT-T, CD-R, FV-C, IR-S, and AM-A obtained the samples and performed the clinical evaluation and following of the patients. LH-P did the pathological evaluation of the samples. AC-T, MC-V, MP-L, RA-L, and EH-C performed all the *in-silico* validation cohort-related analysis and the *in-silico* prediction of the sponge regulation. MR-R, MC-V, and AC-T performed statistical analysis, survival analysis, differential expression analysis, and risk analysis. All authors interpreted and discussed the data. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Mexican National Council of Science and Technology 480751/282036 grant (Scholarship), and Mexican National Council of Science and Technology Basic Science grant (CONACYT grant number 258936).

ACKNOWLEDGMENTS

We want to thank all the patients that accepted to participate in our research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.628027/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: 27 April 2021
doi: 10.3389/fonc.2021.656675

Inferring Cell Subtypes and LncRNA Function by a Cell-Specific CeRNA Network in Breast Cancer

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

Received: *21 January 2021*
Accepted: *30 March 2021*
Published: *27 April 2021*

Citation:
Chen X, Xu J, Zeng F, Yang C, Sun W, Yu T, Zhang H and Li Y (2021) Inferring Cell Subtypes and LncRNA Function by a Cell-Specific CeRNA Network in Breast Cancer. Front. Oncol. 11:656675. doi: 10.3389/fonc.2021.656675

Single-cell RNA sequencing is a powerful tool to explore the heterogeneity of breast cancer. The identification of the cell subtype that responds to estrogen has profound significance in breast cancer research and treatment. The transcriptional regulation of estrogen is an intricate network involving crosstalk between protein-coding and non-coding RNAs, which is still largely unknown, particularly at the single cell level. Therefore, we proposed a novel strategy to specify cell subtypes based on a cell-specific ceRNA network (CCN). The CCN was constructed by integrating a cell-specific RNA-RNA co-expression network (RCN) with an existing ceRNA network. The cell-specific RCN was built based on single cell expression profiles with predefined reference cells. Heterogeneous cell subtypes were inferred by enriching RNAs in CCN to the estrogen response hallmark. Edge biomarkers were identified in the early estrogen response subtype. Topological analysis revealed that NEAT1 was a hub lncRNA for the early response subtype, and its ceRNAs could predict patient survival. Another hub lncRNA, DLEU2, could potentially be involved in GPCR signaling, based on CCN. The CCN method that we proposed here facilitates the inference of cell subtypes from a network perspective and explores the function of hub lncRNAs, which are promising targets for RNA-based therapeutics.

Keywords: cell-specific network, ceRNA, estrogen regulation, lncRNA, subtype

INTRODUCTION

The incidence of breast cancer has increased at a rate of 0.3% per year from 2012 to 2016 in the United States, largely because of the rising rates of local stage and hormone receptor-positive disease (1). As estrogen plays a predominant role in breast cancer, understanding the mechanisms of estrogen regulation holds profound significance in breast cancer research and treatment. The transcriptional regulation of estrogen receptor (ER) is an intricate network of signaling and functional processes that is still largely unknown at the single cell level. Recently, the intra-cell line heterogeneity of breast cancer has been comprehensively characterized through single-cell RNA

sequencing (scRNA-seq), revealing transcriptomic subpopulations within cell lines (2). Therefore, investigating the heterogeneity of estrogen regulation at the single cell level could shed more light on estrogen mechanisms and potential breast cancer therapeutics.

As an active metabolite of estrogen, 17β-estradiol (E2) is essential for both normal breast cells and malignant breast cancer cells. Zhu et al. performed scRNA-seq on estrogen receptor alpha positive breast cancer cells stimulated by E2. Their research revealed a dynamic transcriptional network in which estrogen signaling promotes breast cancer cell survival and growth by mediating a metabolic switch (3). They also provided valuable data resources to explore the heterogeneous response of cells from the same cell line upon estrogen stimulation.

Dai et al. proposed a probability theory-based method to construct a cell-specific network for individual cells (4), which innovatively characterized each cell from the perspective of a 'stable' gene network rather than 'unstable' gene expression. This prompted us to propose a novel strategy to characterize single cells from the perspective of networks. The RNAs interact in a complicated manner within cells. For example, RNA functions as microRNA (miRNA) sponges by competitively binding to the same miRNA, reducing the repression or degradation effect of the miRNA on the target genes. These RNAs are competing endogenous RNAs (ceRNAs). Evidence from studies indicates that long non-coding RNAs (lncRNAs) act as ceRNAs to compete with miRNAs with mRNAs. For example, *NEAT1* was reported to serve as a ceRNA of *ZEB1*, which competes with miR-448 in breast cancer (5). *PTEN*, a well-known tumor suppressor, regulates *MALAT1* expression by potentially sponging oncogenic miRNAs, including *miR-17* and *miR-20a* in breast cancer (6). Therefore, it is adequate for the characterization of single cells from the viewpoint of the ceRNA network. Wang et al. applied the cell-specific network method developed by Dai et al. and integrated public ceRNA regulations to build a database named LnCeCell, which comprised the predicted lncRNA-associated ceRNA networks at single-cell resolution (7). In this study, we aimed to investigate cell heterogeneity upon estrogen stimulation, from the perspective of the ceRNA network.

Liu et al. developed a sample-specific network (SSN) method to construct a personalized network for individual patients, based on the expression profile of these patients (8). Inspired by the SSN method, we designed a novel strategy to construct a cell-specific ceRNA network (CCN) by integrating a cell-specific RNA–RNA co-expression network (RCN) with an existing ceRNA network. The cell-specific RCN was first constructed from single cell expression profiles with the aid of predefined reference cells, provided by the SSN method. After incorporating public ceRNA networks into the RCN, the CCN was obtained. To dissect the heterogeneity of the cell response to estrogen, RNAs in CCN were enriched with estrogen response hallmarks. The edge biomarkers for the early estrogen response subtype were also identified in the CCN; *NEAT1* had high average degree among the early response cells, and ceRNA survival analysis indicated that NEAT1 and its ceRNAs could predict patient survival. Moreover, we inferred the function of another hub lncRNA, *DLEU2*, which might be involved in GPCR signaling, based on both Gene Ontology (GO) and REACTOME pathways. In summary, we established a novel method to construct a CCN and provide single-cell network-related insights into estrogen regulation in breast cancer.

Abbreviation: ESR1, estrogen receptor alpha; RCN, RNA–RNA co-expression network; ceRNAs, competing endogenous RNAs; CCN, cell-specific ceRNA network; scRNA-seq, single-cell RNA sequencing; lncRNAs, long non-coding RNAs; E2, 17β-estradiol; ERGs, estrogen regulated genes; GO, Gene Ontology; ER, estrogen receptor; SSN, sample-specific networks; CGC, Cancer Gene Census; GAD, Genetic Association Database; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; DEGs, differentially expressed genes; GEO, Gene Expression Omnibus; FPKM, fragments per kilo base of transcript per million reads mapped.

MATERIALS AND METHODS

Data Pre-Processing

We downloaded the scRNA-seq data from Gene Expression Omnibus (GEO) (accession number: GSE107858). Following the filtering process described in the paper (3), we performed further analysis on 84 MCF-7 cells. RNAs with fragments per kilo base of transcript per million reads mapped (FPKM) >1 in at least 25% ($84 \times 0.25 = 21$) of the cells were used for further analysis. The filtering parameter is referred to the paper (9). The dataset GSE107863 for T47D was an independent validation cohort to support the findings obtained using MCF-7 cells.

CeRNA Network From starBase

The ceRNA network was downloaded (10) (http://starbase.sysu.edu.cn/) using the Web API. The ceRNAs for all mRNAs, lncRNAs, and pseudogenes were downloaded using default parameters. The ceRNA network contained 308,266 ceRNA pairs composed of 18,942 RNAs. The complete information is presented in **Table S1**.

Gene Sets for Markers

We obtained the known cancer-related genes from the Cancer Gene Census (CGC) database (http://cancer.sanger.ac.uk/cosmic/census), which contains 576 genes (11). Other 876 cancer-related genes were also downloaded from the Genetic Association Database (GAD) database (http://geneticassociationdb.nih.gov/).

Functional gene sets "HALLMARK_ESTROGEN_RESPONSE_EARLY" and "HALLMARK_ESTROGEN_RESPONSE_LATE" were downloaded and extracted from the hallmark gene sets of MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/, v7.2). REACTOME pathways and biological processes information of GO was also downloaded from MSigDB.

We downloaded the transcript annotation from Ensembl and obtained 215,307 annotations. The transcript ID, transcript type, and HUGO Gene Nomenclature Committee (HGCN) symbols were downloaded from Ensembl. Further, the annotations whose transcript type belonged to "lincRNA" or "antisense" were extracted as the lncRNAs. We obtained a total of 1,794 lncRNAs. In addition, we also downloaded the lncRNA annotation file lncipedia_5_0_hg19.gtf (full database) from LNCipedia (12). Considering that

some lncRNAs had alternative names, we extracted the Ensembl ID, gene_alias and gene_id for each lncRNA. The lncRNA information from either Ensembl or LNCipedia was used to annotate the lncNRAs.

ER is the most important hormone receptor in breast cancer. We also screened the differentially expressed genes (DEGs) between ER-positive and ER-negative patients from public cohorts and denoted as ER_DEGs markers. The raw read counts for breast cancer was downloaded from The Cancer Genome Atlas (TCGA). The R package DESeq2 (13) was used for differential analysis. 22,946 DEGs were identified with adjusted p <0.05. The Z-score scaled expression profile of Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) was also downloaded. T-test was used as the statistical method to calculate the p value of gene expression difference between ER+ *vs* ER– samples. The p value was then adjusted by fdr method using the R package fdrtool (14). As a result, we obtained 2,951 DEGs with fdr adjusted p <0.05.

Constructing a CCN Based on Reference Cells

The SSN method was developed by Liu et al. to construct a personalized network for individual patients based on their expression profiles (8). Briefly, a reference network can be constructed using the Pearson correlation coefficient (PCC) between molecules based on the expression data of the reference samples. After a new sample is added to the reference samples, the perturbed network can be similarly constructed. Then, the differential network is constructed by the edges with significantly changed correlation between the reference and perturbed networks.

The changed correlation follows a new type of distribution, which is called the "volcano distribution". The tail areas of this distribution are similar to those of the normal distribution based on the Kolmogorov–Smirnov test with random sampling. Therefore, the statistical hypothesis Z-test was used to evaluate the significance level of the changed correlation because of the central limit theorem (15).

Liu et al. selected 8–17 normal samples as reference samples to construct an SSN. They also ensured that the SSN is robust and stable for the different reference sample sizes. Inspired by the SSN method, 20 MCF-7 cells captured at 0 h were selected as the reference cells in this study (**Figure 1A**). The reference network was constructed using the PCC. The RNA–RNA correlation was deemed significant with a p-value <0.05. For cells at 3, 6, or 12 h, we added one cell to the reference cells and recalculated the RNA–RNA correlation (**Figure 1B**). We retained a correlation network, named the perturbed network, containing significant RNA–RNA relationships with a p-value <0.05.

Next, we compared the significant RNA–RNA interactions in the perturbed network and reference network to keep only the edges with significantly changed correlations (**Figure 1C**). **Figure 1D** shows how to test the significance of the changed correlation ($\Delta Correlation_N$) between a pair of RNAs. According to the SSN theory proposed by Liu et al. (8), the differential correlation followed a normal distribution, and the significance could be evaluated based on the Z-test.

$$Z = \frac{\Delta Correlation_N - \mu_{\Delta Correlation}}{\sigma_{\Delta Correlation}} = \frac{\Delta Correlation_N}{\frac{1-Correlation_N^2}{N-1}},$$

where N is the number of reference cells.

From the expression perspective, the ceRNAs were positively correlated. Therefore, we considered only the positive and significant differential RNA–RNA interactions as candidate ceRNAs (**Figure 1E**). The ceRNA network from StarBase was further used to filter the ceRNA network to ensure its biological importance (**Figure 1F**).

Functional Enrichment of Genes in the CCN

The hypergeometric test was used to evaluate whether the genes in the CCN were significantly enriched in functional gene sets.

$$P = \sum_{x \geq n} \frac{C_N^x \cdot C_{M-m}^{m-x}}{C_M^m}$$

where M is the total number of genes in the background network, N is the number of genes in a functional gene set, m is the number of genes in the CCN, and n is the number of CCN genes shared by the functional gene set.

Topology Analysis of CCN

The R package igraph was used to calculate the topological features of RNAs in the CCN (R 4.0.2). The degree of RNA is the number of direct neighbors in the ceRNA network. RNAs with a high degree can be termed as hub RNAs, which play a pivotal role in maintaining the ceRNA–ceRNA relationships within CCN. The betweenness of RNA i can be calculated with the formula

$$\frac{B_i = \sum_{s \neq i \neq i} \delta_{st}(i)}{d_{st}},$$

where RNA s and t are RNAs in the CCN different from RNA i, d_{st} represents the number of the shortest paths from s to t, and $\delta_{st}(i)$ is the number of the shortest paths from s to t that i lies on. For RNA s and t, the ratio is the proportion of the shortest path that RNA i lies on. The sum of the ratios of all RNA pairs is the betweenness centrality of RNA i. The closeness coefficient is the average closeness of RNA i to other RNAs in the network. It is calculated as

$$C(i) = \frac{1}{d_i} = \frac{n-1}{\sum_{s \neq i} d_{si}},$$

where d_{si} represents the distance between RNA i and other RNAs.

Survival Analysis of CeRNAs

A recently published paper (7) has provided a web tool, "ceRNA survival", to perform survival analysis for ceRNA composed of

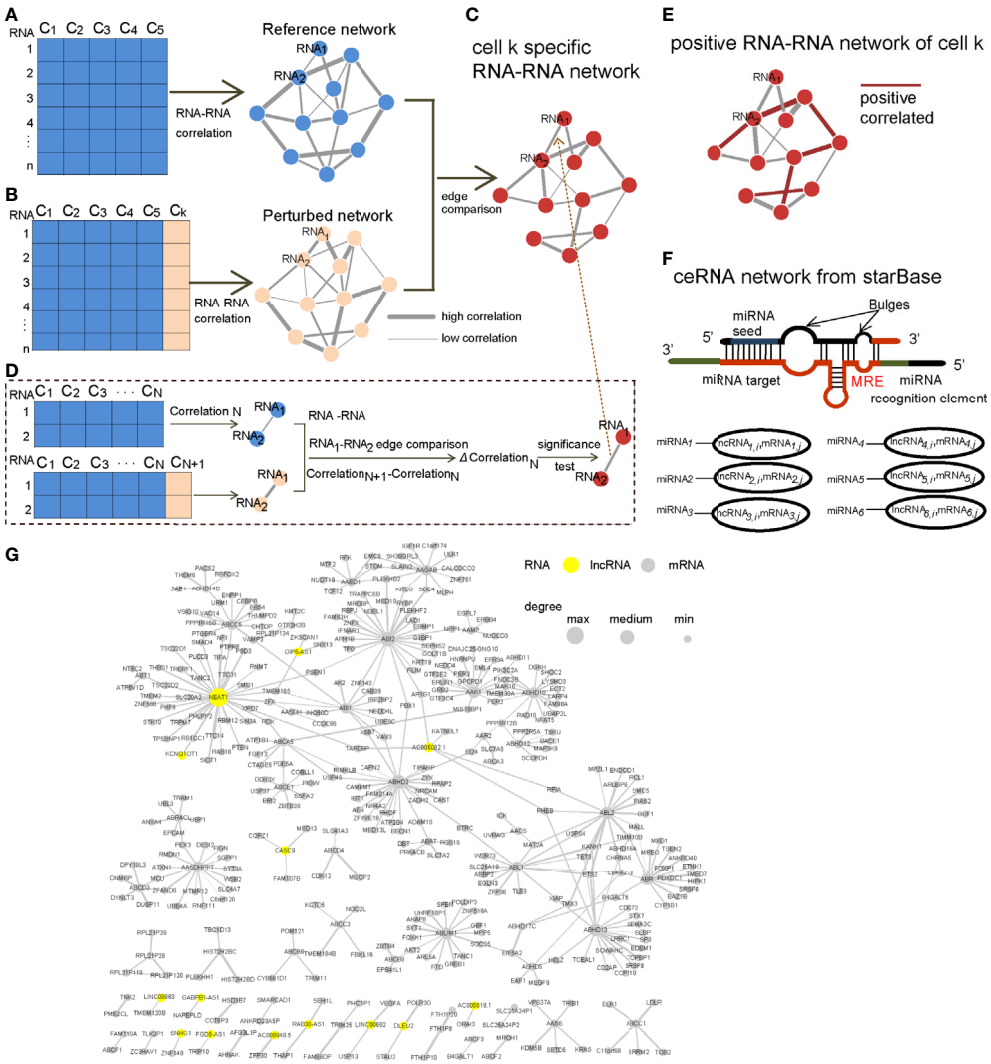

FIGURE 1 | Workflow of constructing the CCN. **(A)** Reference cells were selected, and the corresponding RCN was constructed and referred to as "Reference network". **(B)** One cell k was added to the reference cells, and the corresponding RCN was constructed and referred to as the "Perturbed network". **(C)** Edges were compared between the "Reference network" and "Perturbed network" to obtain a cell k-specific RNA–RNA network. **(D)** One edge composing of two RNAs was compared and tested for its significance level, based on the differential correlation ($\Delta Correlation_N$). **(E)** Only the positive edges in the cell k-specific RNA–RNA network were candidate edges in the CCN. **(F)** We downloaded the ceRNA network from starBase. **(G)** An example of a CCN. The yellow nodes represent lncRNAs, and node size is proportional to its degree in the CCN.

lncRNA–miRNA–mRNA, based on The Cancer Genome Atlas (TCGA) datasets. The web tool was used to perform multivariate Cox regression analysis based on miRNA, mRNA, and lncRNA expression, without co-factors.

Gene Set Enrichment Analysis

GSEA (16) was performed using Preranked utility implemented in the standalone version of the GSEA software (v 4.1.0). The RNA sequencing dataset of *DLEU2* knockdown was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), with accession number GSE162677. We ranked the genes according to the fold change in expression (FPKM.siDLEU2/FPKM.siNC). The fold change was log2 transformed before GSEA.

RESULTS

CCN Construction Based on Reference Cells

The CCN was constructed following the workflow shown in **Figure 1** (more details in *Materials and Methods*). Briefly, the edges with significant differential correlation between the reference network (**Figure 1A**) and perturbed network (**Figure 1B**) were used to construct a cell-specific RCN (**Figure 1C**). miRNA targets are negatively regulated by miRNAs. RNAs competitively bind to the same miRNAs as ceRNAs. Chen et al. generated a ceRNA network for each subtype of breast cancer, based on the principle of positive co-expression and shared miRNAs (17). Therefore, the

positive RCN appeared to be a candidate ceRNA, based on ceRNA theory (**Figure 1E**). The ceRNA network from starBase was further used to reduce false-positive ceRNA relations (**Figure 1F**). As an example, we have demonstrated the ceRNA network for the cell MCF-7 12 h (RHM266) in **Figure 1G**.

Estrogen Receptor Alpha Co-Expressed With Known Marker Genes

ESR1 plays an important role in breast cancer. Therefore, we first examined its interactors in the cell-specific RCN, which is schematically shown in **Figure 1E**. We selected cells at 3, 6, and 12 h, in which *ESR1* had a degree larger than 25. The known estrogen-regulated genes (ERGs), such as *KLF4* (**Figures 2A–F**) and *TSKU* (**Figures 2A, C**), were significantly positively correlated with *ESR1*. Akaogi et al. reported high expression of *KLF4* in ER-α-positive patients. *KLF4* was found to bind to the DNA-binding region of ER-α and inhibit the binding of ER-α to estrogen response elements in the promoter regions (18). Known cancer-related genes from CGC, such as *MYD88* (**Figures 2A, D**), *DDX10* (**Figures 2B, F**), *MLLT6* (**Figure 2D**), *BCL10* (**Figure 2F**), and *KAT6A* (**Figure 2F**) also interacted with *ESR1*. The expression of MYD88 could be modulated in a single nucleotide polymorphism (SNP)- and estrogen-dependent fashion (19). Breast cancer-related genes from GAD were also identified as *ESR1* interactors, including *TP53BP1* (**Figure 2B**), *SRA1* (**Figure 2D**), and *BBS4* (**Figure 2E**). Low expression of TP53BP1 is associated with increased local recurrence in breast cancer patients treated with conserving surgery and radiotherapy (20). In addition to these protein-coding genes, *ESR1* was also co-expressed with non-coding RNAs such as *MIR302B* (**Figure 2C**) and *MIR4426* (**Figure 2E**). LncRNAs *MIR181A1HG* (**Figure 2A**), *ATP1A1OS* (**Figure 2B**), and *LINC00094* (**Figure 2D**) were also shown to cross-talk with *ESR1*. ER_DEGs frequently interacted with *ESR1* within individual cells (**Figures 2A–F**).

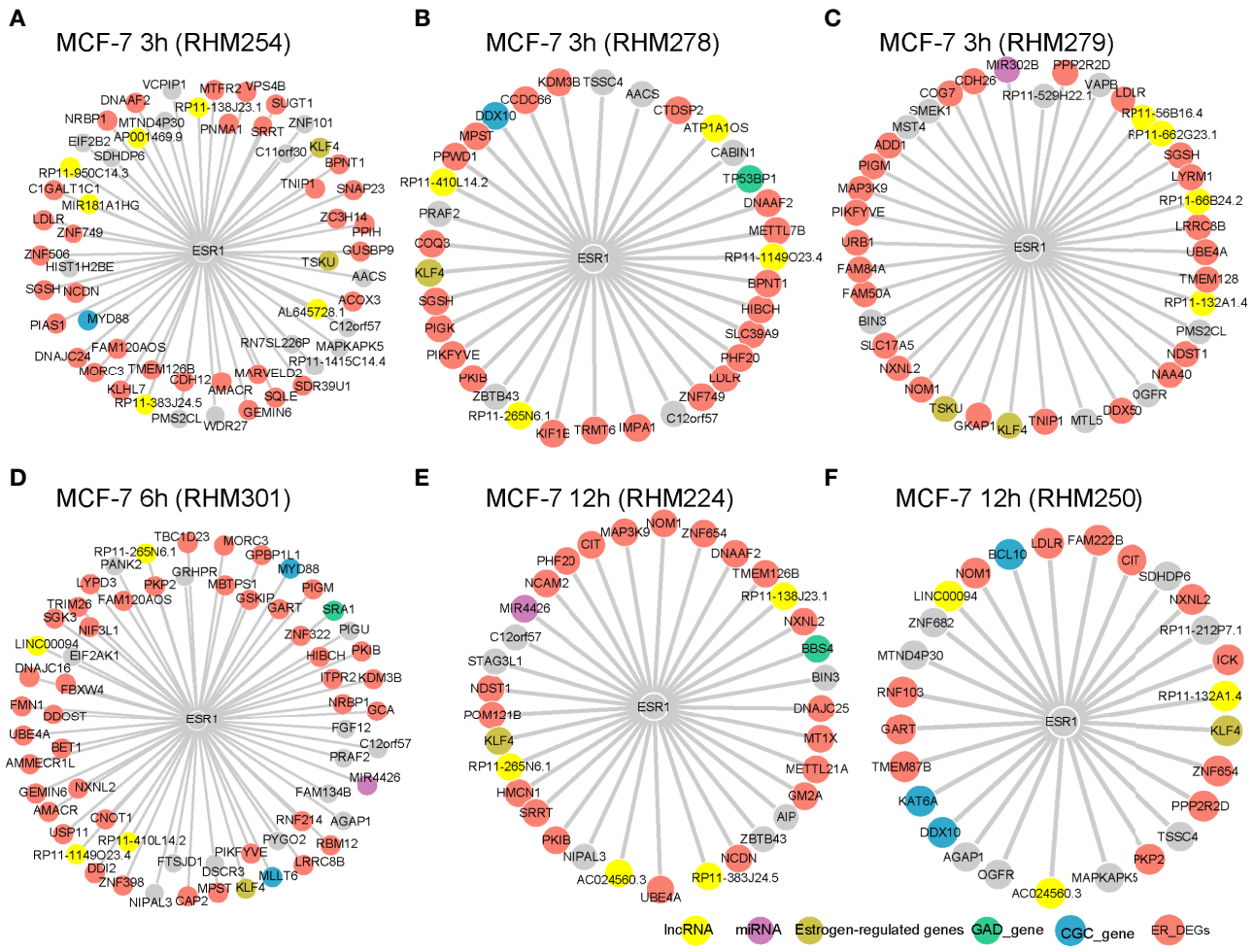

FIGURE 2 | ESR1 interactors in cell-specific RCN. The yellow nodes represent lncRNAs, purple nodes represent miRNAs, green nodes represent GAD genes, blue nodes represent CGC genes, khaki nodes represent estrogen regulated genes, salmon nodes represent DEGs between ER+ *vs* ER– patients from either TCGA or METABRIC, and gray nodes represent genes with an unknown "biological" label. The RCN is shown for the **(A)** MCF-7 3 h (RHM254), **(B)** MCF-7 3 h (RHM278), **(C)** MCF-7 3 h (RHM279), **(D)** MCF-7 6 h (RHM301), **(E)** MCF-7 12 h (RHM224), and **(F)** MCF-7 12 h (RHM250) cells.

These results indicate that the cell-specific RCN reflect the genes' regulations of breast cancer.

We also constructed a cell-specific RCN based on the expression profiles of the T47D dataset. *ESR1* was also co-expressed with known marker genes, including known ERGs, cancer-related genes from CGC, ER-DEGs, and breast cancer-related genes from GAD (**Figure S1**).

The CCN was then constructed by integrating the RCN with the starBase ceRNA database, which was developed based on CLIP-Seq data. The average numbers of edges and lncRNAs in the CCN are shown in **Table 1**. The average number of edges decreased after E2 stimulation, from 882 edges (3 h) to 662 edges (12 h). Meanwhile, the average number of lncRNAs decreased from 16 (3 h) to 14 (12 h). In contrast, there were more than 1,600 edges on average in the CCN from the T47D dataset. However, on average, five lncRNAs were involved in the CCN.

Cell Subtypes Inferred by CCN

Cell type classification assumes high importance in single cell heterogeneity. Therefore, we defined cell subtypes by integrating CCN and the estrogen response hallmark. We retrieved estrogen early response and late response hallmarks from MSigDB. For each CCN, we extracted all the RNAs and performed a hypergeometric test to evaluate the extent of RNA enrichment occurring in these hallmark stages.

We selected one CCN at each time point as an example. The CCN was significantly enriched in the early response hallmark for MCF-7 3 h (RHM223, **Figure 3A**, p = 0.0002), MCF-7 6 h (RHM300, **Figure 3B**, p = 6.61E-8), and MCF-7 12 h (RHM265, **Figure 3C**, p = 3.69E-5). Among all 64 cells (3, 6, and 12 h), 41 were enriched in the estrogen early response hallmark (**Figure 3D**, $p < 0.05$). Three out of 64 cells were enriched in the estrogen late response hallmark (**Figure S2**). Similarly, the cells T47D 3 h (T47D_3 h_2B6), T47D 6 h (T47D_6 h_2H8), and T47D 12 h (T47D_12 h_D8) were enriched in the early response hallmark (**Figures S3A–C**). In the T47D dataset, seven cells were classified as early response cells (**Figure S3D**).

We further classified all 64 cells into two subtypes: early response ones and others. Traditionally, the nodes (RNAs in the network) were screened for biomarker identification. As a complex disease, breast cancer is induced by a set of dysregulated and synergetic genes rather than a single gene. Therefore, network biomarkers are more advantageous for characterizing disease states. Here, we explored the edge markers of both the cell subtypes. We selected the top 20 ceRNA–ceRNA relationships that only appeared in one cell subtype. From the heatmap, we could clearly distinguish the early response subtype from the other subtype (**Figure 3E**). A similar result is shown in **Figure S3E** for the T47 dataset.

TABLE 1 | Average number of edges and lncRNAs in CCN.

Group	Average number of edges	Average number of lncRNAs
3 h	882	16
6 h	809	16
12 h	662	14

CeRNAs of Hub LncRNA Can Predict Patient Survival

Topological characterization of the CCN is crucial for identifying the pivotal genes that substantially contribute to gene regulation upon E2 stimulus. For all the CCNs, we analyzed the topological features, including degree, betweenness, and closeness coefficients. We focused on lncRNAs in the CCN with a high degree in the early response subtype. The top five lncRNAs are listed in **Table 2**. The average degree for all lncRNAs in the early response subtype is in **Table S3**.

The role of *NEAT1* in breast cancer has been widely investigated. It is also a hub lncRNA in the CCN of early response cells with an average degree as high as 5.14. Cells with a degree ≥20 for *NEAT1*were selected. *SMAD4* and *NF1*, the known cancer-related genes in the CGC database, are the common ceRNAs of *NEAT1* in all six CCNs (**Figures 4A–F**). Another CGC gene, *PTEN*, appears in five of these six ceRNA networks. *WWTR1*, a CGC gene, is the ceRNA of *NEAT1* in cell MCF-7 3 h (RHM254, **Figure 4A**) and MCF-7 6 h (RHM271, **Figure 4B**). The GAD genes *PRKCA*, *PRLR*, and *POLK* function as ceRNAs of *NEAT1* in cell MCF-7 3 h (RHM254, **Figure 4A**), MCF-7 6 h (RHM271, **Figure 4B**), and MCF-7 12 h (RHM265, **Figure 4C**), respectively. For cell MCF-7 3 h (RHM255, **Figure 4D**), we identified the ERGs *XRCC1* and *RAPGEFL1* as ceRNAs of *NEAT1*. For cell MCF-7 6 h (RHM271), ERGs *RAPGEFL1* and *SLC7A2* are ceRNAs of *NEAT1*. *TET2*, a CGC gene, is the ceRNA of *NEAT1* in cell MCF-7 6 h (RHM250, **Figure 4E**).

Among *NEAT1* ceRNAs in the six CCNs, we noted that the ceRNA–ceRNA relationship of *NEAT1* and *ZFX* in MCF-7 12 h (RHM266, **Figure 4F**) has been recently validated (21). According to a previous study (21), *NEAT1* and *ZFX* competitively bind to *miR-138-5p*. Next, we performed multivariate Cox regression analysis for the *NEAT1–ZFX–miR-138-5p* regulation axis using the "ceRNA survival" tool of LnCeCell (7). We divided all breast cancer patients from TCGA into two groups, based on the median expression value of *NEAT1–ZFX–miR-138-5p*. Patients with high *NEAT1–ZFX–miR-138-5p* expression had worse overall survival than those with low expression (**Figure 4G**). Moreover, we curated from starBase that *miR-493-5p* and *miR-513a-5p* are significantly shared by *NEAT1* and *ZFX*. Because the expression of *miR-513a-5p* is not available in the TCGA dataset of breast cancer, we tested the prognostic potential of *NEAT1–ZFX–miR-493-5p*. As shown in **Figure 4H**, the *NEAT1–ZFX–miR-493-5p* axis was also an unfavorable prognostic marker of breast cancer.

As *NEAT1* is one of the hubs in CCN, we further used the web tool Kaplan–Meier Plotter (https://kmplot.com/analysis) to perform relapse-free and metastasis-free survival analysis for *NEAT1*. Three probes from the microarrays were mapped to *NEAT1*. The mean expression of the probes was used for the survival analysis of *NEAT1*. High and low *NEAT1* expression levels were divided according to the median expression level. As shown in **Figure 4I**, *NEAT1* was a prognostic marker for breast cancer, based on relapse-free survival analysis. However, *NEAT1* was not a predictor of metastasis-free survival in breast cancer (**Figure 4J**).

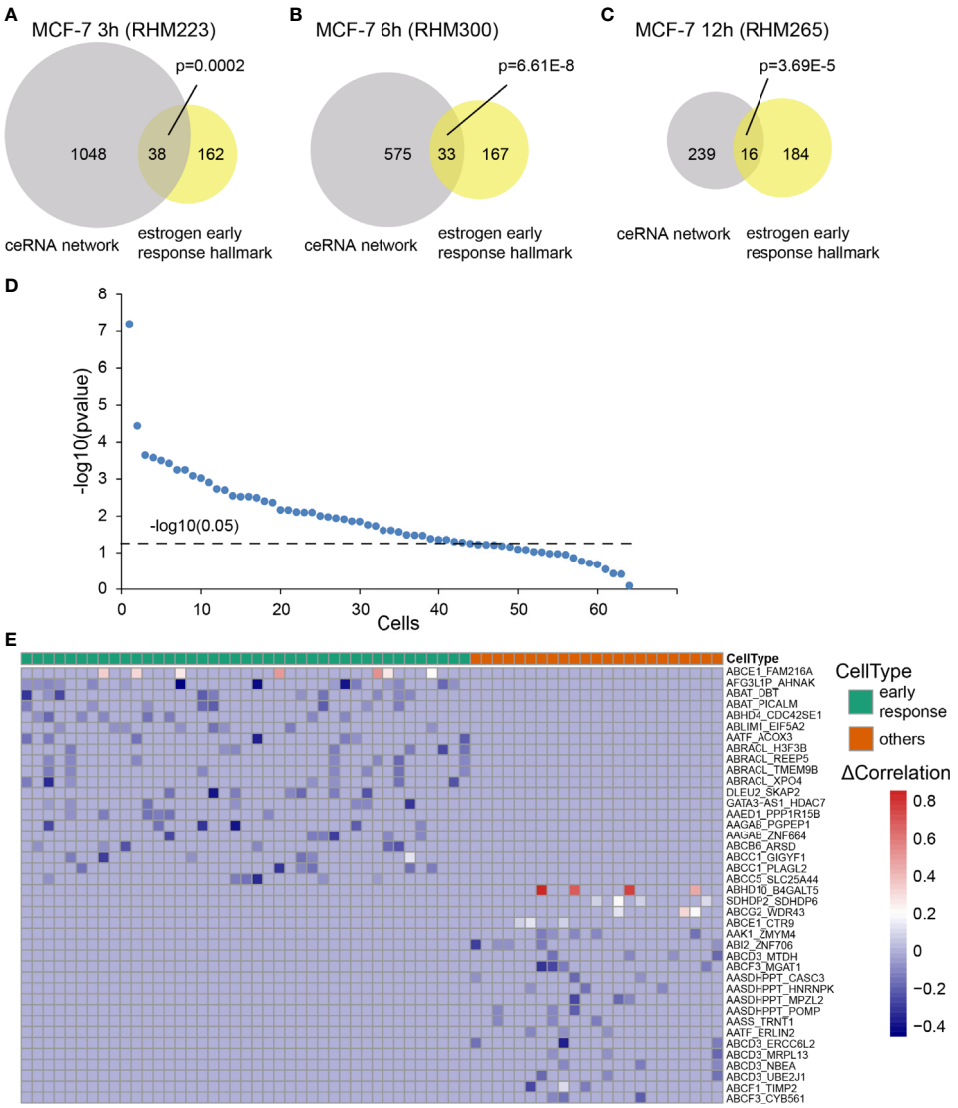

FIGURE 3 | Cell subtypes inferred by the CCN. The RNAs in the CCN were enriched into estrogen early response hallmark, as determined by a hypergeometric test. We showed significant enrichment of RNAs in the CCN of the **(A)** MCF-7 3 h (RHM223), **(B)** MCF-7 6 h (RHM300), and **(C)** MCF-7 12 h (RHM265) cells to the early response hallmarks. **(D)** The minus log10 transformed p-value calculated by a hypergeometirc test for all cells at 3, 6, and 12 h. The dashed line represents the significance threshold, p = 0.05. **(E)** The heatmap of differential correlation (*ΔCorrelation*) in all cells that were classified into two subtypes: early response cells *vs.* others. Blue represents loss of correlation in the "Perturbed network", while red refers to the gain of correlation in the "Perturbed network".

We also constructed CCNs based on the T47D dataset. *NEAT1* is not a hub lncRNA in the CCNs of T47D cells. The top five lncRNAs in the early response cells are shown in **Table S2** for the T47D dataset. Topping the list is *PITPNA-AS1*. But there is no available siLncRNA dataset for *PITPNA-AS1*. Therefore, we focus on MALAT1, which has the second largest degree. *MALAT1* has been widely investigated for its role in breast cancer. Cells with a degree ≥10 for *MALAT1* were selected (**Figures S4A–C**). *MALAT1* interacts with known ERGs, cancer-related genes from CGC, ER-DEGs, and breast cancer-related genes from GAD, which is consistent with the results from the MCF-7 dataset. The ceRNA–ceRNA relationships of *MALAT1–ZFP36L2*, *MALAT1–PGRMC2*, and *MALAT1–PDS5B* were shared among the three cells (**Figures S4A–C**). The ceRNA survival analysis revealed that they were unfavorable prognostic markers for breast cancer (**Figures S4D–F**). Survival analysis of the hub lncRNA *MALAT1* demonstrated that *MALAT1* was a prognostic marker of relapse-free survival (**Figure S4G**) but not metastasis-free survival (**Figure S4H**).

Function of the Hub LncRNA Can Be Inferred With CCN

Function prediction and interpretation of lncRNAs are important factors to dissect their biological mechanisms. Therefore, we tried to infer the function of the hub lncRNA *DLEU2*, which has not been characterized well in breast cancer.

TABLE 2 | LncRNAs with top degree in early response subtype.

Official_Symbol	Average_degree
OIP5-AS1	5.36
NEAT1	5.14
DLEU2	4.06
GABPB1-AS1	3.43
DLEU1	2.77

The silencing or overexpression is a commonly used measure of lncRNA function inference. We searched the GEO database for RNA-sequencing datasets generated by siLncRNA or overexpression of *DLEU2*. As a result, we found that the dataset for siDLEU2 (GSE162677) matched our criteria.

Differential expression analysis is a commonly used method to explore the function of lncRNAs, especially for *in silico*

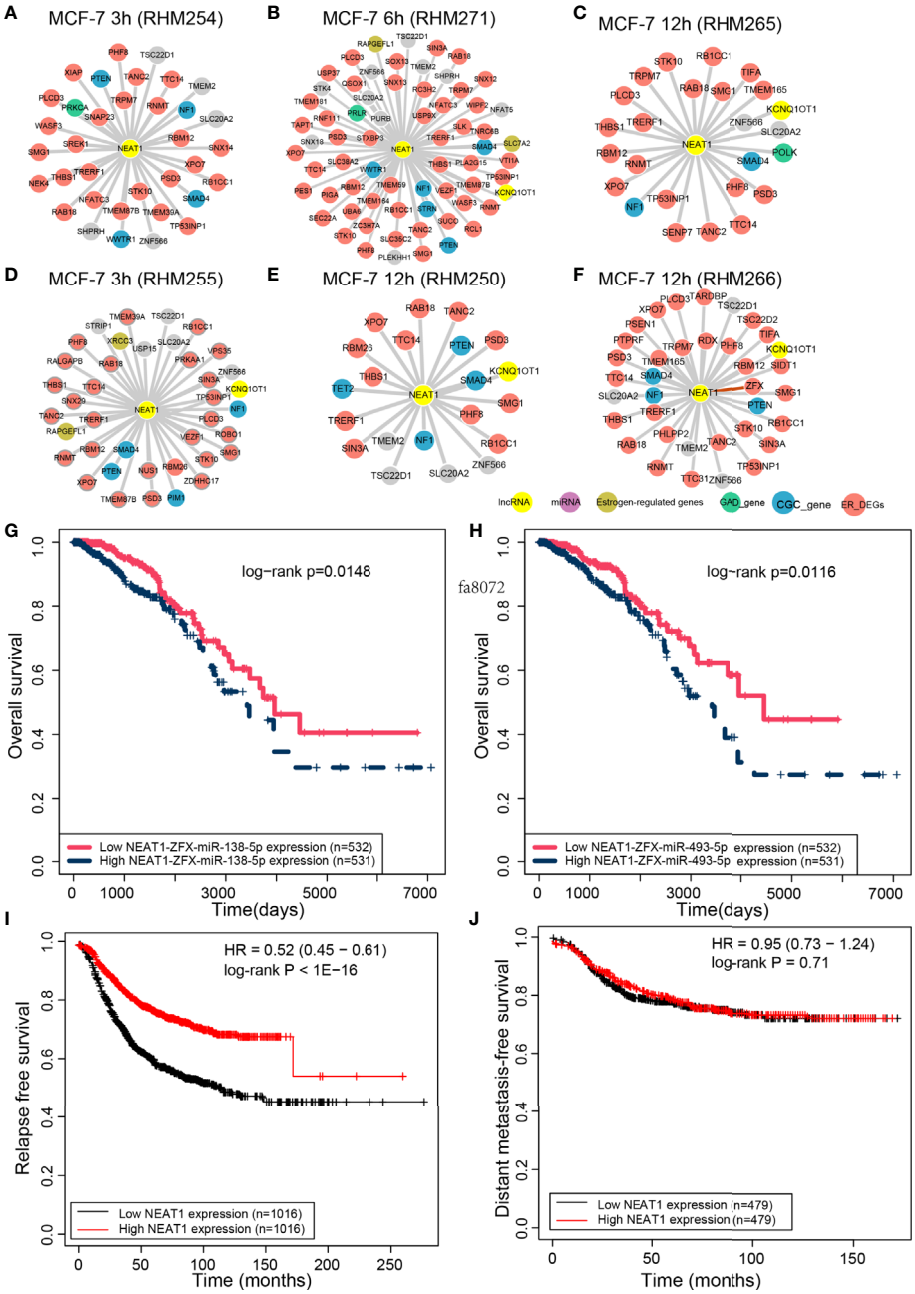

FIGURE 4 | NEAT1 interactors in the CCN. The interactions between NEAT1 and its ceRNAs in the **(A)** MCF-7 3 h (RHM254), **(B)** MCF-7 6 h (RHM271), **(C)** MCF-7 12 h (RHM265), **(D)** MCF-7 3 h (RHM255), **(E)** MCF-7 12 h (RHM250), and **(F)** MCF-7 12 h (RHM266) cells. The survival analysis of NEAT1 and its ceRNA ZFX binding to **(G)** miR-138-5p and **(H)** miR-493-5p. The relapse-free **(I)** and metastasis-free survival analysis **(J)** performed by Kaplan–Meier Plotter for NEAT1.

experiments. The significantly affected biological functions associated with *DLEU2* expression could be theoretically identified based on functional enrichment of the genes affected by *DLEU2*. However, the dataset GSE162677 is generated from a cervical cell line. Thus, it is not suitable for the functional interpretation of *DLEU2* in estrogen regulation in breast cancer.

In MCF-7 cells at 12 h (RHM227), *DLEU2* had the highest number of ceRNAs. GSEA was used to enrich RNAs in the CCN. The RNAs in RHM227 were significantly enriched in the up-regulated genes after siDLEU2 (**Figure 5A**), which indicates that the genes in the CCN may have similar expression changes after siDLEU2 in breast cancer. Therefore, we further predicted *DLEU2* function based on the genes in the CCN and used the hypergeometric test to explore the function of *DLEU2* by functional enrichment of RNAs in the CCN of RHM227. The functional terms of the GO and REACTOME pathways were downloaded from MSigDB (v7.2). The top 10 most significant biological processes from GO and pathways from REACTOME are shown in **Figures 5B, C**, respectively. The most significant GO and REACTOME pathway was GPCR signaling. It should be noted that the biological process "ION_TRANSPORT" was also significantly enriched by RNAs in the CCN of RHM227.

Next, we focused on the ceRNAs of *DLEU2* in the CCN of RHM227. *SOS1* is involved in GPCR signaling from both GO and REACTOME, while *GPR180* participates in GPCR signaling from GO. *TSPAN13*, a ceRNA of *DLEU2*, is also a known marker

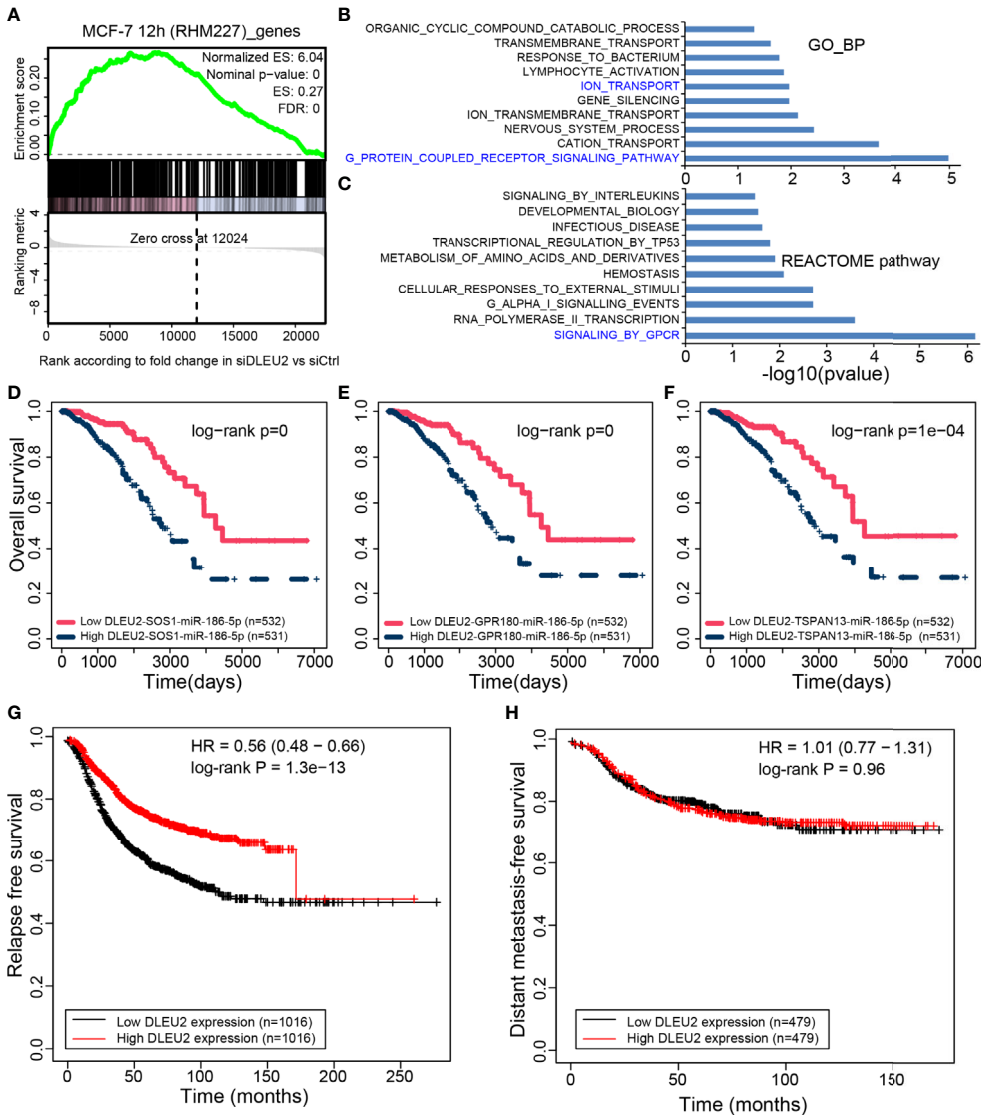

FIGURE 5 | Function inference of DLEU2 *via* the CCN. **(A)** GSEA of RNAs in MCF-7 12 h (RHM227) cell to RNAs affected by siDLEU2. The top 10 functional terms from the **(B)** biological process of GO (GO_BP) and **(C)** REACTOME pathways enriched by RNAs in the CCN of MCF-7 12 h (RHM227) cell, as determined by the hypergeometric test. The interested terms are colored in blue. DLEU2 and its ceRNAs **(D)** SOS1, **(E)** GPR180, and **(F)** TSPAN13 have prognostic potential for breast cancer. The relapse-free **(G)** and metastasis-free survival analysis **(H)** performed by Kaplan–Meier Plotter for DLEU2.

of late estrogen response. It synchronizes with other genes to facilitate ion transport. We retrieved the miRNAs shared by *DLEU2*, *SOS1*, *GPR180*, and *TSPAN13*. Multivariate Cox regression analysis of ceRNAs demonstrated their prognostic potential in breast cancer (**Figures 5D–F**). Furthermore, relapse-free and metastasis-free survival analysis using Kaplan–Meier Plotter (https://kmplot.com/analysis) revealed that *DLEU2* can predict relapse-free survival (**Figure 5G**) but not metastasis-free survival (**Figure 5H**).

To validate the possibility of inferring lncRNA functions *via* the CCN, we also predicted the function of *MALAT1*, based on the CCN of T47D cells. The dataset GSE110239 is an mRNA profile generalized by RNA-sequencing for the mammary tumor mouse model PyMT. Mouse genes were mapped to human gene symbols using the R package biomaRt. The genes in the cell T47D_6 h_2C12 was significantly enriched in the up-regulated genes after *MALAT1* KO (**Figure S5A**). Further functional analysis of RNAs in the CCN showed that they were enriched in the pathway of "fatty acid metabolism" ($p = 0.015$). The "fatty acid metabolism" has also been reported to be enriched by DEGs between BPA exposure and control in mouse liver. Several DEGs were key drivers, such as *Apoa2*, *Akr1c12*, and *Malat1* (22). This provides additional support for the function inference of lncRNAs *via* the CCN.

DISCUSSION

The scRNA-seq technique has become a powerful tool for the elucidation of intra-tumor and intra-cell line heterogeneity in breast cancer (2, 23). Estrogen regulation generally involves not only individual molecules but also molecular networks. Therefore, identifying the CCN upon E2 stimulus is crucial to elucidate the cellular heterogeneity of estrogen regulation at the system level. The CCN is directly constructed based on the single-cell gene expression profile to avoid the bias caused by subjective cluster information. Moreover, Dai et al. demonstrated that gene associations, rather than gene expression, can stably portray biological processes in individual cells (4). We merged ceRNA relations with a cell-specific RCN, which reduced the false-positive RNA–RNA associations.

ESR1 is a pivotal regulator of breast cancer. From the cell-specific RCN, we found that *ESR1* interacted with known ERGs, such as *KLF4* and *TSKU*. Other known cancer-related genes from CGC and GAD were also significantly correlated with *ESR1*. In addition to these protein-coding genes, miRNAs (for example, *MIR302B* and *MIR4426*) and lncRNAs (for example, *MIR181A1HG*, *ATP1A1OS*, and *LINC00094*) were predicted to interact with *ESR1*. *MiR-302* (including *miR-302b*) sensitizes MCF-7 cells to adriamycin and mitoxantrone (24, 25). *LINC00094* has been reported as a super-enhancer-associated ce-lncRNA that promotes cell growth in esophageal squamous cell carcinoma (26). It should be noted that GSE107858 only performed polyA RNA sequencing, without miRNA or lncRNA sequencing. The miRNAs or lncRNAs in the expression profile come from the process of mapping reads to the reference genome.

Heterogeneous cell subtypes upon E2 stimulus were inferred by enriching RNAs in CCN to the estrogen response hallmark. The results showed that 68.7% (44/64) of the cells were responsive to E2 stimulation, and 93.2% (41/44) of them were early response cells. We then classified the cells into two subtypes: early response cells and the remaining cells. The correlation differences of the top 20 edges are shown for each subtype in **Figure 3E**. Among these edge markers, some gene components are not differentially expressed along the time series, which means that they cannot be identified by traditional differential analysis based on gene expression. Regarding the edges of *GATA3-AS1* and *HDAC7*, neither is a differentially expressed gene. However, the edge of *GATA3–AS1* and *HDAC7* had a significant correlation difference in several early response cells. *GATA3–AS1* has been reported to be involved in triple-negative breast cancer progression and immune escape by stabilizing the PD-L1 protein and degrading the *GATA3* protein (27). In contrast, the edge of *AATF* and *ERLIN2* showed no correlation difference in early response cells but had a correlation difference in other cells. Although *AATF* and *ERLIN2* are not DEGs, *AATF* silencing may be utilized to evoke apoptosis and regulate the expression of ERs in MCF-7 cells (28). *ERLIN2* has been reported to promote cell survival by regulating endoplasmic reticulum stress in breast cancer. Moreover, its regulation by *miR-410* is ER-dependent (29).

LncRNA-associated ceRNAs have been investigated in breast cancer. To explore such key lncRNAs and their ceRNAs, topological features such as degree were utilized to identify lncRNAs that function as hub nodes in the CCN. *NEAT1* is the top hub gene observed in the early response subtype, indicating its pivotal role in estrogen regulation. The ceRNAs contain ERGs or cancer-related genes. Intriguingly, *NEAT1* and its ceRNAs can also serve as prognostic markers for breast cancer, which further reveals that the constructed CCN has potential clinical applications.

CCN was used to predict lncRNA function. The RNAs in the CCN of one T47D cell were significantly enriched in the up-regulated genes after *MALAT1* KO (**Figure S5A**). Functional enrichment results implied that RNAs in the CCN participated in the pathway of "fatty acid metabolism", which has also been reported to be associated with BPA exposure, mainly driven by RNAs including *MALAT1* (22). This provides evidence for the functional inference of lncRNA *via* the CCN. We noticed that the hub lncRNA *DLEU2* had not been functionally characterized well in breast cancer. Therefore, the public siRNA datasets of *DLEU2* were selected to infer the function of *DLEU2*. The GSEA results (**Figure 5A**) indicate the feasibility of the functional interpretation of lncRNAs *via* RNAs in the CCN. Functional terms from GO and REACTOME both demonstrated that *DLEU2* is involved in GPCR signaling. In addition, the ceRNAs of *DLEU2* can also predict patient survival in breast cancer. These results can facilitate the speculation of the biological functions of hub lncRNAs, which have not been characterized.

The current CCN method had several limitations. We used the gene expression profile in FPKM, which was biased when comparing gene expression among samples. We also did not consider the impact of inter-sample normalization on our results. In this study, we considered only the positive and

significantly differential RNA–RNA interactions as candidate ceRNAs, in view of direct miRNA targets. Negative correlations are also important because they might be translated as indirect targets of miRNAs sponged by particular ceRNAs. Therefore, the anti-correlated and significantly differential RNA–RNA interactions were added to the candidate ceRNAs. As a result, the anti-correlation will increase the size of cell-specific RCN (**Figure S6** and **Table S4**) and CCN (**Figure S7** and **Table S4**), but do not consequentially increase the significance of CCN enrichment in estrogen early response hallmarks (**Figure S8**). Moreover, it does not affect the function inference of lncRNAs *via* CCN (**Figure S7**).

To conclude, we proposed a novel strategy for constructing a CCN by integrating reference cell-based cell-specific RCNs and public ceRNA networks. This CCN provides new insights into the inference of cell subtypes by incorporating functional gene set information. Hub lncRNAs in the early response subtype and their ceRNAs could be potential prognostic markers for overall survival and relapse-free survival. This CCN also provides a new perspective to infer the functions of uncharacterized hub lncRNAs, which are potential targets for RNA-based therapeutics.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XC and JX conceived the study. YL supervised the project. FZ, CY, and HZ performed the computational analysis. XC and JX drafted the manuscript. WS, TY, YL, and HZ revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported in part by grants from the National Natural Science Foundation of China [Grant No. 62003094 and 82003615] and Natural Science Foundation of Guangdong Province [Grant No. 2018A0303130080 and 2019A1515011377].

ACKNOWLEDGMENTS

The authors are grateful to Haoteng Feng and Xiaoli Wu for their discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.656675/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: 27 May 2021
doi: 10.3389/fonc.2021.687594

The Use of Zebrafish Xenotransplant Assays to Analyze the Role of lncRNAs in Breast Cancer

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Breast cancer represents a great challenge since it is the first cause of death by cancer in women worldwide. LncRNAs are a newly described class of non-coding RNAs that participate in cancer progression. Their use as cancer markers and possible therapeutic targets has recently gained strength. Animal xenotransplants allows for *in vivo* monitoring of disease development, molecular elucidation of pathogenesis and the design of new therapeutic strategies. Nevertheless, the cost and complexities of mice husbandry makes medium to high throughput assays difficult. Zebrafishes (*Danio rerio*) represent a novel model for these assays, given the ease with which xenotransplantation trials can be performed and the economic and experimental advantages it offers. In this review we propose the use of xenotransplants in zebrafish to study the role of breast cancer lncRNAs using low to medium high throughput assays.

Keywords: lncRNAs, breast cancer, xenotransplant, zebrafish, non-coding RNAs

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

Received: *29 March 2021*
Accepted: *04 May 2021*
Published: *27 May 2021*

Citation:
Zampedri C, Martínez-Flores WA and Melendez-Zajgla J (2021) The Use of Zebrafish Xenotransplant Assays to Analyze the Role of lncRNAs in Breast Cancer. Front. Oncol. 11:687594. doi: 10.3389/fonc.2021.687594

INTRODUCTION

Breast Cancer

Breast cancer is the most frequent malignancy in women worldwide and the leading cause of malignancy-related death (1). Whereas early breast cancer is considered curable in 70 to 80% of patients, metastatic disease is considered incurable with our current therapeutic options. The tumor characteristics that lead a breast cancer to become metastatic are not fully understood; however, great efforts are currently being made to elucidate the early mechanisms involved in metastasis, and find early molecular markers and new therapeutic targets related to progression. This malignancy is a heterogeneous group of diseases that deserves our attention and focus on finding new markers that can better discriminate among different subtypes and/or to individualize molecular characteristics of these tumors, allowing for a more reliable prognosis and precise treatments (2). Gene expression profiling of breast cancer has improved the understanding of breast cancer's heterogeneity on the genomic level, challenged the current classification of breast cancer, served as an important prognostic indicator; and most important, begun to guide the treatment in women with early breast cancer (2). It is of pivotal importance to find noninvasive biomarkers with high sensitivity and specificity, which can be used for breast cancer detection at an early stage and monitor of response to therapy (3). Recent advances in technologies, such as microarray and high-throughput sequencing, represented a deeper understanding of molecular biology, especially long noncoding RNA (lncRNA).

THE ROLE OF lncRNAs IN CANCER PROGRESSION

Through a varied repertoire of interactions, lncRNAs are involved in health and disease, through a diverse array of processes such as differentiation and embryonic development (4–6), innate immunity (7, 8) and cancer progression (9, 10). The world of lncRNAs is constantly growing; today, several databases have information on hundreds of thousands of lncRNAs from human and other species (11, 12). Several studies revealed that lncRNAs are key to cancer initiation and progression. Although the biological function and molecular mechanisms of lncRNAs are not known in detail, many lncRNAs are expressed abnormally in cancer.

The expression dynamics of lncRNAs are finely controlled by epigenetic, transcriptional and post-transcriptional regulation. The characteristic tissue-specific expression and low transcription levels of lncRNAS are epigenetically regulated. Transcription of non-coding RNA genes is regulated by central transcription factors that also regulate nearby coding genes; however, some lncRNA may allow their transcription to be unsynchronized with their near mRNAs. Moreover, lncRNAs are also regulated at the post-transcriptional level, including modulation by miRNAs (13).

lncRNAs are involved in a large number of molecular regulatory mechanisms such as chromatin dynamics, gene expression, growth, differentiation, and development. Consequently, they participate in the maintenance of homeostasis, and thus, in several pathologic states. These molecules are transcribed at sizes ranging from 200 nucleotides to several thousand base pairs with little or no translation potential (14). lncRNAs comprise non-coding RNAs (lncRNAs) previously annotated as antisense transcripts, intronic transcripts, processed pseudogenes, lncRNAs (long intergenic non-coding RNAs), and coding-transcript isoforms that do not translate to a functional protein (15–18). These RNAs are transcribed in the cell nucleus and then transported to the cytoplasm to be edited and directed to their final destination to fulfill the function, either in the cytoplasm, nucleus, local organelles (cell-autonomous function) or outside the cell (non-cell-autonomous function) (18).

On vertebrates, lncRNAs are transported from cells into interstitial spaces and body fluid through exosomes, similar to lipids, proteins, DNA, and mRNA (19, 20). Secreted exosomes circulate in different fluids and can be internalized by neighboring cells (in autocrine and paracrine communication) or distant cells (in endocrine communication). They can also be transferred from one organism to another, thus facilitating genetic and epigenetic information exchange between organisms (21).

LncRNAs act at various gene regulation levels, e.g., modulating methylation at the chromatin level (22, 23) or regulating genes through association with activator or repressor complexes at the transcriptional level (23, 24). They also participate in processes of splicing, transport, translation and mRNA decay, as is the case of the versatile lncRNAs MALAT1 (17, 25, 26). In summary, lncRNAs fulfill the functions by their molecular interaction with other biomolecules, including proteins, DNA and several RNA species (mRNA, small RNA and even other lncRNAs).

LncRNAs mediate the interaction between proteins, RNAs, and lipids, not only in physiological situations but also during cancer progression (9). These interactions regulate two key cancer processes: Cancer Stem Cells (CSCs) maintenance and the tumor cells´ interaction with their microenvironment. These characteristics give lncRNAs important features as molecular markers for the diagnosis, prognosis (27), and prediction (28) of cancer. Additionally, circulating lncRNAs have great potential as molecular markers for non-invasive detection since variations in lncRNA expression can be detected in a serum or body fluid sample, avoiding invasive approaches such as tumor tissue biopsies (29). This advantage makes lncRNAs a promising tool on the road to early cancer detection and drug design.

lncRNAs Involved in Breast Cancer

There is currently an extensive list of lncRNAs associated with breast cancer with either oncogenic or tumor suppressor functions, according to their roles in promoting or inhibiting proliferation, metastasis, invasion, apoptosis, autophagy, inflammation, stemness, and drug resistance (30).

lncRNAs in Breast Cancer Metastasis

Plenty information has recently been generated for some lncRNAs such as MALAT1, HOTAIR (31) and NEAT1 (32) describing their breast cancer progression and metastasis roles; and although knowledge about lncRNAs and their association with breast cancer metastasis is constantly growing (**Table 1**), much remain to be elucidated. In particular, there is a paucity of information regarding the molecular mechanisms by which lncRNAs exert their function and clinical relevance. One of the first mechanisms required to initiate metastasis is the epithelial-mesenchymal transition (EMT) (48), which paves the way for the migration and invasion of cancer cells from the primary tumor site to distant secondary sites (49). More than a dozen lncRNAs are known to be involved in the EMT of breast cancer cells.

lncRNAs Are Involved in Apoptosis Avoidance During Breast Cancer Progression

It is clear that lncRNAs are involved in a wide range of biological and physiological processes during breast cancer progression. One of these is regulated cell death, in particular apoptosis. lncRNA-Zfas1 is an antisense of the 5′end of the gene encoding the Zfas1 protein, which is localized to the ducts and alveoli of the mammary gland. Deletion of lncRNA-Zfas1 in breast cancer cells resulted in increased cell proliferation with a concomitant reduction of Zfas1 expression (50). Thus, Zfas1 is a novel and potential suppressor of breast cancer.

LncRNA-Smad has recently been identified as adjacent to the mouse Smad7 gene (51). LncRNA-Smad7 expression is induced by TGF-beta in all mammary gland epithelial cells and breast cancer cell lines (51). Deletion of this lncRNA neutralized the antiapoptotic function of TGF-β. This finding suggests a tumorigenic role of this lncRNA. LOC554202 is an additional

TABLE 1 | lncRNAs involved in breast cancer metastasis.

lncRNA	Function	Cancer	Reference
ANCR	Tumor suppressor		(33)
NKILA	Tumor suppressor		(34)
XIST	Tumor suppressor		(35)
Linc00052	Tumor suppressor		(36)
NEAT1	Oncogenic		(37)
Linc-ROR	Oncogenic		(38)
UCA1	Oncogenic		(39)
TINCR	Oncogenic		(24)
BORG	Oncogenic		(22)
LincIN	Oncogenic		(40)
Lnc015192	Oncogenic		(23)
LINC01638	Oncogenic		(41)
ARNILA	Oncogenic		(42)
Lnc-BM	Oncogenic		(43)
MALAT1	Tumor suppressor and oncogenic		(44, 45)
HOTAIR	Tumor suppressor and oncogenic		(31, 46, 47)

lncRNAs that have been linked to apoptosis repression through interaction with mir-31 in triple negative breast cancer (52). We envision that there are still much to learn about the role of lncRNAs in the regulation of cell death of breast cancer cells.

lncRNAs and Autophagy in Breast Cancer

Recent studies have shown that the regulation of autophagy is involved in the progression and recurrence of cancer (53), and in the resistance of breast tumors to chemotherapy drugs (54). So, it is not surprising that lncRNA could play a role in the regulation of autophagy in breast cancer cells (55). For example, recent work has identified an autophagy-related lncRNA prognostic signature (ALPS) model composed of five autophagy-related lncRNAs (MAPT-AS1, LINC01871, AL122010.1, AC090912.1, AC061992.1). These results suggested that the autophagy-related lncRNAs are clinically valuable prognostic biomarkers in breast cancer (56).

Role of lncRNAs in Inflammation During Breast Cancer

It has recently become widely accepted that the immune system can prevent tumor growth and promote it, through processes grouped in three phases: elimination, equilibrium and escape (57, 58). Elimination is achieved through the identification and destruction of transformed cells by tumor-inhibiting inflammation. This phase is characterized by the infiltration of cells of the innate and adaptive immune system. The escape phase is maintained by tumor-promoting chronic inflammation, mainly involving immunosuppressive cells (58).

NF-κB is a family of proinflammatory inducible transcription factors that are involved in breast cancer progression (59). Several lncRNAs play pivotal regulatory roles in the NF-κB pathway. LncRNA NKILA was first found up-regulated by the inflammatory cytokine TNF-α through the NF-κB pathway in breast cancer. NKILA could directly bind to the NF-κB/IκB complex and inhibit NF-κB signaling from suppressing breast cancer metastasis (60). In another report, NKILA was shown to be up-regulated by TGF-β to block NF-κB signaling, thereby suppressing the TGF-β-induced tumor metastasis in breast cancer (34).

STAT3 is a component of another important pathway that plays a role in inflammation during breast cancer progression, and several lncRNAs (e.g., HOTAIR and Lnc-BM) participate in this process (43, 46). In breast cancer cells, Lnc-BM increased the STAT3-dependent expression of ICAM1 and CCL2, which regulated vascular co-option and recruitment of macrophages in the brain, respectively (43).

The Role of lncRNAs in the Tumor Microenvironment Crosstalk in Breast Cancer

The tumoral microenvironment (TME) is a complex biochemical and physiological system involved in tumorigenesis and metastasis (61–63). It comprises the cancer cells, extracellular matrix, vasculature, non-cancer cells and the tumor's acidic and hypoxic microenvironment. The cellular component consists of cancer-associated fibroblasts (CAFs), adipose cells, endothelial cells, cancer stem cells (CSCs), infiltrated immune cells such as T lymphocytes and natural killer cells (NKs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) (64–66). There is evidence that lncRNAs are involved in the communication between tumor and non-tumoral cells required to induce or maintain cancer hallmarks such as proliferation, migration, and metastasis.

One of the best-known examples of the relationship of lncRNAs in the communication between tumor microenvironment and tumor cells is the HOTAIR lncRNA. In breast cancer, TGF-β1 secreted by CAFs up-regulates HOTAIR expression to promote epithelium- mesenchyme transition (EMT) and metastasis (67). HOTAIR inhibits miR-7 in CSCs of MCF-7 and MDA-MB-231 breast cancer cell lines and thus promote the overexpression of SETDB1, STAT3, c-Myc, twist, and miR-9 (46) and repression of E-cadherin (46, 68) to the benefit of the EMT process. HOTAIR also contributes to EMT through regulation of VEGF, MMP-9, β-cantenin and Vimentin (69). Also, in breast cancer HOTAIR up-regulates SNAIL expression, as a master regulator of the EMT

pathway (31). HOTAIR also mediates the establishment of the SNAIL/HOTAIR/EZH2 tripartite complex by inhibiting the expression of epithelial genes (such as HNF4a, HNF1a, and E-cadherin) through chromatin remodeling in favor of EMT (70).

Another essential component of the stroma required for EMT and subsequent metastasis is tumor vasculature. There is clear evidence that dysregulation of a group of lncRNAs can trigger changes in endothelial cells that favor angiogenesis and metastasis of breast cancer cells. For example, the lncRNA NR2F1-AS1 promotes breast cancer angiogenesis by activating the IGF-1/IGF-1R/ERK pathway (71). Similarly, overexpression of MEG3 suppresses breast cancer angiogenesis through the AKT pathway (72). M2 macrophage-induced lncRNA PCAT6 facilitates angiogenesis of triple-negative breast tumors through modulation of VEGFR2 (73).

CSCs are a subpopulation of cancer cells that can self-renew and proliferate limitlessly. They may be responsible for cancer initiation, progression, and even treatment resistance (74). Several lncRNAs act by modulating the self-renewal and differentiation of CSCs, such as lncH19 and HOTAIR. LncH19 acts as a lncRNA sponge for miRNA let-7, inhibiting its function and favoring the maintenance of CSCs in breast cancer (75). HOTAIR, on the other hand, also regulates the self-renewal of CSCs in breast cancer, inhibiting miR-34a and thus positively regulating Sox2 (76). Interestingly, lncRNAs can also modulate the development, activation and differentiation of T cells, which have both tumor-promoting and tumor-suppressive functions (77). Regulatory T cells (Treg) are a subset of CD4+ T lymphocytes that contribute to the inhibition of anti-tumor immunity of the TME (78, 79). The lncRNA SNHG1 promotes Treg differentiation, and the knockdown of this long noncoding lncRNA inhibits Treg differentiation through increased expression of miR-448 and indoleamine 2,3-dioxygenase (IDO) inhibition, preventing immune escape in breast cancer (80). LncRNAs also modulate immunosuppression and cancer progression through the regulation of ROS (reactive oxygen species), NO (nitric oxide), and ARG1 (arginase 1) production in MDSCs. MDSCs are generated in the bone marrow and have been shown to promote EMT and play an important role in cancer progression by suppressing the immune response (81, 82).

TAMs are also key players in cancer progression through invasion and metastasis regulation. Two functional types of macrophages have been identified, classically activated macrophages (M1) and alternatively activated macrophages (M2) (65). M1 macrophages participate in the Th1-type inflammatory response and have anti-tumor activity, and M2 macrophages are anti-inflammatory macrophages and have a proto-oncogenic role (65, 83, 84). Recently, several studies have shown that lncRNAs can modulate M2 macrophage polarization and by this induce tumor cell migration and invasion in several types of cancer. The lncRNA associated with breast cancer brain metastases (BCBMs), lnc-BM, was found to be overexpressed in breast cancer cells, and associated with the induction of brain metastasis in murine models (43). In breast cancer, lnc-BM increased JAK2 kinase activity to mediate oncostatin M- and IL-6-triggered STAT3 phosphorylation, promote ICAM1 and CCL2 expression, and mediate macrophage recruitment to the brain and consequently metastasis. lnc-BM and JAK2 promote BCBMs by mediating communication between breast cancer cells and the brain microenvironment. Thus, lnc-BM could be a promising therapeutic target for invasive breast cancer (43).

LncRNAs are a diverse set of molecules that can perform their functions intracellularly, travel free in the extracellular matrix, affect distant cells' function and even be transported by exosomes during intercellular communication. Tumor-derived exosomal lncRNAs affect the TME by generating changes in the transferred cells, E.g. stromal cells, endothelial cells, macrophages, and mesenchymal stem cells, leading to induction of proliferation, angiogenesis and metastasis (85, 86).

lncRNAs in Breast Cancer Drug Resistance

Besides its involvement with classic cancer hallmarks, a group of lncRNAs has also been linked to drug treatment resistance. The expression of a diverse array of lncRNAs changes dynamically in response to various drugs contributing to anti-tumor drug resistance through various mechanisms, such as cell cycle arrest, inhibition of apoptosis, DNA damage repair (87–89), EMT (90), transport and internalization of drugs by cancer cells (91, 92), and drug metabolism. lncRNAs involved in breast cancer cell drug-resistance are UCA1 in doxorubicin resistance (93), PANDA in anthracycline resistance (94), ARA in adriamycin resistance (95), CCAT2 in 5-fluorouracil (96), and BCAR4, HOTAIR, and M41 in tamoxifen resistance (97–99). Since lncRNAs aberrant expression is a marker of drug resistance (100), they are potential targets of new therapeutic strategies.

ZEBRAFISH XENOTRANSPLANTS TO STUDY THE ROLE OF lncRNAs IN BREAST CANCER

Although studies in cancer cell lines have advanced our knowledge of lncRNAs functions at the molecular level, the use of animal models provides a rich context in which to investigate the phenotypic impact of these molecules in the breast cancer.

The involvement of lncRNAs in the breast cancer tumor phenotype can be modeled *in vivo* by genetic modifications in an animal, altering the expression of a lncRNA and studying the effects on cancer development. However, breast cancer´s multigenic and multifactorial nature requires an integrative approach in which the genetic landscape that drives the development of the disease is present.

Xenotransplantation, which is generated by implanting human tumor cells into an animal host, allows the study of the effects of altering a particular gene in the development of breast cancer through genetic manipulation of human cell lines before transplantation. Mouse xenotransplants were the first to be used, but zebrafish have recently emerged due to the experimental, economic, and visualization advantages they offer. In recent years, several breast cancer cell lines have been successfully

xenotransplanted in zebrafish, such as MDA-MB-468 (74), MDA-MB-231 (101–104), MDA-MB-435 (105, 106), MDA-MB-23 (102), HCC1806 (101), MCF-7 (36, 107–109) and BT-474 (106) among others. These experiments allowed the exploration of the participation of various genes in pathways related to proliferation-tumorigenesis, apoptosis (109), macrophage-mediated tumor cell invasion (104), migration-metastasis, angiogenesis, drug resistance, stem cell maintenance (106) and tumor microenvironment crosstalk (102, 103).

Zebrafish xenotransplantation represents a step forward in modeling the complexity of breast cancer tumors, and the involvement of a particular gene in each of the events that accompany cancer, as cells are implanted into a living organism in which many types of dynamic interactions can occur. Xenotransplanted cancer cells do not depend on the artificial addition of nutrients, serum, cytokines, and growth factors. In zebrafish, with all functional organs, tumors can engage in both local and systemic cell-cell interactions, shaping tumor progression. These interactions occur between tumor and host and vice versa, with long-distance communication, allowing recapitulation of cancer features such as cell migration, invasion, metastasis, angiogenesis, and immune evasion that are not possible to observe *in vitro*. When breast cancer tumor cells are implanted, many different zebrafish cells are recruited to the tumor site following tumor instructions (102, 103). The zebrafish xenotransplantation model allows simultaneous single-cell resolution monitoring of tumorigenesis at various steps *in vivo*, including tumor vascularization, localized tumor growth, tumor invasion, and micrometastasis formation. Zebrafish xenotransplantation of breast cancer cells enabled the discovery of a new mechanism of metastatic niche formation, and the roles of macrophages in this process were described. The experimental advantages offered by zebrafish also allowed the discovery that physiological migration of neutrophils controls tumor invasion by conditioning the collagen matrix to facilitate the metastatic niche (102).

Finally, drug sensitivity profiling of breast cancer cells using the zebrafish xenotransplantation model allows the assessment of pharmacokinetics, pharmacodynamics and toxicity in a whole living organism, and in a short time. *In vivo* testing has great advantages over *in vitro* assays. E.g., to produce *in vivo* phenotypes, compounds must be absorbed, reach targets, circumvent elimination, and cannot be too toxic, otherwise the animal will not survive. The complexity of *in vitro* models is given by the experience of the investigator, whereas in *in vivo* models, the complexity is built according to the dynamic instructions and signals of the tumor itself. Zebrafish xenotransplantation also allows *in vivo* evaluation at the single cell level of the cell autonomous and non-cell autonomous effects of a drug on the different hallmarks of cancer (110).

There are several methodological advantages for using zebrafish, such as their rapid and external development, the transparency of their embryos (111), the availability of fluorescent cell reporter lines (112), the ease of genetic manipulation (113), and pharmacological approaches (114). Moreover, its wide range of growth temperatures that allows xenotransplantation experiments to be carried out at temperatures close to human physiological ones. These characteristics make the zebrafish an excellent *in vivo* model to visualize the tumor cell behavior and interactions with the host microenvironment.

In addition to facilitating *in vivo* assays related to the breast tumor itself, zebrafish help study functional aspect related to particular molecules such as lncRNAs in breast cancer hallmarks. Xenotransplantation of breast cancer cell lines in zebrafish makes it possible to study human lncRNAs' role in the tumor phenotype and microenvironment, giving a comprehensive *in vivo* perspective of the functions of this molecule.

Zebrafish xenotransplant facilitates the study of signaling mechanisms involved at the whole organism level during cancer initiation and progression. Furthermore, there is significant conservation of oncogenes and tumor suppressor genes between zebrafish and humans, so the data obtained from zebrafish are relevant to humans (115). The xenotransplantation platform in zebrafish is also helpful for drug discovery in the context of breast cancer research (116). Zebrafish cell xenotransplantation studies have the advantage of maintaining the effects of the microenvironment in cell communication and cancer progression, even when there are inter species differences.

Zebrafish present ideal characteristics that allow multiple statistically robust experiments to be performed simultaneously; however, the zebrafish xenotransplantation platform is not without limitations. On the one hand, the lack of an adaptive immune response is beneficial for initial transplantation and injection, but could become a limitation for translation of findings, as adaptive immune cells may play vital roles in promoting or inhibiting breast cancer progression and the effects of some treatments (117, 118).

The zebrafish and human genomes are 70% similar based on the conservation of individual genes, including cancer-related coding and non-coding genes. However, zebrafish are not mammalian, so some important pathways in breast cancer tumor development are absent, including BRCA1, p16 (CDKN2A), Leukemia Inhibitory Factor (LIF), oncostation M (OSM) and interleukin 6 (IL6) (119). These absent pathways pose several challenges when studying the functions of these "missing" genes or the pathways in which they play a role. Furthermore, when foreign tissues and cells are introduced into fishes, there is no guarantee that all molecular mechanisms linking the recipient tissue and xenograft are fully conserved, which could affect interactions between host cells and the cancerous xenograft. This issue is especially relevant to the study of breast cancer as there is no orthotopic site in fish. However, it may be possible to "add" the necessary cells or growth signals to mitigate this problem during xenotransplantation, or to "humanize" the fish by creating transgenic animals that express appropriate human growth factors, receptors and/or cytokines, as has been done in mice (120).

Zebrafish offers two options for cancer modeling by xenotransplantation of breast cancer cell lines or patient-derived tumor cells by microinjection, (**Figure 1**). In 48 hpf (hours post-fertilization) embryos, into the yolk sac, duct of Cuvier (common cardinal vein), caudal vein, or perivitelline space. In adults, into the intraperitoneal cavity, (**Figures 1A–C**). Either option may result in

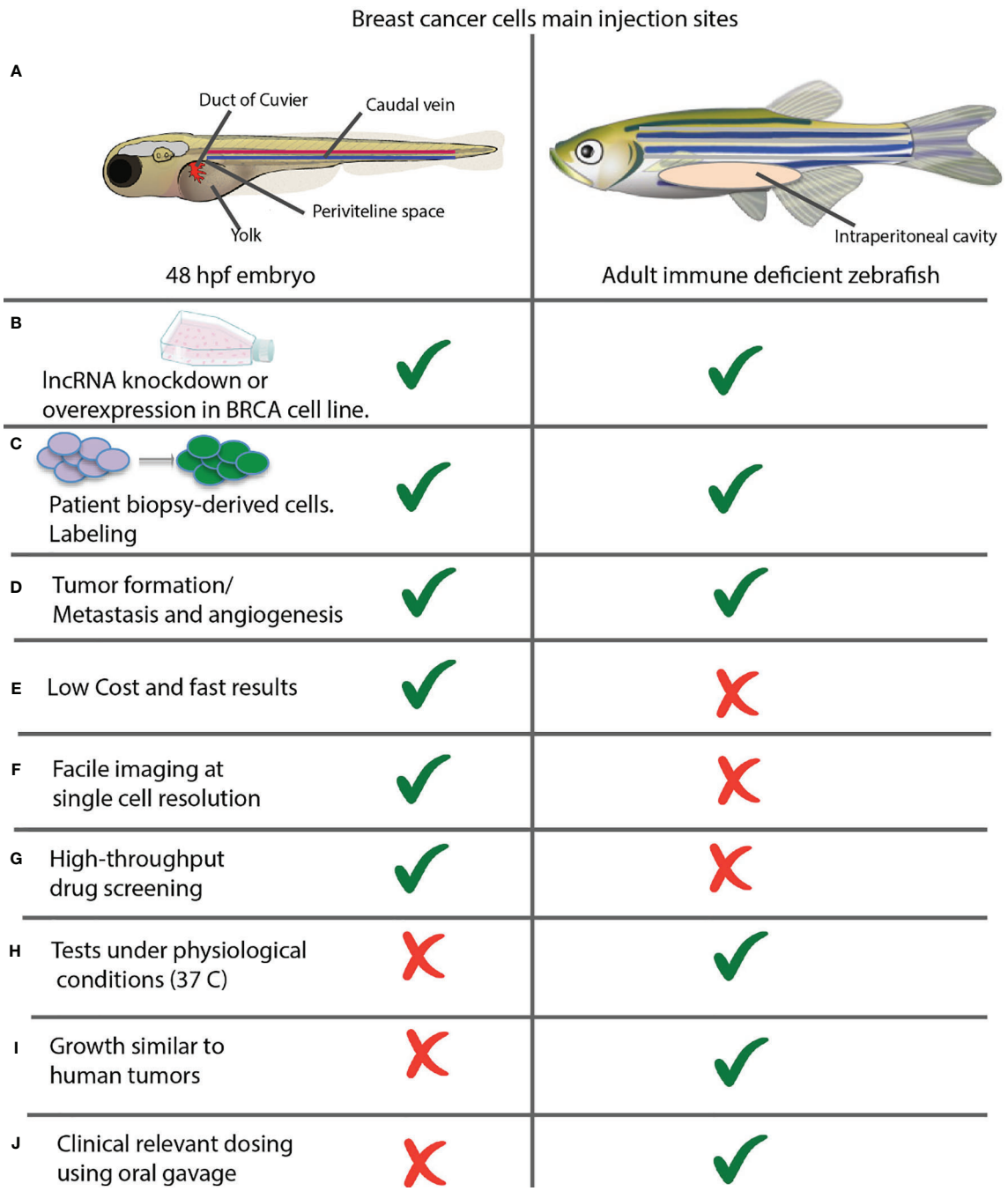

FIGURE 1 | Comparison between xenotransplantation assays in zebrafish embryos and adult animals. **(A)** Common sites of injection. Shown are the most commonly used injection sites for xenotransplantation of a zebrafish in two different stages of development. Left: 48 hpf Stage. The yolk sac is the most common site of injection, but hindbrain ventricle, caudal vein; previteline space and duct of cuvier can be used also. Right: Juvenile Adult. The majority of xenografts occur within the intraperitoneal cavity, and hinbrain ventricle can be used also. **(B)** Xenotransplantation of cancer cell lines and **(C)** patient-derived cells can be performed in both embryos and adults. **(D)** Xenotransplantation allows evaluating the rate of tumor formation, metastasis and angiogenesis. **(E–J)** Advantages and disadvantages of xenotransplantation in embryos versus adult fish assays.

tumor masses, which induce a neo-vascular response around the tumor and consequent migration and metastasis, (**Figure 1D**). Embryo assays are the top choice in most work, given their methodological advantages for fast results, low-cost experiments, compatibility for microscopic imaging, and drug screening potential taking advantage of their small size, (**Figures 1E–G**) (121).

On the other hand, adult animal assays are ideal for studying human physiological temperature-dependent characteristics

such as tumor growth rate or determining the dose using oral gavage, (**Figures 1H–J**) (121, 122).

The use of fishes with fluorescent vasculatures such as Tg(*Fli : EGFP*) or *VEGFR2:G-RCFP* allows the visualization of angiogenesis *in vivo* (123–125). In xenotransplantation, the zebrafish provides the necessary signals for the transplanted cells to integrate into the organs, migrate, proliferate and interact with the zebrafish microenvironment (126, 127).

Xenotransplantation has evolved as one of the most valuable strategies to study breast cancer's discrete aspects, as evidenced by the growing volume of scientific publications on this subject over the last 15 years, (**Supplementary Table 1**). Xenotransplants of breast cancer cell lines in zebrafish allow rapid *in vivo* testing of coding and non-coding genes, pathways involved in tumorigenesis, migration, angiogenesis, or screening for new drugs. Moreover, in recent years, *in vivo* modeling by zebrafish xenotransplantation revealed important information on the role of some lncRNAs in breast cancer hallmarks (36, 107, 128, 129) (**Supplementary Table 1**, blue data). We recently uncovered the role of lncRNA-HAL in promoting the stemness in breast cancer cells; the action of lncRNA LINC00052 in the suppression of migration, as well as the role of LncMat2B in the induction of breast cancer cell invasiveness using *in vivo* xenotransplantation assays in zebrafish (36, 107, 129). Likewise, Peperstrate et al. showed that lncRNA H19 increases breast cancer cells' invasive capacities in xenografted transgenic zebrafish models (128). In this work, breast cancer cell lines were modified to alter the expression of lncRNAs, then stained or labeled with reporter genes and transplanted into zebrafish embryos. Tumor cells that migrated to distant sites within the fish embryos, and the growth of the transplanted mass, or the development of tumors at secondary sites, were related to the different hallmarks of cancer to infer the involvement of the lncRNA ones in these events.

In conclusion, zebrafish xenotransplants allow the *in vivo* functional study of the involvement of lncRNAs in breast cancer in short timescales.

ZEBRAFISH XENOTRASPLANT FOR THE STUDY OF lncRNAs IN BREAST CANCER TUMOR MICROENVIRONMENT

One of the most attractive advantages of using an *in vivo* model for the study of breast cancer as a complement of an *in vitro* model is the possibility of representing the complex context of the tumor microenvironment.

As discussed above, lncRNAs are involved in a various molecular pathways related to communication from the tumor microenvironment to the tumor cells themselves to promote cancer establishment and progression. The zebrafish xenotransplantation platform for breast cancer will facilitate the discovery of functional information of lncRNAs in the complex process of communication with the tumor microenvironment. Zebrafish xenotransplantation allows visualization of *in vivo* events in a real time and cellular level, such as cell-cell interaction. Together with assays to alter the expression of lncRNAs in xenotransplanted cells, the zebrafish xenotransplantation model could provide valuable information on the participation of lncRNAs in this complex process. As previously stated, zebrafish xenotransplantation models are efficient for providing information about several breast cancer hallmarks, e.g. tumor progression, angiogenesis, spread, metastasis or drugs response, revealing the existence of interactions between cancer cells and cellular and non-cellular components of the host inter-species microenvironment.

Xenotransplantation assays in zebrafish have shown that it is possible to investigate the mechanisms and biological implications of tumor-host cell crosstalk. A clear example is the molecular interaction between breast cancer cells and zebrafish host cells. They allow the recapitulation of cancer hallmarks such as angiogenesis in CXCR4 chemokine signaling across zebrafish and humans in xenotransplantation experiments. Tullota et al. showed that human cancer cells expressing CXCR4 responded to the zebrafish Cxcl12 ligand, and zebrafish cells expressing Cxcr4 migrated to the human CXCL12 ligands (130). On the other hand, substantial evidence supporting the molecular interrelationship between human and zebrafish, and involving a lncRNA, is the resistance to tumor formation of a zebrafish knockout of *Thor* (THOR-/-) (an oncogenic lncRNA conserved between zebrafish and human) after xenotransplantation with NRAS61K melanoma cells (131).

The interaction between cancer cells and zebrafish immune cells was discovered by experiments transplanting cancer cells directly into the blood circulation through the duct of Cuvier or the perivitelline space. Neutrophil and macrophage infiltration surrounding the tumor was observed by using transgenic zebrafish strains with labeling in immune system cells (Tg (mpx:GFP)^{i114} (132) in neutrophils, Tg(mpeg1:eGFP)^{gl22} (133) and Tg(mpeg1: mCherry)^{UMSF001} (134) for macrophages (135); and the interaction of cancer cells with endothelium using vessel-tagged strains such as Tg(fli:eGFP)^{y1} (112), Tg(flk1:eGFP)^{s843} (136) and Tg(flk1:mCherry) (137). The zebrafish immune cells are recruited and localized near the breast cancer cells at the primary tumor growth and secondary micrometastasis sites. Also, it was observed that the non-disseminated tumor cells associated with the endothelium of the duct of Cuvier and remodeled it, forming new vessel-like structures and then forming functional vasculature. Subsequently, by knocking down the expression of myeloid differentiation transcription factors in zebrafish, the suppression of tumor vascularization, invasion and micrometastasis was observed (102), showing the dynamic interaction of zebrafish immune cells with human breast cancer cells.

On the other hand, using vasculature-tagged reporter strains, cancer cells injected into the yolk of zebrafish embryos were shown to interact with the endothelium of the embryos blood vessels, migrate through them and form secondary tumors (138). The induction of angiogenesis mediated by the interaction between zebrafish immune cells and transplanted human breast cancer cells was also confirmed through the positive correlation between the expression levels of vascular endothelial growth factor A (VEGFA) secreted by transplanted

breast cancer cell line, the number of immune cells recruited around the tumor, the interaction of macrophages with the vessels, and the induction of new vessel formation around the tumor in zebrafish (103).

In recent work, it was observed that one of the mechanisms by which the interaction between host cells and tumor cells occurs is through the transfer of cytoplasm from zebrafish macrophages to the transplanted tumor cells. Although it is unknown what components are exchanged, it is presumed that it could be RNA molecules (139).

Zebrafish xenotransplantation assays of breast cancer cells, coupled with single cell transcriptional analyses, could facilitate the elucidation of the molecular mechanisms and lncRNAs involved in the communication between the tumor micro environment and cancer cells.

lncRNAs AND ZEBRAFISH PATIENT-DERIVED XENOTRANSPLANTATION (zPDX) IN THE SEARCH FOR PERSONALIZED BREAST CANCER TREATMENTS

Due to breast cancer's genomic advances, one of the greatest challenges in translational and personalized medicine is quick, cheap and reproducible *in vivo* disease modeling. Although molecular breast cancer markers and pharmacogenomics analyses help to predict the best treatment option, many patients do not respond as expected. This event is probably due to the heterogeneity of breast tumors, in which there are non-responding cells immersed in a large group of responding cells, which will escape treatment.

LncRNAs have been associated to breast cancer progression by modulating a large number of oncogenic processes. These results point toward the possibility that they could be useful as future targets for therapeutic intervention against breast cancer (129, 140, 141). In addition, Several studies have proposed lncRNA signatures that could potentially be used for predictive and prognostic value in response to breast cancer treatments (142–144).

LncRNAs can be targeted for inhibition through multiple mechanisms, such as antisense oligonucleotides (ASOs), short hairpin RNAs (shRNAs), short interfering RNAs (siRNAs), aptamers, CRISPR-Cas approaches and small molecule inhibitors (145, 146). There is evidence that ASOs could be a potential targeted therapy for cancer-associated lncRNAs (147). It was recently reported that ASOSs directed against the breast cancer-associated lncRNA MALAT1 effectively suppressed cancer spread to the lung in a murine model of breast cancer xenotransplantation (148).

The use of avatars or patient derived xenotransplantation (PDX) breast cancer models may help evaluate *in vivo* the global response of tumor cells, detect those that escape the drug, and find and decide the most appropriate treatment for that patient. Zebrafishes offer suitable characteristics for breast cancer modeling using PDX, such as the embryo transparency, the possibility of real time visualization, and the short time to obtain results (149). Patient derived xenotransplantation in zebrafish (zPDX) consists of obtaining a very small fraction of the patient's tumor by biopsy and transplanting it into zebrafish directly or after obtaining a primary culture of cells, (**Figures 2A, C**).

The combination of zPDX with the lncRNAs based transcriptomic analysis-guided drug screening assays would enable the finding of efficient and personalized anti-breast cancer treatments, (**Figures 2B–D**). The zebrafish xenograft model allows rapid sensitivity profiling to new anticancer drugs but is also ideal for determining the effects of different therapeutic combinations on tumorigenesis, metastasis, and angiogenesis, in a timeframe compatible with the clinical decision-making process (110), (**Figures 2D–F**). More important, these assays maximize the use of the small amount of breast cancer tissue available after a biopsy, which can be a limiting factor in precision medicine.

ZEBRAFISH CONSERVED lncRNAs IN THE FUTURE DIRECTIONS FOR BREAST CANCER MODELING BY XENOTRANSPLANTATION

LncRNAs are key players in the communication between tumor cells and the surrounding microenvironment, actively participating in cancer progression. These non-coding RNAs can travel free or *via* exosomes to neighbor cells in the tumor microenvironment and carry out their function in a cell-non-autonomous manner (19). Breast cancer xenotransplantation assays in zebrafish showed that there is interspecies molecular communication that allows the development and progression of cancer in the animal model. Despite the low conservation of interspecies sequences of lncRNAs, the knowledge of those that are conserved between human and zebrafish will allow the study of their cell-non-autonomous function, and to test their potential as therapeutic targets.

LncRNAs can be evolutionarily conserved through sequence, structure, function, and expression of the locus of synthesis. In general, lncRNAs do not have high sequence conservation across the full-length sequence because partial sequences or local spatial structures mainly mediate their biological functions. The speed of base change in lncRNA sequences exceeds the evolutionary time scale. It follows that lncRNAs evolve faster than protein coding genes, suggesting that nucleotide sequence conservation is not essential for preserving lncRNAs functionality (150, 151). lncRNAs follow different conservation criteria than those of protein coding genes (150, 152). Identity concentrates on short sections and the secondary structure, unlike coding genes that focus on conservation in all their length to preserve the open reading frame and ensure similarity in amino acid sequence (150). In order to find these conserved segments, diverse groups have generated tools that allow us to study their evolution and to estimate the functional conservation of lncRNAs across species, for example PLAR (153), Gencode V7 (154), Lncipedia (12), and ZFLNC (11). Currently, there are databases focused mainly on

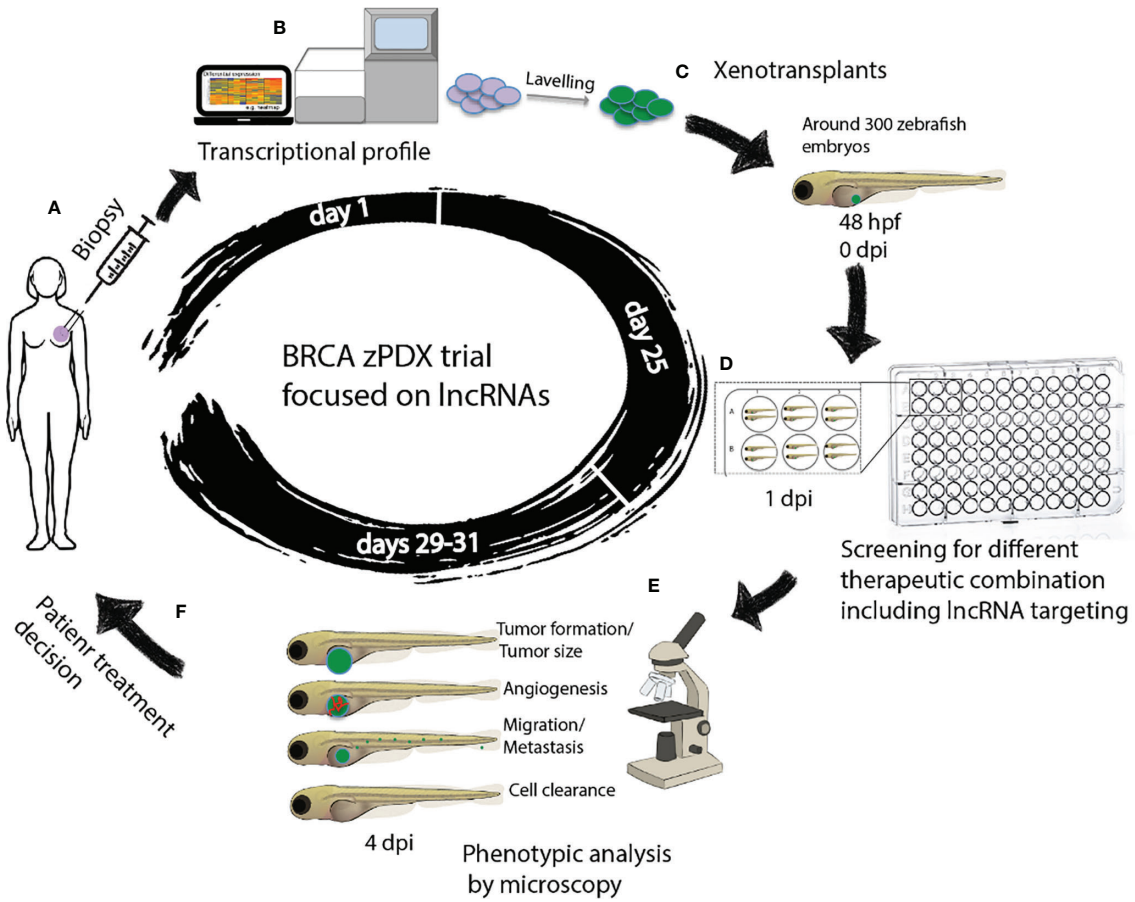

FIGURE 2 | Workflow of zAVATARS in the context of personalized medicine. **(A–C)** Experimental setup for generating zebrafish xenotransplant models. Cells derived from itumor biopsy are analyzed by RNAseq, subsequently labeled and microinjected in the 2dpf larvae. **(D)** One day after injection, larvae are screened for successful injection and distributed in groups for testing chemo-, and/or biological therapies. **(E)** Three days after treatment, larvae are processed for in vivo microscopy for analysis of proliferation, cell death, angiogenesis, and metastatic potential. **(F)** Treatment decision of breast cancer patients based on response of zebrafish cancer hallmarks in drug screening. hpf: hours post fertilization. dpi: days post injection. BRCA zPDX trials: Trial of patient-derived xenotransplants in zebrafish for the study of breast cancer.

zebrafish lncRNAs annotation and expression profiles, such as ZFLNC and zflncRNApedia, for a total of 13,604 genes that transcribe 21,128 lncRNAs (11), of which 1,890 are conserved in human.

One of the most important characteristics observed in the expression patterns of zebrafish lncRNAs is the strong dynamics of temporal expression compared to protein-coding genes (155). This feature is undoubtedly relevant to consider these molecules as indicators or markers in disease processes. Also, it has been found that not only specific gene regions of the lncRNAs are conserved, but there is also significant conservation into the upstream regulatory regions (156), and the epigenetic regulatory mechanisms in the lncRNAs between zebrafish and human (155), suggesting that additional conserved non-coding RNAs have not been identified. Knockdown assays have revealed functional conservation between zebrafish and human lncRNAs. For example, morphological defects generated by *Cyrano* and *Megamind* knockdown in zebrafish (lncRNAs involved in the development of the nervous system) were rescued with mature RNA from their corresponding human orthologous (157). Similarly, *Tuna* knockdown resulted in fish with motor and locomotion defects revealing functional conservation with their human counterpart, known as lncRNAs involved in Huntington's disease (158). These results suggest that despite the evolutionary distance between zebrafish and humans, and the discrete conservation of these molecules in sequence, lncRNAs are essential in homeostasis and health maintenance throughout evolution.

Given the functional and expression conservation of zebrafish and human lncRNAs, these molecules are likely to play a crucial role in developing cancer in zebrafish. We found 15 lncRNAs annotated in the zebrafish genome, orthologous to 18 human lncRNAs associated with breast cancer, using the Gencode (154), Lnc2Cancer v2.0 (30), LNCipedia (12), and ZFLNC (11) databases. The 18 human lncRNAs conserved in the zebrafish genome participate in oncogenic processes such as cell cycle

control, proliferation, differentiation, migration, invasion, metastasis, angiogenesis and maintenance of cancer stem cells, according to CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA), LncTarD and Lnc2Cancer v2.0 data (**Table 2**).

Zebrafish offer experimental advantages for manipulating of gene expression, facilitating the study of functional aspects of genes. Altering the expression of conserved lncRNAs in zebrafish by knockdown with morpholinos or genomic editing by CRISPR cas9, will allow the study of the non-autonomous functions of lncRNAs in the tumor microenvironment during breast cancer xenotransplantation trials, as previously carried out in the melanoma cell xenotransplants (131).

CONCLUSION

Addressing breast cancer in a comprehensive manner, which involves early diagnosis through sensitive tumor markers and advancing personalized treatment design, are two of the most critical challenges in breast cancer medicine and biomedical research. Breast cancer study through xenotransplantation in zebrafish is a valuable tool given the speed with which tumors are obtained and experiments are concluded. Transplantation of human breast cancer cells in fish allows the discriminated and efficient study of aspects related to disease development, such as tumorigenesis, migration, metastasis, angiogenesis and response to drugs.

TABLE 2 | Functional relationship between conserved lncRNAs and breast cancer hallmarks.

Zebrafish name	Human lncRNA name	lncRNA type	Activate	Inhibited	O or S	Expression
ZFLNCT16634 (LOC103909273 un-characterized)	DLX6-AS1	Antisense	P, I, EMT, Mi, T	Ap, CellC, D, DNAd, DNAr, H, Inf, Q	O	Up-regulated
ZFLNCT02505 (ENSDART00000153684.2, CR751227.1-201)	HAGLR (HOXD-AS1)	Antisense,	An, D, Inf, Me	DNA r, I, Q	O	_
ZFLNCT08532 (NONDRET012204.1)	HOTAIR	Antisense, lincRNA	D, Mi, Me, I, T	DNAr, EMT, I, Ap	O	Up-regulated
ZFLNCT02498 (ENSDART00000155072.3, ENSDART00000155419.2, ENSDART00000155896.2, LOC103910246)	HOXA11-AS	Antisense, sponge	CellC, Inf, Q, P, I, Me, Mi	DNAd, DNAr, I, Ap	O/S in OC	Up-regulated
	HOXA-AS2	Antisense, ceRNA, sponge	Ch, P, I, Mi, T, Prog, EMT	D, Ap	O	–
	HOXA-AS3	Antisense, sponge	P, Mi, I, S, Me	EMT, Inf, Me, An, Ap	O	Up-regulated
	HOXB-AS1	Antisense, ceRNA	P, CellC, I, Mi	Ap	O	Up-regulated
ZFLNCT19656 (si:dkey-81p22.11)	HOXC-AS3	Sponge	P, H, T, I, Mi, Me	Q	O	Up-regulated
ZFLNCT01281	LINC00649	Antisense		An, Ap, cellC, D, EMT, H, I, Me, P, Q	_	Down-regulated
ZFLNCT17432 (ENSDART00000153409.2)	LINC00324	lincRNA	P, I, Me, Mi, S	Ap	O	Up-regulated
ZFLNCT14004 (ENSDART00000149569.3)	LINC00461	lincRNA, ceRNA, Sponge	P, cellC, DNAd, S, Mi, I, Me, T, EMT	An, H, Q, Ap	O	Up-regulated
ZFLNCT12716 (lnc2_zgc:194285, lnc1_zgc:194285)	MALAT1	Sponge, ceRNA, lincRNA	Mi, I, EMT, An, H, S, P, Me, Ap	An, CellC, DNAd, DNAr, EMT, Inf, I, Me, Q, P	O and S	_
ZFLNCT01941 (lnc_ghrhra)	MAPT-AS1	Antisense	CellC, S. In ER negative BRCA induce P and Mi.	Ap, DNAd, DNAr, EMT, H, I, Me, P, Q	S	_
ZFLNCT11804 (oip5-as1-202, Cyrano)	OIP5-AS1 (Linc-OIP5, Cyrano)	ceRNA	P, Mi, I, T, CellC, DNAd, S, Me, EMT	P, RR (CRC)	O ans S	Up-regulated
ZFLNCT11748 (NONDRET002400.1)	PAX8-AS1	Processed transcript	Ap	Ap, CellC, DNAd, DNAr, H, Inf, I, Me, P, Q, S	S	Down-regulated
ZFLNCT19020 (PF102167.1-201, ENSDART00000149948.3, sox2ot, si:ch73-334e23.1, LOC101883930)	SOX2-OT	Sense overlapping	P, I, Me	Ap, CellC, D, DNAd, DNAr, H, Q	O	Up-regulated
ZFLNCT09180 (BX571737.1-201)	TTN-AS1	Antisense, ceRNA, sponge, lincRNA	DNAd, P and Me in ESCC. P, I, EMT and Mi in BRCA and CRC	Ap, CellC, DNAd, DNAr, EMT, H, Inf, I, Me	O	Up-regulated
PTENP1	PTENP1	ceRNA, sponge	Ap	Mi, P, I	S	Down-regulated

P, proliferation; An, angiogenesis; Me, metastasis; CellC, Cell cycle; D, differentiation; DNAd, DNA damage; H, Hypoxia; Inf, Inflamation; Q, quiescence; I, Invasion; Mi, Migration; EMT, Epithelial-Mesenchymal transition; T, Tumorigenesis; S, Stemness; Ch, Chemoresitance; Prog, Progression; DR, Drug resistance; RR, Radio resistance; BRCA, Breast cancer; O, oncogenic roll; S, Tumor suppressor roll.

Today, these assays are mainly used to validate *in vitro* assays; however, the significant finding of conservation of zebrafish's lncRNAs and their expression change during the zebrafish cancer process broadens the perspective. We propose that this *in vivo* model will allow the study of the functional impact of lncRNA dysregulation in the host microenvironment allowing a simultaneous search for new breast cancer-associated lncRNAs through transcriptional studies. The fine dynamics of lncRNAs expression and their relationship with the alteration of the tumor microenvironment show that these molecules are excellent candidates for the prediction and prognosis assays and possible therapeutic targets in the area of drug development.

The advantages of xenotransplantation experiments in zebrafish compared to other models suggest their potential in the personalized approach to the breast cancer treatment. One of the main strategies is zPDXs modeling, and subsequent drug screening. Subtle variations in lncRNAs expression could effectively predict the response of cancer cells to drugs and may in turn serve as new targets in the development of new treatments. The combination of the ease of performing drug screening, including lncRNAs targeted drugs, on zebrafish embryos after xenotransplantation, and the possibility of evaluating the functional *in vivo* response through the real time microscopy study, increases the robustness of xenotransplantation models. In addition, the conservation in cancer-related pathways between humans and zebrafish and the existence of interspecies molecular crosstalk during xenotransplantation support the use of knockdown and knockout zebrafish for conserved lncRNAs to determine the nature of the molecular pathways that respond to lncRNAs signaling. In the same way, xenotransplantation assays on a knockout or overexpressing cancer-related lncRNAs in zebrafish could reveal the non-autonomous function of lncRNAs in the tumor microenvironment. Besides, drug-screening trials targeting zebrafish lncRNAs or related pathways are another area that could benefit from xenotransplantation assays. An additional benefit is that the zebrafish transparency will elucidate the relationship of each of the cancer cellular phenotypes (migratory, proliferative, angiogenic) with specific zebrafish lncRNAs expression, facilitating the interpretation and analysis of the results.

AUTHOR CONTRIBUTIONS

CZ reviewed the literature, wrote the initial abstract, drafted the manuscript, figures, and tables, and revised the second draft following feedback from JM-Z. WAM-F reviewed the lncRNAs databases, and contributed to the second draft review. JM-Z reviewed the literature, proposed an article outline, contributed sections of the initial draft, and made editorial suggestions for the second draft. All authors contributed to the article and approved the submitted version.

FUNDING

CZ was supported by a post-doctoral fellowship from the CONACYT grant A1-S-8462, and this study is part of her postdoctoral work. The work at JM-Z laboratory is supported by CONACYT grant A1-S-8462.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.687594/full#supplementary-material

Supplementary Table 1 | Growing scientific output of work on breast cancer modeling by xenotransplantation in zebrafish.

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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: 31 May 2021
doi: 10.3389/fonc.2021.692170

Unraveling Immune-Related lncRNAs in Breast Cancer Molecular Subtypes

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Breast cancer (BRCA) is the most leading cause of cancer worldwide. It is a heterogeneous disease with at least five molecular subtypes including luminal A, luminal B, basal-like, HER2-enriched, and normal-like. These five molecular subtypes are usually stratified according to their mRNA profile patterns; however, ncRNAs are increasingly being used for this purpose. Among the ncRNAs class, the long non-coding RNAs (lncRNAs) are molecules with more than 200 nucleotides with versatile regulatory roles; and high tissue-specific expression profiles. The heterogeneity of BRCA can also be reflected regarding tumor microenvironment immune cells composition, which can directly impact a patient's prognosis and therapy response. Using BRCA immunogenomics data from a previous study, we propose here a bioinformatics approach to include lncRNAs complexity in BRCA molecular and immune subtype. RNA-seq data from The Cancer Genome Atlas (TCGA) BRCA cohort was analyzed, and signal-to-noise ratio metrics were applied to create these subtype-specific signatures. Five immune-related signatures were generated with approximately ten specific lncRNAs, which were then functionally analyzed using GSEA enrichment and survival analysis. We highlighted here some lncRNAs in each subtype. LINC01871 is related to immune response activation and favorable overall survival in basal-like samples; EBLN3P is related to immune response suppression and progression in luminal B, MEG3, XXYLT1-AS2, and LINC02613 were related with immune response activation in luminal A, HER2-enriched and normal-like subtypes, respectively. In this way, we emphasize the need to know better the role of lncRNAs as regulators of immune response to provide new perspectives regarding diagnosis, prognosis and therapeutical targets in BRCA molecular subtypes.

Keywords: immune response, MEG3, LINC01871, EBLN3P, LINC02613, XXYLT1-AS2

OPEN ACCESS

Edited by:
Wenwen Zhang, Nanjing Medical University, China

Reviewed by:
Liuzhi Zhou, Zhejiang University, China
Ryuichi Sugino, Saitama Cancer Center, Japan

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

Received: *07 April 2021*
Accepted: *06 May 2021*
Published: *31 May 2021*

Citation:
Mathias C, Muzzi JCD, Antunes BB, Gradia DF, Castro MAA and Carvalho de Oliveira J (2021) Unraveling Immune-Related lncRNAs in Breast Cancer Molecular Subtypes. Front. Oncol. 11:692170. doi: 10.3389/fonc.2021.692170

INTRODUCTION

Breast cancer (BRCA) is a molecular and histological heterogeneous disease with at least five intrinsic molecular subtypes (1, 2). Based on gene expression, BRCA can be mainly classified into luminal A (LumA), luminal B (LumB), HER2-enriched (Her2), basal-like (Basal), and normal-like (Normal) (3, 4). These subtypes have a distinct prognosis and also differ according to therapeutic

response. LumA and LumB tumors respond well to hormonal interventions, while HER2+ tumors respond effectively when anti-HER2 therapy is used (5). Basal tumors are very aggressive and associated with the shortest survival times, with no current molecular-based targeted therapies available (6).

Immunotherapy brought a new line of action in cancer care; however its response varies across cancer types and patients. The immune system response in the tumor microenvironment may help to guide immunotherapy drug discovery and clinical decisions (7). In general, tumors more responsive to immune checkpoint inhibitors are related to high levels of leukocyte fraction in the tumor microenvironment (8). Besides gene expression differences in BRCA molecular subtypes, they differ significantly concerning the composition of cells that form the tumor microenvironment, especially the immune system's cells. A substantial proportion of natural killer cells and neutrophils have been found in luminal tumors. In contrast, in these tumors, cytotoxic T cells (T CD8+) and naïve and memory T cells are found less frequently. In BRCA Basal tumors, T regs, associated macrophages 2, and activated mast cells form a significant portion of the immune infiltrate cells. The immune infiltrate composition is not widely described in the Her2 subtype. It is known that it is mainly formed by dendritic cells, mast cells, γδ T lymphocytes, T regs and neutrophils (9).

A landscape of tumors' immune microenvironment was characterized from immunogenomics data by Thorsson and colleagues (8). In this study, using an integrated analysis, they could classify solid tumors (from The Cancer Genome Atlas) into six major immune subtypes, which they called C1-C6. These subtypes have distinct immune signature sets, which could also be related to prognosis. C1 (wound healing) exhibited elevated expression of angiogenic genes, a high proliferation rate, and a Th2 cell bias to the adaptive immune infiltrate. C2 (IFN-γ dominant) had a strong T CD8+ signal, the greatest TCR diversity, and a high proliferation rate. C3 (inflammatory) was the subtype that presented high Th17 and Th1 genes and low to moderate tumor cell proliferation. C4 (lymphocyte depleted) displayed a more prominent macrophage signature with Th1 suppressed and high M2 response. C5 (immunologically quiet) was enriched by brain tumors and exhibited the lowest lymphocyte and most increased macrophage responses. Finally, C6 (TGF-β dominant) displayed the highest TGF-β signature and a high lymphocytic infiltrate with an even distribution of type I and type II T cells (8).

According to this approach, BRCA could be classified into five subtypes (C1, C2, C3, C4 and C6), being C2 (n=345) the most representative subtype, followed by C1 (n=320). Immune subtypes were also described according to BRCA molecular subtypes, and as expected, the subtypes varied significantly according to these immune groups. For example, LumA was more representative of the C1 subtype, while Basal samples of C2 (8).

Gene expression sets based on mRNAs were used for the classification and determination of molecular and immune subtypes. However, it is already known that the most abundant part of the human genome is not translated into proteins. These transcripts are organized in a class called "non-coding RNAs." Non-coding RNAs can be classified into two major categories based on their size: small non-coding RNAs (<200 nucleotides) and long non-coding RNAs (>200 nucleotides) (10). LncRNAs are usually transcribed by RNA polymerase II, polyadenylated, and capped. They exhibit high tissue specificity and great regulatory versatility, acting at different gene expression regulation levels (11, 12).

Due to its high tissue specificity, lncRNAs can be evaluated as potential disease biomarkers, including BRCA (13–15). Based on this, we looked for molecular subtype specific lncRNAs signatures that could help differentiate the immune profiles described in Thorsson (8). These lncRNAs were also analyzed if impact the patient's overall survival and progression free interval and were also investigated in differential expression and enrichment analysis to explore other possible biological roles of these lncRNAs in BRCA molecular and immune subtypes.

MATERIAL AND METHODS

Breast Cancer Immune Data

BRCA molecular and immune subtypes, leukocyte fraction, and survival information were downloaded from Thorsson et al. (8) **Supplementary Material**. According to samples' barcode expression and immune type, data were integrated to perform the analysis. In **Supplementary Table 1**, we organized data according to breast cancer molecular and immune subtypes.

Breast Cancer RNA-Seq Data

Log2 normalized FPKM RNA-Seq data from The Cancer Genome Atlas (TCGA) breast cancer cohort was downloaded from XenaBrowser (https://xenabrowser.net/datapages/), and primary tumor samples were selected and merged with Thorsson et al. (8) master table using patients' barcode. The lncRNAs and protein-coding gene expression profiles were filtered from the RNA-Seq data using the R package biomaRt v 2.46.3 (16). For lncRNAs, when available, HGNC Symbol was used; otherwise, Ensembl gene name was used. All Ensembl and HGNC Symbols from lncRNAs used in this study are available in **Supplementary Table 2**. The non-tumoral samples were selected based on TCGA barcodes ending with 11A or 11B. The molecular BRCA subtypes were defined as described in Thorsson et al. (8) **Supplementary Material**, based on PAM50.

The expression profiles of immunomodulatory genes listed (8) (https://www.cell.com/cms/10.1016/j.immuni.2018.03.023/attachment/8d3ffc74-4db4-4531-a4ad-389dfc8bb7ec/mmc7.xlsx) previously were obtained from the gene expression matrix. Of the 75 immune modulators, only one (C10orf54) was not found in the expression matrix. For heatmap construction using *ComplexHeatmap* package (17), samples were displayed in columns and genes in the rows. Column-wise z-score was calculated for gene expression values, and maximum and minimum values were limited to +2 and −2 standard

deviations, respectively. Samples were clustered within each BRCA subtype.

The BRCA lncRNAs expressions were further filtered above the first quartile for lncRNA expression sum, which means lncRNAs with expression sum above 8.04 log2 FPKM in the whole BRCA cohort (1,060 samples). Signal-to-noise ratio (SNR) was calculated for each molecular subgroup individually as follow:

$$SNR = \frac{\mu 1 - \mu 2}{\sigma 1 + \sigma 2}$$

being μ_1 the mean of lncRNA expression in the group analyzed and μ_2 the mean of lncRNA expression in the patients out of the group analyzed. σ refers to the standard deviation from the respective groups mentioned. We selected the lncRNAs above the SNR 0.95 quantile for each BRCA molecular subtype, that means the lncRNAs with higher expression in the subtype analyzed compared with the rest of the cohort. Venn diagram was constructed using *InteractiVenn* (http://www.interactivenn.net/) demonstrating the intersection of lncRNA between groups. Then, we calculated the SNR within each molecular subgroup based on the immune subtypes described previously (8). We considered for the analysis the immune groups with more than five patients in each molecular subtype. In this way, Basal and Her2 samples were divided into C1 and C2 subtypes; LumA and Normal into C1, C2, C3, C4, and C6 subtypes and LumB into C1, C2, C3, and C4 subtypes. After absolute SNR sum calculation, we selected lncRNAs considered in 0.98 quantile, which means the lncRNAs with the most significant variation within the immune subtypes for each molecular subgroup. For histogram construction, the absolute SNR sum was scaled using z-score. The 53 lncRNAs selected from this analysis are presented in **Supplementary Table 3**.

Leucocyte Fraction Correlation

The lncRNAs expression was used to calculate Spearman correlation with the leucocyte fraction observed in each BRCA subtype, and the p-value was calculated with AS 89 algorithm (18) using cor. test function from stats R package v.4.0.4 (19) and adjusted by False Discovery Rate (FDR) method.

Survival Analysis

Survival analysis was firstly performed using the *coxph* function available in the survival R package v3.2-10 (20) based on lncRNA expression for both Overall Survival (OS) and Progression-Free Interval (PFI) for each lncRNA individually in its respective BRCA molecular subgroup. The univariate Cox p-value for each lncRNA was calculated and adjusted by the FDR method; values below 10% FDR level were considered significant.

One lncRNA was selected on each BRCA subtype based on Cox results or Leukocyte Fraction correlation for further analysis. Kaplan-Meier was calculated, and patients were divided by the median lncRNA expression value in High Expression and Low Expression; p-value was calculated by log-rank test.

Enrichment Analysis

The protein-coding genes expression profile was filtered for each molecular subgroup by patients' barcode, and genes with zero sum expression were removed. Patients were divided by the median lncRNA expression value, and High versus Low Expression groups were used for SNR calculus. Genes were ordered by SNR value, and gene set enrichment analysis (GSEA) was inferred using fgsea R package v1.16.0 (21) with MSigDb v7.2 Hallmarks gene sets (22, 23) for 10,000 permutations.

RESULTS

Immune Modulator Genes Expression Demonstrates Distinct Patterns Within BRCA Molecular Subtypes

Based on the gene expression profile related to immune response, Throsson et al. (8) analyzed over 10,000 TCGA samples, from which 1,087 were from BRCA samples and clustered them in six immune subtypes. We merged the barcodes with the gene expression matrix downloaded from XenaBrowser, remaining 1,060 BRCA primary tumor samples. The molecular classification as presented in Thorsson et al. (8) master table as TCGA Subtype was used, dividing the samples into five groups, LumA (n=499), LumB (n = 184), Basal (n=169), Normal (n=136) and Her2 (n=72). In **Supplementary Table 1**, we represented the number of samples according to each molecular subgroup's immune subtype.

Supplementary Figure 1 shows a distinct pattern of immune modulators gene expression in all molecular subtypes. In general, most genes seem to be upregulated in the same samples (columns of the heatmap) independent of its classification as an inhibitor or stimulator of the immune system. Overall, the immune activation seen in the gene expression follows the rising in Leukocyte Fraction and tends to group the C2 immune subtype. Simultaneously, the inverse is observed for a low expression pattern associated with low Leukocyte Fraction and C1 immune subtype. Basal and Her2 demonstrate a more apparent separation of C1 and C2. These two immune subtypes are the major representatives in these molecular groups; for instance, C1 and C2 represent 95.9% and 93.1% of all Basal and Her2 samples, respectively (**Supplementary Table 1**).

SNR Highlights Specific lncRNAs for Each BRCA Molecular Subgroups Related to Immune Subtypes Distinction

The first step of our strategy is to search for lncRNAs that could be associated with immune subtypes in BRCA patients. For this, lncRNAs with greater distinct expression patterns among the five molecular subgroups were selected (**Figure 1**). After selecting the 0.95 quantile in SNR values and including the only 5% more differentially expressed in each subtype, we obtained 528 lncRNAs for each BRCA molecular subtype. **Figure 2A** shows how these lncRNAs were intersected between the five subgroups. None lncRNA was shared between all subgroups, and most of

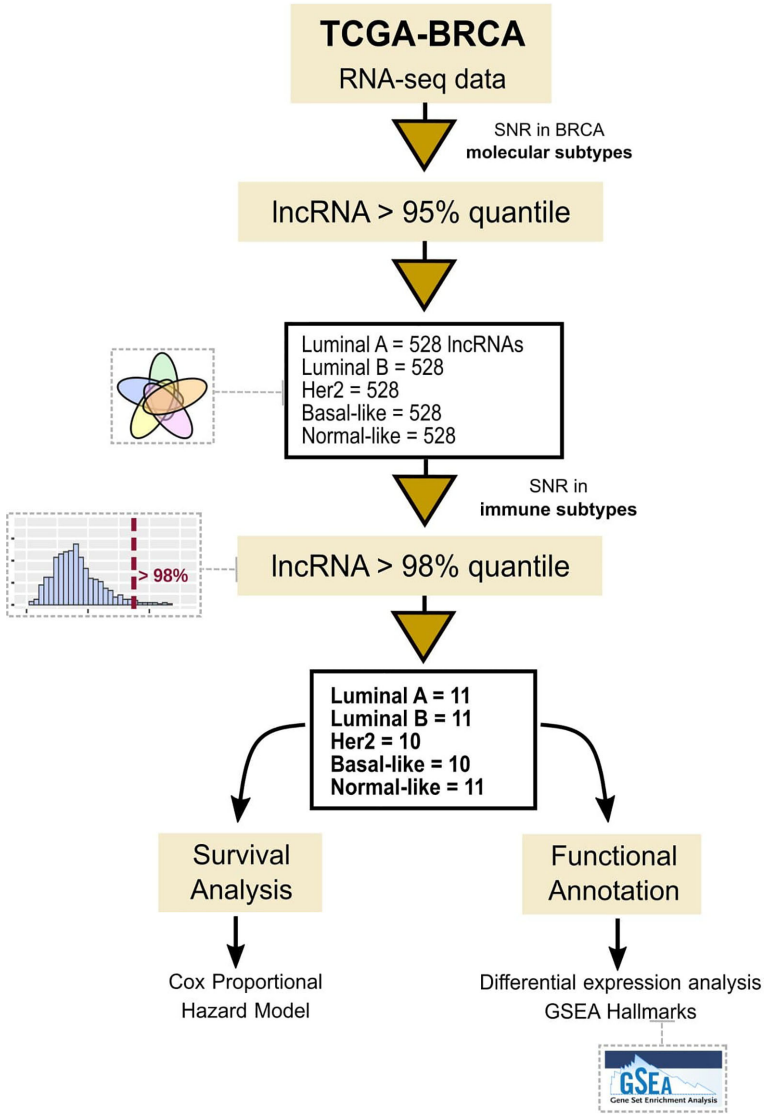

FIGURE 1 | Workflow representation of the used methodology to study immune-related lncRNAs.

them (over 80%) were specific for each molecular subtype. The most significant intersections were seen between LumA and LumB (44 lncRNAs), LumB and Her2 (33 lncRNAs), Basal and Normal (30 lncRNAs) and Basal and Her2 (26 lncRNAs), which represents less than 10% of the 528 lncRNAs defined for each subgroup. Even when selecting the 0.90 quantile in SNR values, none lncRNA was shared between all molecular subtypes (**Supplementary Figure 2**) which shows that the SNR was able to distinguish specific lncRNAs for each molecular subtype.

Secondly, we calculated the absolute SNR sum for the immune subtypes for these 528 lncRNAs selected for each BRCA molecular subgroup. **Figure 2B** shows the distribution of absolute SNR sum for the lncRNAs. Z-score was calculated to allow comparison between groups. The five groups demonstrated different distributions, being Basal and Her2, characterized for most lncRNAs with slight variation between the immune subtypes, while LumA and Normal presented higher variation. After selecting the 0.98 quantile (**Figure 2B** and **Supplementary Table 3**), 11 lncRNAs remained, of which only one was shared between Her2 and Basal, the lncRNA KLHDC7B-DT (ENSG00000272666). This lncRNA was removed for further analysis as we looked for a specific lncRNA signature related to each BRCA molecular subgroup. Eleven specific lncRNAs were selected for LumA, LumB and Normal and ten for Her2 and Basal. All results for the first and the second SNR calculation as well as the quantile for each lncRNA is presented in **Supplementary Table 3**.

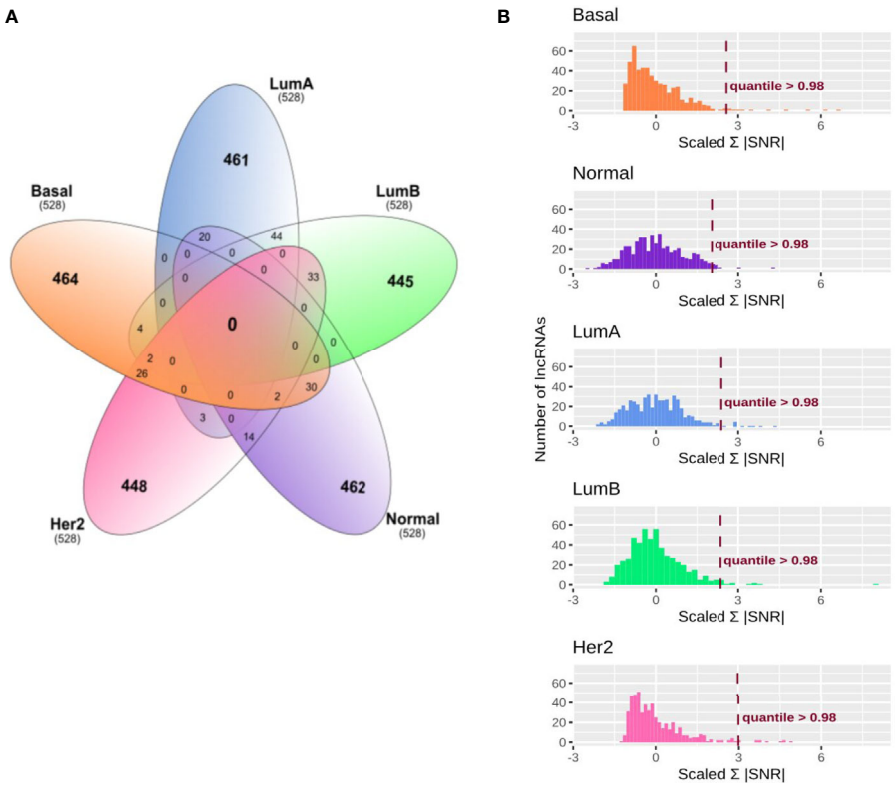

FIGURE 2 | Immune related lncRNAs filter and selection in breast cancer (BRCA) molecular subtypes. **(A)** Venn diagram representing specific and shared immune related lncRNAs in breast cancer molecular subtypes. After filtering for 0.95 quantile in signal to noise ratio (SNR) for the BRCA molecular subtypes, 528 lncRNAs were selected for the next analysis. **(B)** Absolute SNR sum filter selection. SNR was calculated within each BRCA molecular subtype for the immune subtypes. Only groups with more than five patients were used. The histograms represent the amount of lncRNAs in each range of absolute SNR sum for the BRCA molecular subtypes. X-axis was scaled for z-score to allow comparison. The lncRNAs were filtered according to the 0.98 quantile as represented as the dashed line on the histograms.

Specific lncRNAs in BRCA Molecular Subgroups Are Associated With Immune Subtypes Differentiation

The remaining specific lncRNAs related to immune subtypes are presented in **Figure 3**; only immune subtypes with more than five patients are shown. These were the groups used for SNR analysis. For LumA, the lncRNAs clearly distinguished the C4 and C6 subtypes while demonstrating a mixed pattern in C1, C2 and C3. Nevertheless, EWSAT1, LINC00271 and AC105285.1 show higher activation in C3 than in C2, for example. No significant correlation with OS or PFI was observed for lncRNAs expression in Cox univariate analysis in LumA patients (**Figure 3A** and **Supplementary Table 4**).

For LumB (**Figure 3B**), a clear distinction can be seen between C3 and C4 in the gene expression pattern, while LINC02620 and mainly AL445490.1 showed a higher expression pattern in C3. Only EBLN3P correlated with a good prognosis in both OS and PFI for Cox univariate analysis (**Figure 3B** and **Supplementary Table 4**). In the Her2 samples (**Figure 3C**), all ten specific lncRNAs selected demonstrated a higher expression pattern in C2 and a strong positive correlation with Leukocyte Fraction. From the Cox univariate analysis, after p-values adjustment, half of the ten lncRNAs were associated with good prognosis in PFI, but none related to OS (**Figure 3C** and **Supplementary Table 4**).

In Normal (**Figure 3D**), all 11 lncRNAs were suppressed in the C4 subtype and, in general, showed a higher activation pattern in C3 and less evidently in C2. Despite C6 being represented by only six patients, HLX-AS1 and AL133371.2 appeared highly expressed in the C6 group. In Cox analysis, after p-adjustment, six from the 11 lncRNAs had a hazard ratio (HR) < 1 for OS, although none presented significant values for PFI. Finally, in the Basal group (**Figure 3E**), nine lncRNAs presented a higher expression in C2 and a lower expression in C1, while APCDD1L-DT presented an inverse pattern. Only LINC01871 showed a significant correlation with PFI, but not for OS (**Figure 3E** and **Supplementary Table 4**).

lncRNAs Functional Annotations and Survival Analysis

We used MSigDb Hallmarks gene sets for GSEA analysis to infer possible biological roles associated with the specific lncRNAs

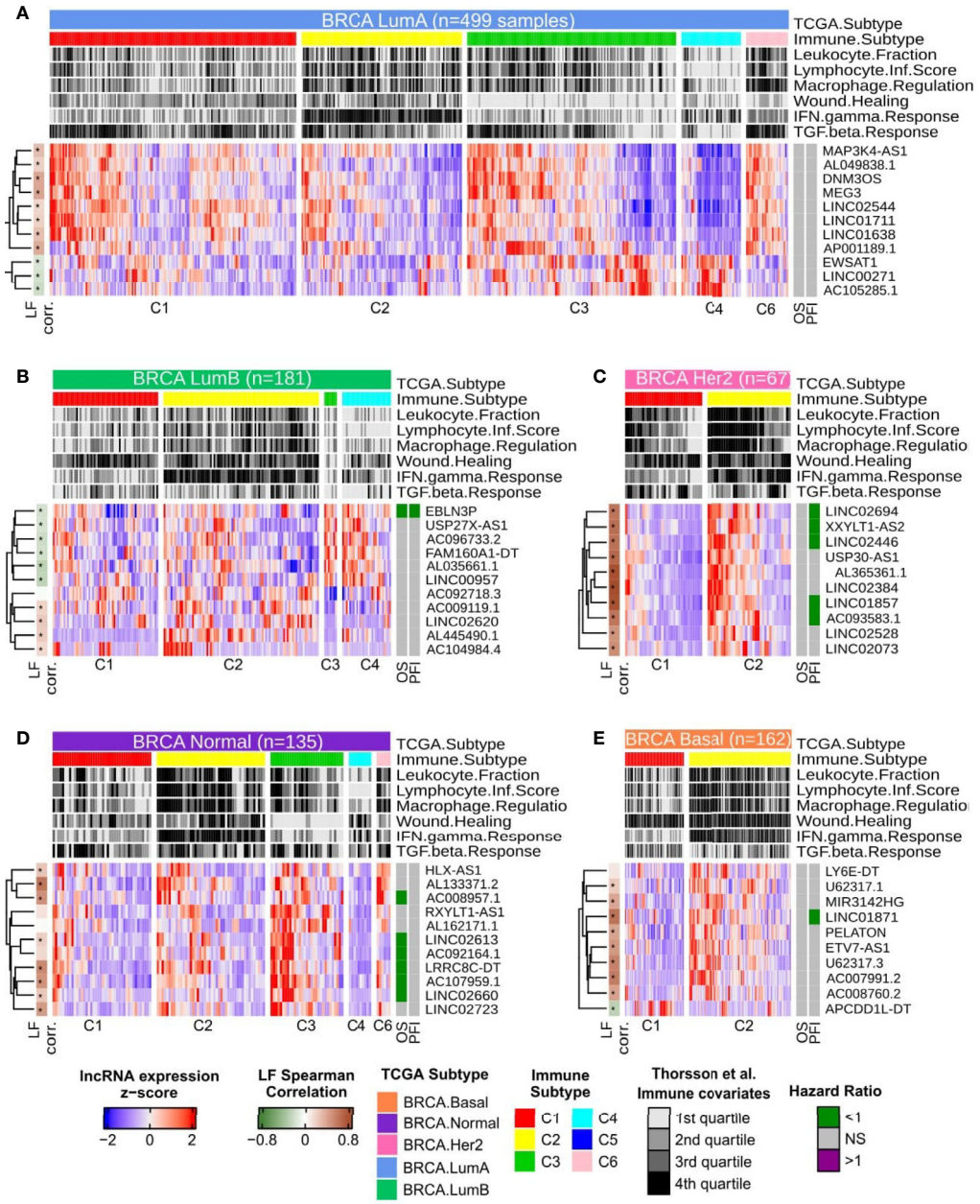

FIGURE 3 | Heatmap with column-wise z-scores for immune related lncRNAs for each BRCA molecular subtype. For color gradient, maximum and minimum z-scores were set to +2 and –2 respectively. Each column represents a sample and were semi-supervised clustered within the immune subtypes. Immune subtypes groups with less than 5 patients are not represented as they were not used in SNR calculation. The top annotations present the molecular subtype with the number of samples, the immune subtype and the immune covariates as described by Thorsson et al. (8). The immune covariates are presented as quartiles in all TCGA-BRCA primary tumor samples. The left annotation shows the Spearman's correlation for each lncRNAs with Leukocyte Fraction, being dark green for negative and brown for positive ρ values, asterisks represent Spearman's correlation adjusted p-values values below 0.05. The right annotation represents the Hazard Ratio (HR) inferred by Cox Univariate analysis for Overall Survival (OS) and Progression Free Interval (PFI). Cox results with adjusted p-values greater than 0.1 are considered non significant and are colored in grey, otherwise, HR values above 1 are colored purple while HR values below 1 are colored in green. **(A)** LumA samples. **(B)** LumB samples. **(C)** HER2-enriched samples. **(D)** Normal-Like samples. **(E)** Basal-Like samples.

expression (**Figure 3**); the results for all 53 lncRNAs are presented in **Supplementary Tables 5–9** and **Supplementary Figure 3**. We selected one lncRNA from each BRCA molecular subgroup to focus. For LumB, Her2, Basal and Normal, we chose the smallest p-value in Cox univariate analysis. As LumA did not present any significant p-values in Cox univariate analysis, we selected MEG3 as it showed a higher correlation with Leukocyte Fraction (Spearman's ρ=0.4, p-adjusted < 2.2×10^{-16}).

BRCA Basal-Specific lncRNAs Are Associated With Interferon Gamma Response and Allograft Rejection Gene Sets. LINC01871 Is More Expressed in Basal and Relates to Better OS and PFI

The GSEA analysis for the selected lncRNAs from BRCA Basal group revealed immune related gene sets, like Interferon Gamma Response, Allograft Rejection and Interferon Alpha Response, enriched in all ten lncRNAs, being negatively associated only with APCDD1L-DT (**Supplementary Table 5** and **Supplementary Figure 3A**), which was also the only one from the ten lncRNAs negatively related to Leukocyte Fraction (**Supplementary Table 4**).

Focusing on LINC01871, this lncRNA presented a higher expression in Basal than all other BRCA molecular subtypes (**Figure 4A**) and was mainly associated with immune activation, for instance, enriched in Interferon Gamma Response, Allograft Rejection, Interferon Alpha Response and Inflammatory Response gene sets (**Figure 4D**). Within Basal samples, it appears significantly suppressed in C1 (**Figure 4B**), while in

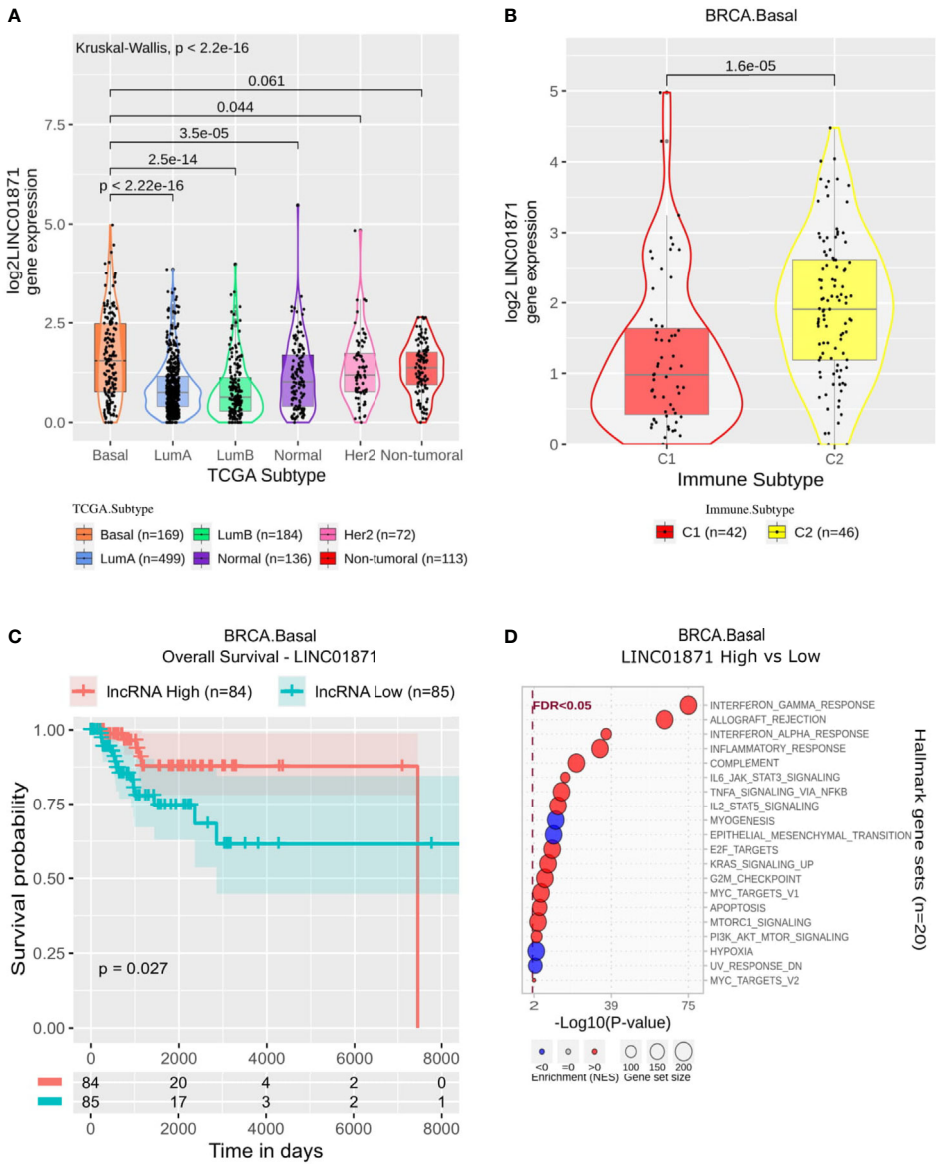

FIGURE 4 | LINC01871 panel representation in basal-like subtype. **(A)** Box-plots representing LINC01871 expression among all breast cancer molecular subtypes. The Kruskal-Wallis test (p-value represented in the panel left top position) was used to differentiate expression between groups, followed by the Wilcoxon test (p-values represented emphasizing comparisons between subtypes). **(B)** Box-plot representing LINC01871 in C1 and C2 immune subtypes in basal-like patients. **(C)** LINC01871 overall survival curves. To define the two groups, LINC01871's median expression classified the patients into "high" and "low" groups. Logrank p-value represented as 0.027. **(D)** Enrichment analysis using Hallmarks gene sets. Red circles refers to activation while blue circles to inactivation. In the X axis, a p-value scale is represented. The circle size varies according to the number of genes in the identified gene set.

Kaplan-Meier analysis, it was associated with better OS and PFI (**Figure 4C** and **Supplementary Figure 4A**).

BRCA Normal Specific lncRNAs Are Associated With Suppression of Proliferation Gene Sets. LINC02613 Is Suppressed in C4 and Relates to OS

The 11 BRCA Normal specific lncRNAs were related to the suppression of gene sets like G2M checkpoint, E2F and MYC targets associated with proliferation (**Supplementary Table 6** and **Supplementary Figure 3B**). Mainly AL133371.2, AC008957.1, LRRC8C–DT, AC107959.1, LINC02660 and LINC02723 presented immune-related gene sets positively enriched (Allograft Rejection, Coagulation, Complement, IL6 JAK STAT3 Signaling, Inflammatory, Interferon Alpha and Interferon Gamma Response). LINC02613 follows this enrichment pattern (**Figure 5D**). It is worth noting that Estrogen Response Early and Late gene sets appear suppressed only for this lncRNA and AC092164.1. Although its expression significantly differs from BRCA Normal to all other molecular subtypes (**Figure 5A**), it is more expressed in non-tumoral samples followed by BRCA Basal and Normal. At the same time, it seems suppressed in LumA, LumB and Her2.

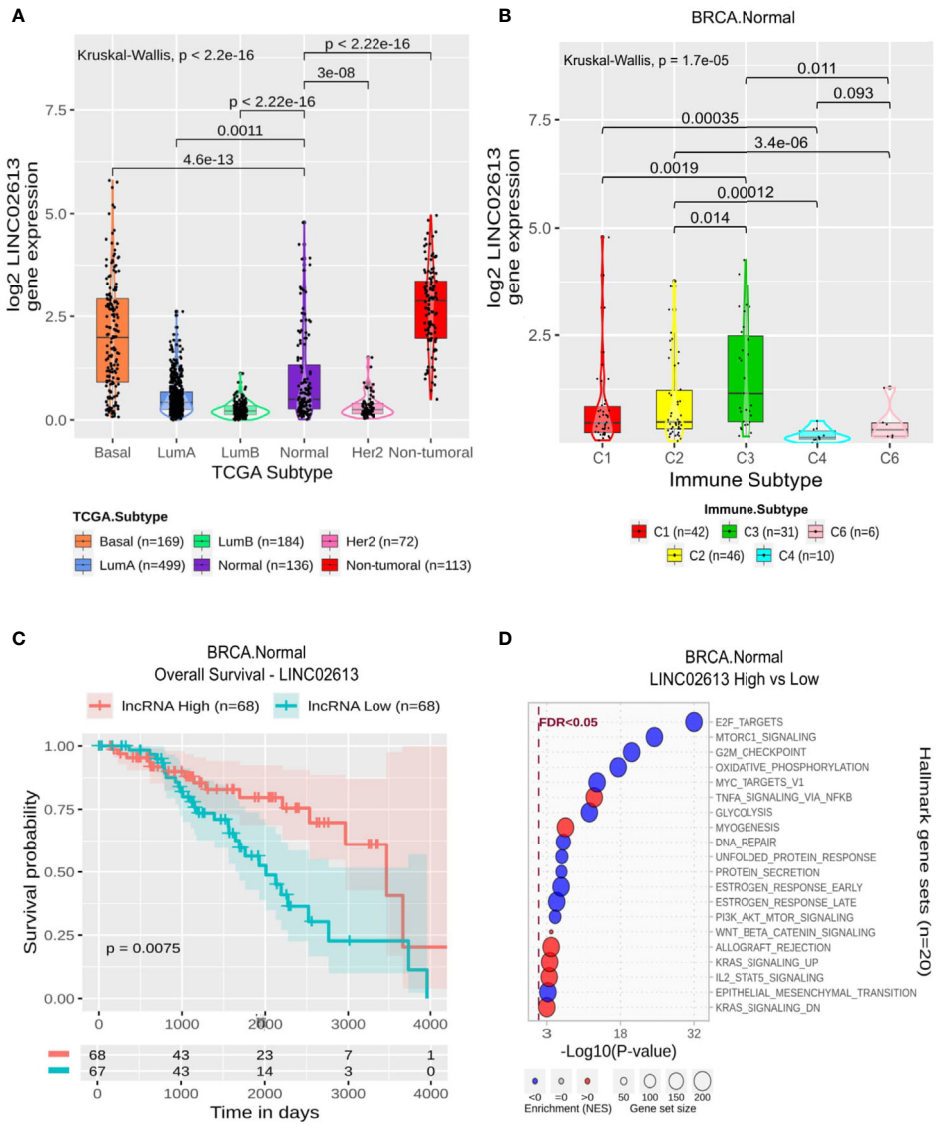

FIGURE 5 | LINC02613 panel representation in normal-like subtype. **(A)** Box-plots representing LINC02613 expression among all breast cancer molecular subtypes. The Kruskal-Wallis test (p-value represented in the panel left top position) was used to differentiate expression between groups, followed by the Wilcoxon test (p-values represented emphasizing comparisons between subtypes). **(B)** Box-plot representing LINC02613 in C1,C2, C3, C4 and C6 immune subtypes in normal-like patients. **(C)** LINC02613 overall survival curves. To define the two groups, LINC02613's median expression classified the patients into "high" and "low" groups. Logrank p-value represented as 0.0075. **(D)** Enrichment analysis using Hallmarks gene sets. Red circles refers to activation while blue circles to inactivation. In the X axis, a p-value scale is represented. The circle size varies according to the number genes in of the identified gene set.

Within Normal samples, it is more expressed in C3 and significantly suppressed in C4 (**Figure 5B**) and correlates with a better OS (**Figure 5C**) but not in PFI (**Supplementary Figure 4B**).

LumA-Specific lncRNAs Relate to Epithelial-Mesenchymal Transition, Immune and Proliferation Gene Sets. MEG3 Is Suppressed in BRCA Compared With Non-Tumoral Samples

For LumA (**Supplementary Table 7** and **Supplementary Figure 3C**), the Epithelial-Mesenchymal Transition (EMT) module was enriched in all 11 lncRNAs, being EWSAT1, LINC00271, and AC105285.1 associated negatively with this module and the other eight lncRNAs positively. In general, this pattern was followed for the immune-related gene sets and the correlation with Leukocyte Fraction (**Figure 3** and **Supplementary Table 4**), that is to say, EWSAT1, LINC00271, and AC105285.1 with the negative association and the other lncRNAs with a positive one. Gene sets associated with proliferation (E2F targets, Myc targets V1 and V2) were overall suppressed in all lncRNAs. Oxidative phosphorylation appeared suppressed in the lncRNAs positively associated with immune gene sets (MAP3K4-AS1, AL049838.1, DNM3OS, MEG3, LINC02544, LINC01711, LINC01638, and AP001189.1).

MEG3 presented a lower expression on BRCA compared with non-tumoral samples and a significant difference when comparing its expression in LumA with other BRCA molecular subtypes except Normal (**Figure 6A**). Within LumA samples, the immune subtypes comparisons resulted in a p-value of 3.2×10^{-14} in the Kruskal-Wallis test with a higher expression pattern in C3 and C6. At the same time, C4 stands out with a significantly lower pattern compared with all other immune subtypes (**Figure 6B**). In general, the GSEA analysis resulted in modules associated with immune response enriched with MEG3 overexpression and modules related to proliferation enriched with MEG3 suppression (**Figure 6D**). As expected by the Cox analysis, the Kaplan-Meier did not significantly impact MEG3 expression on OS or PFI (**Figure 6C** and **Supplementary Figure 4C**).

Most Specific lncRNAs in LumB Are Associated With Immune Pathways. EBLN3P Is Associated With a Good Prognosis in OS and PFI

LumB specific lncRNAs demonstrated a higher variation in the enriched modules (**Supplementary Table 8** and **Supplementary Figure 3D**). AC009119.1, LINC02620, AL445490.1, and AC104984.4 high expressions were positively related with immune gene sets, while with exception of LINC02620, no clear relation was observed for the proliferation ones. These four lncRNAs were also positively correlated with Leukocyte Fraction (**Supplementary Table 4**). AC092718.3, which did not present a significant correlation with Leukocyte Fraction, was also not related to the enrichment of immune gene sets; otherwise, its enrichment revealed proliferation modules upregulated. USP27X-AS1, AC096733.2, FAM160A1-DT, AL035661.1 and LINC00957 were associated with suppressing gene sets like Allograft Rejection, Interferon Gamma Response and Inflammatory Response; they also related negatively with Leukocyte Fraction (**Supplementary Table 4**).

EBLN3P is also related negatively to Leukocyte Fraction and with some immune modules. Still, its enrichment revealed a mixed pattern of modules suppressed like Epithelial-Mesenchymal Transition, Oxidative Phosphorylation, Myogenes and Myc Targets (**Figure 7D**). In LumB, its expression was higher in C3 and C4 than C1 and C2 (**Figure 7B**). In comparison, it presented a significantly higher expression in LumB samples than in the other BRCA molecular subtypes except for LumA (**Figure 7A**). In LumB samples, the higher expression of EBLN3P was related to a better outcome in OS but not with PFI in Kaplan-Meier analysis (**Figure 7C** and **Supplementary Figure 4D**).

Her2-Specific lncRNAs Are Associated With Immune Activation and XXYLT1-AS2 Associated With Higher PFI

All 10 Her2 specific lncRNAs were related with immune activation as can be seen in their enrichment pattern of Allograft Rejection, Interferon Gamma and Alpha Response, Inflammatory Response, IL6 JAK STAT3 signaling and Complement (**Supplementary Table 9** and **Supplementary Figure 3E**) and in the strong positive correlation with Leukocyte Fraction (ρ range from 0.49 to 0.82 in Spearman's Correlation, p-adjusted from 1.5×10^{-5} to $<2.2 \times 10^{-16}$). LINC02384 and LINC02073 are also related to the suppression of proliferation pathways (E2F Targets, G2M checkpoint and MYC Targets).

XXYLT1-AS2 showed a significantly higher expression in Her2 than in LumA, LumB and Normal (**Figure 8A**). Within Her2 samples, it was significantly more expressed in the C2 immune subtype (**Figure 8B**) and associated with better prognosis in PFI (**Figure 8C**) but not with OS (**Supplementary Figure 4E**). Its enrichment revealed high positive correlation with Allograft Rejection, Interferon Gamma Response, Inflammatory Response and IL6 and IL2 signaling and negative relation mainly with EMT, Hypoxia and Myogenesis (**Figure 8D**).

DISCUSSION

Using breast cancer immunogenomics data already published (8), we propose here five distinct immune-related lncRNAs signature according to BRCA molecular subtypes using a two steps SNR selection. Each molecular subtype presented a specific immune-related lncRNAs signature and in GSEA, in general, these lncRNAs functions varied between proliferation and immune activation or suppression, which demonstrates that our selection methodology was able to filter lncRNAs related to the immune response. The survival impact of the selected lncRNAs diverged across the molecular subtypes, in agreement with the fact that the immune activation also differs in terms of prognosis importance between the molecular subtypes (24). For instance, the tumor-infiltrating lymphocytes (TIL) was not related to prognosis in Estrogen Positive BRCA tumors, which

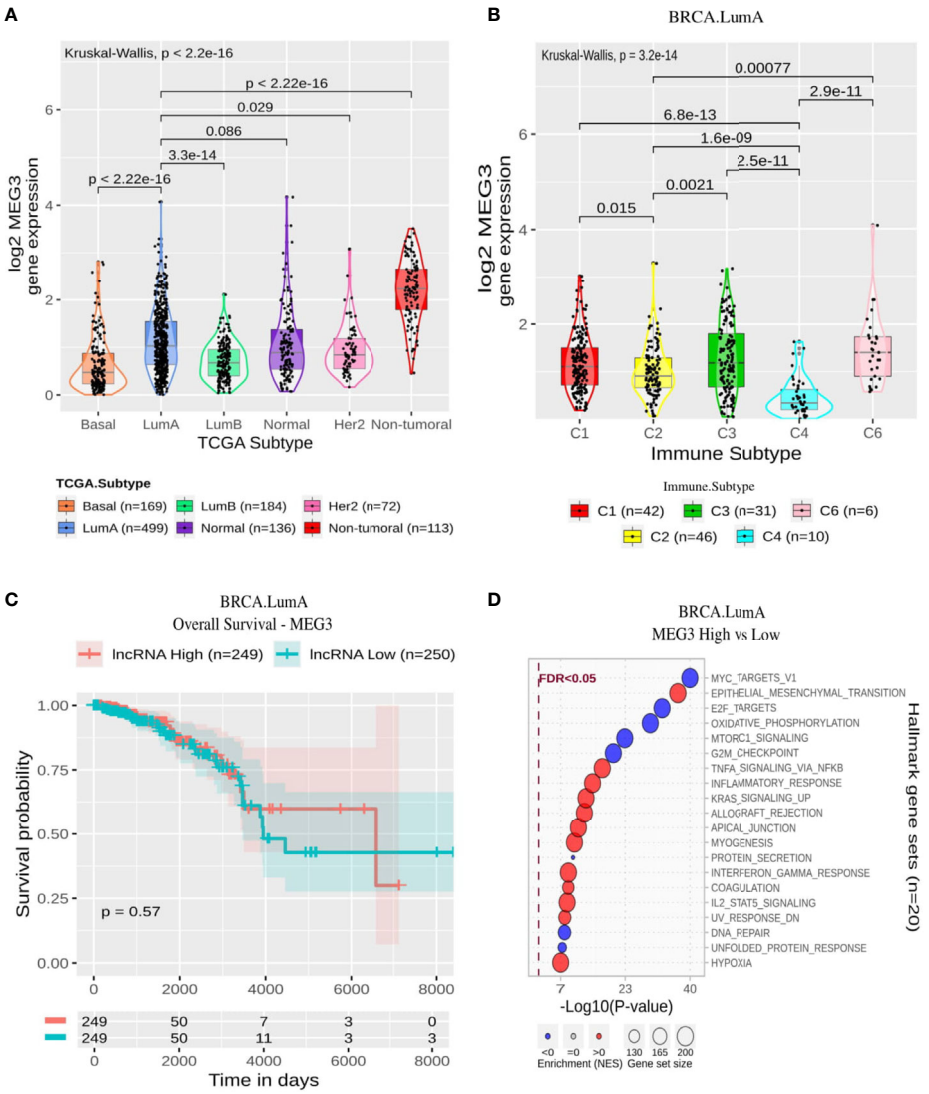

FIGURE 6 | MEG3 panel representation in LumA subtype. **(A)** Box-plots representing MEG3 expression among all breast cancer molecular subtypes. The Kruskal-Wallis test (p-value represented in the panel left top position) was used to differentiate expression between groups, followed by the Wilcoxon test (p-values represented emphasizing comparisons between subtypes). **(B)** Box-plot representing MEG3 in C1,C2, C3, C4, and C6 immune subtypes in LumA patients. **(C)** MEG3 overall survival curves. To define the two groups, MEG3's median expression classified the patients into "high" and "low" groups. Logrank p-value represented as 0.57. **(D)** Enrichment analysis using Hallmarks gene sets. Red circles refers to activation while blue circles to inactivation. In X axis, a p-value scale is represented. The circle size varies according to the number of genes in the identified gene set.

may explain the lack of immune-related lncRNAs associated with OS or PFI in LumA/B (24). Nevertheless, most lncRNAs selected for Her2 and Normal were associated with PFI and OS, respectively, reinforcing BRCA molecular subtypes' well-known heterogeneity. In the article used as a reference (8), the authors elaborated a list of 75 immunomodulatory genes. With this in mind, in **Supplementary Figure 1**, we represented the expression variation (as log2 gene expression z-score) among the molecular subtypes, also considering the immune-related subtype. It is possible to observe a distinct expression pattern in all molecular groups, as expected, regarding breast cancer as a heterogeneous disease. This result reflects the high heterogeneity observed in BRCA molecular subtypes, emphasizing the relevance of characterizing them better molecularly, and we included lncRNA analysis in this complexity.

A specific immune-related signature was proposed to LumA subtype using 11 lncRNAs. Among them, three were suggested related to immune response repression (AC105285.1, LINC00271 and EWSAT1), and none of these were previously investigated under BRCA or immune response aspects (**Supplementary Table 7** and **Supplementary Figure 3C**). However, LINC00271 and EWSAT1 had already been

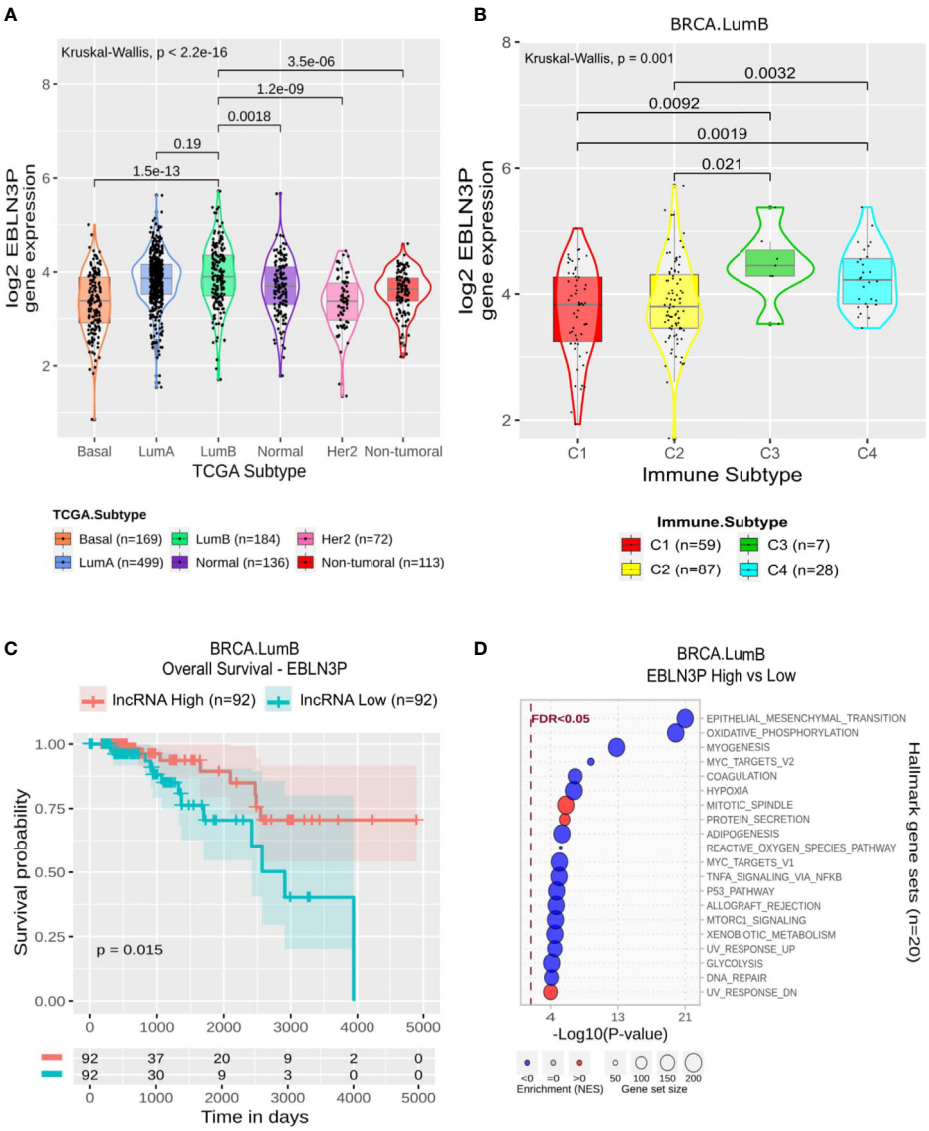

FIGURE 7 | EBLN3P panel representation in LumB subtype. **(A)** Box-plots representing EBLN3P expression among all breast cancer molecular subtypes. The Kruskal-Wallis test (p-value represented in the panel left top position) was used to differentiate expression between groups, followed by the Wilcoxon test (p-values represented emphasizing comparisons between subtypes). **(B)** Box-plot representing EBLN3P in C1,C2, C3, and C4 immune subtypes in LumB patients. **(C)** EBLN3P overall survival curves. To define the two groups, EBLN3P's median expression classified the patients into "high" and "low" groups. Logrank p-value represented as 0.015. **(D)** Enrichment analysis using Hallmarks gene sets. Red circles refers to activation while blue circles to inactivation. In X axis, a p-value scale is represented. The circle size varies according to the number of genes in the identified gene set.

associated with other cancer types. LINC00271 low expression was associated with poor prognosis in papillary thyroid cancer (25) and in adrenocortical tumors (26). EWSAT1 was associated with progression in several cancer types, such as ovarian (27), cervical (28) and colorectal (29).

The lncRNA maternally expressed gene 3 (MEG3) is highlighted here in LumA immune response context. MEG3 is up-regulated in C3 and C6 subtypes and is related to neither overall nor progression aspects (**Figure 6**). The immune subtypes C3 and C6 demonstrate high scores of lymphocyte infiltrate, macrophage regulation and TGF-β response (**Figure 3**). Thus, the increased expression of this lncRNA can be related to immune response activation. Indeed, our functional characterization revealed that MEG3 high expression is associated with several immune hallmarks, such as: "TNFA Signaling Via NFKB," "Inflammatory Response," and "Interferon Gamma Response" (**Figure 6D**). MEG3's role in BRCA immune response is to the best of our knowledge the first time cited here. This lncRNA has only been described in endometrial cancer cells down-regulating *PD-L1* (30). MEG3 is found downregulated in several cancer types,

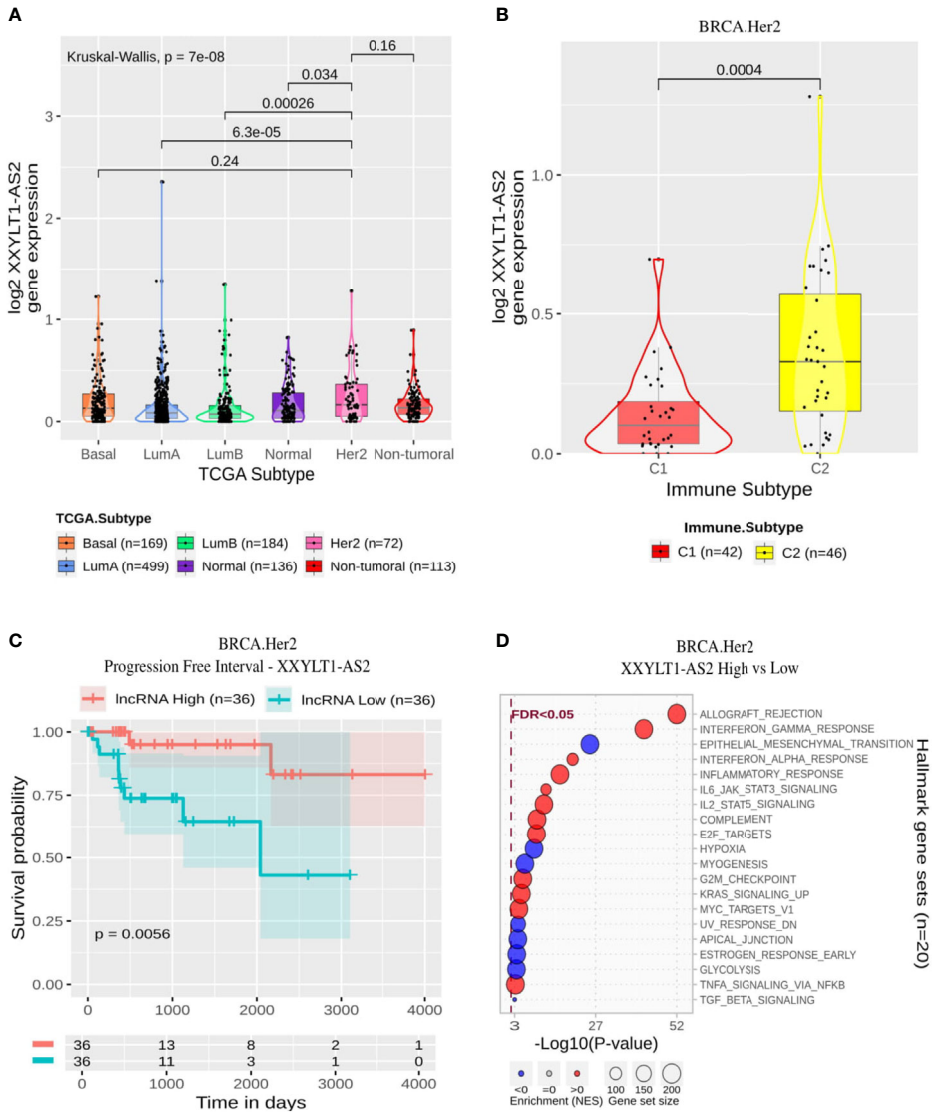

FIGURE 8 | XXYLT1-AS2 panel representation in Her2 subtype. **(A)** Box-plots representing XXYLT1-AS2 expression among all breast cancer molecular subtypes. The Kruskal-Wallis test (p-value represented in the panel left top position) was used to differentiate expression between groups, followed by the Wilcoxon test (p-values represented emphasizing comparisons between subtypes). **(B)** Box-plot representing XXYLT1-AS2 in C1, C2, C3, and C4 immune subtypes in Her2 patients. **(C)** XXYLT1-AS2 progression free interval curves. To define the two groups, XXYLT1-AS2's median expression classified the patients into "high" and "low" groups. Logrank p-value represented as 0.0056. **(D)** Enrichment analysis using Hallmarks gene sets. Red circles refer to activation while blue circles to inactivation. In X axis, a p-value scale is represented. The circle size varies according to the number of genes in the identified gene set.

such as BRCA, liver, colorectal and cervical cancer and was experimentally evidenced as *TP53's* regulator (31). In BRCA, MEG3's downregulation is associated with poor overall survival and tumor staging (32).

In LumB subtype, 11 specific lncRNA were selected, and five of them (LINC00957, AL035661.1, FAM160A1-DT, AC096733.2 and EBLN3P) have their expression related to immune response hallmarks repression (**Supplementary Table 8** and **Supplementary Figure 3D**). These lncRNAs were detected up-regulated in immune subtypes with lower lymphocyte fraction, such as C4. Among these lncRNAs only LINC00957, AL035661.1, and EBLN3P had already been studied in cancer context. LINC00957 high expression was associated with bad prognosis in colorectal cancer (33) and osteosarcoma (34). The lncRNA AL035661.1 was found in a lncRNA profile that managed to efficiently predict early recurrence in hepatocellular carcinoma after curative resection (35) and was associated with EMT in Kidney renal clear cell carcinoma (36).

The lncRNA endogenous Bornavirus-like nucleoprotein 3 (EBLN3P) was highlighted in the LumB subtype. EBLN3P is still not well characterized in the literature since few published

studies have focused on its mechanisms and effects on human diseases (37). Dai and colleagues (38) noted that EBLN3P is expressed in osteosarcoma tissues and cell lines. They pointed out that its overexpression promotes proliferation, migration and invasion. Li et al. (37) have already reported EBLN3P as a novel oncogene for liver cancer for similar aspects (37, 38).

EBLN3P's expression is higher in C3 (Inflammatory) and C4 (Lymphocyte depleted) immune subtypes in LumB samples (**Figure 7B**). In fact, in LumB samples, these immune subtypes exhibit a low TGF-β response score. The role of TGF-β response is still controversial in cancer, depending on the tumor stage. Indeed, TGF-β promotes EMT (39), thus being related to cell proliferation. According to the immune subtypes described by Thorsson et al. (8), the C3 subtype exhibits a low proliferation rate and C4 a moderate one. Using this data with our analysis, we observed a concordance of the data since EBLN3P negatively correlates with TGF-β (**Supplementary Table 8**). In this way, while this lncRNA is highly expressed in these subtypes, TGF-β has a lower expression. The lncRNA EBLN3P's expression was associated with better survival and disease progression outcomes (**Figure 7C**). Regarding its enrichment analysis in LumB samples, the most significant result was achieved considering "Epithelial Mesenchymal Transition" gene sets. In this scenario, EBLN3P's low expression is related to EMT activation.

Interestingly, most LumA and LumB lncRNAs were related positively or negatively to the Interferon Gamma Response module. Recently, a study demonstrated that the activation of the interferon signaling pathway in ER+ BRCA relates to resistance to CDK4/6 inhibitors and immune checkpoint activation (40). Understanding the role of lncRNAs in this context may be the subject of future studies.

A total of 10 Basal lncRNAs were selected after SNR analysis. According to functional annotation analysis of these lncRNAs. (**Supplementary Table 5** and **Supplementary Figure 3A**), only the lncRNA APCDD1L-DT's high expression was related to immune response inhibition. This lncRNA was only evaluated in a competing endogenous RNA network in lung cancer (41), so its immune response regulation role needs to be better understood. The remaining nine lnRNAs were associated with immune response activation (**Supplementary Figure 3A**), and only MIR3142HG was previously studied in cancer context. MIR3142HG polymorphisms were associated with glioma susceptibility and/or prognosis (42). An interesting fact is that MIR3142HG was evidenced as a regulator of IL-1β induced inflammatory response in lung fibroblasts (43). In our analysis, inflammatory response appears to be induced by MIR3142HG (**Supplementary Figure 3A**).

In the Basal, we highlight the lncRNA LINC01871. According to immune subtypes classification, LINC01871 is up-regulated in C2 samples, which is called IFN-γ dominant, and its high expression is associated with a better prognosis (**Figure 4**). Our enrichment analysis (**Figure 4D**) showed a strong relation to immune processes. Considering only Basal samples, LINC01871 was associated with "Allograft rejection," "Interferon Gamma," "Interferon Alpha," and "Inflammatory," for example. In these processes, high expression LINC01871 was associated with the activation of these immune responses. We observe a strong relationship with the leucocyte fraction, which might be related to this observed immune response activation. These results emphasize the relevance of LINC01871 in immune response activation in breast tumor samples, especially in Basal samples, which might be related to better prognosis response.

This lncRNA has already been related to immunity in breast, cervical and gastric cancer. LINC01871 was detected in an immune-related prognosis signature in BRCA and exhibited a strong positive correlation with genes associated with immune response such as *GZMB*, *CTLA4* and *PDCD1*. The authors suggested that this lncRNA may play an important role, mainly related to the above immune processes and immune genes (44). In cervical cancer, LINC01871 was also found in an immune-related prognostic signature being related to immune response and TGF-β signaling pathway (45); additionally, in gastric cancer, LINC01871 expression was positively correlated with CD8+ T cell enrichment levels, cytolytic immune activity, and *CD274* (*PD-L1*) expression levels in TCGA gastric cancer cohort (46).

Taking together our results and background literature is possible to correlate LINC01871 with a cytotoxic immune response. Our enrichment analysis showed an association with Interferon alpha and gamma, which are strongly related to cytotoxic response, going in line with what was observed in other studies (44, 46). In BRCA, CD8+ T cells are reported as prognostic significance in estrogen receptor (ER)-negative BRCA, but not in ER-positive cases, being associated with better clinical outcomes survival and response to treatment (47). Also, CD8+ responses have significantly elevated expression of multiple immune checkpoint molecules, such as programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1) and 2 (PD-L2) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (48). In this way, a stronger infiltration of CD8+ T cells can predict patient response to standard of care chemotherapy and immune checkpoint blockade therapy, such as anti-PD-1 (49).

According to **Figure 3**, it is possible to observe a distinct pattern of expression of LINC01871, both considering immune subtype and Interferon Gamma response. Recently, Interferon Gamma low expression level was associated with worse prognosis in Basal patients (50). In our analysis, LINC01871 high expression was associated with better prognosis and Interferon Gamma response activation (**Figure 4D**), reinforcing the fact that LINC01871 can be used as a good prognosis marker for Basal patients. This can also be evaluated focusing on therapy response, mainly on immunotherapy. Recently, a combination of an immunotherapy drug with chemotherapy was approved for metastatic triple-negative BRCA (51); however, due to this group's high heterogeneity, only a fraction of patients respond well to this treatment. Yang and colleagues (52) defend that an immunity score may be used together with PD-L1 expression to a better design for trials testing immune-checkpoint inhibitors. In this context, lncRNAs like LINC01871 may be used to enhance this selection criterion. However, to be fully applicable as a biomarker, the molecular aspects by which LINC01871 is involved in the immune system activation process need to be better understood.

In the Her2 subtype, ten lncRNAs were evidenced using our selection methodology. All of these lncRNAs were related to immune response activation, as represented in **Supplementary Table 9** and **Supplementary Figure 3E**. Among the ten lncRNAs, four of them had already been related to cancer. LINC02446 is associated with prognosis (53) and EMT activation (54) in bladder cancer. Similarly, USP30-AS1 is associated with prognosis in bladder cancer (54). This lncRNA was also associated with autophagy in ovarian cancer (55), and was related to immune response in cervical cancer (56) and glioblastoma (57). AL365361.1 was associated with good prognosis and immune response in head and neck squamous cell carcinoma (58) and with early recurrence in hepatocellular carcinoma (35). The lncRNA LINC01857 was related to progression in gastric cancer (59), glioma (60), BRCA (61) and B-cell Lymphoma (62). Thus, this lncRNA can be suggested to act as an oncogene. We highlight the lncRNA XXYLT1-AS2, which was associated with progression-free survival in Cox proportional hazard ratio analysis (HR = 0.0011; p-adjusted = 4×10^{-2}). XXYLT1-AS2 is up-regulated in C2 subtype (**Figure 8B**), and its high expression is related to better progression-free interval (p-logrank = 5.6×10^{-3}). According to our functional annotation analysis, XXYLT1-AS2 is associated with immune response activation and EMT repression (**Figure 8D**). This result converges with what we observed concerning the progression-free interval since EMT is one of the main pathways activated during the disease progression process. XXYLT1-AS2 is, to the best of our knowledge, the first time described associated with cancer. This lncRNA was only evaluated in Human umbilical vein endothelial cells (HUVECs), and its up-regulation was related to the inhibition of these cell's proliferation and migration (63).

Finally, for the Normal subtype, a signature composed of 11 lncRNAs was predicted. Most of them are associated with immune response activation and overall survival, and we discuss here LINC02613. This lncRNA is up-regulated in the C3 subtype (**Figure 5B**) and is significantly related to the patient's overall survival. LINC02613's high expression is associated with a better prognosis (p-logrank = 7.5×10^{-3}). The enrichment analysis evidenced that LINC02613 is related to immune response activation and mainly involves cell cycle repression (**Figure 5D**). Indeed, the C3 subtype was described as one with the lowest proliferation rates (8). LINC02613 has not been described in any biological context yet. So we emphasize here the relevance to better characterize this lncRNA.

Considering all the generated and discussed data in this work, we highlight the applied methodology's significance to look for immune-related lncRNAs. The filtering steps followed by functional annotation efficiently got lncRNAs that might be related to immune response. The role of lncRNAs in regulating immune response is increasingly being explored. Our study is limited to *in silico* analysis; however it brings up new lncRNAs candidates as it is a hypothesis generator article.

Another aspect that is important to discuss here is that we found distinct lncRNAs signature for each molecular subtype that may help find important lncRNAs in the immune response process that may be used to guide therapy candidates or as biomarkers; also our results point out that different lncRNAs may be implicated in immune response depending on BRCA molecular subtypes. Our findings are in agreement with what has been discussed about the heterogeneity of BRCA. We identified that different lncRNAs in each molecular subtype are related to the immune system activation. For example, MEG3 and LINC01871 were associated with activation of Interferon Gamma, in LumA and Basal, respectively. This finding highlights the importance of molecularly characterizing each subtype in order to enable increasingly personalized therapeutic approaches.

Nonetheless, the lncRNAs presented here certainly do not cover all important immune-related lncRNAs in BRCA. Our focus was to find lncRNAs that, in some order, could play a significant role in the immune distinction for each molecular subgroup.

CONCLUSION

In conclusion, we present a BRCA specific molecular subtype immune-related lncRNAs signature that may guide future studies aiming to look for important biomarkers in BRCA and highlight the relevance of lncRNAs in the immune subtype's classification.

DATA AVAILABILITY STATEMENT

All scripts, datasets, software and algorithms used to generate results, figures, and tables for this study are available on the GitHub repository (https://github.com/sysbiolab/Sup_Material_Mathias2021) and **Supplementary Table 10**.

AUTHOR CONTRIBUTIONS

CM, JM, and BA conceived the presented idea. CM, JM, and BA developed the theory and performed the computations. DG, MC, and JO verified the analytical methods. DG, MC, and JO supervised the findings of this work. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Public Research Agencies CAPES (001-Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) processo 153771/2018-6. JM received scholarship from the BIG DATA innovation program from the Associação Hospitalar de Proteção à Infância Raul Carneiro-AHPIRAC (2020).

ACKNOWLEDGMENTS

Data used in this manuscript were obtained from The Cancer Genome Atlas (TCGA) BRCA database.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.692170/full#supplementary-material

Supplementary Figure 1 | Heatmap of Immune modulators gene set (n=74) proposed by the reference article Thorsson et al. (8) in breast cancer molecular subtypes. For color gradient, maximum and minimum column-wise z-scores were set to +2 and −2 respectively. Each column represents a sample and were semi-supervised clustered within the molecular subtypes.

Supplementary Figure 2 | Venn diagram representing specific and shared immune related lncRNAs in breast cancer (BRCA) molecular subtypes after filtering for 0.90 quantile in signal to noise ratio (SNR) for the BRCA molecular subtypes. 593 lncRNAs were filtered by this criterion for each molecular subtype.

Supplementary Figure 3 | Enrichment map for immune-related lncRNA signature for each molecular subtype: **(A)** Basal, **(B)** LumA, **(C)** LumB, **(D)** Normal, and **(E)** Her2. The lncRNAs are organized in the X axis and Hallmarks gene sets in the Y axis. Red circles refer to positive enrichment scores while blue circles to negative. The circle size varies according to -log10 (adjusted p-value), bigger circles mean greater significance.

Supplementary Figure 4 | Kaplan-Meier analysis for overall survival for LINC01871 in Basal **(A)** and XXYLT1-AS2 in Her2 **(E)** and for Progression Free Interval for MEG3 in LumA **(B)**, EBLN3P in LumB **(C)** and LINC02613 in Normal **(D)**. The groups of High and Low expressions are based on the median value of its respective lncRNA expression. Confidence interval and logrank p-value are shown and values below 0.05 are considered significant.

Supplementary Table 1 | Breast cancer cohort stratification into the immune subtypes.

Supplementary Table 2 | Ensembl and HGNC Symbol from lncRNAs used in this study.

Supplementary Table 3 | First and second SNR calculated for lncRNAs for each molecular subtype.

Supplementary Table 4 | Cox Proportional Hazard models for the specific immune-related lncRNAs and Leukocyte correlation.

Supplementary Tables 5–9 | GSEA Hallmarks analysis for the specific immune-related lncRNAs organized by the molecular subtype.

Supplementary Tables 10 | Data sets, software and algorithms used in this study.

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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: 11 June 2021
doi: 10.3389/fonc.2021.681946

Prognostic Biomarkers on a Competitive Endogenous RNA Network Reveals Overall Survival in Triple-Negative Breast Cancer

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Edited by:
Wenwen Zhang, Nanjing Medical University, China

Reviewed by:
Lorenzo Sempere, Michigan State University, United States
Feifei Pu, Huazhong University of Science and Technology, China

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

Received: *17 March 2021*
Accepted: *20 May 2021*
Published: *11 June 2021*

Citation:
Qin W, Qi F, Li J, Li P and Zang Y-S (2021) Prognostic Biomarkers on a Competitive Endogenous RNA Network Reveals Overall Survival in Triple-Negative Breast Cancer. Front. Oncol. 11:681946. doi: 10.3389/fonc.2021.681946

The objective of this study was to construct a competitive endogenous RNA (ceRNA) regulatory network using differentially expressed long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and mRNAs in patients with triple-negative breast cancer (TNBC) and to construct a prognostic model for predicting overall survival (OS) in patients with TNBC. Differentially expressed lncRNAs, miRNAs, and mRNAs in TNBC patients from the TCGA and Metabric databases were examined. A prognostic model based on prognostic scores (PSs) was established for predicting OS in TNBC patients, and the performance of the model was assessed by a recipient that operated on a distinctive curve. A total of 874 differentially expressed RNAs (DERs) were screened, among which 6 lncRNAs, 295 miRNAs and 573 mRNAs were utilized to construct targeted and coexpression ceRNA regulatory networks. Eight differentially expressed genes (DEGs) associated with survival prognosis, DBX2, MYH7, TARDBP, POU4F1, ABCB11, LHFPL5, TRHDE and TIMP4, were identified by multivariate Cox regression and then used to establish a prognostic model. Our study shows that the ceRNA network has a critical role in maintaining the aggressiveness of TNBC and provides comprehensive molecular-level insight for predicting individual mortality hazards for TNBC patients. Our data suggest that these prognostic mRNAs from the ceRNA network are promising therapeutic targets for clinical intervention.

Keywords: competitive endogenous RNA, triple-negative breast cancer, prognostic model, differentially expressed genes, nomogram

BACKGROUND

Breast cancer is the most frequently diagnosed cancer and the second most common cause of cancer mortality in women worldwide (1). TNBC, as an aggressive subtype, accounts for 12-18% of all breast cancers (2). Because TNBC patients lack the oestrogen receptor (ER), progesterone receptor (PR) and HER2 receptors, they are not suitable for hormone or anti-HER2 therapy. Targeted therapies cannot significantly improve the survival rate of TNBC patients, and chemotherapy is still the standard treatment. Consequently, exploring the molecular biological mechanism affecting the

prognosis of patients with TNBC and identifying reliable prognostic markers are very valuable for accelerating individual therapies and improving clinical prognoses.

Over the past decade, substantial efforts have been made to classify TNBC into distinct clinical and molecular subtypes to guide treatment decisions. The characterization of genomic, proteomic, epigenomic and microenvironmental changes has expanded our understanding of TNBC. High levels of somatic mutations, frequent TP53 mutations (83%) and complex aneuploidy rearrangement (80%) have been found in TNBC patients *via* deep sequencing studies (3), multiregion sequencing analyses (4) and single-cell sequencing research (5), revealing extensive intratumoural heterogeneity (ITH). However, the molecular mechanism driving TNBC relapse has not been fully elucidated.

The ceRNA hypothesis involves a specific molecular biological regulatory mechanism of posttranscriptional regulation (6). Studies have explored the mechanism of ceRNA biological regulation in TNBC patients. Yuan et al. (7) proposed a ceRNA crosstalk network in triple-negative breast cancer *via* integrative analysis of lncRNAs and miRNAs with coding RNAs. Song et al. (8) established a ceRNA topology network with five specific RNAs: hsa-miR-133a-3p, hsa-miR-1-3p, TRIML2, TERT and PHBP4. However, due to their complex formulas, unclear results, lack of external validation and other reasons, these prediction models do not predict prognostic effects very well. A prognostic model with a simple formula, easily interpretable results and strong external repeatability are very important for optimizing individualized treatment. Therefore, the purpose of this study was to develop and validate a nomogram for determining OS prognoses in patients with TNBC based on gene expression data confirmed by the ceRNA regulatory network and to screen potential therapeutic agents among existing small-molecule inhibitors.

METHODS

Expression Profile Data Screening

The breast cancer expression data (including mRNAs, lncRNAs and miRNAs) were downloaded from The Cancer Genome Atlas (TCGA) database (https://gdc-portal.nci.nih.gov/), and the Illumina HiSeq 2000 RNA platform was used for sequencing. The downloaded data was TCGA TNBC RNA-seq level 3 with a normalized log (FPKM+ 1,2) that can be used for direct analysis. After comparing the clinical information of the downloaded samples (**Table S1**), the following breast cancer samples were retained: patients with known expression levels of mRNAs, lncRNAs and miRNAs; patients negative for ER, PR and HER-2; patients with complete pathological staging and follow-up data; and enrolled patients with TNBC primary disease and no other malignant tumours. A total of 102 samples and 63 control samples were obtained, and these samples were used as the training group. Moreover, the RNA-seq breast cancer data were downloaded from the Molecular Taxonomy of Breast Cancer International Consortium (Metabric) database (http://molonc.bccrc.ca/) and included a total of 1904 breast cancer patients with corresponding clinical information. Based on the above screening criteria, we selected 298 TNBC samples, and this data set was used as the validation group. All of the data sets utilized in this study were from public databases. Ethical approval was not required because researchers are allowed download and analyse data from these public databases for scientific purposes.

Abbreviations: TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; OS, overall survival; ROC-curve, Receiver operating characteristic curve; TNBC, triple-negative breast cancer; lncRNAs, long noncoding RNAs; DERs, differentially expressed RNAs; ceRNA, competitive endogenous RNA.

Data Preprocessing and Screening of Differential Genes

LncRNAs, miRNAs and mRNAs detected in the TCGA samples were annotated. Then, the R language package Limma (version 3.34.0, https://bioconductor.org/packages/release/bioc/html/limma.html) (9) was used to screen for differentially expressed RNAs (DERs) between the TNBC and control (CTRL) groups. The screening threshold was an false discovery rate (FDR) < 0.05 and a $|\log2FC| > 0.5$. The R language package pheatmap (version 1.0.8, https://cran.r-project.org/package=pheatmap) (10) was used to perform bidimensional hierarchical clustering (11, 12) analysis based on the Euclidean distances (13) of the expression levels of the selected DERs, which are displayed in the heatmap.

Construction of a Targeted Regulatory ceRNA Network

We analysed the relationships of differentially expressed (DE) lncRNAs and miRNAs from the DIANA-LncBase database (14) (https://diana.e-ce.uth.gr/lncbasev3) and retained only the pairs with opposite expression trends. The StarBase database (15) (version 2.0, http://starbase.sysu.edu.cn/) was used to search the differentially expressed genes (DEGs) correlated with DEmiRNA regulation, and only the negatively correlated miRNA and mRNA pairs were retained. Based on the above results, a ceRNA regulatory network composed of DElncRNAs, DEmiRNAs, and DEmRNAs was constructed, and the network was visualized with Cytoscape (Version 3.6.1) (https://cytoscape.org/) (16). The regulatory network of ceRNA coexpression was used to calculate the Pearson correlation coefficients (PCCs) between the expression levels of DElncRNAs *vs*. DEmiRNAs, DElncRNAs *vs*. DEmRNAs and DEmiRNAs *vs* DEmRNAs by using the Cor function in R3.6.1 software (http://77.66.12.57/R-help/cor.test.html), and the pairs with significant correlation ($P < 0.05$) were selected. Finally, The Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) (17, 18) (http://metascape.org/) was used to conduct Gene Ontology (GO) biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the DEGs in the ceRNA regulatory network, and $P < 0.05$ was selected as the threshold for determining significant enrichment.

Screening of Prognostic-Related Genes

In the TCGA training set, based on the ceRNA regulatory network and the prognostic information obtained from the samples, univariate Cox analysis was used to screen mRNAs

correlated with significant overall survival (OS) with R software (survival pack, version 2.41, http://bioconductor.org/packages/survivalr/). Multivariate Cox regression analysis was used to screen for mRNAs with significant independent prognostic correlation using the survival package (version 2.41-1) (19) of R3.6.1. A log-rank P value < 0.05 was selected as the threshold for determining significant prognostic mRNAs.

Establishment of the Prognostic Risk Prediction Model

Based on multifactor Cox regression analysis of mRNAs and their expression levels in the TCGA, we constructed the following prognostic score (PS) model:

$$Prognostic\ score\ (PS) = \sum Coef_{mRNAs} \times Exp_{mRNAs}$$

Coef $_{mRNAs}$ represents the prognostic coefficients of mRNAs in the multivariate Cox regression analysis, and Exp $_{mRNAs}$ represents the expression levels of mRNAs in the TCGA dataset.

Statistical Analysis

The t-test or Mann-Whitney U test was used for comparison, and continuous variables are expressed as the mean ± standard deviation. The $\chi 2$ test or Fisher's exact test was used to compare the categorical variables. The prognostic factors were computed using the Cox proportional hazards model, where HR was the 95% confidence interval. OS was defined as the time from the date of diagnosis to the date of last follow-up or death and was analysed by the log-rank test. Nomograms and ROC curves were used to evaluate the predictive performance of the prognostic model. All statistical analyses were computed using SPSS version 22.0 (IBM SPSS Statistics, Chicago, IL, US), and R software (version 3.5.2) with the following packages: "Limma", "pheatmap", "survival", "rmda", "cor", "GOplot", "ROC", "rms". $P < 0.05$ was considered statistically significant.

RESULTS

Data Preprocessing and Screening for Significantly DERs

To explore the significant prognostic correlate factors for triple-negative breast cancer, we selected the patients with triple-negative breast cancer from TCGA and Metabric data sets. There were 102 TNBC patients in the training group and 298 TNBC patients in the validation group. The clinical and demographic characteristics of all patients with TNBC are summarized in **Table 1**. According to the platform annotation information provided in the downloaded data, 14,000 mRNAs, 1778 lncRNAs and 2222 miRNAs were annotated in the data set. A total of 874 DERs were screened, among which 6 lncRNAs, 295 miRNAs and 573 mRNAs met the screening threshold criteria (**Table S2**). The inspection volcano distribution map is shown in **Figure 1A**. The bidirectional hierarchical clustering heat map based on DER expression is presented in **Figure 1B**. The above DERs were identified as candidate prognosis factors.

Construction of the ceRNA Networks and Functional Analysis

To further explore the biological functions and involvement in signaling pathways of DERs, we constructed the ceRNA networks from DERs including lncRNAs, miRNAs and mRNAs. The ceRNA regulatory networks were constructed in two ways: targeted and coexpression regulation. From the binding relationships between DElncRNAs and DEmiRNAs screened in the above step based on the DIANA-LncBasev2 database, we retained only the pairs exhibiting opposite expression trends, yielding total 19 pairs (**Table S3**). Then, we searched the target genes regulated by DEmiRNAs by using the StarBase database and matched the significant DEGs obtained in the first step. Moreover, only pairs with negatively correlated miRNA and mRNA expression levels were retained, yielding 50 total pairs. A targeted ceRNA network composed of lncRNAs, miRNAs, and mRNAs was constructed after synthesizing and collating DElncRNAs, DEmiRNAs, and DEmiRNA-DEmRNAs (**Figure 2A**). Finally, enrichment annotation analyses of GO biological processes and KEGG signalling pathways associated with the mRNAs involved in the ceRNA regulatory network were carried out based on DAVID. We identified 9 significantly related GO biological processes, including neurohypophysis development, synapse assembly, cell differentiation, hypothalamus cell differentiation, regulation of cytokine biosynthetic process, neuron fate specification, positive regulation of filopodium assembly, positive regulation of transcription from RNA polymerase II promoter and transcription, and 4 KEGG signalling pathways, including the ErbB signalling pathway, FoxO signalling pathway, and signalling

TABLE 1 | Clinical characteristics of TNBC patients in the training and validation groups.

Clinical characteristics	Training group N = 102	Validation group N = 298	P value
Age (years, mean ± SD)	57.01 ± 11.75	55.66 ± 13.76	3.38E-01
Pathological M (M0/M1/-)	85/1/16	NA	NA
Pathological N (N0/N1/N2/N3/-)	65/23/11/3	NA	NA
Pathological T (T1/T2/T3/T4)	23/64/11/4	NA	NA
Pathologic stage (I/II/III/IV/-)	16/65/17/1/3	62/130/25/0/81	**2.48E-02**
Radiotherapy (Yes/No/-)	53/40/9	214/84	**1.04E-02**
Recurrence (Yes/No/-)	15/78/9	NA	NA
Death (Yes/No)	16/86	161/137	**3.50E-12**
Overall survival time (months, mean ± SD)	38.95 ± 31.34	113.42 ± 83.37	**2.62E-12**

TNBC, triple-negative breast cancer; NA, not applicable. A P value < 0.05 was considered to be statistically significant. The bold values represent statistically significant.

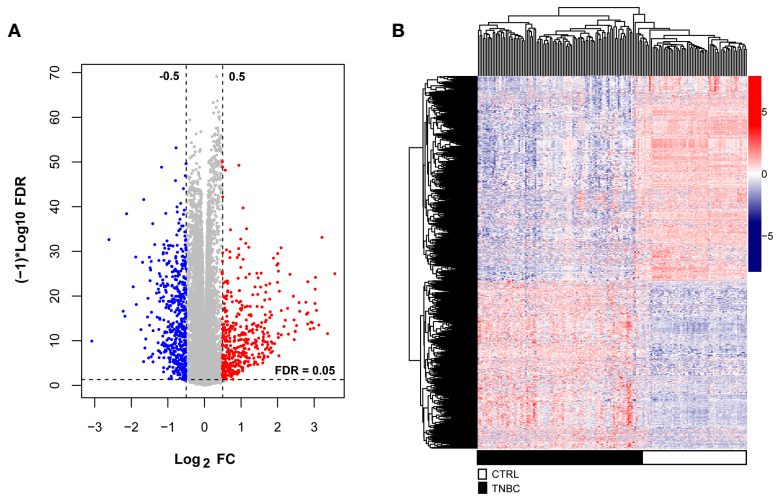

FIGURE 1 | Differentially expressed RNAs, including lncRNAs, miRNAs and mRNAs, from the TCGA dataset. **(A)** The volcano map was constructed. The blue and red dots indicate significantly downregulated and upregulated differentially expressed RNAs, respectively. The horizontal dotted line indicates an FDR < 0.05, and the two vertical dashed lines indicate a | log2FC | > 0.5. **(B)** Bidirectional hierarchical clustering heat map based on the levels of differentially expressed RNAs; the black and white sample bars represent TNBC and control peritumoral tissue patients, respectively.

pathways regulating stem cell pluripotency and proteoglycans in cancer (**Figure 2B**).

Then, we used the Cor function in R to calculate the PCCs between DElncRNAs and DEmiRNAs, DElncRNAs and DEmRNAs and DEmiRNAs and DEmRNAs and selected the significantly correlated pairs (PCC > 0.4, $P < 0.05$). Finally, we obtained 69 pairs between DElncRNAs and DEmiRNAs, 158 pairs between DElncRNAs and DEmRNAs and 369 pairs between DEmiRNAs and DEmRNAs (**Table S4**). The three coexpression relationships were integrated to construct a comprehensive coexpression ceRNA network (**Figure 3A**). Next, a total of 21 significantly related GO biological processes and 6 KEGG signalling pathways were identified by DAVID based on enrichment annotation analysis of mRNAs in the coexpression regulatory network (**Figure 3B**). The top five GO terms related to miRNAs in the coexpression ceRNA network were mainly enriched in proteolysis, regulation of apoptotic processes, responses to lipopolysaccharide, chemical synaptic transmission and cell-cell signalling. The KEGG pathways related to these mRNAs were mainly enriched in ABC transporters, the PPAR signalling pathway, proximal tubule bicarbonate reclamation, neuroactive ligand-receptor interaction, adrenergic signalling in cardiomyocytes and glycolysis/gluconeogenesis.

Screening for DEGs Associated With Survival Prognosis

According to 102 TNBC samples in the TCGA training set, a total of 144 mRNAs were identified based on the mRNAs contained in the two ceRNA regulatory networks. Then, 12 DEGs that were significantly associated with survival prognosis were identified by univariate Cox regression analysis (**Table S5**). Furthermore, multivariate Cox regression analysis was used to identify 8 DEGs associated with survival prognosis, including DBX2, MYH7, TARDBP, POU4F1, ABCB11, LHFPL5, TRHDE and TIMP4 (**Table 2**). Further, we found that ABCB11 involved in ABC transporters pathways and MYH7 involved in Adrenergic signaling in cardiomyocytes pathways. In GO, MYH7 participated in cardiac muscle contraction, POU4F1 participated in synapse assembly and neuron fate specification, TRHDE participated in regulation of blood pressure, proteolysis and cell-cell signaling, and TIMP4 participated in response to lipopolysaccharide. Then, we calculated the PS scores of the 8 DEGs and divided each gene into a high PS score group (PS score higher than or equal to the median PS score) and a low PS score group (PS score lower than the median PS score) according to the median PS score. Survival curves and log-rank tests were used to analyse the prognosis of each gene in patients (**Figures 4A–H**). The results showed that the PS scores of all 8 DEGs were significantly associated with survival prognosis. Therefore, a nomogram based on the prognostic factors that combined the 8 DEGs correlated with overall survival is presented in **Figure 4I**, indicating that the 8 DEGs was able to be an accurate predictor of survival in patients with TNBC.

Evaluation of Effectiveness and Comparison of the Prognostic Models

Based on the prognostic coefficients of the 8 DEGs from the multivariate Cox regression analysis and their expression levels in the TCGA training set obtained in the previous step, we constructed a prognostic score (PS) model and divided all samples from the TGCA training set into a high risk group (PS score equal to or higher than the PS median value) and a low risk group (PS score lower than the PS median value). Moreover, the corresponding mRNA expression levels were extracted from the Metabric validation dataset. Then, we calculated the PS score of each sample and divided them into a high-risk group and a

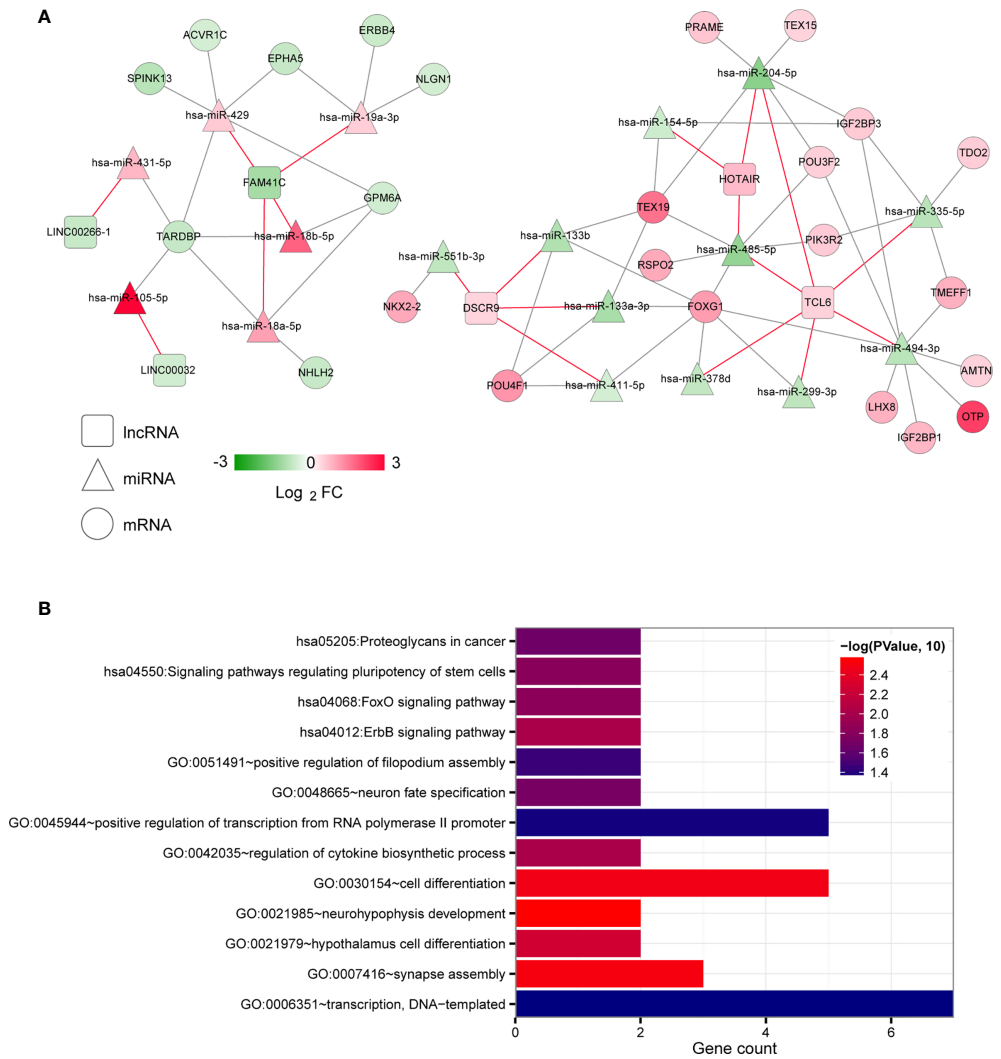

FIGURE 2 | Targeted ceRNA regulatory network. **(A)** The mutual targeting relationships among lncRNAs, miRNAs and mRNAs. The colour change from green to red indicates a change from low to high. The red and grey lines indicate the DElncRNA-DEmiRNA associations and the DEmiRNA-DEmRNA regulatory associations, respectively. **(B)** Bar diagram of the GO biological processes and KEGG signalling pathways significantly correlated with mRNAs in the ceRNA network. The horizontal axis shows the number of genes. The vertical axis shows the entry names. Entries closer to a red colour are more significant.

low-risk group. The survival curve and log-rank test showed a predictability of the PS model groups for actual prognostic information for the disease (**Figures 5A, B** and **Table S6**). The survival rate of the low-risk group was significantly higher than that of the high-risk group in the TGCA training set ($P < 0.01$, HR = 10.06) and the Metabric validation set ($P = 0.02$, HR = 1.437). However, we test this PS model in non-TNBC cases and found that it was not associated with non-TNBC cases prognosis and did not work for non-TNBC (**Figures 5C, D**). Then, we applied ROC curves to assess the precision of the prognostic model. Similar to the nomogram, ROC curve analysis at 1, 3 and 5 years revealed area under the curve (AUC) values of 0.868, 0.911 and 0.860 in the TGCA training set and 0.748, 0.759 and 0.723 in the Metabric validation set, respectively (**Figures 5E, F**). In addition, the calibration curve showed that the nomogram-predicted and actual probability of survival at 3 and 5 years were in good agreement (**Figures 5G, H**). In brief, on the basis of PS model from 8 DEGs, we develop a predictive model for clinical application, which is accurate and effective for TNBC patients.

Correlation of the Prognostic Model With Clinical Factors

To further analysis the accuracy and application of the PS model compared with other clinical prognosis factors, multivariate Cox regression analyses were firstly carried out to evaluate the survival of TNBC patients using the prognostic model. The multivariate Cox regression analyses showed that pathological stage and prognostic model were independent prognostic factors in the TCGA training dataset. In the Metabric validation dataset, multivariate Cox regression analyses identified the patient's age,

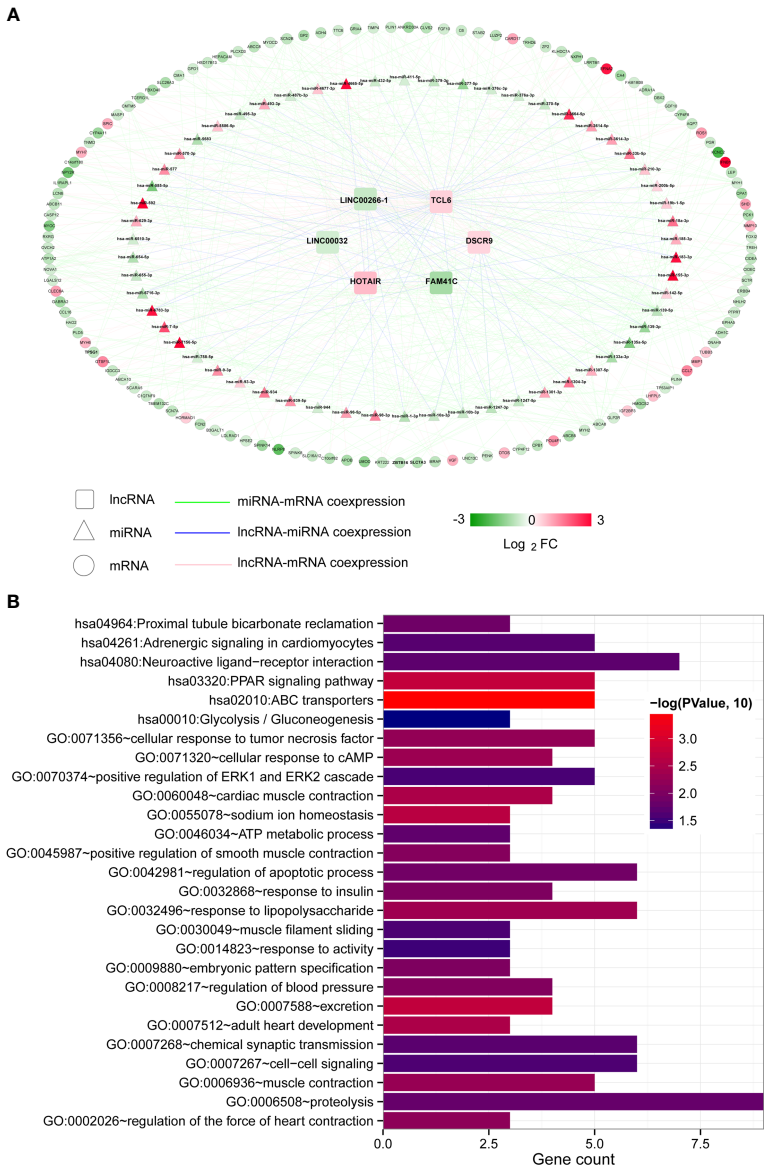

FIGURE 3 | Coexpression ceRNA regulatory network. **(A)** The coexpression relationships among lncRNAs, miRNAs and mRNAs. The colour change from green to red indicates a change from low to high. **(B)** Bar diagram of the GO biological processes and KEGG signalling pathways significantly correlated with mRNAs in the ceRNA network. The horizontal axis depicts the number of genes. The vertical axis shows the entry names. Entries closer to a red colour are more significant.

TABLE 2 | Univariate and multivariate analyses of independent prognostic mRNAs.

Symbol	Univariate analysis			Multivariate analysis			
	HR	95%CI	P value	Coefficient	HR	95%CI	P value
DBX2	0.899	0.807-0.962	2.650E-02	-0.340	0.712	0.553-0.916	8.120E-04
MYH7	0.825	0.728-0.934	1.250E-03	-0.214	0.807	0.676-0.963	1.739E-03
TARDBP	0.887	0.786-0.953	2.550E-02	-0.160	0.852	0.718-0.910	6.511E-03
POU4F1	0.903	0.805-0.941	4.200E-02	-0.147	0.864	0.729-0.923	8.915E-03
ABCB11	0.875	0.768-0.996	2.200E-02	-0.140	0.869	0.689-0.990	2.362E-02
LHFPL5	0.785	0.654-0.934	3.350E-03	0.183	1.200	1.151-1.516	1.245E-02
TRHDE	1.176	1.098-1.412	4.100E-02	0.282	1.326	1.124-1.901	1.258E-02
TIMP4	1.479	1.145-1.910	1.350E-03	0.457	1.579	1.188-2.808	1.196E-02

CI, confidence interval; HR, hazard ratio.

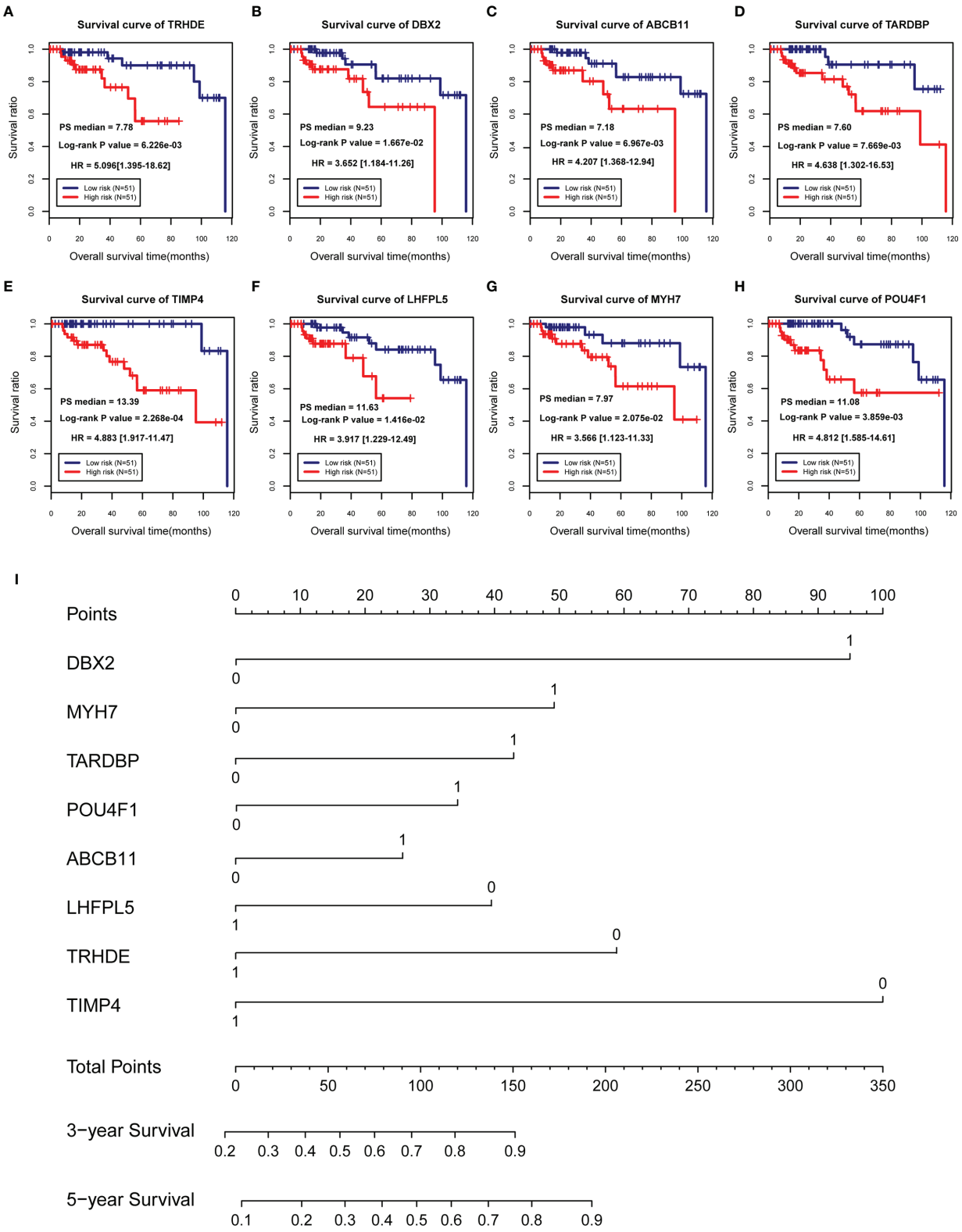

FIGURE 4 | Establishment of a prognostic model for TNBC patients. **(A–H)** Survival curves of patients with TNBC, divided into low-risk and high-risk groups, carrying known prognostic genes. **(I)** Nomogram for the overall survival of TNBC patients.

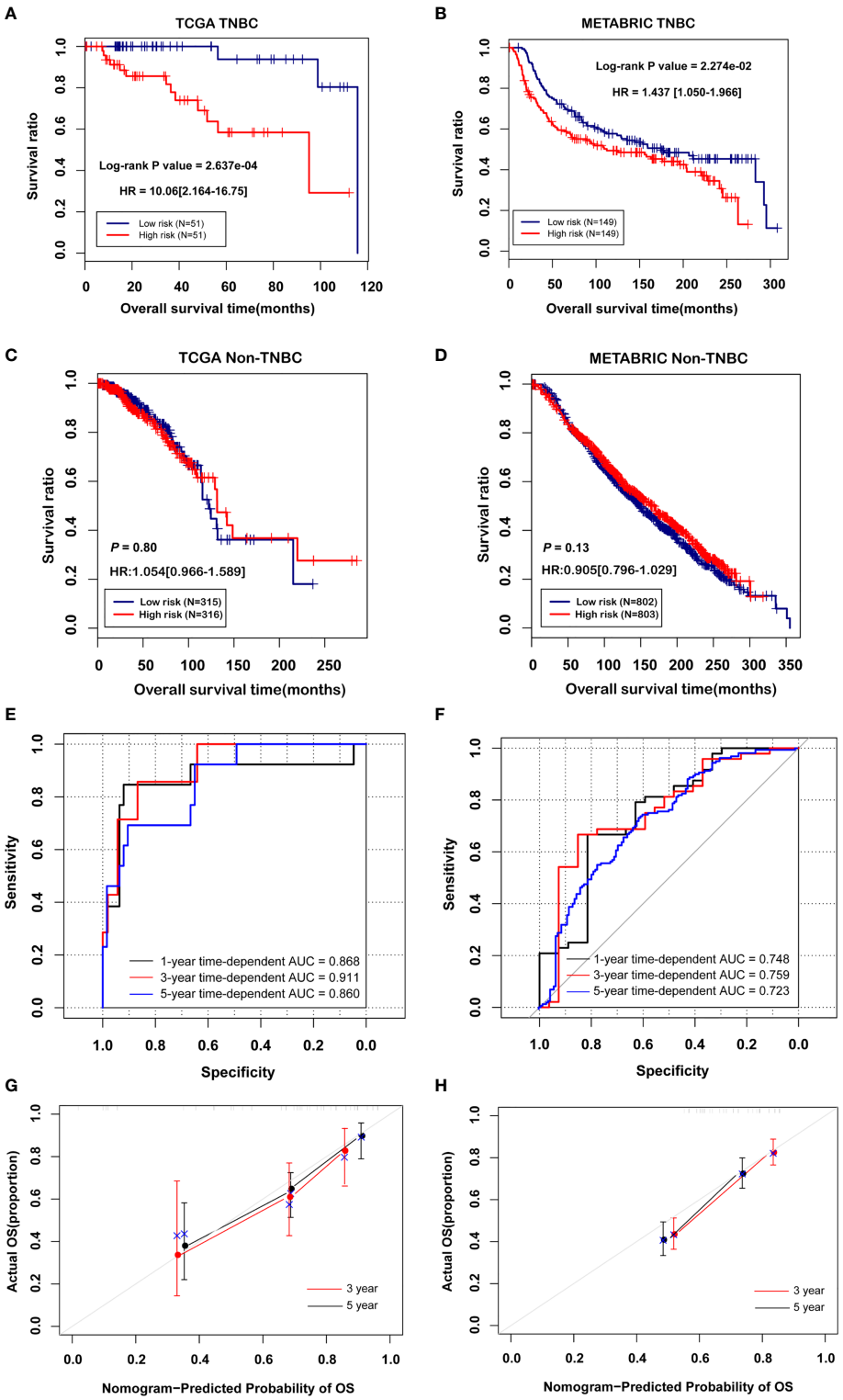

FIGURE 5 | Validation of the prognostic model. **(A, B)** Kaplan-Meier curve based on the PS prediction model and prognostic correlation in TNBC groups. **(C, D)** Kaplan-Meier curve based on the PS prediction model and prognostic correlation in non-TNBC groups. **(E, F)** The ROC curve of the PS forecast model, with the black, red and blue curves representing the 1-year, 3-year and 5-year curves, respectively. **(G, H)** Three- and 5-year calibration curves based on the PS forecast models.

pathologic stage and prognostic model as independent prognostic factors for overall survival (**Table 3**). Therefore, we performed decision curve analysis (DCA) of a single clinical factor, the PS model and clinical factors in combination with the PS model in the TCGA data set to compare the net effects of each variable on survival prognosis. As shown in **Figure 6A**, the prognostic model (red line) yielded a higher net profit than age (green line) and the pathological stage (blue line). DCA implied that the prognostic model combined with clinical factors was more beneficial that both a single clinical factor and the PS model by itself for forecasting the survival rate. Further subgroup analysis showed that the pathological stage was a significant factor influencing the prognosis of TNBC patients. As illustrated in **Figure 6B**, the survival rate in the high-risk group was obviously worse than that in the low-risk group, suggesting that the prognostic model was trustworthy and exhibited a steady predictive performance at distinct pathological subgroup stages. We performed correlation analyses of the 8 DEGs and the prognostic signature with TNBC clinical factors. The outcomes implied that the prognostic signature was considerably correlated with recurrence and the pathological stage (**Figure 6C** and **Table S7**).

Construction of a Network for Small-Molecule Drugs and Characteristic Factors

To explore the underlying treatment value of the PS model from 8 DEGs, we constructed the network of small-molecule drugs targeted 8 DEGs and predicted the possible binding sites. All the relationship data for genes and small-molecule drugs were downloaded from the CTD database, and 12 pairs were obtained from the 8 DEGs selected for construction of the PS model (**Figure 7A** and **Table S8**). Further, the docking possibilities between the target proteins and small molecules were assessed using AutoDock software (20). The results revealed 9 pairs from 3 DEGs and 8 small molecules (**Table 4**). Then, we analysed the local binding sites through PLIP online tools (https://projects.biotec.tu-dresden.de/plip-web/plip/index) and drew 3D structure simulations using PyMOL (version 2.4) (**Figures 7B–J**). According to the above analysis, the combination of fasudil and MYH7 was the best based on the affinity value (-8.2). Moreover, our results also provide a reference for developing clinical drugs according to characteristic factors.

DISCUSSION

Noncoding RNAs (ncRNAs), which account for the majority (98%) of the transcriptome, are defined as gene transcripts with important biological functions (21). Among them, lncRNAs are one of the most important challenges biologists face today and represent potential new biomarkers and drug targets. The hypothesis of Salmena et al. (22) regarding ceRNA networks of multiple molecules involved in posttranscriptional regulation has received extensive attention in recent years. Their research showed that lncRNAs can act as ceRNAs to inhibit the function of miRNAs and communicate with mRNAs by carrying one or more miRNA response elements (MREs). However, the molecular biological mechanisms and effective regulatory networks affecting the progression and prognosis of TNBC remain unclear. It is important and helpful to optimize individualized treatments by exploring the molecular biological regulatory network and prognostic biomarkers of TNBC.

In this work, we used bioinformatic analyses to establish a new ceRNA regulatory network in TNBC that was based on 6 lncRNAs, 295 miRNAs, and 573 mRNAs. The ceRNA regulatory network was constructed in two ways: targeted regulation and coexpression regulation. A total of 144 mRNA nodes were found in the network. Among them, 12 mRNAs were significantly related to survival prognosis as determined by univariate Cox regression analysis, and an 8-mRNA prognostic nomogram was finally obtained *via* further multivariate Cox regression analysis. The 8-mRNA prognostic nomogram was helpful for identifying TNBC patients with a low survival probability. The OS rate in the high-risk group was significantly worse than that in the low-risk group in both the model and validation groups.

Many studies have explored prognostic biomarkers and possible molecular regulatory pathways in breast cancer, but studies on ncRNA-related cancers are commonly concentrated on miRNAs, lncRNAs and circRNAs (23–25). A corresponding lncRNA-miRNA-mRNA prognostic model of OS in TNBC patients has not been constructed. Jiang et al. (26) developed an integrated mRNA-lncRNA signature that effectively classifies

TABLE 3 | Univariate and multivariate analyses of the prognostic model.

Clinical characteristics	Univariate Cox			Multivariate Cox		
	HR	95%CI	P	HR	95%CI	P
Training group (N=102)						
Age (years, mean ± SD)	1.039	0.513-1.994	7.88E-01	1.004	0.964-1.045	8.62E-01
Pathological stage (I/II/III/IV/-)	4.465	2.197-9.824	**4.57E-05**	3.348	1.649-7.355	**1.07E-03**
Radiotherapy (Yes/No/-)	0.877	0.246-3.126	8.40E-01	0.833	0.507-2.730	3.77E-01
PS model status (High/Low)	10.06	2.164-16.75	**2.64E-04**	2.512	1.552-9.978	**1.75E-02**
Validation group (N=298)						
Age (years, mean ± SD)	1.024	1.012-1.036	**9.94E-05**	1.022	1.007-1.037	**3.31E-03**
Pathological stage (I/II/III/IV/-)	1.625	1.201-2.197	**1.57E-03**	1.687	1.249-2.278	**6.46E-04**
Radiotherapy (Yes/No/-)	0.841	0.601-1.177	3.13E-01	0.853	0.564-1.290	4.51E-01
PS model status (High/Low)	1.437	1.050-1.966	**2.27E-02**	1.541	1.071-2.219	**1.98E-02**

CI, confidence interval; HR, hazard ratio. The bold values represent statistically significant (P < 0.05).

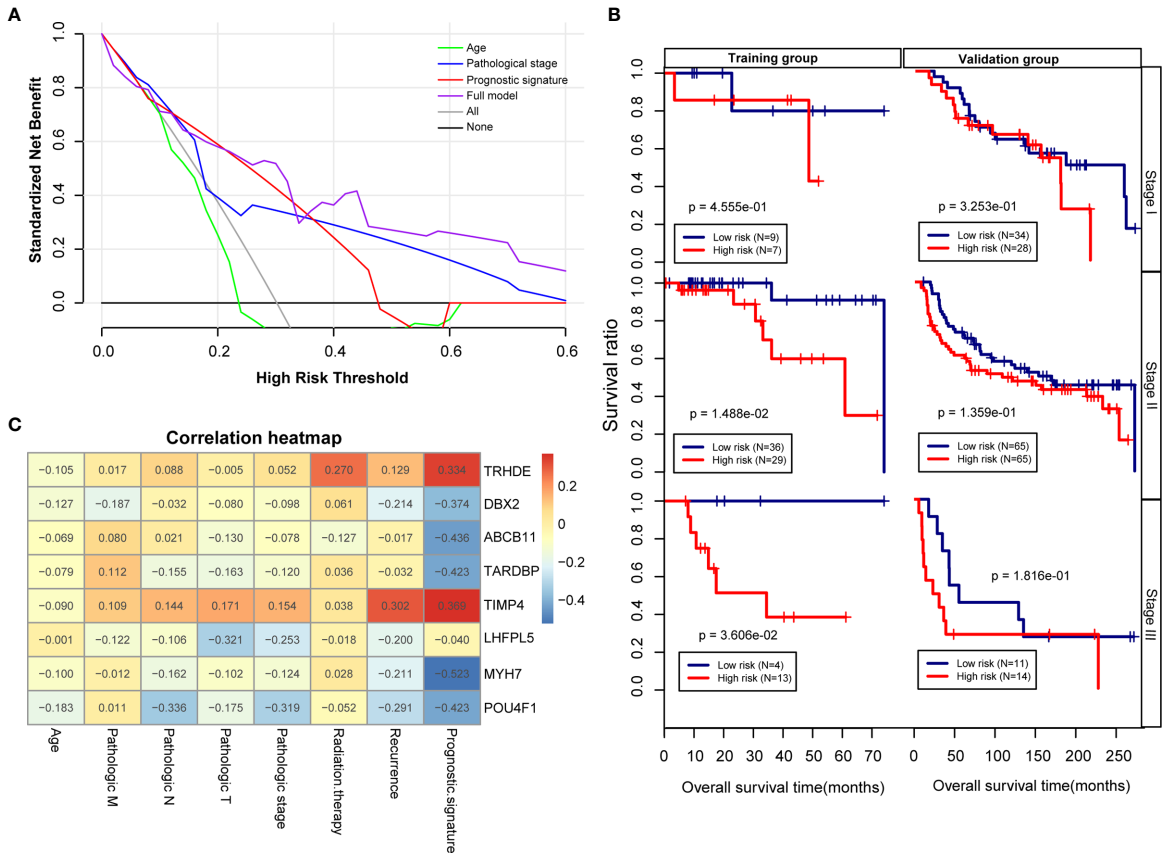

FIGURE 6 | Effectiveness and comparison of the prognostic model. **(A)** Decision curve analysis. The green, blue, red and purple colours represent the decision curves for the patient's age, pathological stage, PS model and combined model, respectively. The grey and black curves show the two extremes. **(B)** Kaplan-Meier curves were analysed by pathological stage stratification in the TCGA training set and Metabric validation set. **(C)** Heat map depicting the significance of correlations between clinical characteristics and 8 factors.

TNBC patients into groups at low and high risk of disease recurrence, but the model is susceptible to the inherent biases of the study format and to the biases of the individuals performing the diagnoses and laboratory experiments. Therefore, we identified prognostic mRNA biomarkers and established a prognostic model based on a TCGA data set, while external verification was carried out using an independent external verification data set from the Metabric database. In this study, 8 potential biomarkers were found to be closely related to the OS of TNBC patients. Through the ceRNA regulatory network, potential lncRNA-miRNA-mRNA regulatory pathways are shown, which may help to clarify the potential biological mechanisms and regulatory pathways related to the OS of patients with TNBC. GO and KEGG pathway enrichments were further assessed to determine the potential molecular mechanism underlying the OS of TNBC patients.

Changes in TIMP4 expression have been reported to contribute to the development of breast cancer (27). In fact, TIMP4 is involved in several processes, including cell invasion and migration, cell proliferation and apoptosis, and angiogenesis (28). It can regulate the proteolytic activity of MMPS and is associated with ECM remodelling, EMT progression, and cancer initiation and progression by directly affecting cellular adhesion (29, 30). Different studies have associated high serum levels of MMPS and TIMPS with poor prognosis (31), and these factors were specifically identified as predictors of adverse outcomes in patients with breast cancer (32, 33). POU4F1 is a member of the POU domain family of transcription factors and plays a key role in regulating cancers. Studies have shown that POU4F1 expression is dramatically increased in breast cancer cells. POU4F1 deletion substantially downregulated the MEK1/2 and ERK1/2 signalling pathways in cancer cells (34, 35). TARDBP was originally considered to be an RNA/DNA binding protein and a regulator of HIV-1 gene expression. Increasing evidence shows that TARDBP may be involved in cell division, apoptosis and microRNA (miRNA) biogenesis (36). It is considered a powerful prognostic indicator of survival in breast cancer (37). In addition, ABCB11 mutations are prevalent in the DNA of patients with primary breast cancers and are considered to be associated with tumour prognosis (38). The results of all of these studies are compatible with the biomarker predictions in our study.

There are a few reports on the effects of other factors, such as DBX2, MYH7, TRHDE and LHFPL5, on TNBC. However, these

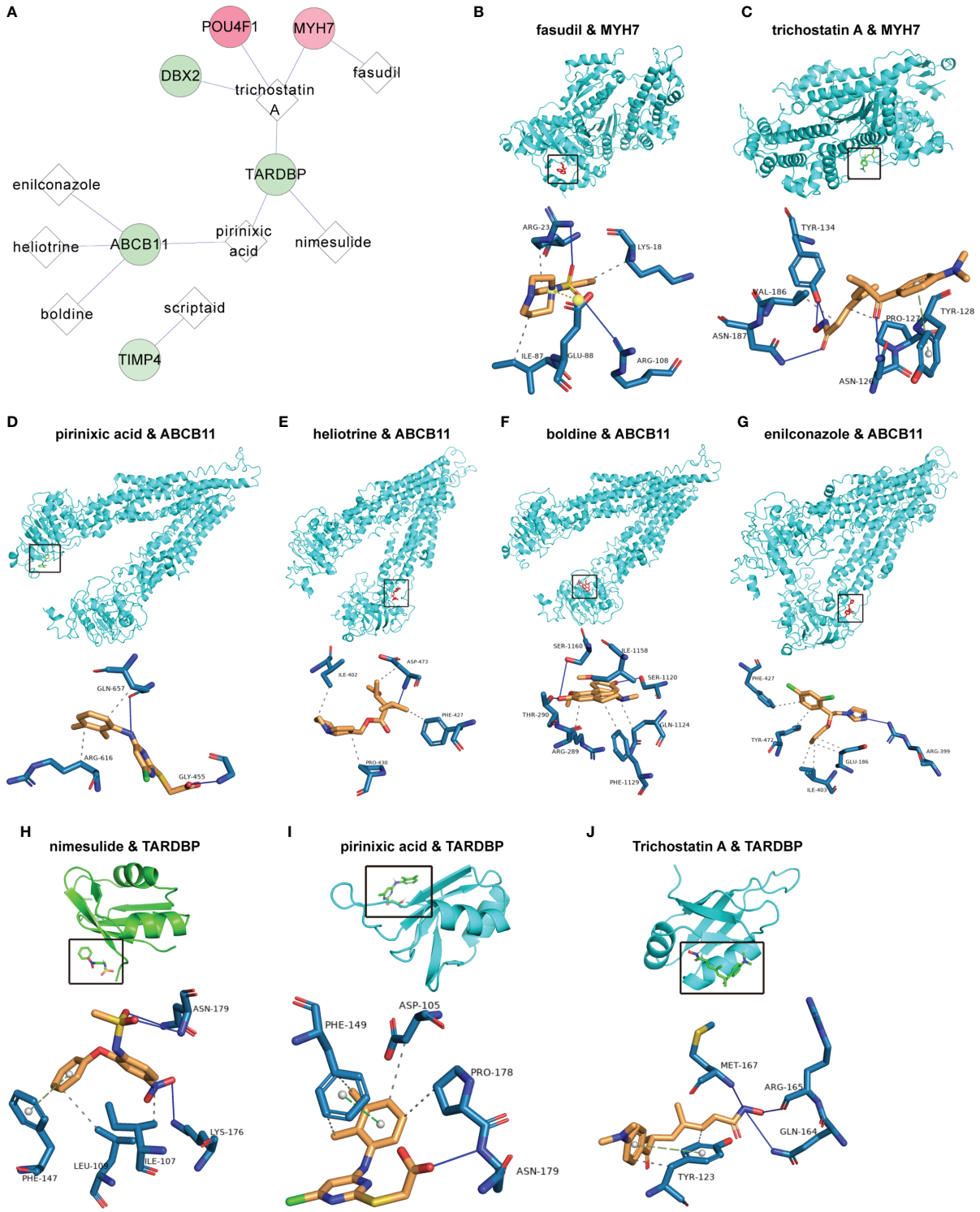

FIGURE 7 | Correlation of the PS model characteristic features with small-molecule inhibitors. **(A)** Network of the associations of characteristic factors with small-molecule drugs. The circles and diamonds represent genes and drug molecules, respectively. **(B–J)** Molecular docking global diagram. Fasudil binding to MYH7 **(B)**, trichostatin A binding to MYH7 **(C)**, pirinixic acid binding to ABCB11 **(D)**, heliotrine binding to ABCB11 **(E)**, boldine binding to ABCB11 **(F)**, enilconazole binding to ABCB11 **(G)**, nimesulide binding to TARDBP **(H)**, pirinixic acid binding to TARDBP **(I)** and trichostatin A binding to TARDBP **(J)** are shown.

TABLE 4 | List of the best molecular docking model scores.

Rank	Molecule	Gene	Affinity
1	fasudil	MYH7	-8.2
2	trichostatin A	MYH7	-8
3	boldine	ABCB11	-6.8
4	pirinixic acid	ABCB11	-6.5
5	enilconazole	ABCB11	-6
6	heliotrine	ABCB11	-5.8
7	trichostatin A	TARDBP	-5.6
8	pirinixic acid	TARDBP	-5.1
9	nimesulide	TARDBP	-4.6

An affinity less than -5 indicates that the docking result was more reliable.

factors are mainly studied in the context of other cancers. DBX2, a hypermethylated gene in the ctDNA of HCC patients, was identified as a potential biomarker and shows great promise for liquid biopsy applications in the future (39). It is significantly upregulated in HCC tissues and plays significant roles in the proliferation and metastasis of HCC cells by activating the Shh pathway (40). MHY7 mutations may play an important role in EBV-associated intrahepatic cholangiocarcinoma (41). TRHDE is reported to be a DNA methylation marker of oral precancer progression (42). Overexpression of the long noncoding RNA TRHDE-AS1 was shown to inhibit the progression of lung cancer *via* the miRNA-103/KLF4 axis (43). LHFPL5 is a member of the lipoma HMGIC fusion partner (LHFP) gene family and can cause deafness in humans. It is proposed to function in hair bundle morphogenesis. Studies have indicated that these biomarkers play important roles in the occurrence and development of tumours. In addition, our study found that some drugs could target these prognostic biomarkers by AutoDock software, including MYH7 targeted by fasudil and trichostatin A, ABCB11 targeted by boldine, pirinixic acid, enilconazole and heliotrine, and TARDBP targeted by trichostatin A, pirinixic acid and nimesulide. Previous studies showed that fasudil, as a ROCK inhibitor, could control tissue mechanics *via* regulation of Capzb in liver homeostasis (44). Trichostatin A is A histone deacetylase inhibitor with anti-breast cancer activity (45). Besides, Bodine has hepatoprotective, cell-protective, antipyretic and anti-inflammatory effects by antagonizing 5-HT3 receptors, and also against glioma cell lines (46, 47). Pirinixic acid inhibit 5-lipoxygenase activity and reduce vascular remodelling in the cardiovascular system (48). Some researches also found that enilconazole inhibited metastatic colorectal cancer through inhibition of TGF-β (49). What is more, nimesulide inhibited lung tumor growth through selective cyclocxygenase-2 (50). Therefore, it is necessary to perform experimental studies to elucidate the relevant pathogenesis and biological regulatory pathways in TNBC. Further studies need to be performed.

The current research has the following advantages. First, the prognostic nomograms provide a convenient way to estimate mortality at different time points. Second, a free network calculator of TNBC patient OS was developed and maintained to facilitate the assessment of individual mortality. Finally, as a noninvasive predictive tool, predictive nomograms provide an alternative for TNBC patients who cannot tolerate or do not want to undergo surgery.

Additionally, this study does have some limitations. First, the current study used gene expression data from the gene chip detection platform and lacks basic experimental verification. Second, the research data were generated on testing platforms, thereby affecting the repeatability of the results in different populations. Third, the model and validation data sets did not contain detailed research information, such as the drug treatment regimens and other postoperative treatments, which may affect the treatment efficacy and clinical prognosis.

Briefly, our research revealed potential molecular biological regulatory pathways and prognostic biomarkers through a ceRNA regulatory network. In particular, our prognostic model based on mRNAs in the ceRNA network showed a substantial ability to improve the prediction of OS in TNBC patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was not required because researchers are allowed to download and analyse data from these databases for scientific purposes.

AUTHOR CONTRIBUTIONS

FQ, Y-SZ, PL and WQ designed the study, analysed the data and wrote the manuscript. FQ and JL collected the follow-up information and performed the clinical data analysis. FQ, JL, and WQ performed the bioinformatics analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (grant nos. 81772590 and 81572395).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.681946/full#supplementary-material

Supplementary Table 1 | Clinical information of the samples obtained from the TCGA and Metabric databases.

Supplementary Table 2 | DERs obtained from meeting the threshold conditions.

Supplementary Table 3 | Connection relationship of targeted ceRNA regulatory network.

Supplementary Table 4 | Connection relationship of coexpression ceRNA regulatory network.

Supplementary Table 5 | mRNAs in ceRNA network associated with survival prognosis.

Supplementary Table 6 | PS score and grouping in training data set and verification data set.

Supplementary Table 7 | Correlation coefficient and *p* value between clinical characteristics and 8 factors.

Supplementary Table 8 | Correlation of the PS model characteristic features with small-molecule inhibitors from CTD database.

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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: 06 July 2021
doi: 10.3389/fonc.2021.695077

Piwi-Interacting RNAs: A New Class of Regulator in Human Breast Cancer

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P-element-induced wimpy testis (Piwi)-interacting RNAs (piRNAs) are a class of germline-enriched small non-coding RNA that associate with Piwi family proteins and mostly induce transposon silencing and epigenetic regulation. Emerging evidence indicated the aberrant expression of Piwil proteins and associated piRNAs in multiple types of human cancer including breast cancer. Although the majority of piRNAs in breast cancer remains unclear of the function mainly due to the variety of regulatory mechanisms, the potential of piRNAs serving as biomarkers for cancer diagnosis and prognosis or therapeutic targets for cancer treatment has been demonstrated by *in vitro* and *in vivo* studies. Herein we summarized the research progress of oncogenic or tumor suppressing piRNAs and their regulatory mechanisms in regulating human breast cancer, including piR-021285, piR-823, piR-932, piR-36712, piR-016658, piR-016975 and piR-4987. The challenges and perspectives of piRNAs in the field of human cancer were discussed.

Keywords: piRNA, tumorigenesis, breast cancer, cancer stem cell, PIWI

OPEN ACCESS

Edited by:
Wenwen Zhang,
Nanjing Medical University, China

Reviewed by:
Keqiang Zhang,
City of Hope National Medical Center, United States
Hamidreza Montazeri Aliabadi,
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Specialty section:
This article was submitted to Cancer Genetics, a section of the journal Frontiers in Oncology

Received: *14 April 2021*
Accepted: *15 June 2021*
Published: *06 July 2021*

Citation:
Qian L, Xie H, Zhang L, Zhao Q, Lü J and Yu Z (2021) Piwi-Interacting RNAs: A New Class of Regulator in Human Breast Cancer. Front. Oncol. 11:695077. doi: 10.3389/fonc.2021.695077

INTRODUCTION

P-element-induced wimpy testis (Piwi)-interacting RNAs (piRNAs) are a class of endogenous non-coding RNA with 26-32nt in length that associate with Piwi family proteins and are specifically expressed and enriched in mammalian germ cells (1). Although piRNA sequences were originally discovered from the repetitive genomic elements of D. melanogaster germline in 2001 by Aravin et al. (2), until 2006 a class of highly abundant small RNAs around 30 nt in length were identified by Girard et al. in mammalian testes, binding to MIWI, a murine Piwi protein, and thereby was named piRNA (3). piRNAs can be derived from transposons, mRNAs or long non-coding RNAs (lncRNAs). Up to date, thousands of piRNA sequences have been identified in germ cells. However, their function mostly remains unknown (4).

Literature has revealed the complexity of the biogenesis of piRNAs, in which a vast amount of proteins are involved (2, 3, 5). There are two approaches have been well demonstrated for the biogenesis of piRNAs including primary biogenesis from cluster transcripts and secondary biogenesis from the ping-pong cycle (5). After transcription from genomic loci carrying transposon fragments, the cluster transcripts are spliced into piRNA precursors (pre-piRNAs), followed by the transit from the inner side to the outer side of the nuclear pore associated with some ribonucleic proteins, such as Nuclear Export Factor 2 (Nxf2) and its co-factor Nuclear Transport Factor 2-like Export Factor 1 (Nxt1) (6). Then pre-piRNAs are either processed through Zucchini (Zuc)-dependent mechanism with Piwi protein involved, or processed through a ping-pong cycle with Aubergine (Aub) and Argonaute3 (Ago3) involved (5). Notably, processing of pre-piRNAs

usually takes place within a specialized subcellular perinuclear structure, termed nuage in mammals and Yb bodies in Drosophila, locating around the mitochondria in the cytoplasm (6, 7). Vret, as one of the most important proteins in both Yb bodies and nuage, has been shown to be required for generation of Piwi-bound piRNAs and localization of Piwi proteins (8).

In germline, a few of piRNAs have been demonstrated to play important roles in regulating germ stem cell maintenance, spermatogenesis, meiosis, transposon silencing, genome rearrangement, and genomic integrity by piRNA-induced silencing complexes (1, 3). In addition, some piRNAs were reported to regulate heterochromatin formation, DNA methylation, and gene expression at transcriptional or post-transcriptional levels (9, 10). In particular, those piRNAs derived from pseudogenes or antisense transcripts usually show regulation of the corresponding endogenous genes (4).

ABERRANT EXPRESSION OF piRNAs AND PIWI MEMBERS IN CANCER

In addition to the enrichment of piRNAs in germ cells, emerging evidence indicates the existence of piRNAs in the somatic stem cells and tumor cells (11, 12). Connections between piRNA/Piwi complex and tumorigenesis have been frequently reported (12–14). Aberrant expression of piRNAs and Piwi proteins in cancer cells may be an indication of the involvement of piRNAs in the regulation of cancer development and progression.

The Ago/Piwi family is not only required for germline development, but also plays central roles in transcriptional and posttranscriptional gene regulation and transposon silencing mediated by piRNAs. In D. melanogaster, the Piwi family consists of Piwi, Aub and Ago3. In the human testis, four homologues Hiwi, Hili, Hiwi2, and Piwil3 have been identified as Piwi family members. Their function in germline has been implicated, but the somatic function remains unclear.

Martinez et al. analyzed 6,260 human piRNA transcriptomes derived from non-malignant and tumor tissues of 11 organs, and discovered 273 and 522 piRNAs with expression in somatic non-malignant tissues and corresponding tumor tissues, respectively, which were able to not only distinguish tissue-of-origin, but also distinguish tumors from non-malignant tissues in a cancer-type specific manner (15). A recent study identified the increased levels of circulating miR-1307-3p, piR-019308, piR-004918 and piR-018569 in the serum exosomes of gastric cancer patients (16). Moreover, higher levels of piR-004918 and piR-019308 were found in the gastric cancer patients with metastasis than non-metastatic patients, indicating that circulating piRNAs in serum are promising non-invasive diagnostic biomarkers for gastric cancer patients and potential markers for monitoring metastasis (16). Wang et al. screened piRNAs in the serum from 7 patients with colorectal cancer and 7 normal controls using small RNA sequencing, followed by QRT-PCR validation of the differentially expressed piRNAs in a training cohort of 140 patients with colorectal cancer (17). As a result, piR-020619 and piR-020450 showed upregulation in the serum of patients. Meanwhile, their expression was also analyzed in 50 patients with lung cancer, 50 with breast cancer, and 50 with gastric cancer, but did not show change in serum of patients with lung, breast, and gastric cancer, indicating the specificity of piR-020619 and piR-020450 as biomarkers for early detection of colorectal cancer (17).

piRNAs also showed regulation by transcriptional factors in human cancer. For example, upregulation of piR-34871 and piR-52200 and downregulation of piR-35127 and piR-46545 by the Ras Association Domain Family Member 1C (RASSF1C) were reported in the lung cancer cell line H1299 and lung tumor tissues (18). Overexpression of piR-35127 and piR-46545 and knockdown of piR-34871 and piR-52200 significantly reduced cell proliferation in both lung cancer cell lines (A549 and H1299) and breast cancer cell lines (Hs578T and MDA-MB-231) (18). Notably, RASSF1C, known as an oncogene, was reported to regulate Piwil1 in lung cancer (19). In MDA-MB-231 breast cancer cells, "cancer-testis gene" Glycerol-3-phosphate acyltransferase-2 (GPAT2) showed regulation on the expression of piRNAs and tRNA-derived fragments. The most GPAT2-regulated piRNAs are single copy in the genome and previously found to be upregulated in cancer cells (20).

Although Piwi-like (Piwil) genes including Piwil1, Piwil2, Piwil3, and Piwil4 have been detected in various types of cancer tumors, such as renal cell carcinoma (21) and breast cancer (22), overexpression of Piwil1 was the most frequently reported in cancers including lung cancer (23), gastric cancer (24), renal cancer (25) and colorectal cancer (26), in which high levels of Piwil1 showed significant correlation with short survival and/or poor prognosis in the patients with cancers (23–26). Piwil2 is highly expressed in glioma, and correlates with poor patient prognosis. Piwil2 plays an important role in the transformation of cervical epithelial cells to tumor-initiating cells by epigenetic regulation (27). In human breast cancer, Piwil2 overexpression was frequently reported to associate with piRNAs (28–30), functioning as an oncogene. Piwil3 showed a tumor-type dependent function as an oncogene or tumor suppressor. In glioma tissues, Piwil3 is downregulated, and negatively associated with pathological grade (31). In gastric cancer, overexpression of Piwil3 promoted cell proliferation, migration, and invasion *via* the JAK2/STAT3 signaling pathway (32). In malignant melanoma, upregulation of Piwil3 was association with cancer aggressiveness and progression (33). For Piwil4, Wang et al. reported high expression of Piwil4 in both breast cancer tissues and MDA-MB-231 breast cancer cell line. Knockdown of Piwil4 in MDA-MB-231 cells dramatically suppressed the cell migration and proliferation through regulating TGF-β and FGF signaling pathways and MHC class II proteins (34). In hepatocellular carcinoma, co-expression of Piwil2/Piwil4 has potential as an indicator for tumor prognosis (35).

REGULATORY FUNCTION OF piRNAs AND PIWI MEMBERS IN CANCER

Although the oncogenic function of Piwil1 in gastric cancer cells was reported to be independent of its partner piRNAs (36), Piwi

proteins have showed significant synergy with related piRNAs in regulating human cancer (28, 29). Complex formed by piRNAs binding to Piwi proteins, such as piR-651/Piwil 1 in gastric cancer (24), piR-54265/Piwil 2 in colorectal cancer (37), piR-932/Piwil 2 in breast cancer (29), have been demonstrated to regulate cell proliferation, invasion and metastasis of cancer cells.

Overexpression of piR-651 was reported in gastric cancer compared to normal control tissues. Gastric cancer cells were arrested at G2/M phase after knockdown of piR-651 (12). Upregulation of piR-823 and its oncogenic function were reported in both esophageal cancer and breast cancer (38, 39). In the luminal subtype of breast cancer cells, overexpression of piR-823 increased the expression of DNA methyltransferase DNMT1, DNMT3A, and DNMT3B, promoted DNA methylation of gene adenomatous polyposis coli (APC), thereby activating Wnt signaling and inducing cancer cell stemness (38). In esophageal squamous cell carcinoma, piR-823 showed significantly upregulation in tumor tissues, compared with matched normal control. The level of piR-823 was significantly associated with lymph node metastasis. In addition, a positive correlation between piR-823 and DNMT3B expression was observed in esophageal cancer (39). However, a recent publication showed tumor repression function of piR-823 in gastric cancer (40), indicating the complexity of the piRNA functions in a cancer type-dependent manner.

In addition to the epigenetic regulation of piRNAs in control of cancer development and progression (15, 22, 38), piRNAs involve in cancer regulation by altering the expression of cancer-related genes in a mechanism similar to microRNA (miRNA). For example, in lung cancer cells piR-55490 was demonstrated to bind with 3′UTR of mTOR mRNA and induce its degradation (41). Like the "seed sequence" of miRNAs, the 5' end of piR-55490 can be complementary to the 3'UTR of mTOR mRNA. In addition, piR-55490 was found to be silenced in lung carcinoma specimens and cell lines, compared with normal controls. Moreover, the expression level of piR-55490 showed a negatively correlation with patients' survival. Restoration of piR-55490 suppressed cell proliferation in lung cancer by suppressing Akt/mTOR pathway (41).

Overall, evidence demonstrating the potential clinical significance of piRNA and Piwi proteins as diagnostic tools, therapeutic targets, and/or prognosis biomarkers in cancer is increasing. The relevant progress in cancer has been recently reviewed (42, 43). Herein, we highlighted the research progress and clinical potential of piRNAs in human breast cancer.

piRNAs IN BREAST CANCER

Breast cancer is the most common cancer and the leading cause of cancer-related death in women all over the world. Abnormal expression of some piRNAs has been reported in breast cancer cells and tissues, showing relevance to the tumor development and progression (**Table 1**). Those aberrantly expressed piRNAs in breast cancer including piR-4987, piR-021285, piR-823, piR-932, piR-36712, piR-016658 and piR-016975 may held potential to be developed as biomarkers and/or therapeutic targets in breast cancer. Our recent study also identified 415 piRNA sequences from the medium of luminal subtype of human breast cancer cell MCF-7, in which 27 piRNAs showed deregulation by pro-oncogene cyclin D1 (28). Huang et al. reported 4 piRNAs including piR-4987, piR-20365, piR-20485 and piR-20582 with upregulation in tumor tissues compared with matched non-tumor tissues in 50 patients with breast cancer (14). Moreover, upregulation of piR-4987 showed association with lymph node positivity (14). Hashim et al. identified over 100 piRNAs in breast cancer cell lines and tumor biopsies by a small RNA-Seq analysis (48). A mRNA targets analysis using piRNome tool further revealed 10 piRNAs with a specific expression pattern in breast tumors targeting key cancer cell pathways (48), in which DQ596670, DQ598183, DQ597341, DQ598252, and DQ596311 were downregulated, while DQ598677, DQ597960, and DQ570994 were upregulated in tumor tissues of breast cancer. Park et al. and Lee et al. applied molecular beacons (a synthetic structured DNA oligonucleotide probe) to detect piR-36026 and/or piR-36743 in a single breast cancer cell, showing their different expression in a cancer cell subtype-dependent manner (49, 50). Kärkkäinen et al. performed small RNA sequencing analysis in 227 fresh-frozen breast tissue samples (51). Three small RNAs annotated as piRNA database entries (DQ596932, DQ570994, and DQ571955) were detected in the tumor samples, all showing upregulation in grade III tumors. Furthermore, patients with estrogen receptor positive and DQ571955 high had shorter relapse-free survival, suggesting DQ571955 as a potential marker for predicting radiotherapy response in estrogen receptor positive

TABLE 1 | Deregulated piRNAs in human breast cancer.

piRNA ID	up/down	Regulatory Function	Mechanism	Reference
piR-021285	up	Promoting invasiveness	DNA methylation	(44)
piRNA-823	up	Promoting cancer cell stemness	DNA methylation/Wnt Signaling	(38)
piRNA-932	up	Promoting EMT and metastasis	DNA methylation	(29)
piR-016658	up	Upregulated in cancer stem cells	N/A	(28)
piR-651	up	N/A	Estrogen regulation	(45)
piR-4987	up	Correlation with lymph node positivity	N/A	(14)
piR-20365, piR-20485, piR-20582	up	N/A	N/A	(14)
piR-36712	down	Suppressing EMT/Related with chemoresistance	Competing endogenous RNAs	(46)
piR-016975	down	Downregulated in cancer stem cell	N/A	(28)
piR-FTH1	down	Promoting chemosensitivity	Targeting FTH1 mRNA	(47)

N/A, not available.

breast cancer. In addition, DQ570994 showed potential for predicting tamoxifen and chemotherapy response (51). In triple negative breast cancer (TNBC), neoadjuvant chemotherapy (NACT) is increasingly applied to the therapy management due to its positive association with response rates of patients. A recent work reported the potential of circulating piR–36743, miR–17, –19b and –30b as diagnostic biomarkers in the monitoring of NACT–driven complete clinical response in TNBC (52). Koduru et al. used public database (small RNA sequencing data) derived from 24 TNBC tumors and 14 adjacent normal tissue samples, and identified a group of differentially expressed miRNAs, piRNAs, lncRNAs and sn/snoRNAs. The top five upregulated piRNAs in tumor tissues are piR-21131, -32745, -21131, -1282, -23672, and top five downregulated piRNAs are piR-23662, -26526, -26527, -30293 and -26528 (53).

Estrogen signaling has been well demonstrated to play important roles in tumor development and progression of breast cancer through interacting with two receptors ERα and ERβ. ERα, as a predominant endocrine regulatory protein in estrogen-induced breast cancer, interacts with non-coding RNAs including piRNAs (54). However, the interaction between ERβ and non-coding RNAs in breast cancer remains unclear. Alexandrova et al. performed sncRNA sequencing analysis on ERβ-expressing TNBC cell lines and 12 ERβ+ and 32 ERβ– TNBC tissue samples (55). A group of ERβ-regulated small ncRNAs was identified in TNBC, including miR-181a-5p and piR-31143 with aberrant upregulation in the ERβ+ tumor samples (55)

Although hundreds of piRNAs have been identified in human breast cancer, only a few of them including piR-021285, piR-823, piR-932, piR-36712, piR-016658 and piR-016975 were determined with the regulatory function and molecular mechanisms, as described in detail below.

piR-021285 in Breast Cancer

piR-021285 is the first piRNA showing regulation of human tumorigenesis in breast cancer *via* an epigenetic mechanism of DNA methylation (44). A genome-wide methylation screening analysis in the piR-021285 mimic-transfected MCF7 cells revealed significant induction of DNA methylation in a number of breast cancer-related genes including ARHGAP11A, which was associated with increased transcription of ARHGAP11A and enhanced invasiveness of MCF-7 cells (44). The expression and/or function of piR-021285 in other types of cancer have yet to be determined.

piRNA-823 in Breast Cancer

piR-823 is another piRNA having regulatory function in human breast cancer through epigenetic mechanism. The carcinogenicity of piR-823 in malignant breast cancer may be related with estrogen status since external administration of estrogen increased piR–823 expression in estrogen receptor negative MDA-MB–231 cells, while reduced the piR–823 levels in estrogen receptor positive MCF-7 cells (45). Further study demonstrated oncogenic function of piR-823 in estrogen receptor positive luminal subtype of breast cancer *via* regulating cancer cell stemness mediated by altered DNA methylation and activated Wnt signaling (38).

In comparison with breast cancer, several other cancer types also showed regulation by piR-823. For example, Increased piRNA-823 expression was associated with late stages and poor prognosis of multiple myeloma. piRNA-823 in the multiple myeloma-derived extracellular vesicles was demonstrated to promote tumorigenesis through re-educating endothelial cells in the tumor microenvironment (56). In colorectal cancer, piRNA-823 increased cell proliferation, invasion and apoptosis resistance through inhibiting the ubiquitination of HIF-1α, thereby upregulating the glucose consumption of cancer cells and inhibiting intracellular reactive oxygen species (57).

piR-932 in Breast Cancer

In addition to piR-021285 and piR-823, piR-932 also showed overexpression in human breast cancer, playing tumor-promoting roles through epigenetic mechanism (29). In Piwil2 positive breast cancer stem cells, piR-932 could bind with Piwil2 to suppress the expression of Latexin by promoting methylation of the CpG island at its promoter region. In view of the negative regulation of cancer stem cells by Latexin (58), piR-932/Piwil2 could be the potential targets for suppressing the progression of breast cancer. Up to date, piR-932 has not been reported in other types of cancer.

piR-36712 in Breast Cancer

piR-36712 was reported having a lower expression level in breast cancer tumors than that in normal tissues, functioning as a tumor suppressor but dependent on the expression of SEPW1 and p53 (46). piRNA-36712 inhibited SEPW1 through interacting with its pseudogene SEPW1P, which thereafter suppressed p53, leading to the upregulation of Slug but downregulation of p21 and E-cadherin, thus performing oncogenic function in human breast cancer. In addition, piR-36712 showed involvement in chemo-sensitivity of breast cancer cells in response to paclitaxel or doxorubicin (46). There is still no literature about piR-36712 study in other cancers.

piR-016658 and piR-016975 in Breast Cancer

Our previous study found upregulation of piR-016658 and downregulation of piR-016975 by cyclin D1 in human breast cancer (28). Further analysis indicated the correlation of piR-016658 and piR-016975 with breast cancer cell stemness. High levels of piR-016658 were found in the basal-like breast cancer cells, as well as Aldehyde dehydrogenase 1 (ALDH1) positive breast cancer stem cells isolated from breast cancer tumors. In contrast, lower levels of piR-016975 were determined not only in basal-like subtype of breast cancer cells compared to luminal subtype, but also in breast cancer stem cells compared to non-stem breast cancer cells. In view of the germ stem cell maintaining function of piRNAs in germline, this study demonstrated that piRNAs are able to regulate cell stemness characteristics in human breast cancer, thereby regulating tumor growth and progression.

piR-FTH1 in Breast Cancer

Ferritin heavy chain (FTH1), as a key regulator of iron metabolism, was recently identified as a favorable prognostic gene for patients with TNBC. Fth1 levels are associated with the progression of breast cancer and chemo-sensitivity of breast cancer cells (59). Balaratnam et al. discovered a human piRNA piR-FTH1 with sequence

complementary to Fth1 mRNA in human somatic cells. In the tested cancer cells, piR-FTH1 and Fth1 showed inverse correlation in expression. In addition, piR-FTH1 can downregulate the Fth1 expression at post-transcriptional level in TNBC cells *via* a HIWI2/HILI mediated mechanism (47).

FUTURE PERSPECTIVE

It has been widely acknowledged that the cell of origin of cancer is a deregulated somatic cell that loses normal regulatory mechanism and reproduces itself without control. The primitive cancer life cycle contains primary cancer stem cells, somatic cells and reproductive cells (60). In view of the similarity between cancer stem cells and germ cells of stemness properties and reproductive ability, there are some regulatory mechanisms in common shared by the two cell types. Although piRNAs were originally identified as a group of germline-specific non-coding small RNAs, emerging evidence revealed their aberrant expression in human cancers. Functional assays indicated the regulation of cancer cell proliferation, EMT, metastasis and cancer stem cells by piRNAs. Overall, piRNAs may have potential to be developed as biomarkers for cancer diagnosis and prognosis, and/or as therapeutic targets for cancer treatment.

Although the studies of piRNAs and Piwi family members in cancer are adding a novel page in the history of cancer research, currently the majority of piRNAs remains unclear of their function in regulating human cancer. There are several key challenges about piRNAs that we are facing, and need to solve, including 1) whether biogenesis of piRNAs in cancer cells shares similar approaches with that in germline; 2) what is the main regulatory mechanism of piRNAs in control of cancer development and progression; 3) how to predict the target DNAs of piRNAs when epigenetically regulating gene expression. It is hoped that with these challenges addressed and the regulatory mechanisms revealed, along with the application of piRNAs in suppression of tumorigenesis and cancer progression in the animal models, piRNAs and Piwi proteins may lead to novel therapeutic approaches in treatment of cancer, including breast cancer.

AUTHOR CONTRIBUTIONS

LQ, HX, LZ, QZ, and JL wrote a part of the draft. ZY designed the work and revised the draft. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grant from the National Key Research and Development Program of China Stem Cell and Translational Research (2016YFA0101202); grants 81772810 and 81972476 (YZ), 82002789 (JL) from the National Natural Science Foundation of China; Grant 20JC1410400 from Science and Technology Commission of Shanghai Municipality; grant 19DZ2251000 from Shanghai Engineering Research Center of Artificial Heart and Heart Failure.

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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

SYSTEMATIC REVIEW
published: 09 July 2021
doi: 10.3389/fonc.2021.691317

The Regulation Network and Clinical Significance of Circular RNAs in Breast Cancer

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OPEN ACCESS

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

Received: *06 April 2021*
Accepted: *23 June 2021*
Published: *09 July 2021*

Citation:
Xu J, Chen X, Sun Y, Shi Y, Teng F, Lv M, Liu C and Jia X (2021) The Regulation Network and Clinical Significance of Circular RNAs in Breast Cancer. Front. Oncol. 11:691317. doi: 10.3389/fonc.2021.691317

Breast cancer is one of the most common malignant tumors in women worldwide. Circular RNA (circRNA) is a class of structurally stable non-coding RNA with a covalently closed circular structure. In recent years, with the development of high-throughput RNA sequencing, many circRNAs have been discovered and have proven to be clinically significant in the development and progression of breast cancer. Importantly, several regulators of circRNA biogenesis have been discovered. Here, we systematically summarize recent progress regarding the network of regulation governing the biogenesis, degradation, and distribution of circRNAs, and we comprehensively analyze the functions, mechanisms, and clinical significance of circRNA in breast cancer.

Keywords: circRNA, breast cancer, biogenesis, degradation, distribution, oncogenic, tumor suppressive, biomarker

INTRODUCTION

Breast cancer is the most common female malignancy worldwide. According to authoritative cancer statistics in 2021 (1), breast cancer has become the most prevalent of all cancers. An abundance of epidemiological studies has led to the identification of a variety of risk factors for developing breast cancer, including age, family history, early menarche, late pregnancy and menopause, high estrogen level, and excessive dietary fat intake (2).

Breast cancer can be generally divided into four molecular subtypes according to the status of the expression of the joint hormone receptors (estrogen receptors [ER] and progesterone receptors [PR]) and of the human epidermal growth factor receptor 2 (HER2). These subtypes include Luminal A, in which hormone receptors are expressed but HER2 is not (ER+PR+HER2−); Luminal B, in which hormone receptors and HER2 are expressed (ER+PR+HER2+); HER2 overexpression, in which hormone receptors are absent but HER2 is expressed (ER-PR-HER2+); and triple negative breast

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; BC, breast cancer; LA, luminal A; TNBC, triple-negative breast cancer; NMGT, normal mammary gland tissues; BCLM, breast cancer liver metastases; IPSC, induced pluripotent stem cells; HR, hormonal receptor; TNM, topography, lymph node, metastasis; OS, overall survival; DFS, disease free survival; RFS, recurrence-free survival; BCSC, breast cancer stem cell; EMT, epithelial–mesenchymal transition; PTX, paclitaxel; AUC, area under curve; DALI, distal-Alu-long-intron; SRD, structure-mediated RNA decay; RBP, RNA binding proteins; IRES, internal ribosome entry site; AUC, area under curve.

cancer (TNBC), in which all of the noted receptors are absent (ER-PR-HER2−). Endocrine and targeted therapies can be effective for ER+ or HER2+ breast cancer patients. These treatments include the use of tamoxifen to block the effects of estrogen and trastuzumab to target HER2 receptors on breast cancer cells (3, 4). However, such treatments are ineffective for TNBC, and chemotherapy is regarded as the main systematic treatment for TNBC. Because the efficacy of chemotherapy is unsatisfactory, there exists a desperate need for effective therapies for TNBC (5). Therefore, it is important to explore new treatment regimens to improve current therapeutic strategies, especially for TNBC patients.

One potentially underappreciated class of biological molecules that may yield effective therapeutic targets is circular RNAs (circRNA). CircRNAs generally form single-stranded covalently closed circular structures by the joining of the 3′ and 5′ ends (6). Two key pathways leading to circRNA formation have been proposed in recent authoritative articles. One mechanism involves the backsplicing of exons during transcriptional activities; this splicing is facilitated by base pairing of reverse repeat elements, such as *Alu* elements, located in flanking introns or by RNA binding proteins (RBP). In another key mechanism, circRNAs can be generated from lariat precursors formed in exon-skipping events, as well as from intronic lariat precursors escaping from debranching (7–9). Approximately 80% of circRNAs come from the backsplicing of exons from precursor mRNA or lncRNA (10), but the first circRNA molecules to be discovered, viroids, were found more than 40 years ago to be produced independent of a backsplicing mechanism (11). No matter the mechanism of formation, circRNAs tend to have the following characteristics: (1) CircRNAs are widely and abundantly present in tissues and bodily fluids, and some circRNAs accumulate to a higher level than their respective linear counterparts because of their stable covalently closed structure (9). (2) Many circRNAs are evolutionarily conserved in eukaryotes, in part because they exert critical biological functions (8). (3) Specific circRNAs are typically expressed in a tissue- or cell-specific manner (8, 12).

Improvements in RNA sequencing technology have led to the identification of several types of circRNAs, including exonic circRNAs, which consist of only exon(s), exon-intron circRNAs (EIciRNA), which consist of both exon(s) and intron(s), and intronic circRNAs (CiRNA), which consist of only intron(s) (**Figure 1**). Cytoplasmic exonic circRNAs tend to have much

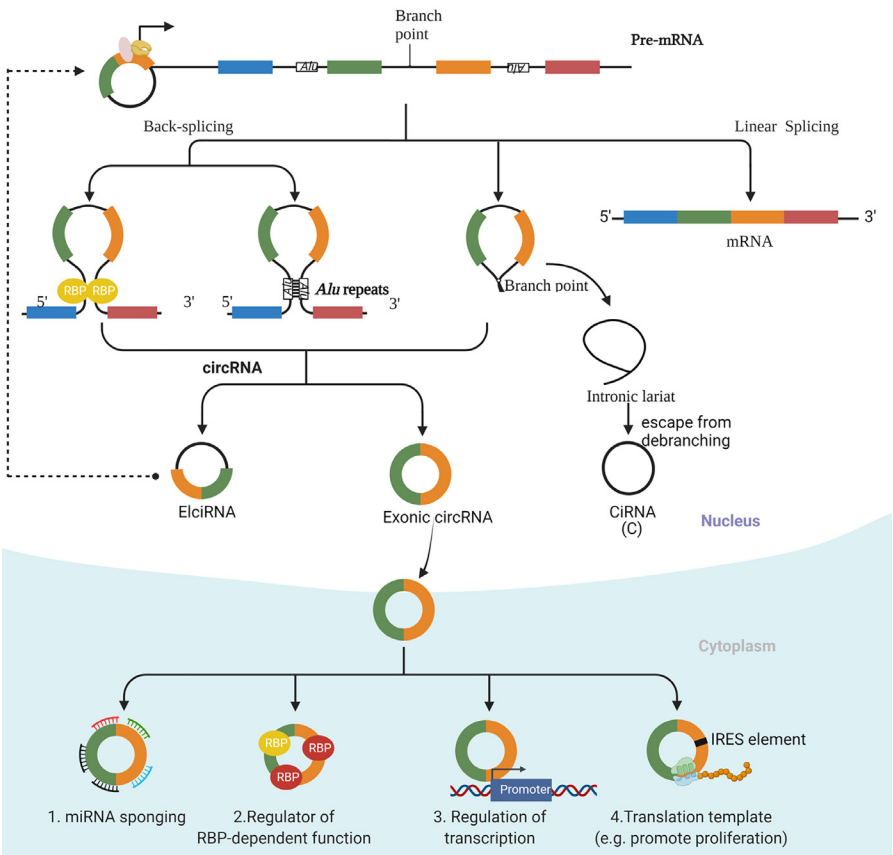

FIGURE 1 | The biogenesis and function of circRNAs. There are three main types of circRNAs, including exonic circRNAs, which consist of only exon(s), exon-intron circRNAs (ElciRNA), which consist of both exon(s) and intron(s), and intronic circRNAs (CiRNA), which consist of only intron(s). An exon-intron circRNA can regulate the expression of its parental gene through binding to its promoter. The mechanism of regulation by exonic circRNAs include serving as a decoy for binding to microRNAs (miRNAs); interacting with specific binding elements on RBPs and acting as a sponge to regulate the functions of RBPs and to affect the activities of associated proteins; regulating transcription; and acting as templates to encode proteins.

longer intracellular half-lives compared with their linear counterparts (half-lives of circRNAs average approximately 48 h as opposed to roughly 10 h for many linear RNA molecules). A generally higher intracellular level of circRNAs than their linear counterparts results from this stability, and the stability can be explained at least partly by the resistance of circular RNA molecules to digestion by exonucleases (13, 14).

Other characteristics that are specific to the size and sequence of particular circRNAs have also been discovered among exonic circRNAs. Jeck *et al.* have reported that the length of a given exon appears to influence circularization, a factor that is especially evident for those circles that consist of a single exon (15). Memczak *et al.* has shown that exonic circRNAs described to date generally involve the GT-AG pair of canonical splice sites (16). Moreover, the flanking intron sites that are involved in the backsplicing process generally have relatively long intron sequences, but some exceptions to the lengths of these sequences exist (9). Finally, the sequence of the circRNA can impact cellular localization and activity, as EIciRNA and CiRNA are mainly located in the nucleus and regulate the transcription of specific genes (17, 18).

The most well-established biological function of circRNA involves its serving as a decoy for binding to microRNAs (miRNAs). This activity of circRNA thus modulates the effects of miRNA effects on their target transcripts. In addition, circRNAs can interact with specific binding elements on RBPs and can act as a sponge to indirectly regulate the functions of RBPs and to affect the activities of associated proteins. Finally, circRNAs can recruit specific proteins to certain loci, such as promoter regions, so as to facilitate the transcription of their own host genes (8, 10). Interestingly, under certain circumstances, some circRNAs with internal ribosome entry site (IRES) elements and AUG sites have been reported to be translated into specific peptides, which points to their underestimated cap-independent translational potency (19) (**Figure 1**).

In recent years, regulators of circRNA biogenesis, degradation, and distribution have been identified with increasing rapidity. In addition, various types of circRNA have been found to be abnormally expressed in breast cancer, and their aberrant expression correlates with the occurrence, development, and prognosis of breast cancer; thus, circRNAs may be an important therapeutic target for breast cancer. In this review, we summarize the biogenesis, degradation, and distribution of circRNAs and the mechanisms of action of their regulators. We focus on cutting-edge discoveries concerning functions of circRNAs as well as associated mechanisms and their clinical significance in breast cancer.

THE REGULATORS OF CIRCRNA BIOGENESIS

RNA Splicing Factor Regulates the Cyclization and Biogenesis of circRNA

CircRNAs are generally formed by backsplicing of mRNA from canonical splice sites (19). Because RNA splicing factors are central to this process, these factors are important to the regulation of circRNA biosynthesis. For example, Ashwal-Fluss *et al.* discovered that circRNA exon cyclization and canonical pre-mRNA splicing are mutually exclusive and that this competition is a tissue-specific and conservative mechanism of regulation of circRNA in animals (20). They found that a circular RNA related to the muscleblind gene (circMbl) and its flanking introns contain conserved binding sites for the MBL protein. Therefore, the level of MBL present strongly affects circMbl biosynthesis, and the authors confirmed that this effect depends on specific binding sites for MBL (20). Similarly, Pagliarini *et al.* found that SAM68 can bind to the distal intron inverted repeat *Alu* sequence of the spinal muscular atrophy gene (*SMN*), thereby supporting circRNA biosynthesis *in vivo* and *in vitro* (21). Splicing factor proline/glutamine-rich (SFPQ) is specifically enriched in introns flanking a type of Distal-Alu-Long-Intron (DALI) circRNA that is characterized by distal inverted *Alu* elements and long flanking introns, and depletion of SFPQ has been shown to significantly repress DALI circRNA production (22).

The importance of circRNA and its regulation have been demonstrated for several cancer types. Li *et al.* for example, found a global downregulation of circRNA levels in hepatocellular carcinoma. They further demonstrated that inhibition of the RNA splicing factor nudix hydrolase 21 (NUDT21) in this cancer type can promote the formation of circRNA and the production of UGUA sequences. Notably, UGUA sequences are crucial for circRNA formation, and thus this system establishes a positive feedback acceleration of circRNA production (23). In another carcinoma, oral squamous cell carcinoma, Zhao *et al.* found that circUHRF1 could bind to and inhibit miR-526b-5p and thus up-regulate the expression of c-MYC. Overexpression of c-MYC then facilitates the transcription of TGF-β1 and epithelial splicing regulatory protein 1 (ESRP1). Interestingly, ESRP1 itself targets flanking introns of circUHRF1, thereby accelerating circUHRF1 cyclization and biosynthesis (24). In gliomas, Liu *et al.* found that splicing factor serine and arginine-rich splicing factor 10 (SRSF10) can bind to *Alu* sequences of DNA surrounding a circular RNA related to ataxin-a (circATXN1) to regulate biosynthesis of this circRNA. Circ-ATXN1 levels were, therefore, decreased significantly upon knocking down of SRSF10 (25).

RNA Binding Proteins Regulate the Biogenesis of circRNA

Several other proteins with RNA binding activity have been shown to affect circRNA levels. For example, the immune factor NF90/NF110 contains double-stranded RNA binding domains and can bind to circRNA in the cytoplasm. In addition, however, the production of circRNA has been shown to increase upon association of nuclear NF90/NF110 with intronic RNA pairs flanking circRNA-forming exons. During viral infection, NF90/NF110 translocates from the nucleus to the cytoplasm, leading to a decrease in circRNA formation (26).

These regulatory abilities have, importantly, been shown to be important mediators in several developmental processes. In lung cancer, trinucleotide repeat-containing gene 6A (TNRC6A) can bind to introns near the exons that form circRNA to regulate

production of circ0006916 (27). In induced pluripotent stem cell (iPSC) cells, the RNA binding protein fused in sarcoma (FU) can bind to introns near circular RNA reverse splicing sites to regulate circRNA biosynthesis (28). In prostate cancer, P53 can regulate activation of RNA binding motif protein 25 (RBM25), thus affecting the binding of RBM25 to circAMOTL1L. This binding is important for the induction of the biosynthesis of circAMOTL1L (29), because RBM25 mainly binds to the splicing complex, which in turn regulates selective splicing in combination with poly-G or exon splicing enhancer 5′-CGGGCA-3′ sequences (30, 31).

In TNBC, transcription factor E2F1 binds to the promoter of the gene that encodes septin-9 (SEPT9) and promotes the transcription of SEPT9 and the biogenesis of circSEPT9. Another transcription factor, EIF4A3, also regulates the biogenesis of circSETP9 by binding to the upstream and downstream sequences of the circSEPT9 exon (32). In hepatocellular carcinoma, RBM3 binds to SCD-circRNA 2 and regulates its biosynthesis, though the actual mechanisms underlying this regulation are not yet clear (33).

m^{6}A Modification Regulates circRNA Biogenesis and Stability

Cis factors are also responsible for the control of biogenesis and stability of circRNAs. A recent study found that backsplicing occurs mostly at sites enriched in N-6-methyladenosine (m^{6}A). For example, in male germ cells, approximately half of the circRNA molecules contain an m^{6}A-modified initiation codon (34). Similarly, in macrophages of patients with acute coronary syndrome, knockdown of the N^6-adenosine-methyltransferase METTL3 can down-regulate the m^{6}A modification of hsa_circ_0029589 and promote biogenesis of this circRNA (35). Specifically relating to cancer, in hepatocellular carcinoma, m^{6}A modification has been found to increase the cellular levels of circ_SORE by enhancing its stability (36).

Di Timoteo *et al.* found that m^{6}A modification regulates the accumulation of circ-ZNF609 by regulating the back-splicing of circ-ZNF609, and they found direct correlations among a requirement for the presence of the METTL3 methyltransferase, binding of YTHDC1, and the back-splicing of the m^{6}A modified exon (37), suggesting that methylation-mediated regulation might be a general phenomenon in circRNAs. Zhou *et al.* found that the m^{6}A reader proteins YTHDF1, YTHDF2, and YTHDC1 bind to m^{6}A-modified circRNA and that binding of YTHDF2 in particular reduces the stability of the circRNA (38). Thus, it can be concluded that m^{6}A modification sites can function together with accessory proteins, including m^{6}A writers, erasers, and readers, to control circRNA biogenesis.

THE REGULATION OF CIRCRNA DEGRADATION

The levels of circRNA can be controlled at both the synthesis and degradation levels. It has been universally acknowledged that the degradation of most mRNAs initiates with poly(A)-tail shortening at the 3′ end, whereas some mRNAs undergo interior cleavage by endonucleases. Because of the lack of a 5′ 7-methylguanosine cap or a 3′ poly (A) tail, the cleavage of circRNAs, on the other hand, are generally dependent on endonucleases, which initiate degradation internally (39, 40). Cleavage of circRNA CDR1 by endonuclease AGO2 has been shown to be assisted by miR-671. This discovery served as the first evidence that some circRNAs can be degraded by endonucleases in a sequence-dependent manner (41).

In general, the pathways leading to circRNA degradation can be divided into five categories: miRNA-guided degradation, structure-mediated RNA decay (SRD), decay mediated by GW182 and its human homolog, specific m^{6}A-modified circRNA decay and endoribonuclease RNaseL-mediated decay. Fischer *et al.* reported that the degradation of some highly structured circRNAs can be regulated by UPF1 and G3BP1, both of which recognize and unwind the overall structures of circRNAs (42). The absence of GW182, which is a key component of P body and RNAi complexes, can lead to the accumulation of endogenous circRNA and increase the steady state of cytoplasmic circRNAs, whereas the absence of other factors in the P body or RNAi complex has no similar effect (43). Further study indicated that the MID domain of GW182 protein may mediate the interaction between circRNAs and circRNA decay factors; the absence of TNRC6A, TRNC6B, or TRNC6C, which are the human homologs of GW182, in HEK293 cells, results in the same accumulation of steady-state circRNAs in human cells, indicating a conserved role of P-body and RNAi-mediated degradation of circRNA (43). Park *et al.* reported that m^{6}A-modified circRNA can recruit the m^{6}A reader protein YTHDF2 as well as the adaptor protein HRSP12, and HRSP12 can serve as the bridge to connect YTHDF2 with the endoribonuclease RNase P/MRP, thus enabling downregulation of m^{6}A modified circRNA by RNase P/MRP (44). Liu *et al.* discovered that RNase L, a widely expressed cytoplasmic endoribonuclease, can be activated upon viral infection through an undefined mechanism and degrade the circRNAs with 16 to 26 bp RNA duplex (45) (**Figure 2**).

REGULATORS OF CIRCRNA DISTRIBUTION

Once produced, the majority of circRNAs are transported to the cytoplasm where they exhibit further biological functions, whereas some intronic circRNAs and exon-intron circRNAs are thought to reside in the nucleus (17, 18). Since circRNAs lack the classical RNA transport sequence, the nuclear transport mechanism of circRNA remains unclear. As existing studies have indicated that the location of circRNAs determines their functions, it is important to continue to explore the potential mechanisms of circRNA nucleus export.

In order to explore circRNA nuclear transporting mechanisms, Li *et al.* knocked down 26 reported RNA transport-related proteins in *Drosophila*. Their results showed that one circRNA, with a length of 1120 bp, remained in the

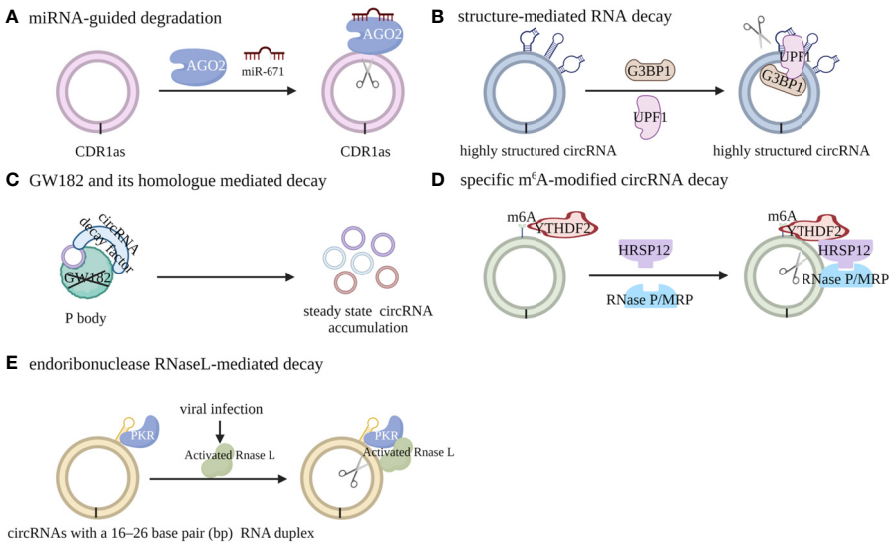

FIGURE 2 | Mechanisms of circRNA degradation. **(A)** CircRNA CDR1 can be cleaved by endonuclease AGO2 with the assistance of miR-671 in a miRNA target sequence-dependent manner. **(B)** The degradation of highly structured circRNAs can be regulated by UPF1 and G3BP1, both of which recognize and unwind the overall structures of circRNAs. **(C)** m^6A-modified circRNA can recruit the m^6A reader protein YTHDF2 as well as the adaptor protein HRSP12, and HRSP12 can serve as a bridge to connect YTHDF2 with the endoribonuclease RNase P/MRP, thus enabling degradation of m^6A modified circRNA by RNase P/MRP. **(D)** The absence of GW182 or its human homologue TRNC6A/B/C, the key component of P body and RNAi complexes, can lead to the accumulation of endogenous circRNA and increase the steady state of cytoplasmic circRNAs. **(E)** Upon viral infection, RNase L can be activated and can degrade circRNAs with RNA duplexes containing 16 to 26 bp through an undefined mechanism.

nucleus, whereas another circRNA, with a length of 490 bp, mainly localized to the cytoplasm after deletion of Hel25E. Both of these circRNAs mainly localize in the cytoplasm in wild-type *Drosophila*. The localizations of 12 other endogenous circRNAs that reside in the cytoplasm in wild-type *Drosophila* were further studied in Hel25E-knockout flies, and it was discovered that circRNAs with lengths of more than 811 bp mainly accumulated in the nucleus, whereas circRNA lengths of less than 702 bp resided in the cytoplasm in cells of the knockout *Drosophila*. An experiment exploring the human Hel25E homologs UAP56 (DDX39B) and URH49 (DDX39A), which share more than 90% similarity with *Drosophila* Hel25E, found that human UAP56 mainly regulates the nuclear transport of long circRNA (more than 1200 bp), whereas URH49 mainly regulates the nuclear export of short circRNA (less than 400 bp). Therefore, the nuclear accumulation of circRNA results mainly from defects of nuclear transport, and the length of circRNA is an important determinant that determines nuclear export (46, 47).

In addition, a very recent study found that m^6A modification can also influence circRNA nuclear export. It was reported that circNSUN2 could bind to the m^6A reader YTHDC1, which interacted with the m^6A binding motif at the GAACU m^6A within the splice junction of circNSUN2 (48). In studies of mRNA splicing, YTHDC1 has been shown to bind to the RNA splicing factors SRSF1, SRSF3, SRSF7, and SRSF10 to regulate mRNA splicing (49). YTHDC1 can also bind to SRSF3 and the classical nuclear receptor NXF1, thereby regulating the metabolism and transport of m^6A modified mRNAs (50), suggesting that circRNA may also share the nuclear transporting mechanisms with mRNA.

CIRCRNA PROFILES IN BREAST CANCER

By using high-throughput RNA sequencing and microarray analysis, profiles of circRNA expression in breast cancer have been comprehensively analyzed. The RNA sequencing or microarray data of circRNA expression profiles in samples related to breast cancer are listed in **Table 1**. Unlike the studies in gastric cancer and hepatocellular carcinoma that found more down-regulated circRNAs than up-regulated ones (30, 66), in breast cancer, more circRNAs have been found to be up-regulated. These results indicated that the regulation of circRNA levels is tissue specific and that circRNAs likely regulate different process in different systems.

THE DIVERSE ROLES OF CIRCRNAS IN BREAST CANCER PROGRESSION AND ASSOCIATED MECHANISMS

Mechanisms Leading to Oncogenic Functions of circRNAs in Breast Cancer

Most reported circRNAs have been proposed to function by acting as molecular sponges that bind to and inhibit the

TABLE 1 | RNA sequencing and microarray analyses of differentially expressed circRNAs in breast cancer.

Samples	Detection method	Differentially expressed circRNAs	References
4 pairs of TNBC and adjacent noncancerous tissues	RNA sequencing	47 were up-regulated and 307 were down-regulated (FC≥2, $P< 0.05$)	(32)
3 pairs of breast cancer and corresponding adjacent non-cancerous tissues	RNA sequencing	85 were upregulated and 67 were downregulated.(FDR ≤ 0.001, FC≥2)	(51)
8 patients' specimens (TNBC, $N = 4$; luminal A, $N = 4$) and 3 normal mammary gland tissues (NMGT)	RNA sequencing	140 upregulated and 95 downregulated circRNA were identified, including 215 and 73 circRNAs for the TNBC and LA subtypes, respectively (FC>1.5, $P<0.05$)	(52)
4 TNBC and 3 NMGT	RNA sequencing	122 upregulated and 93 downregulated circRNA were identified (FC>1.5, $P<0.05$)	(52)
4 LA and 3 NMGT	RNA sequencing	55 upregulated and 18 downregulated circRNA were identified (FC>1.5, $P<0.05$)	(52)
3 pairs of breast cancer tissues with or without metastasis	RNA sequencing	A total of 51 circRNAs were differentially expressed (FC>1.5, P< 0.05)	(53)
4 pairs of TNBC tissues and adjacent non-cancerous tissues	RNA sequencing	47 were upregulated, whereas 307 were downregulated (FC>2.0, $P< 0.05$)	(54)
4 pairs of breast cancer lesions and matched adjacent normal-appearing tissues	microarray	715 circRNAs were upregulated and 440 circRNAs were downregulated (FC≥2, $P<0.05$)	(55)
3 pairs of TNBC tissues and matched normal mammalian tissues	microarray	173 circRNAs were up-regulated, and 77 circRNAs were down-regulated (FC≥1.5, $P<0.05$, FDR< 0.05)	(56)
4 pairs of breast cancer tissues and adjacent noncancerous tissues	microarray	A total of 2,587 circRNAs with 1.5 fold of upregulation or downregulation (FC>1.5, $P<0.05$)	(57)
4 pairs of breast cancer tissues and adjacent normal tissues	microarray	15 circRNAs upregulated and 16 circRNAs downregulated (FC>4.0, $P<0.05$)	(58)
5 pairs of breast tumor tissues and matched non-tumor tissues	microarray	A total of 716 circRNAs that were differently expressed (FC>1.5, $P<0.05$)	(59)
3 pairs of breast cancer tissues and their adjacent non-tumor tissues	microarray	1953 circRNAs were upregulated and 1700 circRNAs were downregulated (FC>2.0 and $P< 0.05$)	(60)
3 pairs of TNBC and adjacent normal tissues	RNA sequencing	1307 circRNAs were up-regulated and 3726 circRNAs were down-regulated (FC≥2, $P< 0.05$)	(61)
5 pairs of breast cancer tissues and corresponding nontumorous tissues	RNA sequencing	30 were up-regulated and 19 were down-regulated (FC>2, $P< 0.001$)	(62)
2 pairs of BCLM tissues and primary tumor tissues	microarray	559 were upregulated and 661 were downregulated (FC>2, $P< 0.05$)	(63)
6 pairs of breast cancer/adjacent tissues	microarray	2,375 were upregulated, whereas 1,995 were downregulated (FC>2.0 or <0.5, $P<0.05$)	(64)
3 pairs of breast cancer tissues and paired noncancerous tissues	microarray	292 were upregulated and 228 were downregulated (FC≥2, P< 0.05)	(65)

BC, breast cancer; LA, luminal A; TNBC, triple-negative breast cancer; NMGT, normal mammary gland tissues; BCLM, breast cancer liver metastases; FC, fold change; FDR, false discovery rate.

functions of miRNA molecules (67, 68). As shown in the RNA sequencing or microarray data, many circRNAs are dysregulated in breast cancer. Among them, the up-regulated circRNAs mainly function as oncogenic factors, whereas the circRNAs down-regulated in breast cancer generally play tumor suppressive roles. The circRNAs that play oncogenic roles in breast cancer are listed in **Table 2**. Here, we discuss several mechanisms through which circRNAs might influence the development or progression of breast cancer.

Acting as Competing Endogenous RNAs (ceRNAs)

A major mechanism that explains impacts of circRNA levels on cancer development involves the interaction of these RNA molecules with miRNA molecules. This binding event, called "sponging," can inhibit the interactions of these miRNA molecules with other cellular molecules, frequently leading to inhibition of the miRNA activity. Thus, circRNAs can serve as ceRNAs. The downstream effect of the competition depends on the ultimate target of the miRNA itself.

Multiple circRNAs can target the same miRNA and thus assist malignant progression of breast cancer. For example, hsa_circ_0006528 and circCDR1as can target CCNE1 and RAF1, respectively, through sponging miR-7. In this way, both of these circRNA molecules promote the proliferation, invasion, migration, and chemoresistance of breast cancer cells (69, 70). Similarly, hsa_circ_0000291, hsa_circRNA_000518, and circ_TFF1 can target ETS1, FGFR1, and TFF1, respectively, through sponging miR-326, to accelerate the proliferation, colony formation, invasion, and migration of breast cancer cells; these activities also serve to reduce cell apoptosis (71, 72, 151). CircZNF609 and circIQCH can target DNMT3A and p70S6K1 by sponging miR-145 to promote the malignant phenotype of breast cancer cells (73, 74). Hsa_circ_0136666 and hsa_circ_0006528 can target CDK6 and CDK8 by sponging miR-1299, respectively, to regulate cell cycle progression, proliferation, apoptosis, invasion, migration, and drug resistance of breast cancer cells (75, 76). Hsa_circ_0052112 and hsa_circ_0001667 mainly regulates ZNF36 and FOXO3a by sponging target miR-125a-5p, respectively, to regulate the

TABLE 2 | CircRNAs that play oncogenic roles in breast cancer.

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
circSEPT9	hsa_circ_ 0005320	645	SEPT6	cytoplasm	promote cell proliferation and migration *in vitro*	miR-637	LIF/STAT3	(32)
circular RNA CDR1as	hsa_circ_0001946	1485	CDR1	\	increase chemosensitivity of 5-FU-resistant BC cells	miR-7	CCNE1	(69)
hsa_circ_0006528	hsa_circ_0006528	496	PRELID2	\	promote adriamycin resistance	miR-7-5p	Raf1	(70)
hsa_circRNA_0006528	hsa_circ_0006528	496	PRELID2	\	promote cell proliferation, invasion, and migration, inhibit apoptosis	miR-7-5p	Raf1-MAPK/ ERK	(71)
hsa_circRNA_0000518	hsa_circ_0000518	150	RPPH1	\	and promote cell proliferation, migration, invasion, inhibit cell cycle arrest, apoptosis, *in vitro*, and promote tumor growth *in vivo*	miR-326	FGFR1	(72)
circIQCH	hsa_circ_0104345	864	IQCH	cytoplasm	promote cell proliferation, migration *in vitro*, and promote tumor growth, and metastasis *in vivo*	miR-145	DNMT3A	(73)
circZNF609	hsa_circ_0000615	874	ZNF609	cytoplasm	promote cell proliferation, invasion and migration *in vitro*, and promote tumor growth *in vivo*	miR-145-5p	p70S6K1	(74)
hsa_circ_0136666	hsa_circ_0136666	477	PRKDC	\	promote cell proliferation	miR-1299	CDK6	(75)
circ_0006528	hsa_circ_0006528	496	PRELID2	\	promote proliferation, migration, invasion, autophagy, and inhibit cell apoptosis of PTX-resistant breast cancer cells	miR-1299	CDK8	(76)
circ_0001667	hsa_circ_0001667	456	HEATR2	\	promote cell proliferation, invasion, and migration *in vitro*	miR-125a-5p	TAZ	(77)
hsa_circ_0052112	hsa_circ_0052112	209	ZNF83	cytoplasm	promote cell migration and invasion *in vitro*	miR-125a-5p	ZNF83	(78)
circHMCU	hsa_circ_0000247	792	MCU	cytoplasm	promote cell proliferation, migration, and invasion *in vitro*, and promote tumor growth and lung metastasis *in vivo*	let-7	MCY, HMGA2, and CCND1	(79)
circ-ABCB10	\	\	ABCB10	\	decrease PTX sensitivity and apoptosis, promote cell invasion and autophagy of PTX-resistant BC cells	let-7a-5p	DUSP7	(80)
circPLK1	hsa_circ_0038632	1708	PLK1	cytoplasm	promote cell proliferation, invasion *in vitro*, and tumor occurrence and metastasis *in vivo*	miR-296-5p	PLK1	(81)
hsa_circ_0000515	hsa_circ_0000515	229	RPPH1	cytoplasm	promote cell proliferation, invasion, angiogenesis, and	miR-296-5p	CXCL10	(82)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
					enhance inflammatory response *in vitro*, promote tumor growth *in vivo*			
hsa_circ_0011946	hsa_circ_0011946	782	SCMH1	\	promote cell migration and invasion	miR-26a/b	RFC3	(51)
circWWC3	hsa_circ_0089866 and hsa_circ_0001910	1068 and 825	WWC3	cytoplasm	promote cell proliferation, migration, and invasion	miR-26b-3p and miR-660-3p	ZEB1	(83)
circ-DNMT1	hsa_circ_0049224	155	DNMT1	cytoplasm	promote cell proliferation	\	interact with p53 and AUF1	(84)
FECR1	\	571	FLI1	both cytoplasm and nucleus	promote cell invasion *in vitro*	\	binds to the FLI1 promoter *in cis* and recruits TET1, binds to and downregulates DNMT1 *in trans*	(85)
circular HER2	hsa_circ_0007766	676	ERBB2	cytoplasm	promote cell proliferation, invasion, and tumorigenesis *in vitro* and *in vivo*	encoded a novel protein, HER2-103	promote homo/ hetero dimerization of EGFR/HER3, sustain AKT phosphorylation	(86)
circIFI30	hsa_circ_0005571	351	IFI30	cytoplasm	promote cell proliferation, migration, invasion, inhibit apoptosis *in vitro*, and promote tumorigenesis and metastasis *in vivo*	miR-520b-3p	CD44	(87)
circAGFG1	hsa_circ_0058514	527	AGFG1	cytoplasm	promote cell proliferation, mobility, and invasion *in vitro*, promote tumorigenesis and metastasis *in vivo*	miR-195-5p	CCNE1	(54)
circKIF4A	hsa_circ_0007255	355	KIF4A	cytoplasm	promote cell proliferation, migration *in vitro*, and promote tumor growth and metastasis *in vivo*	miR-375	KIF4A	(88)
circEPSTI1	hsa_circ_0000479	375	EPSTI1	cytoplasm	promote cell proliferation and inhibit apoptosis	miR-4753 and miR-6809	BCL11A	(56)
circGNB1	hsa_circ_0009362	152	GNB1	cytoplasm	promote cell proliferation, migration *in vitro*, and tumor growth *in vivo*	miR-141-5p	IGF1R	(89)
circ-ABCB10	hsa_circ_0008717	724	ABCB10	\	promote cell proliferation and inhibit apoptosis *in vitro*	miR-1271	\	(57)
hsa_circ_0001982	hsa_circ_0001982	899	RNF111	\	promote cell proliferation, invasion, and inhibit apoptosis	miR-143	\	(59)
circANKS1B	hsa_circ_0007294	459	ANKS1B	cytoplasm	promote cell invasion and migration *in vitro*, promote metastasis *in vivo*	miR-148a-3p and miR-152-3p	USF1-TGF-β1/ SMAD	(61)
hsa_circ_0004771	hsa_circ_0004771	203	NRIP1	\	promote tumor growth and inhibit apoptosis	miR-653	ZEB2	(62)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
circGFRA1	hsa_circ_0005239	427	GFRA1	cytoplasm	promote cell proliferation and inhibit apoptosis *in vitro*	miR-34a	GFRA1	(90)
circRNA_069718	hsa_circ_0069718	590	DCUN1D4	\	promote cell proliferation, invasion *in vitro*	\	reduce the expression of Wnt/β-catenin pathway-related genes	(91)
circular RNA ciRS-7	hsa_circ_0001946	1485	antisense strand of CDR1	cytoplasm	promote cell migration and invasion *in vitro*	miR-1299	MMPs	(92)
circRNA-CER	hsa_circ_0023404	180	RNF121	\	promote cell proliferation and migration *in vitro*	miR–136	MMP13	(93)
circ-RNF111	hsa_circ_0001982	899	RNF111	\	promote cell proliferation, invasion, glycolysis, and PTX resistance in PTX-resistant BC cells *in vitro*, and enhanced PTX sensitivity *in vivo*	miR-140-5p	E2F3	(94)
circACAP2	\	\	\	cytoplasm	promote cell proliferation and motility	miR-29a/b-3p	COL5A1	(95)
circVAPA	hsa_circ_0006990	338	VAPA	cytoplasm	promote cell proliferation, migration, invasion	miR-130a-5p	\	(96)
hsa_circ_002178	\	\	\	cytoplasm	promote cell proliferation, invasion, and migration	miR-1258	KDM7A	(97)
hsa-circ-0083373, hsa-circ-0083374, hsa-circ-0083375	hsa_circ_0083373, hsa_circ_0083374, hsa_circ_0083375	3868,4045,5469	DLC1	\	promote pathogenesis and development of breast cancer	hsa-miR-511	\	(98)
circRNA_100876	\	\	\	\	promote cell proliferation and invasion	miR-4753 and miR-6809	BCL11A	(99)
circ_0103552	hsa_circ_0103552	920	UBR1	\	promote cell proliferation, migration, invasion, and inhibit apoptosis	miR-1236	\	(100)
circMMP11	hsa_circ_0062558	906	MMP11	cytoplasm	promote cell proliferation and migration *in vitro*	miR-1204	MMP11	(101)
circ-UBE2D2	\	\	UBE2D2	\	promote cell proliferation, migration, and invasion *in vitro*	miR-1236 and miR-1287	\	(102)
circ_UBE2D2	hsa_circ_0005728	280	UBE2D2	cytoplasm	promote tamoxifen resistance	miR-200a-3p	\	(103)
circUBE2D2	hsa_circ_0005728	280	UBE2D2	cytoplasm	promote cell proliferation, migration, and invasion, and doxorubicin resistance *in vitro*, promote tumor growth *in vivo*	miR-512-3p	CDCA3	(104)
hsa_circ_0091074	hsa_circ_0091074	373	TCONS_00016926	\	promote cell proliferation and invasion	miR –1297	TAZ/TEAD4	(105)
circ_0000043	hsa_circ_0000043	438	PUM1	\	promote cell proliferation, migration, invasion, and EMT	miR-136	SMAD3	(106)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
hsa_circ_0003645	hsa_circ_0003645	356	C16orf62	cytoplasm	promote cell proliferation and inhibit cell apoptosis *in vitro* and *in vivo*	miR-139-3p	HMGB1	(107)
circCDYL	hsa_circ_0008285	667	CDYL	cytoplasm	promote the malignant progression *in vitro* and *in vivo*	miR-1275	ATG7 and ULK1	(108)
circ_0000520	hsa_circ_0000520	123	RPPH1	cytoplasm	promote cell proliferation, migration, and invasion, but inhibit cell cycle arrest and apoptosis	miR-1296	SP1	(109)
circular RNA KIF4A	hsa_circ_0007255	355	KIF4A	cytoplasm	promote cell migration, invasion, and inhibit apoptosis	miR-152	ZEB	(110)
circABCC4	hsa_circ_0030586	1192	ABCC4	\	promote cell proliferation, migration, invasion, and inhibit apoptosis	miR-154-5p	promote NF-κB and WNT/β-catenin pathways	(111)
circDENND4C	\	\	DENND4C	\	increase glycolysis, migration, and invasion under hypoxia	miR-200b and miR-200c	MMP2, MMP9	(112)
circDENND4C	\	\	\	\	promote cell migration and invasion under hypoxia *in vitro*, promote tumor growth *in vivo*	\	\	(113)
circHIPK3	hsa_circ_0000284	1099	HIPK3	cytoplasm	promote cell proliferation, migration, and invasion *in vitro* and tumor growth *in vivo*	miR-193a	HMGB1/PI3K/AKT	(114)
hsa_circ_001783	\	34460	EBLN3- ZCCHC7	cytoplasm	promote cell proliferation and invasion	miR-200c-3p	\	(115)
circABCB10	hsa_circ_ 0008717	724	ABCB10	\	promote cell proliferation, glycolysis, and increase IR resistance	miR-223-3p	PFN2	(116)
circular RNA PVT1	hsa_circ_0001821	410	PVT1	cytoplasm	promote tumor growth *in vivo*	miR-204-5p	upregulate E-cadherin, downregulate N-cadherin, Vimentin, Slug, and Twist	(117)
circRAD18	hsa_circ_0002453	756	RAD18	cytoplasm	promote cell proliferation and migration, inhibit cell apoptosis *in vitro*, promote tumor growth *in vivo*	miR-208a and miR-3164	IGF1 and FGF2	(118)
circRAD18	hsa_circ_0002453	756	RAD18	cytoplasm	promote cell proliferation, migration, invasion, EMT, and inhibit cell apoptosis	miR-613	HK2	(119)
hsa_circ_0131242	hsa_circ_0131242	12400	MAP3K4	\	promote cell proliferation and migration *in vitro*	miR-2682	\	(120)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
*hsa_circ_0072995	hsa_circ_0072995	435	ARHGEF28	both nucleus and cytoplasm	promote cell migration and invasion	miR-30c-2-3p	ARHGEF28	(121)
circ-TFF1	hsa_circ_0061825	492	TFF1	cytoplasm	promote cell proliferation, migration, invasion, EMT *in vitro*, and tumor growth *in vivo*	miR-326	TFF1	(122)
circ_0000291	hsa_circ_0000291	311	CD44	\	promote cell proliferation, migration, and invasion	miR-326	ETS1	(123)
circular RNA 0007255	hsa_circ_0007255	355	KIF4A	cytoplasm	promote oxygen consumption, colony formation, and cell mobility *in vitro*, inhibit tumor growth *in vivo*	miR-335-5p	SIX2	(124)
circEIF3M	hsa_circ_0003119	361	EIF3M	cytoplasm	promote cell proliferation, migration, and invasion	miR-33a	cyclin D1	(125)
hsa_circRNA_002178	hsa_circ_0000519	98	RPPH1	cytoplasm	promote cell proliferation, energy metabolism, and angiogenesis	miR-328-3p	COL1A1	(126)
circ-PGAP3	hsa_circ_0106800	537	PGAP3	cytoplasm	promote cell proliferation and invasion	miR-330-3p	Myc	(127)
circIRAK3	hsa_circ_0005505	754	IRAK3	cytoplasm	promote cell migration and invasion *in vitro*, promote metastasis and *in vivo*	miR-3607	FOXC1	(128)
hsa_circ_0008039	hsa_circ_0008039	462	PRKAR1B	cytoplasm	promote cell proliferation and migration *in vitro*	miR-432-5p	E2F3	(129)
hsa_circ_0008039	hsa_circ_0008039	462	PRKAR1B	\	promote cell proliferation, migration, and invasion *in vitro*, and promote tumor growth *in vivo*	miR-515-5p	CBX4	(130)
circular RNA circ-ZEB1	\	\	\	\	promote cell proliferation and inhibit apoptosis	miR-448	eEF2K	(131)
circular RNA-100219	hsa_circ_0004619	377	FAF1	\	promote cell proliferation and migration *in vitro*	miR-485-3p	NTRK3	(132)
circMYO9B	hsa_circ_0000907	898	MYO9B	\	promote cell proliferation, migration, and invasion *in vitro*, and promote tumor growth *in vivo*	miR-4316	FOXP4	(133)
circRNF20	hsa_circ_0087784	499	RNF20	cytoplasm	promote cell proliferation, inhibit cell apoptosis *in vitro*, promote tumor growth *in vivo*	miR-487a	HIF1α-HK2	(134)
hsa_circ_0007534	hsa_circ_0007534	400	DDX42	\	promote cell proliferation, invasion, and inhibit apoptosis *in vitro*	miR-593	MUC19	(135)
circ_DCAF6	\	\	\	cytoplasm	promote cell proliferation and stemness	miR-616-3p	GLI1	(136)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
circ_0005230	hsa_circ_0005230	3958	DNM3OS	\	promote cell invasion, migration *in vitro*, and promote cell growth *in vivo*	miR-618	CBX8	(137)
circZFR	hsa_circ_0072088	693	ZFR	\	promote cell proliferation, migration, invasion, glycolysis, and inhibit cell apoptosis *in vitro*, promote tumor growth *in vivo*	miR-578	HIF1A	(138)
circ-UBAP2	hsa_circ_0001846	747	UBAP2	cytoplasm	promote cell proliferation, migrate, and inhibit cell apoptosis *in vitro*, and promote tumor growth and metastasis *in vivo*	miR-66	MTA1	(139)
circFBXL5	hsa_circ_0125597	912	FBXL5	cytoplasm	promote cell proliferation and migration *in vitro*, promote tumor growth and metastasis *in vivo*	miR-660	SRSF6	(140)
circTP63	\	295	TP63	cytoplasm	promote cell proliferation, invasion, migration *in vitro*, promote tumor growth *in vivo*	miR-873-3p	FOXM1	(141)
hsa_circRPPH1_015	hsa_circ_0000517	88	RPPH1	cytoplasm	promote cell proliferation and aggressiveness *in vitro*, promote tumor growth *in vivo*	miR-326	ELK1	(142)
#circANKRD12	hsa_circ_0046841 or \	286 or 925	ANKRD12	cytoplasm	promote cell proliferation, invasion, migration, and alter cell metabolism	\	CCND1	(143)
circCNOT2	\	\	\	\	increase cell proliferation	\	\	(144)
circAMOTL1	\	\	\	\	promote cell proliferation and invasion, and inhibit cell apoptosis when exposed to PAX *in vitro*	\	AKT	(145)
circ_103809	\	\	\	\	promote cell proliferation	\	PI3K/AKT signaling	(146)
hsa_circ_0008673	hsa_circ_0008673	689	BRCA1	\	promote cell proliferation and migration	\	\	(147)
hsa_circ_001569	\	\	\	\	promote cell proliferation, migration, invasion, and inhibit cell apoptosis *in vitro*	\	PI3K/AKT signaling	(148)
circBACH2	hsa_circ_0001627	2995	BACH2	cytoplasm	promote cell proliferation, invasion, and migration *in vitro*, promote tumor growth and metastasis *in vivo*	miR-186-5p/miR-548c-3p	CXCR4	(149)

(Continued)

TABLE 2 | Continued

CircRNA name	Circbase ID	Length	Gene Name	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
circPGR	\	\	PGR	cytoplasm	promote ER-positive breast cancer cell proliferation, invasion, and migration *in vitro*, promote tumor growth *in vivo*	miR-301a-5p	Cell cycle genes	(150)

*EMT, epithelial mesenchymal transition; PAX, paclitaxel; *The circRNA can simultaneously regulate an miRNA and genes, but the relationship between miRNA and genes is not clear. #Two circular isoforms of 286 bp (hsa_circ_0046841) and 925 bp (not found in the circbase) lengths share the same backsplice junction of ANKRD12, and the study did not distinguish these two circular isoforms.*

invasion and migration of breast cancer cells (77, 78). Circ-ABCB10 and circHMCU can sponge let-7 then regulate the expression of MCY, HMGA2, CCND1, and DUSP7, respectively, to promote the proliferation, invasion, migration, and chemoresistance of breast cancer cells (79, 80). CircPLK1 and hsa_circ_0000515 can target miR-296-5p to regulate the expression of PLK1 and CXCL10, respectively, to promote the growth, proliferation, migration and inflammatory response of breast cancer cells (81, 82). And surprisingly, hsa_circ_0011946 can interact with miR-26a/b to regulate RFC3, to promote the invasion and migration of breast cancer cells, whereas circWWC3 can interact with miR-26b-3p and miR-660-3p to regulate the expression of ZEB1, and thus promote the proliferation, invasion and migration of breast cancer cells (51, 83).

It is important to note the potential for ceRNA-acting circRNA molecules to improve patient outcomes in addition to their roles in cancer progression. For example, TV-circRGPD6 can interact with miR-26b. This interaction serves to regulate the expression of YAF2 and thus leads to a better prognosis (152).

Protein Decoy or Scaffolding Functions

In some cases, circRNAs may modulate the progression of breast cancer through direct or indirect interactions with RBPs. These interactions may alter the functions of the RBPs either through competition, with the circRNA serving as a decoy, or through the recruitment of other interacting factors, with the circRNA serving a scaffolding role. For example, in breast cancer cells with wild-type p53, circ-CCNB1 can interact with both p53 and H2AX, and Bclaf1 is free to bind to Bcl2. In p53-mutant breast cancer cells, on the other hand, H2AX is not able to interact with p53, and circ-CCNB1 is thus free to form a complex with H2AX and Bclaf1 and to thus to slow the development of p53 mutation-induced breast cancer progression (153). Similarly, circ-Dnmt1 can interact with p53 and AUF1 to facilitate their nuclear translocation, and this nuclear translocation of p53 induces autophagy. The nuclear translocation of AUF1 can increase mRNA stability and protein expression of Dnmt1, whereas the nuclear translocation of Dnmt1 further inhibits p53 transcription (84). In addition, CircSKA3 can interact with Tks5 and integrin-β1 to promote the invadopodium formation, thereby promoting breast cancer invasion (154).

Transcriptional Regulation

In at least one important case, circRNA has been shown to impact cellular biological functions *via* a direct impact on transcription in breast cancer. Specifically, circRNA FECR1 (a circRNA consisting of exons 2, 3, and 4 of the Friend leukemia integration 1 (*FLI1*) gene) has been shown to bind to the promoter of the gene encoding DNA methyltransferase 1 (*DNMT1*) and to down-regulate DNMT1 transcription. Similarly, circRNA FECR1 has been shown to recruit the methylcytosine dioxygenase TET1 to the promoter of FLI1 and thus to induce demethylation at the FLI1 promoter (85).

Encoding of Functional Peptides

One circRNA that is related to the growth factor receptor HER2 (circHER2) encodes a 103- amino acid peptide known HER2-103. This peptide promotes the binding of EGFR with HER2 and increases EGFR kinase activity. This activity has been shown to correlate with increased proliferation and invasion of breast cancer cells and to enhance sensitivity to pertuzumab (86).

Mechanisms Leading to Tumor Suppressive Functions of circRNAs in Breast Cancer

Consistent with the smaller number of circRNAs that are down-regulated in breast cancer, the number of known tumor suppressive circRNAs is much fewer than the oncogenic circRNAs in breast cancer. Similar to the mechanisms leading to enhancement of tumor development, these circRNAs may act as ceRNAs, protein decoys or scaffolds as well as encode functional peptides to suppress breast cancer progression. The known tumor suppressive circRNAs are listed in **Table 3**.

ceRNAs

Both CircRNAs 000554 and circRNA_0025202 can sponge miR-182 to regulate the expression of ZNF36 and FOXO3a, respectively, and thus inhibit cellular proliferation, invasion, migration, and sensitivity to chemotherapy agents (155, 156). Similarly, circRNA-0001283 functions to sponge miR-187 to suppress HIPK3 expression, thus inhibiting the proliferation and invasion and promoting the apoptosis of breast cancer cells (157). CircTADA2A-E6 mainly regulates suppressor of cytokine signaling (SOCS) expression by sponging miR-203a-

TABLE 3 | CircRNAs with tumor suppressive functions in breast cancer.

CircRNA name	CircBase ID	Length	Gene	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
TV-circRGPD6	\	\	\	\	inhibit tumor-initiating properties *in vitro*, inhibit tumor growth and metastasis *in vivo*	miR-26b	YAF2	(152)
circ-Ccnb1	hsa_circ_0072758	342	CCNB1	nucleus	inhibit cell proliferation, increase cell apoptosis *in vitro*, inhibit tumor growth and extend mouse viability *in vivo*	\	interact with H2AX, p53, and BCLAF1	(153)
circular RNA 000554	hsa_circ_0000376	48782	PRH1-PRR4	cytoplasm	inhibit cell invasion and migration *in vitro*, inhibit tumor growth *in vivo*	miR-182	ZFP36	(155)
circRNA_0025202	hsa_circ_0025202	495	GAPDH	cytoplasm	inhibit cell proliferation, migration, increase cell apoptosis and sensitivity to tamoxifen in HR(+) BC cells *in vitro*	miR-182-5p	FOXO3a	(156)
circular RNA-0001283	hsa_circ_0001283	1400	WDR48	cytoplasm	inhibit cell proliferation, invasion, and promote cell apoptosis	miR-187	HIPK3	(157)
circTADA2A-E6 and circTADA2A-E5/E6	hsa_circ_0006220 hsa_circ_0043278	158 and 250	TADA2A	cytoplasm	inhibit cell proliferation, migration, invasion *in vitro*	miR-203a-3p	SOCS	(52)
circASS1	hsa_circ_0089105	241	ASS1	both nucleus and cytoplasm (majority)	inhibit cell invasion and migration *in vitro*	miR-4443	ASS1	(158)
circKDM4C	hsa_circ_0001839	292	KDM4C	cytoplasm	inhibit cell proliferation, invasion, migration, doxorubicin resistance, promote cell apoptosis *in vitro*, inhibit tumor growth and metastasis, *in vivo*	miR-548p	PBLD	(53)
circBMPR2	hsa_circ_0003218	342	BMPR2	cytoplasm	inhibit cell proliferation, migration, and invasion and tamoxifen resistance	miR-553	USP4	(159)
circular RNA 0001073	hsa_circ_0001073	473	ACVR2A	both nucleus and cytoplasm	inhibit proliferation, migration, invasion, and promote apoptosis *in vitro*, inhibit tumor growth *in vivo*	\	HuR	(160)
circ-Foxo3	\	\	FOXO3	\	inhibit tumor growth and extend mouse lifespan *in vivo*	\	increase Foxo3 protein level but decreasep53 level	(161)
circular RNA –MTO1	hsa_circ_0007874	318	MTO1	both nucleus and cytoplasm	inhibit cell proliferation, promote monastrol-induced cell cytotoxicity and reverse monastrol resistance *in vitro*	\	TRAF4/Eg5	(162)
circFBXW7	hsa_circ_0001451	1227	FBXW7	cytoplasm	inhibit cell proliferation, migration *in vitro* and inhibit tumor growth and lung metastasis *in vivo*	miR-197-3p and FBXW7-185aa	FBXW7	(163)
circRNA-000911	\	\	\	\	inhibit cell proliferation, migration, and invasion, and promote cell apoptosis	miR –449a	NOTCH1/NF-κB	(59)
circEHMT1	\	\	EHMT1	\	inhibit cell migration and invasion *in vitro* and inhibit lung metastasis *in vivo*	miR-1233-3p	KLF4/MMP2	(164)
circNFIC	hsa_circ_0002018	311	NFIC	cytoplasm	inhibit cell proliferation and migration *in vitro*, inhibit cell growth and lung metastasis *in vivo*	miR-658	UPK1A	(165)
circ_0000442	hsa_circ_0000442	7273	MED13L	\	inhibit cell proliferation *in vitro* and inhibit tumor growth *in vivo*	miR-148b-3p	PTEN/PI3K/AKT	(166)
circCDYL	hsa_circ_0008285	667	CDYL	cytoplasm	inhibit cell proliferation, migration, invasion, and promote cell apoptosis	miR-190a-3p	TP53INP1	(167)
circ-ITCH	\	\	ITCH	\	inhibit cell proliferation, migration, and invasion *in vitro*, and inhibit tumor growth and metastasis *in vivo*	miR-214 and miR-17	ITCH	(168)
Circular RNA BARD1	hsa_circ_0001098	1204	BARD1	\	inhibit cell proliferation and promote cell apoptosis *in vitro*, inhibit tumor growth and metastasis *in vivo*	miR-3942-3p	BARD1	(169)
circAHNAK1	hsa_circ_0000320	384	AHNAK1	cytoplasm	inhibit cell proliferation, migration, and invasion *in vitro*, inhibit tumor growth and metastasis *in vivo*	miR-421	RASA1	(170)
hsa_circ_0072309	hsa_ circ_0072309	580	LIFR	cytoplasm	inhibit cell proliferation, migration, and invasion *in vitro*, promote tumor growth *in vivo*	miR-492	\	(171)

(Continued)

TABLE 3 | Continued

CircRNA name	CircBase ID	Length	Gene	Distribution	Phenotype	Target	Downstream genes/ pathways	Reference
circRNA_103809	\	\	ZFR	\	inhibit cell proliferation, invasion, and migration, EMT *in vitro*	miR-532-3p	\	(172)
circDDX17	hsa_circ_0002211	927	DDX17	cytoplasm	inhibit cell proliferation and promote cell apoptosis	miR-605	CDK1 and p21	(173)
hsa_circ_0068033	hsa_circ_0068033	900	NAALADL2	cytoplasm	inhibit cell proliferation, invasion, migration, and promote apoptosis *in vitro*, inhibit tumor growth *in vivo*	miR-659	\	(174)
hsa_circ_0001785	hsa_circ_0001785	467	ELP3	cytoplasm	inhibit cell proliferation, migration, and invasion *in vitro*, inhibit tumor growth *in vivo*	miR-942	SOCS3	(175)
circSMARCA5	hsa_circ_0001445	269	SMARCA5	nucleus	increase cisplatin sensitivity *in vitro* and *in vivo*	\	SMARCA5	(176)
circSCYL2	hsa_circ_0006258	508	SCYL2	\	inhibit cell migration and invasion	\	EMT	(177)
circ-LARP4	\	\	LARP4	\	increase the doxorubicin sensitivity *in vitro*	\	\	(178)
circular RNA VRK1	hsa_circ_0141206	852	VRK1	\	inhibit cell proliferation, promote cell apoptosis *in vitro*	\	\	(179)
circular RNA VRK1	hsa_circ_0141206	852	VRK1	cytoplasm	inhibit cell proliferation, promote cell apoptosis, and inhibit self-renewal capacity *in vitro*	\	\	(180)
circUSP42	hsa_circ_0007823	451	USP42	\	inhibit cell invasion and migration	miR-4443	ASS1	(181)

3p, and this sponging effect correlates with inhibition of breast cancer proliferation, invasion, and migration (52). CircASS1 mainly inhibits breast cancer invasion and migration by sponging miR-4443; it has also been found that the expression of circASS1 is inversely related with that of its parental gene arginosuccinate synthase 1 (ASS1), which indicates that the splicing of ASS1 mRNA and circASS1 may compete with each other (158). CircKDM4C mainly regulates the expression of phenazine biosynthesis like protein domain containing (PBLD) by sponging miR-548p, thereby inhibiting breast cancer growth, metastasis, and drug resistance (53). CircBMPR2 regulates ubiquitin specific peptidase 4 (USP4) expression by sponging miR-553, to inhibit breast cancer proliferation, invasion, migration, and tamoxifen resistance (159).

Protein Decoys or Scaffolds

Some tumor suppressive circRNAs function through interacting with proteins. For example, circ-Ccnb1 interacts with p53, inhibits tumor growth and increase the survival time of mice (153). Yi *et al.* reported the direct interaction between circRNA 0001073 and the RNA binding protein HuR (160). Experiments with the apoptosis-related protein receptor-interacting protein (RIP) revealed that the tumor suppressive circRNA circ-FOXO3, which is enriched in normal cells, can interact with MDM2, p53, and FOXO3, facilitating the formation of the MDM2-P53 complex while inhibiting MDM2-FOXO3 binding. This results in promotion of p53 ubiquitination and inhibition of FOXO3 degradation and thus the induction of apoptosis of breast cancer cells (161). Circular RNA-MTO1 can block the interaction between TRAF4 and Eg5, and suppress the translation of Eg5, to inhibit breast cancer growth and reverse the resistance of breast cancer cells to monosterol (162).

Encoding of Functional Peptides

circFBXW7 not only can interact with miR-197-3p, but also encodes a 185-amino-acid peptide that regulates the expression of FBXW7 and the degradation of c-Myc, thus inhibiting proliferation and migration of ovarian cancer cells (163).

ANALYSIS OF CIRCRNAS IN THE DIAGNOSIS AND PROGNOSIS OF BREAST CANCER

Although circRNAs in plasma are not as stable as those within cells, circRNAs are still considered important diagnostic and prognostic biomarkers for cancer. CircRNAs that are particularly important diagnostic or prognostic biomarkers in breast cancer are listed in **Table 4**.

As an example of this role, the analyses of several circRNAs can distinguish breast cancer tissues from noncancerous tissues. These distinctions have been shown to occur with favorable statistics, including having area under the curve (AUC) values greater than 0.7. These diagnostic circRNAs include the circular RNA VRK1 (AUC: 0.720, n = 350) (179), circIFI30 (AUC: 0.733, n = 38) (87), circSEPT9 (AUC: 0.711, n = 60) (32) and circAGFG1 (AUC: 0.767, n = 40) (54). In addition, the combination of hsa_circ_006054, hsa_circ_100219, and hsa_circ_406697 has proven useful in diagnosis (AUC: 0.82, n = 51) (55). Although these circRNAs have been shown to be useful as tissue markers, to date, only hsa_circ_0001785 in the plasma has been shown to serve as potentially non-invasive biomarker for breast cancer (AUC: 0.771, n = 20; AUC: 0.784, n = 57) (182).

TABLE 4 | CircRNAs with diagnostic and prognostic value in breast cancer.

CircRNA name	Circbase ID	Clinicopathological Association	Tumor type (Sample number)	Reference
circSEPT9	hsa_circ_0005320	TNM stage, T stage, N stage, OS, diagnosis	TNBC (cohort2: n=80; cohort1: n=60)	(32)
circZNF609	hsa_circ_0000615	lymph node metastasis, TNM stage, OS	(n=143)	(74)
circHMCU	hsa_circ_0000247	lymph node metastasis, T stage, N stage, histological grade, OS	(n=267)	(79)
circPLK1	hsa_circ_0038632	tumor size, lymph node metastasis, TNM stage, DFS, OS	TNBC (n=240)	(81)
hsa_circ_0000515	hsa_circ_0000515	OS	(n=340)	(82)
circWWC3	hsa_circ_0089866 and hsa_circ_0001910	clinical stage, OS	(n=156)	(83)
circRGPD6	\	DFS, OS	(n=165)	(152)
circular HER2	hsa_circ_0007766	OS	TNBC (n=59)	(86)
circRNA_0025202	hsa_circ_0025202	lymph node metastasis, histological grade	HR positive breast cancer (n=230)	(156)
circular RNA 0001073	hsa_circ_0001073	tumor size, distant metastasis, TNM stage, RFS, diagnosis	(n=132)	(160)
circFBXW7	hsa_circ_0001451	tumor size, lymph node metastasis, DFS, OS	TNBC (n=473)	(163)
circular RNA VRK1	hsa_circ_0141206	tumor size, TNM stage, T stage, N stage, histological grade, OS, diagnosis	(n=350)	(179)
*circIFI30	hsa_circ_0005571	clinical stage, histological grade, OS, diagnosis	TNBC (n=78/n=38)	(87)
circAGFG1	hsa_circ_0058514	TNM stage, T stage, N stage, OS, diagnosis	TNBC (n=40)	(54)
hsa_circ_006054, hsa_circ_100219, and hsa_circ_406697	\	diagnosis	(n=51)	(55)
hsa_circ_0001785	hsa_circ_0001785	distant metastasis, TNM stage, histological grade, diagnosis	(n=57)	(182)
circKIF4A	hsa_circ_0007255	tumor size, lymph node metastasis, TNM stage, DFS, OS	TNBC (n=240)	(88)
circEPSTI1	hsa_circ_0000479	tumor size, lymph node metastasis, TNM stage, DFS, OS	TNBC (n=240)	(56)
circGNB1	hsa_circ_0009362	tumor size, TNM stage, DFS, OS	TNBC (n=222)	(89)
circANKS1B	hsa_circ_0007294	lymph node metastasis, clinical stage, OS	TNBC (n=165)	(61)
hsa_circ_0006220	hsa_circ_0006220	lymph node metastasis, pathological type, diagnosis	(n=50)	(65)
circGFRA1	hsa_circ_0005239	tumor size, lymph node metastasis, TNM stage, DFS, OS	TNBC (n=222)	(90)
circRNA_069718	hsa_circ_0069718	lymph node metastasis, TNM stage, OS	TNBC (n=35)	(91)
hsa_circ_002178	\	tumor size, lymph node metastasis, OS	(n=83)	(97)
circRNA_100876	\	OS	(n=50)	(99)
circMMP11	hsa_circ_0062558	lymph node, metastasis, TNM stage	(n=113)	(101)
circUBE2D2	\	tumor size, lymph node metastasis, TNM stage, OS, PFS	(n=80)	(102)
circUBE2D2	hsa_circ_0005728	lymph node metastasis, TNM stage, OS	TNBC (n=66)	(104)
circCDYL	\	DFS, OS	(n=113)	(108)
circ_0000520	hsa_circ_0000520	lymph node metastasis, TNM stage, OS	(n=60)	(109)
circHIPK3	hsa_circ_0000284	lymph node metastasis, TNM stage, OS	(n=50)	(114)
hsa_circ_001783	\	tumor size, metastasis, TNM stage, DFS	(n=136)	(115)
circular RNA PVT1	hsa_circ_0001821	TNM stage, OS	(n=99)	(117)
circRAD18	hsa_circ_0002453	tumor size, TNM stage, T stage, OS	TNBC (n=126)	(118)
hsa_circ_0131242	hsa_circ_0131242	tumor size, TNM stage, OS	TNBC (n=86)	(120)
circ_0000291	hsa_circ_0000291	tumor size, lymph node metastasis	(n=37)	(123)
hsa_circRNA_002178	hsa_circ_0000519	OS	(n=70)	(126)
circ-PGAP3	hsa_circ_0106800	tumor size, lymph node metastasis, TNM stage, DFS,OS	TNBC (n=86)	(127)
circIRAK3	hsa_circ_0005505	RFS	(n=122)	(128)
circMYO9B	hsa_circ_0000907	tumor size, lymph node metastasis, TNM stage, OS	(n=21)	(133)
circRNF20	hsa_circ_0087784	tumor size, lymph node metastasis, OS	(n=50)	(134)
circZFR	hsa_circ_0072088	TNM stage, OS	(n=70)	(138)
circ-UBAP2	hsa_circ_0001846	tumor size, lymph node metastasis, distant metastasis, TNM stage, OS	TNBC (n=78)	(139)
circFBXL5	hsa_circ_0125597	OS	(n=150)	(140)
circCNOT2	\	PFS	(n=84)	(144)
hsa_circ_001569	\	lymph node metastasis, clinical stage, OS	(n=75)	(148)
circBACH2	hsa_circ_0001627	T stage, N stage, TNM stage	TNBC (n=38)	(149)

(Continued)

TABLE 4 | Continued

CircRNA name	Circbase ID	Clinicopathological Association	Tumor type (Sample number)	Reference
circNFIC	hsa_circ_0002018	lymph node metastasis, OS	(n=145)	(165)
circAHNAK1	hsa_circ_0000320	TNM stage, T stage, N stage, DFS, OS	TNBC (n=136)	(170)
hsa_circ_0072309	hsa_circ_0072309	tumor size, lymph node metastasis, TNM stage, OS	(n=32)	(171)
circRNA_103809	\	distant metastasis, TNM stage, OS, HER2 status	(n=65)	(172)
circDDX17	hsa_circ_0002211	lymph node metastasis, TNM stage	\	(173)
hsa_circ_0068033	hsa_circ_0068033	tumor size, lymph node metastasis, diagnosis	(n=36)	(174)
circ-LARP4	\	tumor size, TNM stage, T stage, N stage, DFS, OS	(n=283)	(178)
circUSP42	hsa_circ_0007823	lymph node metastasis, TNM stage, DFS, OS	TNBC (n=30)	(181)

*TNM, tumor node metastasis; DFS, disease free survival; PFS, progression free survival; OS, overall survival; *n = 78 for the study of relationships between circIFI30 expression and clinical stage, histological grade and OS, while n = 38 for the study of the diagnostic value of circIFI30.*

In addition to diagnostic potential, several circRNAs are prognostic biomarkers for breast cancer. For example, the expressions of circPLK1, circKIF4A, circEPSTI1, circGNB1, or circGFRA1 are associated with the tumor size and TNM stage of TNBC, whereas circPLK1, circKIF4A, circEPSTI1, or circGFRA1 are also associated with the lymph node metastasis of TNBC (56, 81, 88, 89). Similarly, high expression of circPLK1, circKIF4A, circEPSTI1, circGNB1, or circGFRA1 is associated with worse disease-free survival and overall survival in TNBC (56, 81, 88, 89).

PROSPECTIVES

Despite the rapid progress of our understanding of circRNA biogenesis and degradation over the last several decades, many important issues remain unresolved. For example, what signals regulate circRNA-specific expression? What are the intracellular or extracellular signals that activate the mechanisms leading to degradation of circRNAs? Why have only a few blood-localized circRNAs been identified as useful diagnostic markers?

Yet another important question involves potential roles of circRNAs in the communication between the tumor cells and the tumor microenvironment? Although many breast cancer-associated dysregulated circRNAs have been discovered and functionally characterized, most related studies have focused only on the roles of circRNAs on the proliferation, migration, invasion, apoptosis, and chemoresistance of tumor cells. However, tumor heterogeneity is a great challenge for therapeutic management, so growing attention has recently been paid to the molecules that mediate communication between tumor cells and cells of the tumor microenvironment, including cancer-associated fibroblasts, tumor-associated macrophages and T cells. Accordingly, further studies regarding the function of circRNA in mediating the dialogs that occur within or between tumor, stromal and immune cells have great future prospects.

Recently, with the development of ribosome nascent-chain complex (RNC) sequencing and ribo-sequencing, several circRNAs with coding potential have been discovered; however, only a few circRNAs with coding potential have been definitively identified, and most of these studies have indicated that the circRNA-encoded peptides, but not the circRNAs themselves, are the functional unit. Given the broad prospects of peptide therapeutics in the pharmaceutical industry, studies on tumor suppressive circRNA-encoded peptides may provide new directions for the development of drugs for breast cancer.

In addition, as noted in this study, different circRNAs can sponge the same miRNA, but these interactions tend to regulate the expression of different targets. These circRNAs, miRNAs, and targets form regulatory networks and thus promote or inhibit the progression of breast cancer. Therefore, the study of the circRNAs, miRNAs, and target networks in different systems is an important direction for future research.

Another important question involves mechanisms leading to the intracellular localizations of circRNAs. By analyzing the distribution of the tumor suppressive and oncogenic circRNAs in breast cancer (**Table 2**, **Table 3**), we noticed that most of the circRNAs with known function are mainly distributed in the cytoplasm. Five circRNAs, with lengths of 241, 318, 435, 473, and 571 bp, are distributed in both the cytoplasm and nucleus, and two circRNAs, of 269 and 342 bp, are mainly distributed in the nucleus. Similarly, at least 22 cytoplasm-localized circRNAs are less than 400 bp, and this phenomenon cannot be explained by transport mechanisms that solely involve the length of the circRNA, indicating that regulation of the localization of circRNAs is more complex than is currently appreciated.

CONCLUSION

CircRNAs form a large class of regulators of breast cancer progression. Clinical studies have also indicated that circRNAs are potential biomarkers for breast cancer diagnosis, prognosis, and therapy. However, the regulation network of circRNAs in breast cancer is still incompletely defined, and this uncertainty impedes the clinical exploitation of circRNAs. Future studies on the precise mechanisms of the key circRNA regulators and clinical relevance to breast cancer will further promote the clinical use of circRNAs and/or their relevant products in the management of breast cancer.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JX wrote the manuscript text and prepared the tables. XC revised the manuscript and prepared the figures. CL and XJ provided advice and revised the manuscript. YuS, YaS, FT, and ML critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The study was financially supported by the National Natural Science Foundation of China (81902651), Jiangsu provincial key research and development program (BE2019621), Research Innovation Program for Graduates of Jiangsu Province (JX10413758, JX10413759).

ACKNOWLEDGMENTS

We thank BioRender for providing the template for our figures.

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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 potential conflicts of interest.

The reviewer [ZW] declared a shared affiliation, with no collaboration, with the authors to the handling editor at the time of review.

frontiers in Oncology

ORIGINAL RESEARCH
published: 19 July 2021
doi: 10.3389/fonc.2021.710337

miRNome and Functional Network Analysis of PGRMC1 Regulated miRNA Target Genes Identify Pathways and Biological Functions Associated With Triple Negative Breast Cancer

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Background: Increased expression of the progesterone receptor membrane component 1, a heme and progesterone binding protein, is frequently found in triple negative breast cancer tissue. The basis for the expression of PGRMC1 and its regulation on cellular signaling mechanisms remain largely unknown. Therefore, we aim to study microRNAs that target selective genes and mechanisms that are regulated by PGRMC1 in TNBCs.

Methods: To identify altered miRNAs, whole human miRNome profiling was performed following AG-205 treatment and PGRMC1 silencing. Network analysis identified miRNA target genes while KEGG, REACTOME and Gene ontology were used to explore altered signaling pathways, biological processes, and molecular functions.

Results: KEGG term pathway analysis revealed that upregulated miRNAs target specific genes that are involved in signaling pathways that play a major role in carcinogenesis. While multiple downregulated miRNAs are known oncogenes and have been previously demonstrated to be overexpressed in a variety of cancers. Overlapping miRNA target genes associated with KEGG term pathways were identified and overexpression/amplification of these genes was observed in invasive breast carcinoma tissue from TCGA. Further, the top two genes (*CCND1* and *YWHAZ*) which are highly genetically altered are also associated with poorer overall survival.

Conclusions: Thus, our data demonstrates that therapeutic targeting of PGRMC1 in aggressive breast cancers leads to the activation of miRNAs that target overexpressed genes and deactivation of miRNAs that have oncogenic potential.

Keywords: PGRMC1, miRNA, miRNome, TNBC, KEGG, REACTOME, Gene Ontology

OPEN ACCESS

Edited by:
Wenwen Zhang,
Nanjing Medical University, China

Reviewed by:
Weifeng Ding,
Nantong University, China
Ammad Ahmad Farooqi,
Institute of Biomedical and Genetic Engineering (IBGE), Pakistan

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

Received: *15 May 2021*
Accepted: *24 June 2021*
Published: *19 July 2021*

Citation:
Pedroza DA, Ramirez M, Rajamanickam V, Subramani R, Margolis V, Gurbuz T, Estrada A and Lakshmanaswamy R (2021) miRNome and Functional Network Analysis of PGRMC1 Regulated miRNA Target Genes Identify Pathways and Biological Functions Associated With Triple Negative Breast Cancer. Front. Oncol. 11:710337. doi: 10.3389/fonc.2021.710337

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women in the U.S (1). Treatment for breast cancers are guided by the identification of hormone receptors, Estrogen receptor (ER), Progesterone receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2) (2, 3). Based on receptor status, breast cancers are categorized into four major molecular subtypes: Luminal A, Luminal B, HER2-enriched, and triple negative/basal-like (3). Among these triple negative breast cancers (TNBCs) are the most aggressive breast cancers with an overall poorer prognosis compared to other subtypes (4, 5). Because TNBC lack ER, PR and HER2, endocrine and antibody-based therapy are ineffective (6–8). Therefore, it is important to identify novel molecular drivers that enable TNBC growth and metastasis and target or reprogram these markers to better treat patients with aggressive metastatic cancers.

Recent evidence in multiple cancers (9–13) including breast cancer (14–16) identify microRNAs (miRNAs) as novel gene expression regulators and potential biomarkers (17–19). miRNAs are small non-coding RNAs approximately 19 to 25 nucleotides in length; they control gene expression by targeting selective-sequences of mRNAs, inducing translational repression or complete mRNA degradation (20). miRNA expression profiles have the ability to identify molecular breast cancer subtypes (21, 22) and can differentiate between basal and luminal subtypes (23). Their effect on hormone receptor expression, regulation, and activity remains in its infant stage. Ongoing studies however, have a major focus for miRNAs that target genes that are altered in aggressive breast cancers while dysregulation of miRNAs has been directly linked to aggressive basal-like breast cancers (24–28). Although one miRNA can target hundreds of genes, treatments that can switch-on specific miRNAs could lead to direct targeted gene suppression of multiple genes that are overexpressed or have oncogenic potential.

PGRMC1 a member of the membrane-associated progesterone receptor (MAPR) family with the ability to initiate non-classical signaling has been described in breast cancers (29–33). PGRMC1 overexpression is observed in more aggressive phenotypes and is associated with poor prognosis in patients diagnosed with ER-negative breast cancers (34). In addition, *in vitro* and *in vivo* studies demonstrate that PGRMC1 possess the ability to promote the growth and survival of human breast cancer cells and xenografted breast tumors (35, 36). Although PGRMC1 expression has been observed in multiple cancers (36–40), it's signaling mechanism remains unknown.

Sequencing and microarray technology has opened new insights into the genetic and genomic landscape of all breast cancers including TNBC (41, 42). For example, amplification of *MYC* and loss-of-function mutation of *BRCA1* are often described in TNBCs (43, 44). Further, the most frequently mutated or amplified genes in TNBCs include *PI3KCA* (55%), *AKT1* (13%) and *CDH1* (13%) (45). These genes can activate downstream cell-cycle regulators that can either activate (cyclin D1) or repress (p53), leading to sustained proliferation and inhibition of apoptosis of breast cancers (46). Our recent work demonstrated that PGRMC1 activates EGFR and PI3K/AKT signaling pathways, leading to increased cell proliferation of TNBC cells (33). While, other studies have demonstrated cell-specific effects between PGRMC1 and AKT signaling (47–49). Historically, the PI3K/AKT pathway is one of the most altered signaling mechanisms in human cancers (50–53). It plays a key role in controlling cellular processes such as cell proliferation and tumor growth (54, 55). Although directly targeting amplified genes such as *PI3KCA* and *AKT1* has proven to be difficult but promising (56, 57), novel genes that behave in a similar fashion should be identified.

To uncover genes and pathways associated with PGRMC1 in TNBCs we performed human miRNome profiling. We impaired PGRMC1 signaling using a chemical inhibitor and RNA interference. Whole human miRNome profiling identified miRNAs that were both up and down regulated following PGRMC1 impairment. Using an array of online databases and datasets we identified direct miRNA target genes. We proceeded to study these genes by identifying their involvement in the different signaling pathways that were altered following PGRMC1 suppression. More importantly, these genes were differentially expressed in human metastatic tumor samples. From all of the miRNA target genes observed, CyclinD1 (*CCND1*) and 14-3-3 protein zeta/delta (*YWHAZ*) had the highest gene expression in human tumors and were involved in various signaling pathways. Patient samples with high expression of either gene were associated with overall poorer survival probability. Increased relative gene expression and copy number variation of *CCND1* and *YWHAZ* was observed in MDA-MB-468 breast cancer cells and silencing PGRMC1 reduced the expression of these genes. Interestingly, multiple miRNAs (miR-224, miR-550a, miR-181a, miR-664a, miR-30b, miR-345, miR-93) that were downregulated upon PGRMC1 impairment are known to be overexpressed in multiple cancers and are described as possible oncogenes. Our results demonstrate that targeting PGRMC1 regulates miRNAs that directly target amplified genes and downregulates oncogenic miRNAs in TNBCs.

MATERIALS AND METHODS

Cell Culture

MDA-MB-468 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 media supplemented with 100 units/mL of penicillin, 100 µg/mL of streptomycin (Life Technologies, Grand Island, NY, USA), and 10% fetal bovine serum (FBS). Cells were incubated at 37°C in 5% CO^2 and maintained at an atmosphere of 95% air.

Treatment With Small Molecule Inhibitor and Gene Silencing

MDA-MB-468 cells were plated in six-well plates at a density of $5x10^5$ cells/well and allowed to attach overnight. Cells were then

either treated with 50 μM AG-205 for 24 h or transfected with PGRMC1 siRNA for 48 h. Using MIrus bio TransIT siQUEST transfection reagent (Mirus Bio) with either a control scrambled-sequence or siRNAs targeting PGRMC1-sequence (Origene). Three different siRNA sequences (A, B and C) and multiple concentrations ranging from 20 to 60 nM were used to effectively silence PGRMC1. To minimize toxicity, the ratio of siRNA to transfection reagent was maintained at 1:1, in accordance with the manufacture's protocol. siRNA sequences used were as follows:

SR323253A-rGrArUrCrArArCrUrUrUrUrArGrUrCrArUrGrArUrGrUrUCT

SR323253B-rCrArArUrUrGrArCrUrUrArArCrUrGrCrArUrGrArUrUrUCT

SR323253C-rUrCrArArCrUrUrUrUrArGrUrCrArUrGrArUrGrUrCrUGT

Quantitative RT-PCR

Total RNA was isolated from MDA-MB-468 breast cancer cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was then reverse transcribed using the RT2 first strand kit (Qiagen; Cat. No. 330401). qRT-PCR was performed using the StepOnePlus real time PCR system (Applied Biosystems, Foster City, CA, USA). The comparative Ct ($2^{-\Delta\Delta CT}$) method was used to analyze the results. The primers used for PGRMC1, CCND1, YWHAZ and 18S are as follows:

PGRMC1

Forward: 5′-CGACGGCGTCCAGGACCC-3′

Reverse: 5′-TCTTCCTCATCTGAGTACACAG-3′

CCND1

Forward: 5′-ATGGAACATCAGCTGCTGT-3′

Reverse: 5′-TCAGATGTCCACATCCCGC-3′

YWHAZ

Forward: 5′-ATGCAACCAACACATCCTATC-3′

Reverse: 5′- GCATTATTAGCGTGCTGTCTT-3′

18S

Forward: 5′-CCTCGATGCTCTTAGCTGAGT-3′

Reverse: 5′-TCCTAGCTGCGGTATCCAG-3′

miRNome Profiling

Global microRNA profiling was generated using the SABiosciences PCR miScript PCR Array Human miRNome (Cat No. MIHS-216Z). Briefly, total RNA was extracted using TRIzol reagent (Life Technologies) from MDA-MB-468 cells treated with 50 μM AG-205 for 24 h or 48 h post siRNA transfection. Human miRNome array was performed following the synthesis of cDNA using miScript II RT kit (SABiosciences). miScript miRNA PCR array was performed using miScript SYBR Green PCR Kit (SABiosciences). All of the differentially expressed miRNAs were well-characterized in the human genome as annotated by miRNet (http://www.mirnet.ca/).

Identifying Pathways Altered by PGRMC1 Using KEGG, Gene Ontology and Reactome

Using KEGG and gene ontology terms we analyzed the signaling pathways that were significantly altered following PGRMC1 disruption. The Reactome Analysis Tool (http://reactome.org) (58, 59) was used to visualize the genome-wide hierarchy of enriched pathways in response to PGRMC1. The most significantly enriched pathways are represented as yellow and are maintained in the middle of the circular representation and the less or non-significantly enriched pathways are labeled in grey. A list of all the miRNA target genes was uploaded into the Reactome database and significantly enriched pathway analysis was defined by FDR < 0.05.

Determining PGRMC1-Induced Genetic Alterations Using In Silico Analysis

To study possible genetic alterations such as inframe, missense, truncating mutations as well as gene amplification and deep deletion of the miRNA target genes observed following PGRMC1 disruption. We uploaded the DEG dataset onto the cbioportal (http://www.cbioportal.org/) database and analyzed it in reference to the cancer genome atlas (TCGA). Oncoprint diagrams were used to visualize genetic alterations from invasive breast carcinoma samples (60). Because we impaired PGRMC1 in TNBC cells, using the xena platform (https://xenabrowser.net) database, we studied the altered gene expression in response to PGRMC1 disruption. More specifically we obtained data from the breast cancer cell line Heiser 2012 (54 breast and breast cancer cell lines), breast cancer cell line encyclopedia (68 breast and breast cancer cell lines) as well as TCGA Breast Cancer (BRCA) dataset (n = 1,247 samples).

Assessing PGRMC1 Signaling and Overall Survival in Breast Cancer Patients Using KM Plotter and Interaction of miRNA Target Genes Using Genemania

The cBioportal (http://www.cbioportal.org/) database was used to study overall cumulative survival of patients with high and low expression of the miRNA target genes observed following PGRMC1 impairment. Kaplan-Meier plots were generated from TCGA breast invasive carcinoma samples (n=817). To study the impact of individual genes on overall survival probability, we used the KM plotter (http://kmplot.com/) database and generated Kaplan-Meier plots from ER-negative/HER2-negative breast cancer samples (n=869). Finally, using genemania 3 (http://genemania.org) we explored the interconnection between miRNA target genes involved in the pathways that were significantly altered following PGRMC1 impairment.

Statistical Analysis

All data are expressed as the mean ± SD. The differences between control and experimental groups were compared using Student's *t*-test. $P < 0.05$ was considered to be statistically significant. Statistical analysis was conducted using GraphPad Prism 7 software, version 7.0 (GraphPad Prism Software, San Diego, CA, USA).

RESULTS

Disrupting PGRMC1 Signaling the Human miRNome

To identify miRNAs regulated by PGRMC1, whole human miRNome profiling was performed using a miScript miRNA PCR array (miRNome V16) where a total of 1,084 mature miRNAs including their respective controls were measured. MDA-MB-468 breast cancer cells were treated with 50 μM AG-205. AG-205 is known to disrupt the downstream signaling of PGRMC1 possibly causing it to accumulate in the membrane. Therefore, it was not surprising to observe an increase in PGRMC1 mRNA expression (**Figure 1A**) as earlier studies have shown increased protein expression of PGRMC1 following AG-205 treatment (33, 38). Human miRNome profiling following AG-205 treatment identified alterations in the expression of various miRNAs (**Figure 1B**). The 20 most upregulated and downregulated miRNAs were observed (**Figures 1C, D**). Because AG-205 increased PGRMC1 mRNA expression, we proceeded to silence PGRMC1 to further study its impact on miRNA expression (**Figure 1E**). Following successful PGRMC1 silencing, human miRNome profiling identified alterations to 776 miRNAs (**Figure 1F**). Here again, the 20 most upregulated and downregulated miRNAs, were identified (**Figures 1G, H**). We then identified the target genes for the 20 most altered miRNAs using the miRNet database. Following AG-205 treatment the 20 most upregulated miRNAs targeted 2,898 genes while the 20 most downregulated miRNAs targeted 2,501 genes (**Figure 1I** and **Supplementary Tables 1, 2**). Similarly, the top 20 most upregulated miRNAs accounted for 1,788 target genes. While, the 20 most downregulated miRNAs targeted 3,029 genes after PGRMC1 was silenced (**Figure 1J** and **Supplementary Tables 3, 4**).

PGRMC1 Signal Disruption Alters miRNAs Involved in Pathways Associated With Cancers

Since our earlier analysis with the top 20 miRNAs altered by PGRMC1 resulted in a large number of target genes, we proceeded to study the network analysis of the top 10 most upregulated and downregulated miRNAs following AG-205 treatment. Network analysis of the top 10 most upregulated miRNAs (hsa-miR-523-3p, hsa-miR-3167, hsa-miR-3176, hsa-miR-570-3p, hsa-miR-410-3p, hsa-miR-646, hsa-miR-1256, hsa-miR-576-3p, hsa-miR-378a-5p and hsa-miR-1224-5p) identified 1,479 target genes (**Figure 2A** and **Supplementary Table 5**) while the top 10 most downregulated miRNAs (hsa-miR-3681-5p, hsa-miR-3617-5p, hsa-miR-34a-5p, hsa-miR-101-5p, hsa-miR-224-5p, hsa-miR-550a-3p, hsa-miR-181a-3p, hsa-miR-1914-3p, hsa-miR-664a-3p and hsa-miR-3605-3p) targeted 1,402 genes (**Figure 2B** and **Supplementary Table 6**). Studying

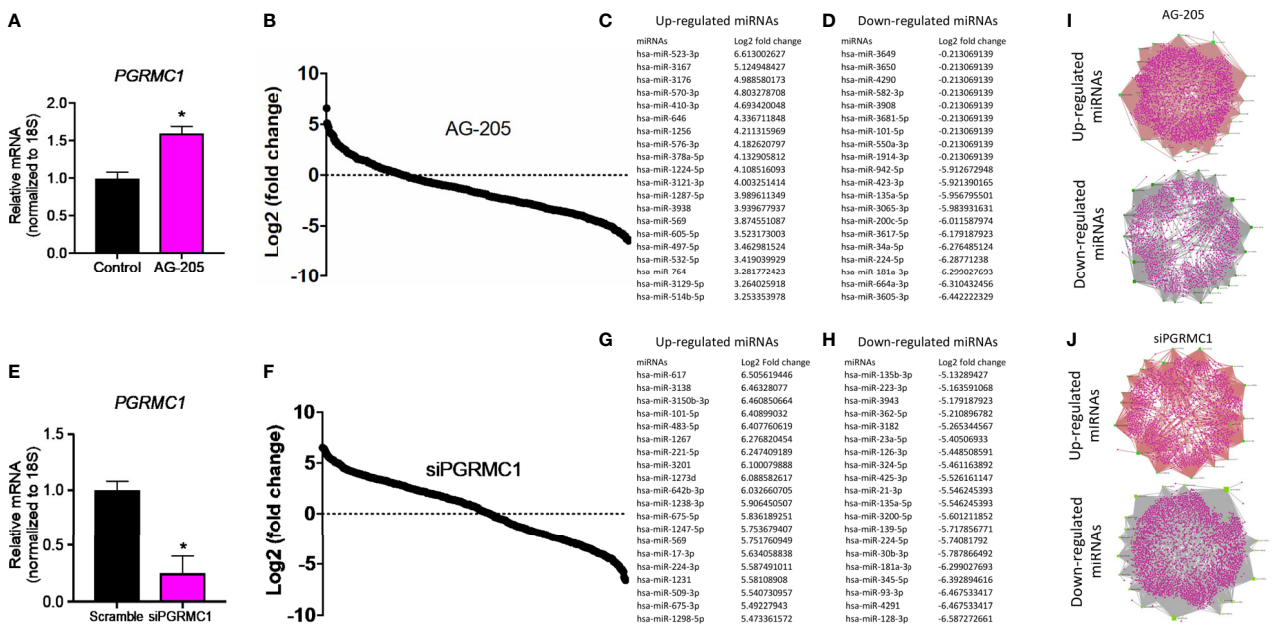

FIGURE 1 | Human miRNome profiling identified differentially regulated miRNAs following PGRMC1 signal disruption and silencing. **(A)** Relative mRNA expression of PGRMC1 in MDA-MB-468 breast cancer cells following 50 μM AG-205 after 24 h. **(B)** Whole human miRNome profiling identified differentially expressed miRNAs following signaling disruption by AG-205 treatment. **(C)** The top 20 most upregulated miRNAs were identified all which had a log2 (fold change) greater than 3. **(D)** The 20 most downregulated miRNAs, all which had a log2 (fold change) less than 1. **(E)** Relative mRNA expression of PGRMC1 in MDA-MB-468 cells following PGRMC1 silencing after 48 h. **(F)** miRNome profiling identified differentially expressed miRNAs following PGRMC1 silencing. **(G)** The 20 most upregulated miRNAs with a log2 (fold change) greater than 5. **(H)** The 20 most downregulated miRNAs were identified all which had a log2 (fold change) less than -5. **(I)** Interaction network hubs of the top 20 up and downregulated miRNAs and their mRNA target genes following AG-205 treatment. **(J)** Interaction network hubs of the top 20 up and downregulated miRNAs and their mRNA target genes following PGRMC1 silencing. Four individual networks are demonstrated with miRNAs illustrated in green, miRNA-mRNA interacting nodes in brown and target genes represented in pink. *P < 0.05.

the top miRNAs made our study more focused on miRNAs that may be more effectively regulated by PGRMC1. To identify miRNA target genes that could have a significant impact, we narrowed down our search by performing KEGG and gene ontology analysis. KEGG terms of the computed 1,479 target genes allowed us to pin-point and identify target genes of PGRMC1 altered miRNAs that are uniquely involved within the top signaling pathways, which interestingly included, p53 signaling pathway, cell cycle and pathways in cancers (**Figure 2C**; **Supplementary Figure 1** and **Supplementary Table 7**). Interestingly, the downregulated miRNAs also significantly altered pathways in cancer, cell cycle and p53 signaling pathways (**Figure 2D**; **Supplementary Figure 2** and **Supplementary Table 8**). Further, gene functions including kinase binding, single-stranded DNA binding, gene silencing, intrinsic apoptotic signaling pathway, regulated program cell death, enzyme binding, and nucleotide binding were classified using gene ontology based molecular functions and biological processes of both up and downregulated miRNAs (**Figures 2E, F**). The candidate 10 most up and downregulated miRNAs following AG-205 treatment and their respective target genes were listed (**Tables 1**, **2**).

miRNAs Regulated Signaling Pathways Identified Following PGRMC1 Silencing

Network analysis following PGRMC1 silencing identified 1,015 genes as targets of the 10 most upregulated miRNAs (hsa-miR-617, hsa-miR-3138, hsa-miR-3150b-3p, hsa-miR-101-5p, hsa-miR-483-5p, hsa-miR-1267, hsa-miR-221-5p, hsa-miR-3201, hsa-miR-1273d and hsa-miR-642b-3p) (**Figure 3A** and **Supplementary Table 9**). While, 2,010 genes were identified to be direct targets of the top 10 most downregulated miRNAs (hsa-miR-135a-5p, hsa-miR-3200-5p, hsa-miR-139-5p, hsa-miR-224-5p, hsa-miR-30b-3p, hsa-miR-181a-3p, hsa-miR-345-5p, hsa-miR-93-3p, hsa-miR-4291 and hsa-miR-128-3p) (**Figure 3B** and **Supplementary Table 10**). KEGG analysis of the upregulated (**Figure 3C**; **Supplementary Figure 4** and **Supplementary Table 11**) and downregulated (**Figure 3D**; **Supplementary Figure 5** and **Supplementary Table 12**) miRNAs following PGRMC1 silencing identified enrichment to similar KEGG terms observed in the AG-205 treatment group, such as p53 signaling pathway, cell cycle and pathways in cancers. Gene ontology terms, identified important molecular functions and biological processes including protein kinase binding, transcription factor binding, MAPK kinase activity, inactivation of MAPK activity, intrinsic apoptotic signaling pathway, purine nucleotide binding, adenyl nucleotide binding, protein phosphorylation, and regulation of phosphorylation (**Figures 3E, F**). The candidate 10 most up and downregulated miRNAs following PGRMC1 silencing and their respective target genes were listed (**Tables 3**, **4**).

PGRMC1 Signal Disruption and Silencing Alters miRNAs That Target Genes Involved in Breast Cancers

Once we identified the altered pathways following PGRMC1 signal disruption by AG-205 treatment we wanted to identify if the genes that are directly involved within these pathways are observed in breast cancer patient samples. Therefore, the identified genes were taken and computed into the xenabrowser database. TCGA data

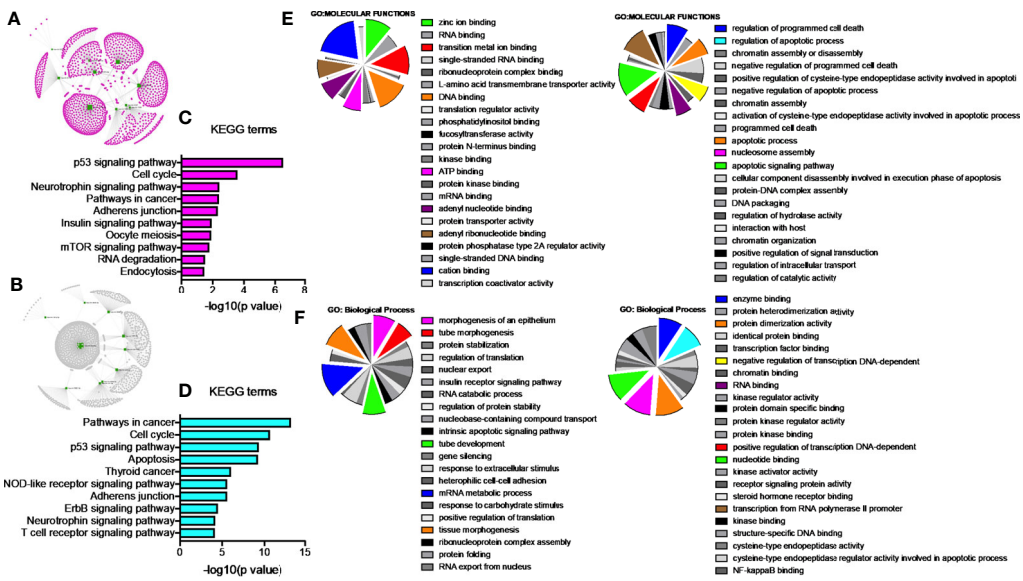

FIGURE 2 | Network analysis identifies mRNA target genes involved in altered pathways following AG-205 treatment. **(A)** The top ten upregulated miRNAs depicted in green, identify target genes highlighted in pink. **(B)** The top ten downregulated miRNAs are also depicted in green with their respective target genes highlighted in grey. **(C)** and **(D)** KEGG pathway analysis identified the top 10 significantly enriched pathways (non-disease related) involved within the miRNA network hub, adjusted $p < 0.05$. **(E, F)**. GO: term Molecular functions and Biological process involved within the observed miRNAs.

TABLE 1 | Upregulated miRNAS and target genes in response to AG-205.

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-3167	MIMAT0015042	CALM2	805	PAR-CLIP	23592263
hsa-mir-3167	MIMAT0015042	AURKA	6790	PAR-CLIP	26701625
hsa-mir-3167	MIMAT0015042	VPS4A	27183	PAR-CLIP	22012620
hsa-mir-3167	MIMAT0015042	WASF2	10163	HITS-CLIP	23824327
hsa-mir-3176	MIMAT0015053	ZNF274	10782	HITS-CLIP	23824327|27418678
hsa-mir-3176	MIMAT0015053	CYCS	54205	HITS-CLIP	19536157
hsa-mir-3176	MIMAT0015053	TTC37	9652	HITS-CLIP	23824327
hsa-mir-3176	MIMAT0015053	ANAPC7	51434	HITS-CLIP	23824327
hsa-mir-3176	MIMAT0015053	LSM3	27258	HITS-CLIP//PAR-CLIP	23446348|23824327
hsa-mir-3176	MIMAT0015053	RAB11FIP4	84440	PAR-CLIP	23446348
hsa-mir-3176	MIMAT0015053	ACTB	60	CLASH	23622248
hsa-mir-570-3p	MIMAT0003235	HHIP	64399	PAR-CLIP	22100165
hsa-mir-570-3p	MIMAT0003235	CALM3	808	PAR-CLIP	23592263
hsa-mir-570-3p	MIMAT0003235	PMAIP1	5366	PAR-CLIP	23592263|21572407
hsa-mir-570-3p	MIMAT0003235	RAC1	5879	PAR-CLIP	23592263
hsa-mir-570-3p	MIMAT0003235	TGFBR2	7048	HITS-CLIP	19536157
hsa-mir-570-3p	MIMAT0003235	ETS1	2113	PAR-CLIP	22012620
hsa-mir-570-3p	MIMAT0003235	CDKN1A	1026	PAR-CLIP	26701625|27292025
hsa-mir-570-3p	MIMAT0003235	TPM3	7170	PAR-CLIP	21572407
hsa-mir-570-3p	MIMAT0003235	TNFRSF10B	8795	PAR-CLIP	22012620|21572407
hsa-mir-570-3p	MIMAT0003235	GRK5	2869	PAR-CLIP	23592263
hsa-mir-570-3p	MIMAT0003235	IGF1R	3480	HITS-CLIP	23313552
hsa-mir-410-3p	MIMAT0002171	VEGFA	7422	PAR-CLIP	23446348
hsa-mir-410-3p	MIMAT0002171	CRK	1398	PAR-CLIP	21572407
hsa-mir-410-3p	MIMAT0002171	CHEK1	1111	HITS-CLIP	23824327
hsa-mir-410-3p	MIMAT0002171	HHIP	64399	HITS-CLIP	21572407
hsa-mir-410-3p	MIMAT0002171	PPP2R5E	5529	HITS-CLIP//PAR-CLIP	21572407
hsa-mir-410-3p	MIMAT0002171	CNOT6	57472	PAR-CLIP	23446348
hsa-mir-410-3p	MIMAT0002171	MET	4233	Luciferase reporter assay//qRT-PCR//Western blot	22750473
hsa-mir-410-3p	MIMAT0002171	CUL2	8453	HITS-CLIP//PAR-CLIP	23446348|22012620|21572407|20371350|23313552
hsa-mir-410-3p	MIMAT0002171	CDK1	983	PAR-CLIP	21572407
hsa-mir-410-3p	MIMAT0002171	LDLR	3949	HITS-CLIP//PAR-CLIP	23446348|21572407|20371350
hsa-mir-410-3p	MIMAT0002171	MDM2	4193	Luciferase reporter assay//qRT-PCR//Western blot	25136862
hsa-mir-410-3p	MIMAT0002171	PRKCD	5580	PAR-CLIP	23446348|21572407
hsa-mir-410-3p	MIMAT0002171	BTG3	10950	PAR-CLIP	23446348|22012620|21572407
hsa-mir-410-3p	MIMAT0002171	NTRK3	4916	HITS-CLIP//PAR-CLIP	23446348|21572407
hsa-mir-410-3p	MIMAT0002171	YWHAZ	7534	HITS-CLIP//PAR-CLIP	23446348|21572407|20371350|23824327|23313552
hsa-mir-410-3p	MIMAT0002171	RAB11FIP1	80223	PAR-CLIP	23446348|21572407
hsa-mir-410-3p	MIMAT0002171	FZD5	7855	HITS-CLIP//PAR-CLIP	23446348|21572407
hsa-mir-410-3p	MIMAT0002171	CCNB1	891	Luciferase reporter assay//qRT-PCR	26125663
hsa-mir-410-3p	MIMAT0002171	TFDP1	7027	PAR-CLIP	23446348|21572407|20371350
hsa-mir-410-3p	MIMAT0002171	THBS1	7057	PAR-CLIP	23592263
hsa-mir-410-3p	MIMAT0002171	TRAF6	7189	PAR-CLIP	22100165
hsa-mir-410-3p	MIMAT0002171	ADCY9	115	HITS-CLIP//PAR-CLIP	23446348|21572407|20371350
hsa-mir-410-3p	MIMAT0002171	GSK3B	2932	HITS-CLIP//PAR-CLIP	23446348|22012620|21572407|23313552
hsa-mir-410-3p	MIMAT0002171	SNAI1	6615	Luciferase reporter assay//qRT-PCR//Western blot	27221455
hsa-mir-410-3p	MIMAT0002171	PIK3CG	5294	HITS-CLIP//PAR-CLIP	21572407|23313552
hsa-mir-410-3p	MIMAT0002171	TRIP10	9322	HITS-CLIP	23824327
hsa-mir-646	MIMAT0003316	ZMAT3	64393	PAR-CLIP	24398324|22012620|21572407|20371350
hsa-mir-646	MIMAT0003316	CCND1	595	PAR-CLIP	24398324
hsa-mir-646	MIMAT0003316	CHEK1	1111	HITS-CLIP	23313552
hsa-mir-646	MIMAT0003316	CRK	1398	PAR-CLIP	21572407

(Continued)

TABLE 1 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-646	MIMAT0003316	VEGFA	7422	HITS-CLIP//PAR-CLIP	23592263|24398324|23446348|22012620|21572407|20371350
hsa-mir-646	MIMAT0003316	BTG2	7832	PAR-CLIP	24398324|20371350|26701625
hsa-mir-646	MIMAT0003316	PPP2R5C	5527	PAR-CLIP	21572407|20371350
hsa-mir-646	MIMAT0003316	DDX6	1656	PAR-CLIP	22012620
hsa-mir-646	MIMAT0003316	CSNK2A1	1457	HITS-CLIP	23313552
hsa-mir-646	MIMAT0003316	ORC4	5000	PAR-CLIP	24398324|23446348|21572407|20371350|27292025
hsa-mir-646	MIMAT0003316	PRKAR2A	5576	PAR-CLIP	23592263|23446348|21572407|20371350
hsa-mir-646	MIMAT0003316	RBL1	5933	PAR-CLIP	20371350
hsa-mir-646	MIMAT0003316	BIRC5	332	PAR-CLIP	23446348|21572407|20371350
hsa-mir-646	MIMAT0003316	WEE1	7465	PAR-CLIP	21572407|20371350
hsa-mir-646	MIMAT0003316	CDK6	1021	PAR-CLIP	20371350
hsa-mir-646	MIMAT0003316	STK11	6794	PAR-CLIP	26701625
hsa-mir-646	MIMAT0003316	PRDM4	11108	PAR-CLIP	21572407
hsa-mir-646	MIMAT0003316	PTPRF	5792	HITS-CLIP	19536157
hsa-mir-646	MIMAT0003316	PIK3R1	5295	HITS-CLIP//PAR-CLIP	23446348|21572407|23824327|23313552
hsa-mir-646	MIMAT0003316	CCNE2	9134	PAR-CLIP	23446348|21572407|20371350
hsa-mir-646	MIMAT0003316	MAP3K7	6885	PAR-CLIP	20371350
hsa-mir-646	MIMAT0003316	AKT3	10000	PAR-CLIP	23592263|21572407
hsa-mir-646	MIMAT0003316	CCNE1	898	PAR-CLIP	21572407|20371350
hsa-mir-646	MIMAT0003316	FGF2	2247	PAR-CLIP	23446348
hsa-mir-646	MIMAT0003316	PHKA1	5255	HITS-CLIP//PAR-CLIP	23446348|21572407|20371350
hsa-mir-646	MIMAT0003316	CNOT6L	246175	PAR-CLIP	20371350
hsa-mir-646	MIMAT0003316	CCND2	894	PAR-CLIP	21572407|20371350
hsa-mir-1256	MIMAT0005907	MKNK2	2872	PAR-CLIP	23592263|20371350
hsa-mir-1256	MIMAT0005907	WNT2B	7482	HITS-CLIP	27418678
hsa-mir-1256	MIMAT0005907	CHMP2B	25978	PAR-CLIP	21572407
hsa-mir-1256	MIMAT0005907	STK4	6789	PAR-CLIP	26701625
hsa-mir-1256	MIMAT0005907	WASL	8976	PAR-CLIP	23446348
hsa-mir-1256	MIMAT0005907	PABPC1	26986	PAR-CLIP	21572407|20371350|26701625
hsa-mir-576-3p	MIMAT0004796	PMAIP1	5366	PAR-CLIP	23592263
hsa-mir-576-3p	MIMAT0004796	PPP2R5E	5529	PAR-CLIP	23592263
hsa-mir-576-3p	MIMAT0004796	CCDC6	8030	PAR-CLIP	20371350
hsa-mir-576-3p	MIMAT0004796	SESN3	143686	PAR-CLIP	22100165
hsa-mir-576-3p	MIMAT0004796	SH2B3	10019	PAR-CLIP	23592263
hsa-mir-576-3p	MIMAT0004796	HIF1A	3091	PAR-CLIP	21572407
hsa-mir-576-3p	MIMAT0004796	YWHAQ	10971	PAR-CLIP	23446348
hsa-mir-378a-5p	MIMAT0000731	CYCS	54205	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	CCND2	894	PAR-CLIP	22012620
hsa-mir-378a-5p	MIMAT0000731	YWHAB	7529	CLASH	23622248
hsa-mir-378a-5p	MIMAT0000731	TPR	7175	CLASH	23622248
hsa-mir-378a-5p	MIMAT0000731	ATM	472	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	PPP1R3B	79660	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	FGF19	9965	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	SMURF2	64750	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	PYGB	5834	PAR-CLIP	20371350
hsa-mir-378a-5p	MIMAT0000731	RNF41	10193	PAR-CLIP	21572407
hsa-mir-378a-5p	MIMAT0000731	RPS6	6194	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	BRAF	673	CLASH	23622248
hsa-mir-378a-5p	MIMAT0000731	ACTN4	81	CLASH	23622248
hsa-mir-378a-5p	MIMAT0000731	SUFU	51684	Luciferase reporter assay//qRT-PCR//Western blot	18077375
hsa-mir-378a-5p	MIMAT0000731	WNT7B	7477	HITS-CLIP	23824327
hsa-mir-378a-5p	MIMAT0000731	CDK4	1019	HITS-CLIP	23824327

(Continued)

TABLE 1 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-378a-5p	MIMAT0000731	XIAP	331	HITS-CLIP	23824327|22927820
hsa-mir-378a-5p	MIMAT0000731	BBC3	27113	PAR-CLIP	23592263|24398324
hsa-mir-378a-5p	MIMAT0000731	PPARGC1A	10891	CLASH	23622248
hsa-mir-378a-5p	MIMAT0000731	DCP2	167227	HITS-CLIP	19536157
hsa-mir-378a-5p	MIMAT0000731	F2R	2149	HITS-CLIP	22927820
hsa-mir-378a-5p	MIMAT0000731	ZMAT3	64393	PAR-CLIP	22012620
hsa-mir-1224-5p	MIMAT0005458	WASF2	10163	CLASH	23622248
hsa-mir-1224-5p	MIMAT0005458	ZMAT3	64393	PAR-CLIP	22100165

from primary and metastatic tumor samples was downloaded and plotted. Genes from p53 signaling pathway, cell cycle neutrophin signaling pathways, pathways in cancer, adherens junction, insulin signaling pathway, oocyte meiosis, mTOR signaling pathway, RNA degradation, and endocytosis were differentially expressed in both metastatic and primary tumor tissue samples (**Figure 4**). Target genes of downregulated miRNAs were also differentially expressed in similar pathways including pathways in cancer, cell cycle, and p53 signaling pathway (**Supplementary Figure 5**). Identified genes involved within each pathway following PGRMC1 silencing were similarly computed into the xenabrowser database. TCGA data analyzed from metastatic tumor samples identified upregulated miRNA target genes to be involved in pathways in cancer, T cell receptor signaling pathway, cell cycle, p53 signaling pathway, B cell receptor signaling pathway, MAPK signaling pathway, JAK-STAT signaling pathway, ErbB signaling pathway, NOD-like receptor signaling pathway, and mRNA surveillance pathway (**Figure 5**). Intriguingly, downregulated miRNAs had similarly altered miRNA target genes in pathways in cancer, p53 signaling pathway, T cell receptor signaling pathway and ErbB signaling pathway (**Supplementary Figure 6**). However, some miRNA target genes were also observed in adherens junctions, focal adhesion, neurotrophin signaling pathway, regulation of actin cytoskeleton, aldosterone-regulated sodium reabsorption and chemokine signaling pathway (**Supplementary Figure 6**).

PGRMC1 Regulates miRNAs Involved in Cell Cycle, Disease Signal and Transduction Processes

Gene network analysis allowed us to identify novel target genes and we were able to classify them using KEGG term enrichment following AG-205 treatment of PGRMC1 silencing. We employed the Reactome database to study pathway-topology analysis using the miRNA target genes from KEGG and GO analysis. Using the Reactome pathway identifier we were able to observe genes that are mapped to pathways and over-represented within those pathways (58, 61). Following AG-205 treatment, we identified over-representation of miRNA target genes in pathways involved in cell cycle, gene expression (Transcription), disease, and signal transduction (**Figure 6A**). Similarly, following PGRMC1 silencing we observed over-representation of miRNA target genes in pathways involved in immune system, signal transduction, gene expression (transcription), and cell cycle (**Figure 6B**).

Functional Annotation Analysis of PGRMC1 Altered miRNA Target Genes in Invasive Breast Carcinomas Samples Using TCGA Dataset

TCGA data was used to study possible genetic alterations of the miRNA target genes due to miRNA alterations in response to PGRMC1 disruption. From the miRNA target genes observed, the top 22 that displayed increased mRNA expression within the spectrum of signaling pathways identified by KEGG were further analyzed. Using the cBioportal database we were able to observe and differentiate between the miRNA target genes based on genetic alteration. Using oncoprint we visualized the genetic alterations in the 22 miRNA target genes (*CCND1, YWHAZ, TPM3, BTG2, PABPC1, IGF1R, RAB11FIP1, PRKDC, MAPKAPK2, MAPK3, THBS1, CALM2, PIK3R1, RPS6, ACTB, PTPRF, ITGB1, RHOA, MAPK1, BCL2L1, RAC1* and *PPP2R1A*) (**Figure 7A** and **Supplementary Figure 7**). However, the percentage of genetic alteration varied within each gene and most miRNA target genes that displayed an alteration in > 5 percent were mainly amplified (**Figure 7A**). Patients that displayed high expression of these genes had a cumulative lower survival rate (**Figure 7B**). Network analysis by the Genemania database demonstrated that these amplified genes have tight interactions within signaling pathways. The light-red lines connect genes that are known to directly interact with one another within signaling pathways that are well studied (**Figure 7C**). Although, cumulatively these genes displayed a lower survival rate, only high expression of *CCDN1* and *YWHAZ* in ER-negative breast cancer patients displayed significant overall lower survival probability (**Figure 7D** and **Supplementary Figure 8**). Finally, gene expression data analysis from the breast cancer cell line dataset and copy number variation from the cancer cell line encyclopedia dataset similarly demonstrated increased expression/CN variation of *CCND1* and *YWHAZ* in TNBC cell lines (**Figure 7E**). Further, we also confirmed the decreased expression of *CCND1* and *YWHAZ* in PGRMC1 silenced MDA-MB-468 cells (**Figure 7F**). Overall, our *in vitro* and in silico analysis demonstrates that PGRMC1 plays a major role in influencing the miRNome in such a way that these alterations favor breast tumor growth and progression.

DISCUSSION

TNBCs account for approximately 12-14% of breast cancers diagnosed in the United States, with most exhibiting BRCA1/2

TABLE 2 | Downregulated miRNAS and target genes in response to AG-205.

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-181a-3p	MIMAT0000270	ARHGDIA	396	PAR-CLIP	26701625
mir-3605-3p	None				
hsa-mir-664a-3p	MIMAT0005949	TPR	7175	PAR-CLIP	22012620
hsa-mir-664a-3p	MIMAT0005949	CTBP1	1487	PAR-CLIP	24398324|21572407|26701625|27292025
hsa-mir-664a-3p	MIMAT0005949	MAPK8	5599	PAR-CLIP	24398324
hsa-mir-664a-3p	MIMAT0005949	WNT7A	7476	PAR-CLIP	22012620
hsa-mir-664a-3p	MIMAT0005949	WEE2	494551	HITS-CLIP	23824327
hsa-mir-664a-3p	MIMAT0005949	CALM1	801	PAR-CLIP	21572407
hsa-mir-664a-3p	MIMAT0005949	RPS6KA5	9252	PAR-CLIP	21572407
hsa-mir-1914-3p	MIMAT0007890	YWHAE	7531	PAR-CLIP	23592263
hsa-mir-1914-3p	MIMAT0007890	PLCG1	5335	CLASH	23622248
hsa-mir-1914-3p	MIMAT0007890	E2F3	1871	PAR-CLIP	23592263
hsa-mir-1914-3p	MIMAT0007890	STAT5B	6777	PAR-CLIP	22291592
hsa-mir-1914-3p	MIMAT0007890	TAB2	23118	PAR-CLIP	23592263
hsa-mir-1914-3p	MIMAT0007890	NRG4	145957	PAR-CLIP	23592263
hsa-mir-1914-3p	MIMAT0007890	CALM3	808	PAR-CLIP	23446348|26701625
hsa-mir-3617-5p	MIMAT0017997	CDKN1A	1026	PAR-CLIP	26701625
hsa-mir-3617-5p	MIMAT0017997	CDKN2B	1030	HITS-CLIP	23313552
hsa-mir-3617-5p	MIMAT0017997	MAPK10	5602	HITS-CLIP	23824327|27418678
hsa-mir-3617-5p	MIMAT0017997	MDM2	4193	PAR-CLIP	21572407|26701625
hsa-mir-3617-5p	MIMAT0017997	CDK1	983	PAR-CLIP	21572407
hsa-mir-3617-5p	MIMAT0017997	PMAIP1	5366	PAR-CLIP	27292025
hsa-mir-3617-5p	MIMAT0017997	CALM3	808	PAR-CLIP	21572407|20371350|26701625
hsa-mir-224-5p	MIMAT0000281	CCND1	595	PAR-CLIP	26701625
hsa-mir-224-5p	MIMAT0000281	BCL2	596	Microarray//qRT-PCR//Western blot	22989374
hsa-mir-224-5p	MIMAT0000281	CASP3	836	Luciferase reporter assay//Western blot	26307684
hsa-mir-224-5p	MIMAT0000281	IGF1R	3480	PAR-CLIP	20371350
hsa-mir-224-5p	MIMAT0000281	SMAD4	4089	Luciferase reporter assay//qRT-PCR//Western blot	20118412|23922662|25804630
hsa-mir-224-5p	MIMAT0000281	PDGFRB	5159	Microarray//Northern blot	16331254
hsa-mir-224-5p	MIMAT0000281	CDC42	998	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	20023705|24817781|22989374
hsa-mir-224-5p	MIMAT0000281	MTOR	2475	/Luciferase reporter assay//qRT-PCR//Western blot	27315344
hsa-mir-224-5p	MIMAT0000281	GSK3B	2932	Luciferase reporter assay	25588771
hsa-mir-224-5p	MIMAT0000281	HSP90AA1	3320	PAR-CLIP	23446348|20371350|26701625
hsa-mir-224-5p	MIMAT0000281	MAP2K2	5605	HITS-CLIP	23824327
hsa-mir-224-5p	MIMAT0000281	RAC1	5879	Luciferase reporter assay	27222381
hsa-mir-224-5p	MIMAT0000281	TPR	7175	PAR-CLIP	22012620
hsa-mir-224-5p	MIMAT0000281	GSK3B	2932	Luciferase reporter assay	25588771
hsa-mir-224-5p	MIMAT0000281	SERPINE1	5054	PAR-CLIP	22012620
hsa-mir-224-5p	MIMAT0000281	CASP7	840	Luciferase reporter assay//qRT-PCR//Western blot	26307684
hsa-mir-224-5p	MIMAT0000281	KRAS	3845	qRT-PCR//Western blot	23667495
hsa-mir-224-5p	MIMAT0000281	CDH1	999	/qRT-PCR//Western blot	22989374|25804630
hsa-mir-224-5p	MIMAT0000281	YES1	7525	PAR-CLIP	22012620
hsa-mir-224-5p	MIMAT0000281	PAK2	5062	Microarray//qRT-PCR//Western blot	22989374
hsa-mir-224-5p	MIMAT0000281	PAK2	5062	Microarray//qRT-PCR//Western blot	22989374
hsa-mir-550a-3p	MIMAT0003257	MAPK3	5595	/Luciferase reporter assay//qRT-PCR//Western blot	27462780
hsa-mir-550a-3p	MIMAT0003257	HSP90AA1	3320	PAR-CLIP	21572407
hsa-mir-550a-3p	MIMAT0003257	MDM2	4193	PAR-CLIP	20371350
hsa-mir-550a-3p	MIMAT0003257	MAPK1	5594	/Luciferase reporter assay//qRT-PCR//Western blot	27462780
hsa-mir-550a-3p	MIMAT0003257	TPM3	7170	PAR-CLIP	26701625
hsa-mir-550a-3p	MIMAT0003257	TRAF1	7185	HITS-CLIP	19536157
hsa-mir-550a-3p	MIMAT0003257	YWHAE	7531	PAR-CLIP	23592263
hsa-mir-101-5p	MIMAT0004513	FOS	2353	Luciferase reporter assay//qRT-PCR//Western blot	27485165
hsa-mir-101-5p	MIMAT0004513	VEGFA	7422	Luciferase reporter assay//qRT-PCR//Western blot	26870229
hsa-mir-101-5p	MIMAT0004513	RAC1	5879	Luciferase reporter assay//qRT-PCR//Western blot	26697839
hsa-mir-101-5p	MIMAT0004513	STK4	6789	PAR-CLIP	26701625
hsa-mir-101-5p	MIMAT0004513	ATM	472	Luciferase reporter assay//qRT-PCR	20617180
hsa-mir-101-5p	MIMAT0004513	PRKDC	5591	Luciferase reporter assay//qRT-PCR	20617180

(Continued)

TABLE 2 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-101-5p	MIMAT0004513	PMAIP1	5366	PAR-CLIP	23446348|22012620|21572407|20371350
hsa-mir-3681-5p	MIMAT0018108	FZD6	8323	HITS-CLIP//PAR-CLIP	24398324|21572407|23313552
hsa-mir-3681-5p	MIMAT0018108	GRAP2	9402	HITS-CLIP	19536157
hsa-mir-3681-5p	MIMAT0018108	MALT1	10892	PAR-CLIP	23592263
hsa-mir-34a-5p	MIMAT0000255	AKT1	207	Flow//qRT-PCR//Western blot	27073535
hsa-mir-34a-5p	MIMAT0000255	BIRC2	329	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	BIRC3	330	Microarray//Northern blot	17540599
hsa-mir-34a-5p	MIMAT0000255	XIAP	331	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	BIRC5	332	/PCR array//qRT-PCR//Western blot	23264087|24068565|25436980|26318298|28097098
hsa-mir-34a-5p	MIMAT0000255	FASLG	356	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	AR	367	qRT-PCR//Western blot	23145211
hsa-mir-34a-5p	MIMAT0000255	BAX	581	Luciferase reporter assay//Western blot	27610823
hsa-mir-34a-5p	MIMAT0000255	CCND1	595	/Reporter assay//Sequencing//Western blot	18406353|19461653|20309880|20371350|27220728
hsa-mir-34a-5p	MIMAT0000255	BCL2	596	/qRT-PCR//QRTPCR//Reporter assay//Western blot	26802970|27939626|26406332|25910896
hsa-mir-34a-5p	MIMAT0000255	BCL2L1	598	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CASP3	836	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CASP8	841	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CASP9	842	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CDK4	1019	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	21240262|21128241|24504520
hsa-mir-34a-5p	MIMAT0000255	CDK6	1021	/PAR-CLIP//qRT-PCR//Reporter assay//Western blot	19773441|21240262|23035210|23592263
hsa-mir-34a-5p	MIMAT0000255	CDKN1B	1027	PAR-CLIP	23446348
hsa-mir-34a-5p	MIMAT0000255	CDKN2A	1029	Western blot	21128241
hsa-mir-34a-5p	MIMAT0000255	CSF1R	1436	Luciferase reporter assay//qRT-PCR	24198819
hsa-mir-34a-5p	MIMAT0000255	CTNNB1	1499	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	DAPK1	1612	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	E2F1	1869	/Luciferase reporter assay//qRT-PCR//Western blot	17875987|21128241|27704360|28293146
hsa-mir-34a-5p	MIMAT0000255	E2F3	1871	//Microarray//PAR-CLIP//qRT-PCR//Western blot	23954321|23298779|26802970|28389657|25675046
hsa-mir-34a-5p	MIMAT0000255	ERBB2	2064	Luciferase reporter assay//Western blot	27813227
hsa-mir-34a-5p	MIMAT0000255	FOS	2353	ChIP//mRNA decay//qRT-PCR//Western blot	27513856
hsa-mir-34a-5p	MIMAT0000255	GRB2	2885	Sequencing	20371350
hsa-mir-34a-5p	MIMAT0000255	HDAC1	3065	/qRT-PCR//Reporter assay//Western blot	21566225|23836017|26035691|28123637
hsa-mir-34a-5p	MIMAT0000255	IGF1R	3480	CLASH	23622248
hsa-mir-34a-5p	MIMAT0000255	ITGA6	3655	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	KIT	3815	Luciferase reporter assay//Western blot	24009080|27056900
hsa-mir-34a-5p	MIMAT0000255	SMAD4	4089	//PAR-CLIP//qRT-PCR//Western blot	20371350|28348487|26077733
hsa-mir-34a-5p	MIMAT0000255	MET	4233	/Northern blot//qRT-PCR//Western blot	24983493|26313360|26238271|27513895|28250026
hsa-mir-34a-5p	MIMAT0000255	MYC	4609	/Reporter assay//Sequencing//TRAP//Western blot	21297663|22159222|20371350|24510096|25572695
hsa-mir-34a-5p	MIMAT0000255	NFKB1	4790	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	PDGFRA	5156	//Microarray//qRT-PCR//Western blot	22479456|23805317|24837198|27302634
hsa-mir-34a-5p	MIMAT0000255	PDGFRB	5159	/Luciferase reporter assay//qRT-PCR//Western blot	23805317|24837198|26324236
hsa-mir-34a-5p	MIMAT0000255	PIK3CG	5294	Flow//qRT-PCR//Western blot	27073535
hsa-mir-34a-5p	MIMAT0000255	PLCG1	5335	Proteomics	21566225

(Continued)

TABLE 2 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-34a-5p	MIMAT0000255	MAPK3	5595	CLASH	23622248
hsa-mir-34a-5p	MIMAT0000255	MAP2K1	5604	Luciferase reporter assay//Northern blot//qRT-PCR//Western blot	20299489
hsa-mir-34a-5p	MIMAT0000255	RALB	5899	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	SPI1	6688	Luciferase reporter assay//Reporter assay	20598588
hsa-mir-34a-5p	MIMAT0000255	STAT1	6772	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	TCF7	6932	/Luciferase reporter assay//qRT-PCR//Western blot	25436980
hsa-mir-34a-5p	MIMAT0000255	TGFBR2	7048	PAR-CLIP	22012620
hsa-mir-34a-5p	MIMAT0000255	TP53	7157	/Northern blot//qRT-PCR//QRTPCR//Western blot	23292869|26406332|26403328|26177460
hsa-mir-34a-5p	MIMAT0000255	TRAF2	7186	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	TRAF3	7187	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	VEGFA	7422	ELISA//Luciferase reporter assay	18320040
hsa-mir-34a-5p	MIMAT0000255	WNT1	7471	//Luciferase reporter assay//Microarray//qRT-PCR//Western blot	19336450|19398721|28199987
hsa-mir-34a-5p	MIMAT0000255	CCNE2	9134	Luciferase reporter assay//Microarray//PAR-CLIP//Western blot	19461653|17914404|23446348
hsa-mir-34a-5p	MIMAT0000255	LEF1	51176	/Microarray//Proteomics//qRT-PCR//Reporter assay//Western blot	21566225|25587085|28098757
hsa-mir-34a-5p	MIMAT0000255	CYCS	54205	PCR array	28097098
hsa-mir-224-5p	MIMAT0000281	KRAS	3845	qRT-PCR//Western blot	23667495
hsa-mir-34a-5p	MIMAT0000255	CCND3	896	Western blot	18406353
hsa-mir-34a-5p	MIMAT0000255	CDC20	991	CLASH//Proteomics	21566225|23622248
hsa-mir-34a-5p	MIMAT0000255	CDC25A	993	Western blot	18406353
hsa-mir-34a-5p	MIMAT0000255	CDC25C	995	Microarray	19461653
hsa-mir-34a-5p	MIMAT0000255	CDK4	1019	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	19461653|17914404|21240262|21128241|24504520
hsa-mir-34a-5p	MIMAT0000255	CDK6	1021	Microarray//PAR-CLIP//qRT-PCR//Reporter assay//Western blot	17914404|19773441|21240262|23035210|23592263
hsa-mir-34a-5p	MIMAT0000255	CDKN1B	1027	PAR-CLIP	23446348
hsa-mir-34a-5p	MIMAT0000255	CDKN2A	1029	Western blot	21128241
hsa-mir-34a-5p	MIMAT0000255	CDKN2C	1031	qRT-PCR//Reporter assay	21128241
hsa-mir-34a-5p	MIMAT0000255	GADD45A	1647	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	E2F1	1869	/Luciferase reporter assay//qRT-PCR//Western blot	17875987|21128241|27704360|28293146
hsa-mir-34a-5p	MIMAT0000255	E2F3	1871	/Luciferase reporter assay//Microarray//PAR-CLIP//qRT-PCR//Western blot	23954321|23298779|26802970|28389657|25675046
hsa-mir-34a-5p	MIMAT0000255	E2F5	1875	Microarray	19461653
hsa-mir-34a-5p	MIMAT0000255	SFN	2810	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	HDAC1	3065	/Proteomics//qRT-PCR//Reporter assay//Western blot	21566225|23836017|26035691|28123637
hsa-mir-34a-5p	MIMAT0000255	SMAD4	4089	/Luciferase reporter assay//PAR-CLIP//qRT-PCR//Western blot	20371350|28348487|26077733
hsa-mir-34a-5p	MIMAT0000255	MCM2	4171	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	MCM3	4172	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	MCM4	4173	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	MCM5	4174	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	MCM6	4175	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	MCM7	4176	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	CDC23	8697	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	CCNE2	9134	Luciferase reporter assay//Microarray//PAR-CLIP//Western blot	19461653|17914404|23446348
hsa-mir-34a-5p	MIMAT0000255	STAG2	10735	Proteomics	21566225
hsa-mir-34a-5p	MIMAT0000255	FZR1	51343	PAR-CLIP	26701625
hsa-mir-34a-5p	MIMAT0000255	ANAPC5	51433	CLASH	23622248
hsa-mir-34a-5p	MIMAT0000255	CASP8	841	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CASP9	842	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	TNFRSF10B	8795	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	CYCS	54205	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	AKT1	207	Flow//qRT-PCR//Western blot	27073535

(Continued)

TABLE 2 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-34a-5p	MIMAT0000255	BIRC2	329	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	BIRC3	330	Microarray//Northern blot	17540599
hsa-mir-34a-5p	MIMAT0000255	XIAP	331	PCR array	28097098
hsa-mir-34a-5p	MIMAT0000255	FASLG	356	PCR array	28097098

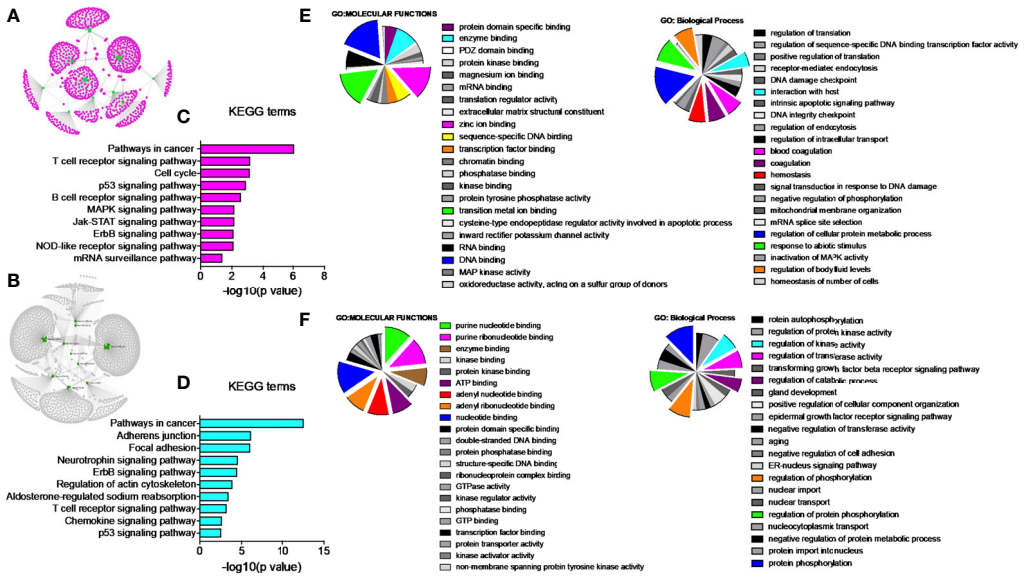

FIGURE 3 | PGRMC1 silencing alters pathways that are have miRNA target genes involved. Silencing PGRMC1 upregulates different miRNAs (from AG-205 treatment) that target similar miRNA target genes which are also upregulated in metastatic breast cancer samples. **(A)** Target genes highlighted in pink of the top ten most upregulated miRNAs highlighter in green. **(B)** The top ten most downregulated miRNAs highlighted in green and their direct targets highlighted in grey. **(C)** and **(D)** The top 10 most significantly enriched pathways (non-disease related) were identified by KEGG analysis, adjusted $p < 0.05$. **(E, F)** miRNA target genes show involvement in GO: terms Molecular functions and Biological process.

and p53 germline mutations (62, 63). TNBCs are the most aggressive type of breast cancer and most patients do not respond well to conventional chemotherapy (64, 65). The concept of gene therapy has been brought up as an alternative to chemotherapy to treat these aggressive cancers (66, 67) in this case RNAi could be used to target mutated proteins which are a product of missense mutations, leading to high constitutive expression of mutated proteins such as TP53 (68). However, suppressing genes with RNAi requires effective delivery methods, which have proven to be effective in some cases but difficult in both *in vivo* and *in vitro* systems (69–71). Therefore, other means of gene targeting therapies could be valued options.

miRNAs have emerged as important biological regulators of normal development (72) and evidence suggest that they play a major role in human cancers (73). miRNAs are abundantly found in multiple human cells and have the ability to regulate gene expression of approximately 60% of all mammalian genes (74, 75) hence they promote themselves as an attractive therapeutic option. Several miRNAs have been shown to be altered in TNBCs (24–28). Two examples of this are through the activation of STAT3, a transcription factor that is well documented in cancers (76). Activation of STAT3 is observed in TNBC tumors where epigenetic suppression of miR-146b leads to constitutive STAT3 activation and tumor growth (77, 78). Secondly, through the activation of the miRNA-200 family, these miRNAs are known to negatively regulate the epithelial to mesenchymal transition (EMT) and can specifically target ZEB1/2 (79, 80). Thereby, leading to the question, if miRNAs such as miR-14b or the miR-200 family of miRNAs were to be up-regulated could they then target genes that are overexpressed or active like STAT3 and EMT inducers to inhibit tumor growth?

PGRMC1 has been deemed a novel tumor biomarker due to its elevated levels in human cancers (49, 81–84). Because PGRMC1 plays a role in chemoresistance, tumor progression and growth it has become an attractive therapeutic target (36). Intriguingly, PGRMC1 is commonly observed in aggressive TNBC tissue (35). This is particularly interesting because TNBCs lack the classical signaling hormone receptors, ER and PR yet TNBCs that overexpress PGRMC1 could respond to steroid hormones *via* PGRMC1. Our previous studies showed that PGRMC1 is clearly overexpressed in the TNBC cell line MDA-MB-468 and using a known inhibitor (AG-205) and

TABLE 3 | Upregulated miRNAS and target genes in response to silencing PGRMC1.

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-617	MIMAT0003286	PABPC1	26986	HITS-CLIP	19536157
hsa-mir-3138	MIMAT0015006	PPP2R5E	5529	PAR-CLIP	23592263
hsa-mir-3138	MIMAT0015006	PPP2R1A	5518	PAR-CLIP	26701625
hsa-mir-3138	MIMAT0015006	CDC25A	993	PAR-CLIP	23592263
hsa-mir-3138	MIMAT0015006	CDK6	1021	PAR-CLIP	26701625
hsa-mir-3138	MIMAT0015006	FZD6	8323	HITS-CLIP//PAR-CLIP	24398324|21572407|23313552
hsa-mir-3138	MIMAT0015006	PIAS4	51588	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	CBL	867	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	BBC3	27113	PAR-CLIP	23592263
hsa-mir-3150b-3p	MIMAT0018194	WNT7B	7477	PAR-CLIP	23592263|26701625
hsa-mir-3150b-3p	MIMAT0018194	RBM8A	9939	PAR-CLIP	23592263|23446348|22012620|20371350|26701625|27292025
hsa-mir-3150b-3p	MIMAT0018194	YWHAZ	7534	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	SUGT1	10910	PAR-CLIP	23592263|20371350
hsa-mir-3150b-3p	MIMAT0018194	RALBP1	10928	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	CBLB	868	HITS-CLIP	19536157
hsa-mir-3150b-3p	MIMAT0018194	PABPC1L2B	645974	PAR-CLIP	23592263
hsa-mir-3150b-3p	MIMAT0018194	FZD7	8324	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	IKBKG	8517	PAR-CLIP	24398324
hsa-mir-3150b-3p	MIMAT0018194	PLK1	5347	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	PABPC1L2A	340529	PAR-CLIP	23592263
hsa-mir-3150b-3p	MIMAT0018194	BCL2L1	598	PAR-CLIP	23592263|26701625
hsa-mir-3150b-3p	MIMAT0018194	CDK2	1017	PAR-CLIP	23446348|20371350|26701625
hsa-mir-3150b-3p	MIMAT0018194	MAPK1	5594	PAR-CLIP	23592263
hsa-mir-3150b-3p	MIMAT0018194	PABPN1	8106	PAR-CLIP	26701625
hsa-mir-3150b-3p	MIMAT0018194	CACNA1B	774	HITS-CLIP	23824327|27418678
hsa-mir-3150b-3p	MIMAT0018194	CDKN1A	1026	PAR-CLIP	23592263
hsa-mir-101-5p	MIMAT0004513	STMN1	3925	Immunofluorescence//Luciferase reporter assay//qRT-PCR//Western blot	25607713
hsa-mir-101-5p	MIMAT0004513	STK4	6789	PAR-CLIP	26701625
hsa-mir-101-5p	MIMAT0004513	DUSP3	1845	PAR-CLIP	21572407
hsa-mir-101-5p	MIMAT0004513	VEGFA	7422	Luciferase reporter assay//qRT-PCR//Western blot	26870229
hsa-mir-101-5p	MIMAT0004513	ATM	472	Luciferase reporter assay//qRT-PCR	20617180
hsa-mir-101-5p	MIMAT0004513	FOS	2353	Luciferase reporter assay//qRT-PCR//Western blot	27485165
hsa-mir-101-5p	MIMAT0004513	RAC1	5879	Luciferase reporter assay//qRT-PCR//Western blot	26697839
hsa-mir-101-5p	MIMAT0004513	PMAIP1	5366	PAR-CLIP	23446348|22012620|21572407|20371350
hsa-mir-101-5p	MIMAT0004513	PRKDC	5591	Luciferase reporter assay//qRT-PCR	20617180
hsa-mir-101-5p	MIMAT0004513	PABPN1	8106	PAR-CLIP	23592263
hsa-mir-483-5p	MIMAT0004761	CACNG8	59283	HITS-CLIP	23313552
hsa-mir-483-5p	MIMAT0004761	RHOA	387	Luciferase reporter assay//Microarray//PAR-CLIP//qRT-PCR//Western blot	26148871|26701625
hsa-mir-483-5p	MIMAT0004761	NCBP2	22916	HITS-CLIP	21572407
hsa-mir-483-5p	MIMAT0004761	PDGFRA	5156	HITS-CLIP//PAR-CLIP	23446348|23313552
hsa-mir-483-5p	MIMAT0004761	VHL	7428	HITS-CLIP	23824327
hsa-mir-483-5p	MIMAT0004761	TRAF1	7185	PAR-CLIP	21572407
hsa-mir-483-5p	MIMAT0004761	IL21R	50615	PAR-CLIP	20371350
hsa-mir-483-5p	MIMAT0004761	MAPKAPK2	9261	PAR-CLIP	26701625
hsa-mir-483-5p	MIMAT0004761	MAP4K2	5871	HITS-CLIP	23313552
hsa-mir-483-5p	MIMAT0004761	MAPK3	5595	Luciferase reporter assay//Microarray//qRT-PCR//Western blot	22465663|25622783
hsa-mir-483-5p	MIMAT0004761	IFNAR1	3454	HITS-CLIP	23824327|23313552
hsa-mir-483-5p	MIMAT0004761	SRF	6722	Luciferase reporter assay//qRT-PCR//Western blot	21893058
hsa-mir-1267	MIMAT0005921	IL2RA	3559	HITS-CLIP	23824327
hsa-mir-1267	MIMAT0005921	MAPK14	1432	HITS-CLIP	23824327
hsa-mir-1267	MIMAT0005921	CRK	1398	HITS-CLIP	23824327
hsa-mir-1267	MIMAT0005921	CDK4	1019	HITS-CLIP	23824327
hsa-mir-1267	MIMAT0005921	SMAD2	4087	PAR-CLIP	27292025
hsa-mir-1267	MIMAT0005921	RPS6KA5	9252	HITS-CLIP	23824327
hsa-mir-1267	MIMAT0005921	CUL2	8453	HITS-CLIP//PAR-CLIP	21572407
hsa-mir-1267	MIMAT0005921	WEE1	7465	HITS-CLIP	27418678
hsa-mir-1267	MIMAT0005921	NFKBIB	4793	HITS-CLIP	27418678
hsa-mir-1267	MIMAT0005921	CDKN1B	1027	PAR-CLIP	23446348

(Continued)

TABLE 3 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-221-5p	MIMAT0004568	CDKN1B	1027	Chromatin immunoprecipitation//Co-immunoprecipitation//qRT-PCR//Western blot	26153983
hsa-mir-221-5p	MIMAT0004568	ABL1	25	PAR-CLIP	26701625
hsa-mir-221-5p	MIMAT0004568	CDKN1C	1028	Chromatin immunoprecipitation//Co-immunoprecipitation//qRT-PCR//Western blot	26153983
hsa-mir-221-5p	MIMAT0004568	ITGB1	3688	PAR-CLIP	20371350
hsa-mir-221-5p	MIMAT0004568	GRB2	2885	PAR-CLIP	26701625
hsa-mir-221-5p	MIMAT0004568	CARD8	22900	HITS-CLIP	23313552
hsa-mir-221-5p	MIMAT0004568	STAT2	6773	PAR-CLIP	20371350
hsa-mir-221-5p	MIMAT0004568	FZD2	2535	HITS-CLIP	23824327
hsa-mir-221-5p	MIMAT0004568	IL6R	3570	Luciferase reporter assay//qRT-PCR//Western blot	26645045
hsa-mir-3201	MIMAT0015086	LAMC1	3915	PAR-CLIP	23446348|22012620|20371350|26701625|27292025
hsa-mir-3201	MIMAT0015086	SPRED1	161742	PAR-CLIP	23592263
hsa-mir-3201	MIMAT0015086	TNFRSF10B	8795	HITS-CLIP	23313552
hsa-mir-3201	MIMAT0015086	PTEN	5728	PAR-CLIP	23592263
hsa-mir-3201	MIMAT0015086	EGLN1	54583	PAR-CLIP	21572407
hsa-mir-3201	MIMAT0015086	DUSP10	11221	HITS-CLIP	23824327
hsa-mir-3201	MIMAT0015086	CDC25B	994	PAR-CLIP	23592263
hsa-mir-1273d	MIMAT0015090	CBL	867	HITS-CLIP	23824327
hsa-mir-1273d	MIMAT0015090	VAV2	7410	PAR-CLIP	26701625
hsa-mir-1273d	MIMAT0015090	CD4	920	PAR-CLIP	23592263
hsa-mir-1273d	MIMAT0015090	SERPINE1	5054	PAR-CLIP	22012620
hsa-mir-642b-3p	MIMAT0018444	CACNA1B	774	HITS-CLIP	23824327
hsa-mir-642b-3p	MIMAT0018444	CDC25B	994	PAR-CLIP	23592263
hsa-mir-642b-3p	MIMAT0018444	SYK	6850	HITS-CLIP	24906430|19536157
hsa-mir-642b-3p	MIMAT0018444	MAP3K5	4217	PAR-CLIP	21572407|27292025
hsa-mir-642b-3p	MIMAT0018444	NRAS	4893	PAR-CLIP	21572407
hsa-mir-642b-3p	MIMAT0018444	CDKN1A	1026	PAR-CLIP	26701625

PGRMC1 silencing we demonstrated that it promotes TNBC cell proliferation through the EGFR/PI3K/AKT pathway (33). However, our study also focused on signaling pathways associated with ER-positive breast cancers (33). Here, we mainly focused on TNBCs as alternative mechanisms regulated by PGRMC1 in TNBCs should be further explored. To study and uncover novel mechanisms behind PGRMC1 we performed miRNome profiling following AG-205 treatment and PGRMC1 silencing. Studying the human miRNome enabled us to identify miRNAs that were significantly altered following PGRMC1 signal disruption and silencing. This presents itself as an important way to identify signaling pathways and genes involved within these pathways that could be associated with PGRMC1.

Human miRNome profiling identified alteration of 1,008 miRNAs following AG-205 treatment and 776 miRNAs after PGRMC1 siRNA transfection. Using a variety of gene mining platforms (miRNet, xenabrowser, cbioportal, Reactome, Kaplan-Meier plotter and GeneMANIA) we identified miRNA-mRNA network hubs that are altered when PGRMC1 is impaired. Network analysis by miRNet, an all in one, high-performance, analytics tool was used to predict PGRMC1 altered miRNAs targets (85). miRNet, incorporates data from TarBase, miRTarBase, starBase, EpimiR, PharmacomiR, SM2miR, PhenomiR, HMDD, miR2Disease, miRanda and miRecords making it a reliable data mining source (86). The top 10 most upregulated and downregulated miRNAs following AG-205 treatment and PGRMC1 silencing were identified. KEGG pathway analysis identified matching enriched pathways between the two treatment groups which included, pathways in cancer, cell cycle and p53 signaling pathway. In addition, TCGA derived gene expression data analysis taken from metastatic tissue identified the 22 most overexpressed genes in response to PGRMC1 signaling inhibition and silencing. Based on the above data, miRNAs that were upregulated following PGRMC1 impairment directly target and have the capability to suppress genes that are overexpressed in TNBC patient samples. However, because of their function we proceeded to study the downregulated miRNAs but considered them to be possible biomarkers. Interestingly, miR-30b, miR-664a-3p and miR-93-3p, miR-224-5p all which were downregulated following PGRMC1 impairment are commonly observed in multiple cancers including ovarian (87), prostate (88), gastric (89) and metastatic breast cancer (90–92). Furthermore, miR-181a-3p, miR-224-5p, miR-345-5p and miR-93-3p act like oncogenes and all have been associated with chemoresistance, migration, metastasis and stemness (87, 88, 91, 93). Based on the available literature disrupting PGRMC1 downregulates miRNAs that display oncogenic potential.

To get a better understanding of the signaling mechanism involved within the upregulated miRNA target genes we employed the Reactome pathway analyzer. This enabled us to study different signaling pathways that are not associated with the KEGG analysis from the miRNet database. We observed the upregulated genes to be involved in cell cycle and signal transduction mechanisms. This agrees with our previous findings of cell cycle involvement;

TABLE 4 | Downregulated miRNAS and target genes in response to silencing PGRMC1.

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-139-5p	MIMAT0000250	BCL2	596	Luciferase reporter assay//qRT-PCR//Western blot	27244080
hsa-mir-139-5p	MIMAT0000250	FOS	2353	qRT-PCR//Western blot	23001723|27668889
hsa-mir-139-5p	MIMAT0000250	HRAS	3265	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	HSP90AA1	3320	PAR-CLIP	21572407
hsa-mir-139-5p	MIMAT0000250	IGF1R	3480	Luciferase reporter assay//qRT-PCR//Western blot	22580051|24942287|26097570
hsa-mir-139-5p	MIMAT0000250	JUN	3725	/Luciferase reporter assay//qRT-PCR//Western blot	25499265
hsa-mir-139-5p	MIMAT0000250	MET	4233	/Luciferase reporter assay//qRT-PCR//Western blot	26497851
hsa-mir-139-5p	MIMAT0000250	NFKB1	4790	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	PIK3CA	5290	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	WNT1	7471	Luciferase reporter assay//Western blot	25529604
hsa-mir-139-5p	MIMAT0000250	IGF1R	3480	Luciferase reporter assay//qRT-PCR//Western blot	22580051|24942287|26097570
hsa-mir-139-5p	MIMAT0000250	MET	4233	Luciferase reporter assay//qRT-PCR//Western blot	26497851
hsa-mir-139-5p	MIMAT0000250	BCL2	596	Luciferase reporter assay//qRT-PCR//Western blot	27244080
hsa-mir-139-5p	MIMAT0000250	HRAS	3265	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	IGF1R	3480	Luciferase reporter assay//qRT-PCR//Western blot	22580051|24942287|26097570
hsa-mir-139-5p	MIMAT0000250	JUN	3725	Luciferase reporter assay//qRT-PCR//Western blot	25499265
hsa-mir-139-5p	MIMAT0000250	MET	4233	Luciferase reporter assay//qRT-PCR//Western blot	26497851
hsa-mir-139-5p	MIMAT0000250	PIK3CA	5290	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	RAP1B	5908	PAR-CLIP//qRT-PCR//Western blot	24942287|23592263
hsa-mir-139-5p	MIMAT0000250	ROCK2	9475	Luciferase reporter assay//qRT-PCR//Western blot	24942287
hsa-mir-224-5p	MIMAT0000281	BCL2	596	Microarray//qRT-PCR//Western blot	22989374
hsa-mir-224-5p	MIMAT0000281	HSP90AA1	3320	PAR-CLIP	23446348|20371350|26701625
hsa-mir-224-5p	MIMAT0000281	IGF1R	3480	PAR-CLIP	20371350
hsa-mir-224-5p	MIMAT0000281	CCND1	595	PAR-CLIP	26701625
hsa-mir-224-5p	MIMAT0000281	CASP3	836	Luciferase reporter assay//Western blot	26307684
hsa-mir-224-5p	MIMAT0000281	CDC42	998	/Microarray//qRT-PCR//Western blot	20023705|24817781|22989374
hsa-mir-224-5p	MIMAT0000281	MTOR	2475	Luciferase reporter assay//qRT-PCR//Western blot	27315344
hsa-mir-224-5p	MIMAT0000281	GSK3B	2932	Luciferase reporter assay	25588771
hsa-mir-224-5p	MIMAT0000281	KRAS	3845	qRT-PCR//Western blot	23667495
hsa-mir-224-5p	MIMAT0000281	SMAD4	4089	Luciferase reporter assay//qRT-PCR//Western blot	20118412|23922662|25804630
hsa-mir-224-5p	MIMAT0000281	PDGFRB	5159	Microarray//Northern blot	16331254
hsa-mir-224-5p	MIMAT0000281	MAP2K2	5605	HITS-CLIP	23824327
hsa-mir-224-5p	MIMAT0000281	RAC1	5879	Luciferase reporter assay	27222381
hsa-mir-224-5p	MIMAT0000281	TPR	7175	PAR-CLIP	22012620
hsa-mir-224-5p	MIMAT0000281	CDH1	999	Luciferase reporter assay//qRT-PCR//Western blot	22989374|25804630
hsa-mir-224-5p	MIMAT0000281	YES1	7525	PAR-CLIP	22012620
hsa-mir-224-5p	MIMAT0000281	PAK2	5062	Microarray//qRT-PCR//Western blot	22989374
hsa-mir-139-5p	MIMAT0000250	HRAS	3265	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	JUN	3725	Luciferase reporter assay//qRT-PCR//Western blot	25499265
hsa-mir-139-5p	MIMAT0000250	NFKB1	4790	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	PIK3CA	5290	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	RAP1B	5908	PAR-CLIP//qRT-PCR//Western blot	24942287|23592263
hsa-mir-139-5p	MIMAT0000250	ABL2	27	PAR-CLIP	23446348|21572407|20371350
hsa-mir-139-5p	MIMAT0000250	HRAS	3265	Luciferase reporter assay	24158791
hsa-mir-139-5p	MIMAT0000250	ROCK2	9475	Luciferase reporter assay//qRT-PCR//Western blot	24942287
hsa-mir-135a-5p	MIMAT0000428	BCL2	596	Luciferase reporter assay//qRT-PCR	25230140
hsa-mir-135a-5p	MIMAT0000428	BIRC5	332	PAR-CLIP	23446348|21572407|20371350
hsa-mir-135a-5p	MIMAT0000428	E2F1	1869	Microarray//qRT-PCR//Western blot	27683111
hsa-mir-135a-5p	MIMAT0000428	FOXO1	2308	Luciferase reporter assay//qRT-PCR//Western blot	25888950|26261511|27486383
hsa-mir-135a-5p	MIMAT0000428	MYC	4609	PAR-CLIP//Western blot	21572407|20371350|26701625
hsa-mir-135a-5p	MIMAT0000428	PTK2	5747	Luciferase reporter assay//qRT-PCR//Western blot	28415713
hsa-mir-135a-5p	MIMAT0000428	TRAF6	7189	PAR-CLIP	26701625
hsa-mir-135a-5p	MIMAT0000428	DAPK2	23604	Microarray//qRT-PCR//Western blot	27683111
hsa-mir-135a-5p	MIMAT0000428	PIAS4	51588	HITS-CLIP	23824327
hsa-mir-135a-5p	MIMAT0000428	EGFR	1956	Luciferase reporter assay//Western blot	27524492
hsa-mir-135a-5p	MIMAT0000428	SRC	6714	Immunoblot//Microarray	26364608
hsa-mir-135a-5p	MIMAT0000428	ROCK2	9475	Luciferase reporter assay//qRT-PCR//Western blot	25065599
hsa-mir-135a-5p	MIMAT0000428	ROCK1	6093	Luciferase reporter assay//qRT-PCR//Western blot	24465504|25065599
hsa-mir-135a-5p	MIMAT0000428	TRAF6	7189	PAR-CLIP	26701625
hsa-mir-135a-5p	MIMAT0000428	IRS2	8660	Luciferase reporter assay	23579070
hsa-mir-135a-5p	MIMAT0000428	PTK2	5747	Luciferase reporter assay//qRT-PCR//Western blot	28415713
hsa-mir-135a-5p	MIMAT0000428	APC	324	Luciferase reporter assay//qRT-PCR	18632633
hsa-mir-135a-5p	MIMAT0000428	PIP5K1A	8394	PAR-CLIP	22100165

(Continued)

TABLE 4 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-135a-5p	MIMAT0000428	NR3C2	4306	Luciferase reporter assay//qRT-PCR	19944075
hsa-mir-3200-5p	MIMAT0017392	PAX8	7849	PAR-CLIP	23446348
hsa-mir-3200-5p	MIMAT0017392	TGFBR2	7048	HITS-CLIP	19536157
hsa-mir-3200-5p	MIMAT0017392	IGF1R	3480	PAR-CLIP	24398324|21572407
hsa-mir-3200-5p	MIMAT0017392	CCND2	894	PAR-CLIP	22012620
hsa-mir-3200-5p	MIMAT0017392	ENAH	55740	PAR-CLIP	21572407
hsa-mir-3200-5p	MIMAT0017392	PFN2	5217	PAR-CLIP	23446348|21572407|20371350
hsa-mir-128-3p	MIMAT0000424	CASP3	836	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	MTOR	2475	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	BAX	581	Luciferase reporter assay//qRT-PCR//Western blot	23526655
hsa-mir-128-3p	MIMAT0000424	RUNX1	861	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	E2F3	1871	Luciferase reporter assay	18810376|19013014
hsa-mir-128-3p	MIMAT0000424	EGFR	1956	Western blot	22853714
hsa-mir-128-3p	MIMAT0000424	IGF1	3479	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	JAK1	3716	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	SMAD2	4087	Luciferase reporter assay	27087048
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	MAP2K1	5604	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	PTEN	5728	Luciferase reporter assay//qRT-PCR//Western blot	24132591|25250855
hsa-mir-128-3p	MIMAT0000424	PTGS2	5743	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	RET	5979	Flow//Luciferase reporter assay	23022987
hsa-mir-128-3p	MIMAT0000424	RXRA	6256	Microarray//qRT-PCR//Western blot	23990020
hsa-mir-128-3p	MIMAT0000424	SOS1	6654	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	TGFBR1	7046	Luciferase reporter assay//PAR-CLIP//Western blot	20054641|23622248|23592263
hsa-mir-128-3p	MIMAT0000424	HSP90B1	7184	CLASH	23622248
hsa-mir-128-3p	MIMAT0000424	VEGFC	7424	Microarray//qRT-PCR//Western blot	17612493|25001183|26460960
hsa-mir-128-3p	MIMAT0000424	CCDC6	8030	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	FZD9	8326	PAR-CLIP	23446348|21572407|20371350
hsa-mir-128-3p	MIMAT0000424	FADD	8772	Luciferase reporter assay//qRT-PCR//Western blot	24316133
hsa-mir-128-3p	MIMAT0000424	WNT3A	89780	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	EGFR	1956	Western blot	22853714
hsa-mir-128-3p	MIMAT0000424	SMAD2	4087	Luciferase reporter assay	27087048
hsa-mir-128-3p	MIMAT0000424	TGFBR1	7046	Luciferase reporter assay//PAR-CLIP//Western blot	20054641|23622248|23592263
hsa-mir-128-3p	MIMAT0000424	FYN	2534	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	SNAI2	6591	Flow//qRT-PCR//Western blot	23019226
hsa-mir-128-3p	MIMAT0000424	SNAI1	6615	Luciferase reporter assay//qRT-PCR//Western blot	28424413
hsa-mir-128-3p	MIMAT0000424	WASL	8976	PAR-CLIP	23592263
hsa-mir-128-3p	MIMAT0000424	NECTIN4	81607	Luciferase reporter assay//Western blot	27507538
hsa-mir-128-3p	MIMAT0000424	EGFR	1956	Western blot	22853714
hsa-mir-128-3p	MIMAT0000424	IGF1	3479	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	MAP2K1	5604	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	PTEN	5728	Luciferase reporter assay//qRT-PCR//Western blot	24132591|25250855
hsa-mir-128-3p	MIMAT0000424	SOS1	6654	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	VEGFC	7424	/Microarray//qRT-PCR//Western blot	17612493|25001183|26460960
hsa-mir-128-3p	MIMAT0000424	FYN	2534	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	RAP1B	5908	PAR-CLIP	23592263
hsa-mir-128-3p	MIMAT0000424	ARHGAP5	394	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	ILK	3611	PAR-CLIP	23592263
hsa-mir-128-3p	MIMAT0000424	PDPK1	5170	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	RELN	5649	Luciferase reporter assay//qRT-PCR//Western blot	19713529
hsa-mir-128-3p	MIMAT0000424	BAX	581	Luciferase reporter assay//qRT-PCR//Western blot	23526655
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	MAP2K1	5604	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	SOS1	6654	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	RAP1B	5908	PAR-CLIP	23592263
hsa-mir-128-3p	MIMAT0000424	MAPK14	1432	Immunoblot//Luciferase reporter assay//qRT-PCR	23109423
hsa-mir-128-3p	MIMAT0000424	NTRK3	4916	Luciferase reporter assay	19370765|21143953
hsa-mir-128-3p	MIMAT0000424	PDK1	5163	Luciferase reporter assay//qRT-PCR//Western blot	26949090
hsa-mir-128-3p	MIMAT0000424	YWHAZ	7534	HITS-CLIP	23824327
hsa-mir-128-3p	MIMAT0000424	RPS6KA5	9252	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	BEX3	27018	PAR-CLIP	23592263|24398324
hsa-mir-128-3p	MIMAT0000424	MTOR	2475	Luciferase reporter assay//Microarray//qRT-PCR	27893811

(Continued)

TABLE 4 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-128-3p	MIMAT0000424	EGFR	1956	Western blot	22853714
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	MAP2K1	5604	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	SOS1	6654	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	NCK2	8440	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	EGFR	1956	Western blot	22853714
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	MAP2K1	5604	Sequencing	20371350
hsa-mir-128-3p	MIMAT0000424	SOS1	6654	HITS-CLIP	23313552
hsa-mir-128-3p	MIMAT0000424	WASL	8976	PAR-CLIP	23592263
hsa-mir-128-3p	MIMAT0000424	GNG12	55970	PAR-CLIP	24398324|21572407|20371350
hsa-mir-128-3p	MIMAT0000424	IGF1	3479	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	PIK3R1	5295	Luciferase reporter assay//Microarray//qRT-PCR	27893811
hsa-mir-128-3p	MIMAT0000424	PDPK1	5170	Microarray	17612493
hsa-mir-128-3p	MIMAT0000424	FXYD2	486	Microarray	17612493
hsa-mir-93-3p	MIMAT0004509	CDC42	998	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	MAP2K1	5604	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	HSP90AB1	3326	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	LAMA4	3910	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	STAT5B	6777	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	NCOA4	8031	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	CUL2	8453	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	SUFU	51684	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	CYCS	54205	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	FYN	2534	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	ACTB	60	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	ACTN1	87	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	FER	2241	HITS-CLIP	23824327
hsa-mir-93-3p	MIMAT0004509	PARD3	56288	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	PPP1R12A	4659	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	IRAK1	3654	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	EIF4EBP1	1978	PAR-CLIP	20371350
hsa-mir-93-3p	MIMAT0004509	TIAM1	7074	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	ENAH	55740	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	ATP1A1	476	CLASH	23622248
hsa-mir-93-3p	MIMAT0004509	NEDD4L	23327	Luciferase reporter assay//qRT-PCR//Western blot	26581907
hsa-mir-30b-3p	MIMAT0004589	IGF1	3479	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	CDKN1A	1026	PAR-CLIP	26701625
hsa-mir-30b-3p	MIMAT0004589	XIAP	331	HITS-CLIP//PAR-CLIP	23446348|23824327
hsa-mir-30b-3p	MIMAT0004589	BCL2L1	598	PAR-CLIP	26701625
hsa-mir-30b-3p	MIMAT0004589	CRKL	1399	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	ITGA3	3675	HITS-CLIP	23706177|23313552
hsa-mir-30b-3p	MIMAT0004589	MDM2	4193	PAR-CLIP	27292025
hsa-mir-30b-3p	MIMAT0004589	PDGFRA	5156	HITS-CLIP//PAR-CLIP	23446348|23313552
hsa-mir-30b-3p	MIMAT0004589	RARA	5914	PAR-CLIP	23592263
hsa-mir-30b-3p	MIMAT0004589	STK4	6789	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	WNT7B	7477	PAR-CLIP	23592263
hsa-mir-30b-3p	MIMAT0004589	YES1	7525	PAR-CLIP	27292025
hsa-mir-30b-3p	MIMAT0004589	CTNND1	1500	PAR-CLIP	23592263|26701625
hsa-mir-30b-3p	MIMAT0004589	COL5A1	1289	PAR-CLIP	23592263
hsa-mir-30b-3p	MIMAT0004589	ITGB3	3690	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	TLN1	7094	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	YWHAZ	7534	PAR-CLIP	26701625
hsa-mir-30b-3p	MIMAT0004589	YWHAB	7529	PAR-CLIP	27292025
hsa-mir-30b-3p	MIMAT0004589	IRAK3	11213	HITS-CLIP//PAR-CLIP	21572407|20371350|23824327
hsa-mir-30b-3p	MIMAT0004589	MSN	4478	PAR-CLIP	23592263
hsa-mir-30b-3p	MIMAT0004589	MYH9	4627	HITS-CLIP//PAR-CLIP	23824327|23313552|26701625
hsa-mir-30b-3p	MIMAT0004589	ARPC3	10094	PAR-CLIP	20371350
hsa-mir-30b-3p	MIMAT0004589	ABI2	10152	HITS-CLIP	23824327
hsa-mir-30b-3p	MIMAT0004589	ATP1B4	23439	HITS-CLIP	23824327
hsa-mir-345-5p	MIMAT0000772	CDKN1A	1026	Luciferase reporter assay//qRT-PCR//Western blot	20190813
hsa-mir-345-5p	MIMAT0000772	PAX8	7849	PAR-CLIP	23446348
hsa-mir-345-5p	MIMAT0000772	CDKN1A	1026	Luciferase reporter assay//qRT-PCR//Western blot	20190813

(Continued)

TABLE 4 | Continued

miRNA ID	Accession	Target Gene	Target ID	Experiment	Literature PubMed ID
hsa-mir-345-5p	MIMAT0000772	NTRK3	4916	Luciferase reporter assay	19370765
hsa-mir-4291	MIMAT0016922	CDKN1A	1026	PAR-CLIP	26701625
hsa-mir-4291	MIMAT0016922	LAMA4	3910	PAR-CLIP	23592263
hsa-mir-4291	MIMAT0016922	CDK6	1021	PAR-CLIP	23446348|21572407|20371350
hsa-mir-4291	MIMAT0016922	FGF2	2247	PAR-CLIP	23446348|21572407|20371350
hsa-mir-4291	MIMAT0016922	RAF1	5894	PAR-CLIP	21572407
hsa-mir-4291	MIMAT0016922	TRAF1	7185	PAR-CLIP	23592263
hsa-mir-4291	MIMAT0016922	FZD6	8323	PAR-CLIP	22100165
hsa-mir-4291	MIMAT0016922	LAMA4	3910	PAR-CLIP	23592263
hsa-mir-4291	MIMAT0016922	RAF1	5894	PAR-CLIP	21572407
hsa-mir-4291	MIMAT0016922	VASP	7408	PAR-CLIP	26701625
hsa-mir-4291	MIMAT0016922	RAF1	5894	PAR-CLIP	21572407
hsa-mir-4291	MIMAT0016922	CDKN1A	1026	PAR-CLIP	26701625
hsa-mir-4291	MIMAT0016922	RAF1	5894	PAR-CLIP	21572407
hsa-mir-4291	MIMAT0016922	RAF1	5894	PAR-CLIP	21572407
hsa-mir-181a-3p	MIMAT0000270	ARHGDIA	396	PAR-CLIP	26701625

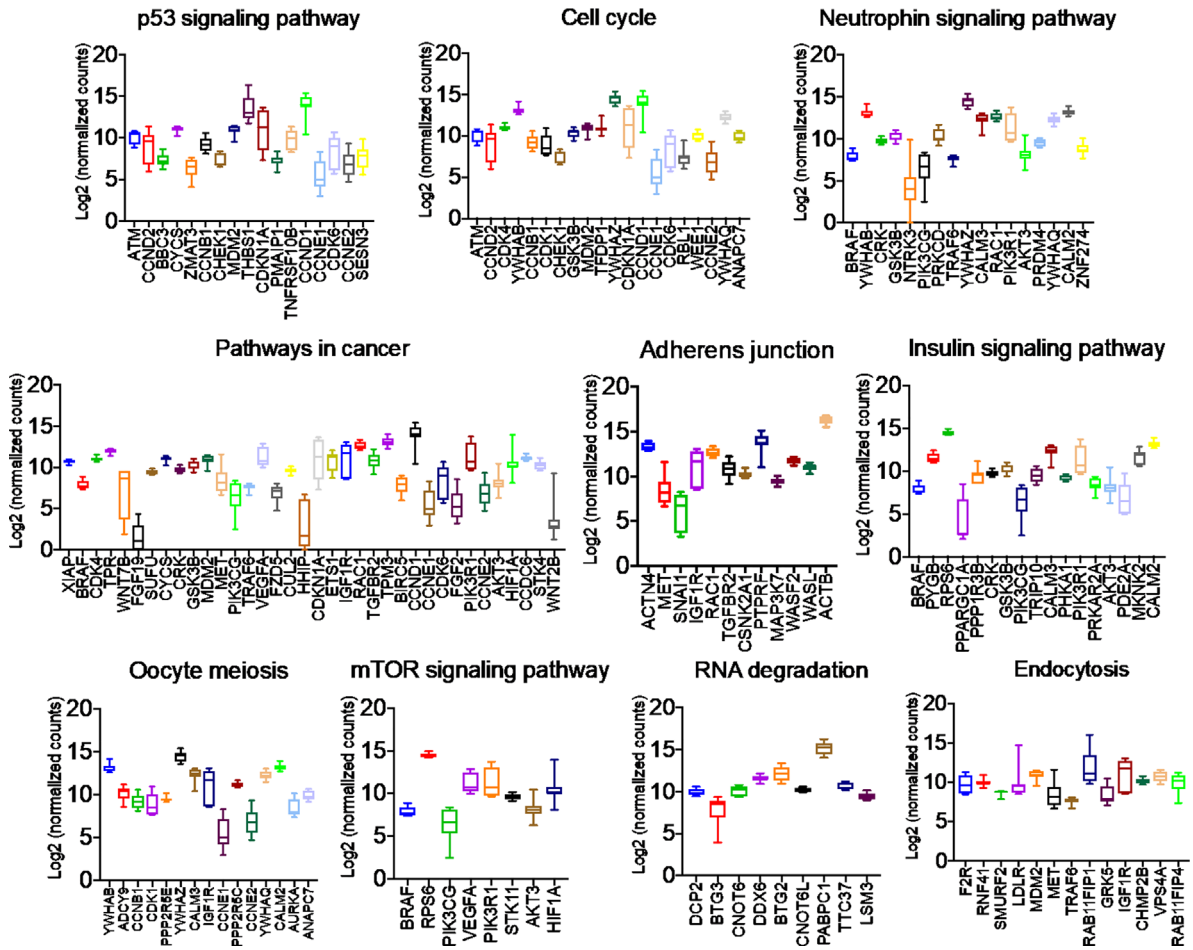

FIGURE 4 | Network analysis identified miRNA target genes to be upregulated in breast cancers following AG-205 treatment. miRNAs target differentially expressed genes miRNA target genes that are upregulated in metastatic breast tumor samples. A Log2 (normalized_counts) expression of upregulated miRNA target genes in metastatic breast tumor samples downloaded from TCGA database. miRNA target genes are involved in term pathways identified by KEGG analysis and are direct targets of the top miRNAs.

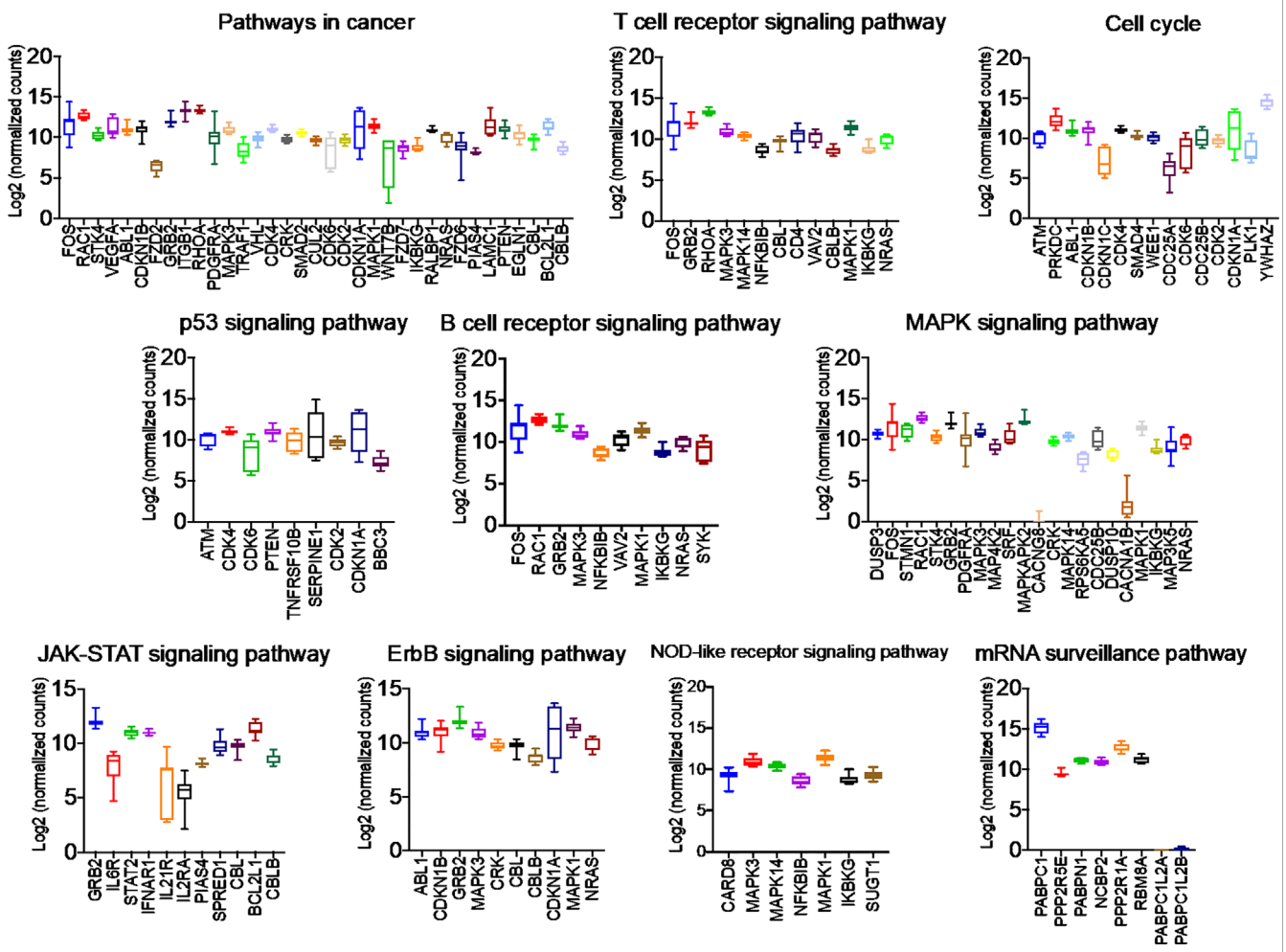

FIGURE 5 | Network analysis identified miRNA target genes to be upregulated in breast cancers following AG-205 treatment. The top upregulated miRNA target genes involved in KEGG pathway analysis have upregulated Log2 (normalized_counts) expression in metastatic breast tumor samples obtained from TCGA database.

interestingly upregulated genes involved in signal transduction mechanisms could be directly regulated by PGRMC1, as signal transduction mechanisms are known to be directly involved in cellular membranes where PGRMC1 is primarily located (94). To further study the clinical impact of these genes, we studied genetic alterations using OncoPrint. It was particularly interesting to see that only 10 genes displayed significant genetic alteration among the 22 genes that were overexpressed. However, of the ten genes the top two most genetically altered, *CCND1* and *YWHAZ* seemed to be overexpressed due to amplification and had overall lower survival probability. *CCND1* has long been considered an oncogene and has been demonstrated to be amplified in 10-20% in one study while in another study *CCND1* amplification was seen in 78.6% of breast cancer cases (95–97). *CCND1* is thought to play a major role in ER-positive but not in ER-negative breast cancers (98). One of the reasons could be because it is a known downstream target of PR that can promote breast cancer cell proliferation (99, 100). One interesting thought could be that in TNBCs that overexpress PGRMC1, it could be enhancing the transcription of *CCND1* even in tumors that lack ER and PR making it a potential target in TNBCs. The *YWHAZ* gene has been described in multiple cancers including non-small lung cancer (101), hepatocellular carcinoma (102), gastric cancer (103), bladder cancer (104), and in breast cancers (105). Overexpression of *YWHAZ* in breast cancers has been associated with chemoresistance to anthracyclines particularly associated with metastatic recurrence (105). This is also extremely interesting as PGRMC1 has been linked to chemoresistance (106) and it would be strongly warranted to further explore the possibility of a PGRMC1/YWHAZ axis in metastatic breast cancers that do not respond to chemotherapy.

CONCLUSION

In summary, our study identified that impairing PGRMC1 can alter miRNAs, specifically hsa-mir-646 that directly targets *CCND1* (107) as well as hsa-mir-410-3p and hsa-mir-3150b-3p

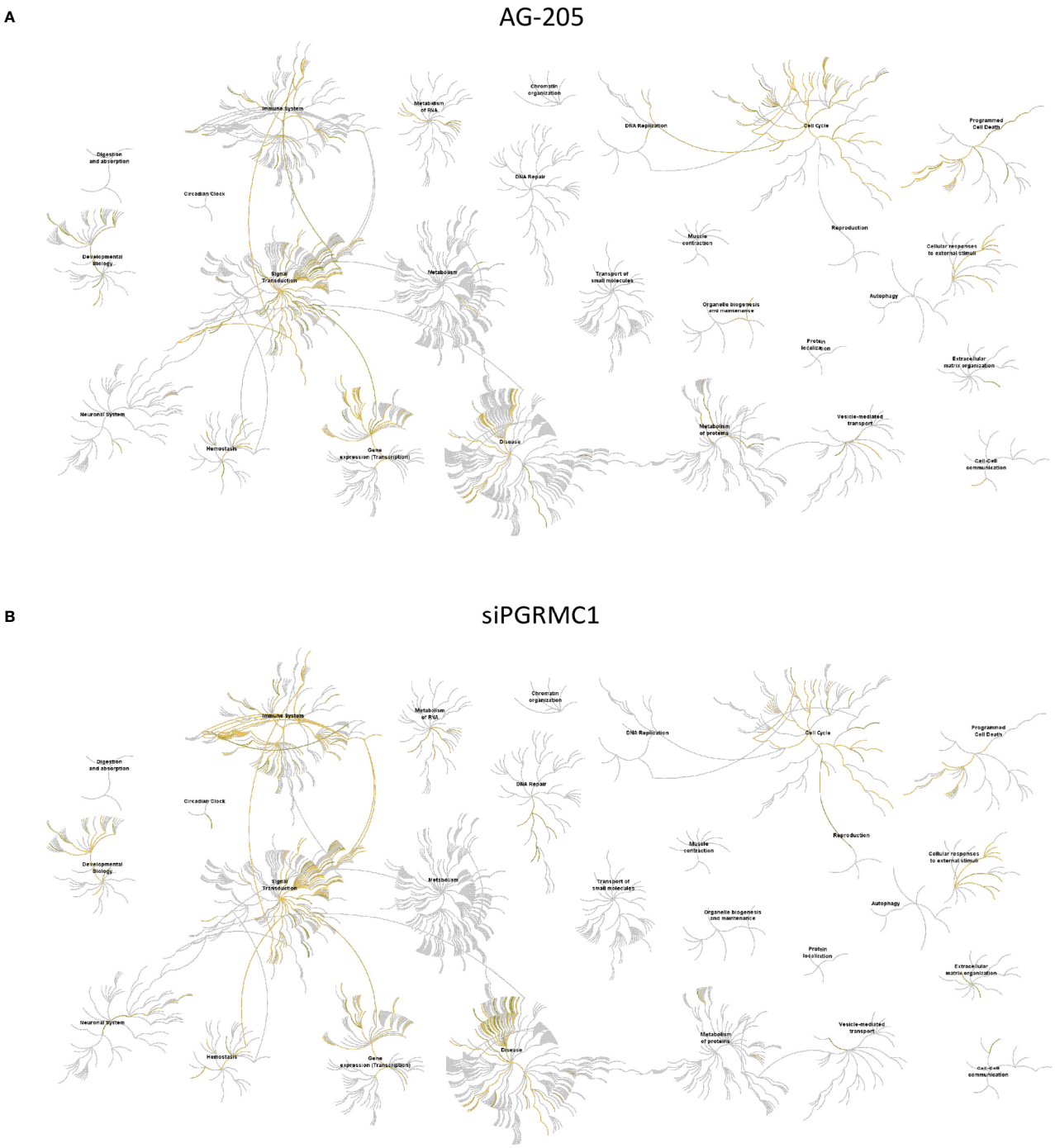

FIGURE 6 | Reactome pathway analysis of the genes identified by KEGG term analysis. **(A)** Reactome pathways analysis of the miRNA target genes (n = 112) identified following AG-205 treatment illustrates increased pathway involvement. **(B)** Top pathways involved within the miRNA target genes (n = 84) observed following PGRMC1 silencing were also mapped. Over-represented pathways are highlighted in yellow. All overexpressed pathways are from gene lists of formerly annotated and published signatures.

which target *YWHAZ* (108–113). Interestingly, both genes were amplified in patients with aggressive TNBCs and patients that express high levels of either gene have lower overall survival probability. Lastly, PGRMC1 impairment downregulates oncogenic miRNAs (miR-30b, miR-664a-3p and miR-93-3p, miR-224-5p, miR-181a-3p and miR-345-5p) in TNBC cells. Therefore, targeting PGRMC1 with AG-205 or a novel compound that can downregulate PGRMC1 expression could

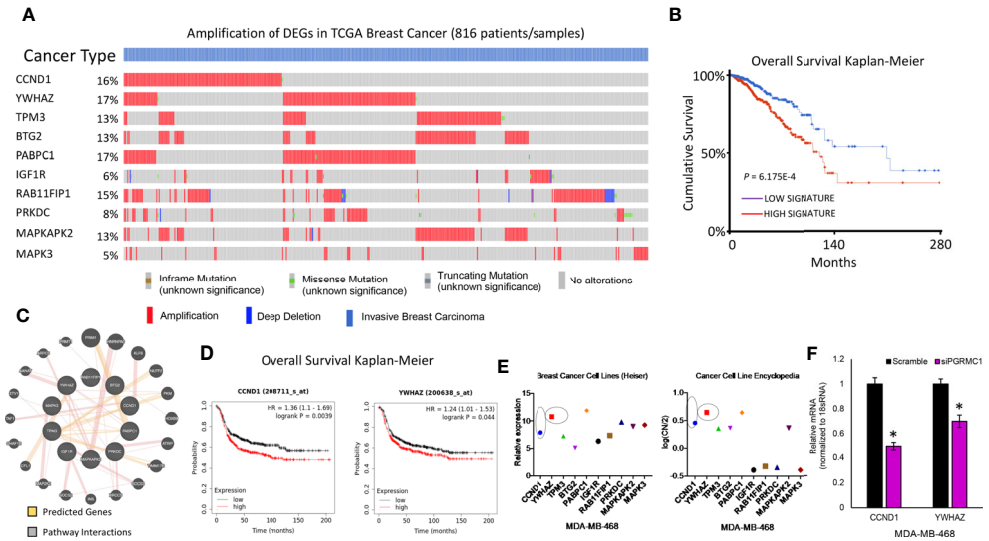

FIGURE 7 | PGRMC1 impairment identified miRNA target genes to be amplified in invasive breast carcinoma patients. **(A)** Oncoprint illustrates genetic alterations such as inframe mutations, missense mutation, truncating mutation, amplification and deep deletion of breast cancer tumor samples (n=816). miRNA target genes that had a greater than 5% genetic alteration were considered for further analysis**. (B)** Cumulatively patient samples that have high signature/expression of miRNA target genes exhibiting > 5% genetic alterations are associated with poorer overall survival. **(C)** Network analysis links the top ten miRNA target genes with associated pathway interactions and predicts interactions within known pathways. **(D)** The top two miRNA target genes, *CCND1* and *YWHAZ* are associated with significantly poorer overall survival in ER-negative breast tumor samples ($P < 0.05$ was considered significant). **(E)** Increased relative gene expression and copy number variation of *CCND1* and *YWHAZ*, are observed in MDA-MB-468 breast cancer cell lines. **(F)** Relative mRNA expression of *CCND1* and *YWHAZ* in PGRMC1 silenced MDA-MB-468 cells. *P < 0.05.

be potential therapeutic options for TNBC patients that overexpress PGRMC1.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Conception and design: RL and DP. Methodology was developed by DP and VR. Data acquisition: DP, MR, and VR. Data was interpreted by RL, DP, MR, VR, RS, and AE. The manuscript was written and/or revised by DP, MR, RS, VM, TG, and RL. This study was supervised by RL. All authors contributed to the article and approved the submitted version.

FUNDING

Breast Cancer Discretionary Fund from Texas Tech University Health Sciences Center El Paso.

ACKNOWLEDGMENTS

We would like to thank Texas Tech University Health Sciences Center El Paso for supporting this research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.710337/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: 06 September 2021
doi: 10.3389/fonc.2021.700853

Biological Role and Clinical Implications of microRNAs in BRCA Mutation Carriers

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OPEN ACCESS

Edited by:
Naoyuki Kataoka, The University of Tokyo, Japan

Reviewed by:
Bryan Raymond George Williams, Hudson Institute of Medical Research, Australia
Saeed Pirouzpanah, Tabriz University of Medical Sciences, Iran

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

Received: *27 April 2021*
Accepted: *20 August 2021*
Published: *06 September 2021*

Citation:
Tommasi C, Pellegrino B, Boggiani D, Sikokis A, Michiara M, Uliana V, Bortesi B, Bonatti F, Mozzoni P, Pinelli S, Squadrilli A, Viani MV, Cassi D, Maglietta G, Meleti M and Musolino A (2021) Biological Role and Clinical Implications of microRNAs in BRCA Mutation Carriers. Front. Oncol. 11:700853. doi: 10.3389/fonc.2021.700853

Women with pathogenic germline mutations in *BRCA1* and *BRCA2* genes have an increased risk to develop breast and ovarian cancer. There is, however, a high interpersonal variability in the modality and timing of tumor onset in those subjects, thus suggesting a potential role of other individual's genetic, epigenetic, and environmental risk factors in modulating the penetrance of BRCA mutations. MicroRNAs (miRNAs) are small noncoding RNAs that can modulate the expression of several genes involved in cancer initiation and progression. MiRNAs are dysregulated at all stages of breast cancer and although they are accessible and evaluable, a standardized method for miRNA assessment is needed to ensure comparable data analysis and accuracy of results. The aim of this review was to highlight the role of miRNAs as potential biological markers for BRCA mutation carriers. In particular, biological and clinical implications of a link between lifestyle and nutritional modifiable factors, miRNA expression and germline *BRCA1* and *BRCA2* mutations are discussed with the knowledge of the best available scientific evidence.

Keywords: miRNAs, breast cancer, nutriepigenomics, BRCA1/2 mutations, breast cancer risk

INTRODUCTION

Breast cancer (BC) is the most common cancer in women, as the cumulative risk of developing a BC during all life is calculated to be about 1 case every 8 women worldwide (1) (2). BC is also the principal cause of cancer death among women worldwide accounting for 25% of cancer cases and 15% of cancer-related deaths (3).

Hereditary breast cancer accounts for about 5-10% of all breast cancers (BCs) and is associated with an increased risk of ovarian cancer (4, 5).

Hereditary Breast and Ovarian Cancer syndrome (HBOC) is related, in about 50% of cases, to pathogenic germline mutations of *BRCA1* and *BRCA2* genes (6, 7). *BRCA1/2* genes are onco-

suppressors involved in homologous recombination repair (HRR) of DNA double-strand breaks (DSBs) and maintenance of genome stability (8). Women who inherit *BRCA1/2* mutations have a lifetime risk to develop breast and ovarian cancer of 45-60% and 10-59%, respectively (9) (10). In these cases, viable prevention strategies include intensive radiologic surveillance, chemoprevention, and prophylactic surgery of breasts and ovaries (11).

Although breast and ovarian cancer risk increases considerably, not all women with *BRCA1/2* mutations develop a neoplasm. There is a high interpersonal variability in the modality and timing of tumor onset in BRCA-mutated subjects, thus suggesting a potential role of other genetics, epigenetics, or environmental individual risk factors in modulating the penetrance of *BRCA1/2* germline mutations (12).

Transcribed, non-coding RNAs (ncRNAs) do not encode for proteins and have a specific biological function (13). NcRNAs have various transcripts' lengths: short ncRNAs are <50 nucleotides (nt), as well as microRNAs (miRNAs); midsize ncRNAs include the ncRNAs between 50 nt and 200 nt. Finally, long ncRNAs (lncRNAs) have a length over 200 nt (13–15).

MiRNAs are involved in post-transcriptional, epigenetic modification of DNA expression (16). They are readily detectable in tissue and blood samples (17), saliva (18) or urine (19). While it is difficult to establish cause-and-effect relationships, several studies indicate that some miRNA expression patterns may be associated with: i) increased breast/ovarian cancer risk; ii) some modifiable nutrition/lifestyle risk factors; iii) *BRCA1/2* mutations (12, 17). Gene panels, which simultaneously evaluate whole miRNAs, are able to identify different miRNA expression profiles between healthy women, women with sporadic BC and women with BRCA-mutated BC (19, 20).

Based on these considerations, the aim of this review is to highlight the role of miRNAs as potential biomarkers for BRCA mutation carriers. Biological and clinical implications of a link between lifestyle and nutritional modifiable factors, miRNA expression and germline *BRCA1* and *BRCA2* mutations are here discussed with the knowledge of the best available scientific evidence.

MECHANISTIC INSIGHTS OF THE INTERACTION OF miRNAs WITH BRCA GENES

MiRNAs are critical regulators of the transcriptome over a number of different biological processes and they may behave as onco-suppressors and onco-promoters (21). MiRNAs can post-transcriptionally suppress gene expression by binding to the 3′-untranslated region (UTR) of messenger RNA (mRNA) (22). However, miRNA interactions with other regions, which include the 5′-UTR, coding sequence, and gene promoters, have also been described (22, 23). Moreover, miRNAs have been shown to trigger gene expression under certain condition (21). Recent studies have demonstrated that miRNAs are transferred between various subcellular compartments to regulate both translation and transcription (21) (22).

BRCA1/2 gene expression can be altered by miRNAs, in addition to deletion or mutation, in a BRCAness-like phenomenon (**Figure 1**) (21). E2F1, a G1/S transition regulator, is targeted by miR-302b in breast cancer cell lines. MiR-302b, by negatively regulating E2F1, downregulates ATM, the principal cellular sensor of DNA damage, that phosphorylases and actives BRCA1. As a result, miR-302b indirectly impairs BRCA1 function (23). Furthermore, various studies have evaluated some miRNAs targeting BRCA genes in breast cancer. MiR-146a binds to the 3′-UTRs of BRCA1and

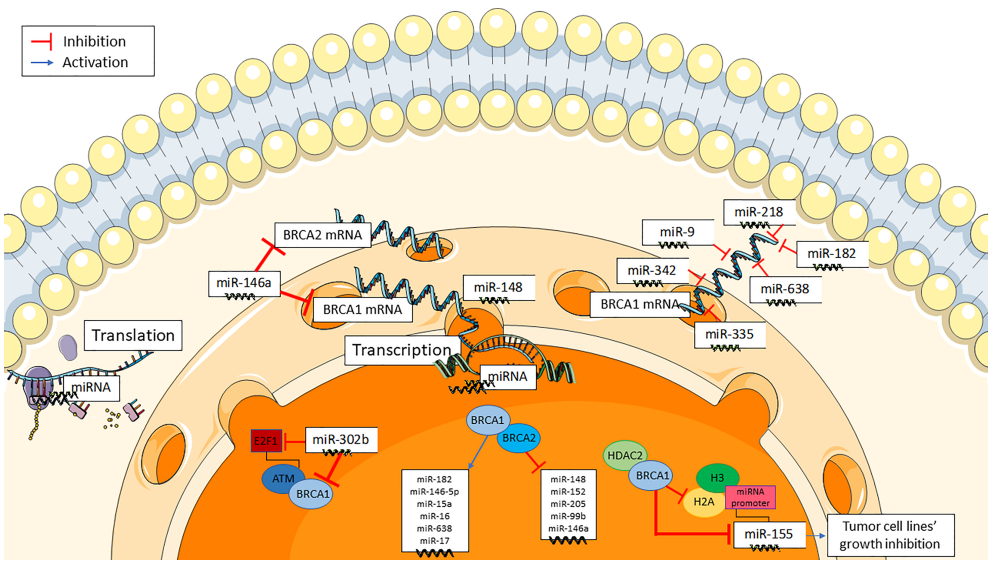

FIGURE 1 | Mechanistic insights of the interaction of miRNAs with BRCA genes.

BRCA2 mRNAs, thus negatively modulating their expression. Interestingly, the binding capacity of miR-146a seems to be dependent from some of its gene polymorphisms (24). In human tumor xenografts, miR-9 has been found to bind to the 3'-UTR of BRCA1 mRNA, downregulate BRCA1 expression, and enhance cancer cell susceptibility to DNA damage (25). Similar findings have been reported for miR-182 (26), miR-155 (27), miR-342 (28), miR-335 (29), miR-218 and miR-638 (23).

BRCA1 and *BRCA2* genes also regulate several miRNAs either by upregulating some of them (i.e. miR-146a, miR-146-5p, miR-182, miR-15a, miR-16, miR-17, and miR-638), or by downregulating some others (i.e. miR-155, miR-152, miR-148, miR-205, miR-146a, and miR-99b) (**Figure 1**) (19). Notably, BRCA1 is involved in the epigenetic control of miR-155, which is a well-known proinflammatory and oncogenic miRNA (27, 30). MiR-155 overexpression stimulates while miR-155 knockdown impairs cancer cell growth. BRCA1 targets the miR-155 promoter, thus suppressing its transcription. More precisely, BRCA1 suppresses miR-155 expression through its association with histone deacetylase 2 (HDAC2), which deacetylates histones H2A and H3 on the miR-155 promoter (27). Some moderate-risk BRCA1 variants (e.g. R1699Q) do not affect DNA repair but abrogate the inhibition of microRNA-155 (27).

ROLE OF miRNAs IN DIFFERENT BREAST CANCER SUBTYPES

Breast cancers are usually grouped into surrogate intrinsic subtypes, defined by routine histology and immunohistochemistry (IHC): luminal A-like tumors are generally low grade, strongly estrogen receptor (ER)/progesterone receptor (PR)-positive, human epidermal growth factor receptor (HER2)-negative and have low proliferation rate. Luminal B-like tumors are ER-positive with variable degrees of ER/PR expression, are higher grade and have higher proliferation rate. HER2-positive tumors are usually high grade, frequently ER/PR-negative, and have high proliferative rate. Triple-negative breast cancers (TNBCs) are high grade, ER/PR-negative, HER2-negative, and have high proliferation rate (1) (2) (3). The expression of numerous miRNAs correlates with different BC subtypes and different prognosis (**Table 1**).

Some highly expressed miRNA clusters have been associated with Luminal A-like and Luminal B-like tumors (31, 32). Interestingly, overexpression of miR-100 in basal-like breast cancer cells leads to stemness loss, expression of luminal markers and sensitivity to endocrine therapy (54). Baseline tumor expression of miR-100 has been associated with response to endocrine treatment in patients with ER-positive/HER2-negative breast cancer. In the METABRIC dataset, high expression of miR-100 was observed in luminal A breast cancers with better overall survival (54).

MiR-21 overexpression has been observed in HER2-positive breast cancers, probably because the corresponding gene is located on chromosome 17 and, thus, co-amplifies with HER2 (35, 55). MiR-21 is also an independent prognostic factor associated with early disease relapse and worse disease-free interval (DFI) (56). Furthermore, low miR-497 expression has been observed to be strictly correlated with HER2-positive status and advanced clinical stage (39). In a retrospective case series of tumor tissues and correspondent normal breast tissues, patients

TABLE 1 | miRNAs as diagnostic and prognostic biomarkers in breast cancer.

miRNA role/function	Identified miRNAs	miRNA detection tissue	Reference
Overexpressed in Luminal-like BC	miR-29; miR-181a; miR-652; miR-342	Serum	(31)
	miR-100; miR-155; miR-126; miR10a; let-7c; let-7f; miR-217; miR-218; miR-377; miR-520f-520c; miR-18a	Tumor tissue	(32–34)
Overexpressed in HER2-positive BC	miR-21; miR-376b	Serum	(35, 34)
Overexpressed in TNBC	miR-210; miR-146a; miR-146b-5p; miR-10b; miR-18a; miR-135b; miR-93v; miR-299-3p; miR-190; miR-135b; miR-520g; miR-527-518a	Tumor tissue	(34, 36–38)
Overexpression associated with endocrine sensitivity	miR-27a; miR-100; miR-375; miR-342; miR-221/222; let-7f	Tumor tissue	(32, 34)
Overexpression associated with better survival	miR-497; miR10a; miR-126; Let-7b; miR-147b; miR-6715a; miR-324-5p; miR-711; miR-375	Tumor tissue	(32–34, 39–41)
	miR-1258	Tumor tissue/serum	(42)
	miR-92a	Plasma/serum	(43)
Overexpression associated with worse survival	miR-21	Tumor tissue/serum	(29, 43)
	miR-210; miR-205; miR-374a; miR-10b; miR-549a; miR-4501; miR-7974; miR-4675; miR-9; miR-18b; miR-103; miR-107; miR-652; miR-27b-3p	Tumor tissue	(32, 36, 38) (42–44)
Overexpressed in BC compared to HC	miR-1; miR-133a; miR-133b; miR-92a; miR-21; miR-16; miR-27; miR-150; miR-191; miR-200c; miR-210; miR-451; miR-155; miR-195; Let-7b; miR-106b; miR-145; miR-425-5p; miR-139-5p; miR-130a; miR-34a	Tumor/normal tissue/serum	(41–43, 45–50)
	miR-1246; miR-1307-3p; miR-4634; miR-6861-5p; miR-6875-5p; miR-1246; miR-146a; miR-18a	Plasma/serum	(34, 43, 47, 51)
Underexpressed in BC compared to HC	miR-145	Tumor/normal tissue/serum	(41)
	miR-30a	Plasma	(52)
	miR-140-5p; miR-497; miR-199a; miR-484; miR-202; miR-181a (Nassar)	Tumor/normal tissue	(36, 41, 53)

miRNA, microRNA; BC, breast cancer; TNBC, triple negative breast cancer; pts, patients; HC, healthy controls.

with low miR-497 expression had worse 5-year disease-free survival (DFS) and overall survival (OS) than the ones with high miR-497 (39).

Approximately 10-15% of breast tumors are known to be of the TNBC subtype, which is considered to have an aggressive clinical history and shorter survival (20, 31, 57, 58). Up to 29% of TNBC patients harbors somatic mutations or epigenetic downregulation of *BRCA1* and *BRCA2* genes (59). Differential miRNA expression could help predict prognosis in patients with TNBC (36, 37). Accordingly, miR-210 is often up-regulated in TNBC tissues and correlates with unfavorable prognosis (38). High levels of miR-146a and miR-146b-5p have also been described in triple negative tumor samples (60). MiR-155 is commonly down-regulated in TNBC, indeed its overexpression is related with good prognosis (61). Interestingly, some miRNAs may play different prognostic roles depending by molecular subtypes: miR-27 is associated with better OS in ER-positive BC patients, while its upregulation is detrimental in ER-negative ones (44).

Several other miRNAs have been shown to influence the prognosis of BC patients (**Table 1**). The deregulation of 5 metastasis-related miRNAs (miR-21, miR-205, miR-10b, miR-210, and let-7a) observed in a series of 84 primary breast tumors significantly correlated with clinical outcome (32, 62). In a sample of 81 postmenopausal, ER-positive BC patients, a higher tumor expression of miR-126 and miR-10a was associated with longer relapse-free survival (RFS) (33). The expression of let-7b in tumor tissues of 80 breast cancer patients was inversely associated with lymph node involvement, OS and RFS (40). MiR-374a expression was significantly elevated in the primary tumors of 33 patients with metastatic disease in comparison with that observed in primary tumors of 133 patients with no evidence of distant metastasis (63). Decreased levels of miR-92a and increased tumor levels of miR-21 were associated with higher tumor stage and presence of lymph node metastases (43). According to other reports, the downregulation of miR-1258 was associated with positive lymph node involvement, later clinical stage, and poor prognosis (42).

ROLE OF MiRNAs IN BREAST CANCER RISK AND DETECTION

Several studies focused on identifying either individual or groups of miRNAs to be used for prediction of BC risk and for BC detection (**Table 2**). The most studied miRNAs were analyzed in plasma/serum and tumor tissues of BC patients in comparison with normal controls (41). However, those studies were conducted in different ethnic groups and used different experimental methodology (43). Some studies detected miRNAs in breast tissues using distinct platforms (real-time quantitative reverse transcription PCR [qRT-PCR], sequencing, microarray) followed by validation in serum/plasma (41, 34). Others produced array panels on plasma samples followed by verification using qRT-PCR (47) or started with qRT-PCR on tissues with subsequent serum qRT-PCR (41). One of the limitations of the previously mentioned platforms is their restriction to known miRNAs. Next-generation sequencing (NGS) technologies provide novel approaches for identification of new miRNAs and confirmation of known ones (34).

In a recent study, miRNAs from paired breast tumors, normal tissue, and serum samples of 32 patients were profiled; serum samples from healthy individuals (n = 22) were also used as controls. Twenty miRNAs including miR-21, miR-10b, and miR-

TABLE 2 | Diagnostic parameters to evaluate breast cancer diagnostic ability of individual and combined studied miRNAs.

miRNA	Sensitivity	Specificity (%)	PPV (%)	NPV (%)	DA (%)	Reference
miR-21	73	81	76	78	77	(50, 64–67)
miR-155	78	75	78	75	77	(50, 64–67)
miR-23a	78	75	88	44	68	(50, 64–67)
miR-130a	83	78	83	78	81	(50, 64–67)
miR-145	78	91	78	91	83	(50, 64–67)
miR-425-5p	70	100	70	100	81	(50, 64–67)
miR-139-5p	76	96	76	95	83	(50, 64–67)
miR-451	73	72	73	72	73	(50, 64–67)
miR-200a	69	62	NR	NR	70	(50, 64–67)
miR-200c	71	67	NR	NR	74	(50, 64–67)
miR-141	68	70	NR	NR	74	(50, 64–67)
miR-10b	NR	NR	NR	NR	85	(50, 64–67)
miR-181a	NR	NR	NR	NR	82	(50, 64–67)
miR-106b	NR	NR	NR	NR	89	(50, 64–67)
miR-34a	85	70	93	70	81	(50, 64–67)
miR-200b + miR-429	77	63	NR	NR	75	(50, 64–67)
miR-145 + miR-425-5p	78	95	78	95	84	(50, 64–67)
miR-21 + miR-23a	95	66	95	66	82	(50, 64–67)
miR-21 + miR-130a	88	78	88	78	84	(50, 64–67)
miR-21 + miR-23a +miR-130a	93	78	93	78	86	(50, 64–67)
miR-145 + miR-139-5p +miR-130a	95	86	95	86	92	(50, 64–67)
miR-145 +miR-139-5p +miR-130a + miR-425-5p	97	91	97	90	95	(50, 64–67)
miR-1246 + miR-206 + miR-24 + miR-373	98	96	NR	NR	97	(50, 64–67)

PPV, positive predictive value; NPV, negative predictive value; DA, diagnostic accuracy; NR, not reported.

145 were found to be differentially expressed in breast cancers. Only 7 miRNAs were overexpressed in both serum and tumors, thus indicating that miRNAs may be selectively released into the serum. MiR-92a, miR-1, miR-133a, and miR-133b were identified as the most significant diagnostic serum markers (45). A combination of 5 serum miRNAs (miR-1307-3p, miR-1246, miR-6861-5p, miR-4634, and miR-6875-5p) were also found to detect breast cancer patients among healthy controls (53).

MiR-21, one of the most common miRNAs in human cells, has been investigated in different diseases including cardiovascular diseases as well as cancers (46). Studies have demonstrated that miR-21 plays an oncogene function in breast cancer by targeting tumor suppressor genes which include programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1), and phosphatase and tensin homolog (PTEN) (68). The diagnostic role of high plasma levels of both miR-1246 and miR-21 was demonstrated by analyzing the contents of circulating exosomes, which are secretory microvesicles that selectively enclose miRNAs (69). Exosomes were collected from the conditioned media of human BC cell lines, murine plasma of patient-derived xenograft models (PDX), and human plasma samples. MiR-21 and miR-1246 were selectively enriched in human BC exosomes and significantly increased in the plasma of BC patients (69). Other studies have suggested a key role for miR-21 in discriminating healthy individuals from BC patients. Overexpression of circulating miR-21 and miR-146a were significantly higher in plasma samples of BC patients, when compared to controls (51). Serum levels of miR-16, miR-21, miR-155, and miR-195 were observed to be higher in stage I BC patients in comparison with unaffected women (51).

Nine miRNAs (miR-16, miR-21, miR-27a, miR-150, miR-191, miR-200c, miR-210, miR-451 and miR-145) were observed to be deregulated in both plasma and tumor tissues from BC patients (47). A validation cohort study reported that a combination of miR-145 and miR-451 was the best biomarker in discriminating breast cancer from healthy controls and other tumor types (47).

The expression of 10 miRNAs (measured by qRT-PCR) has been evaluated in 48 tissue and 100 serum samples of patients with primary BC and in 20 control samples of healthy women (43). The level of miR-92a was significantly lower, while miR-21 was higher in tissue and serum samples of BC patients in comparison with controls. The same expression levels correlated with tumor size and lymph node-positive status (43).

MiR-30a has also been studied as diagnostic biomarker of breast cancer. The median plasma levels of miRNA-30a were significantly lower in a sample of 100 patients with preoperative breast cancer than in 64 age-matched and disease-free controls (52). MiR-497 and miR-140-5p have been shown to be down-regulated in breast tumor samples compared to noncancerous breast tissues, while let-7b seems to be upregulated in BC specimens compared to benign breast diseases (38, 46, 68). Validation of these data in serum or plasma samples is missing.

Another interesting approach is to use miRNAs to unveil BC patients with lymph node involvement (overcoming the prognostic role of the axillary dissection) or to identify patients with metastatic disease. Levels of circulating miR-1258 decreased (42), while miR-10b and miR-373 levels increased (70) in a series of BC patients with lymph nodes metastasis in comparison with non-metastatic patients (70). Furthermore, tumor tissue expression of miR-140-5p decreased in the sequence from stage I to III breast cancer, and was lower in breast tumors with lymph node involvement in comparison with ones without metastasis (71).

Receiver operating characteristics (ROC) analyses have been conducted to evaluate breast cancer diagnostic ability of miRNAs (50, 64–67). Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy for the most studied individual and combined miRNAs are reported in **Table 2**.

ROLE OF MiRNAs IN BRCA MUTATION CARRIERS

Breast cancer susceptibility in *BRCA1/2* mutation carriers may be related to the aberrant expression of certain miRNA clusters (**Table 3**) (78). MiR-3665, miR-3960, miR-4417, miR-4498, and let-7 have been observed to be overexpressed in women with BRCA-mutated breast tumors (75). Conversely, the downregulation of miR-200c has been reported in BRCA-mutated, TNBC (74).

Some studies have also shown that genetic polymorphisms in the gene codifying for miR-146a were associated with early onset in familial cases of breast and ovarian tumors (24). Different expression patterns of six miRNAs (miR-505*, miR-142-3p, miR-1248, miR-181a-2*, miR-340*, and miR-25*) have been found to distinguish between BRCA-mutated and BRCA wild-type (BRCAwt) breast tumors with an accuracy of 92% (24,72). Similarly, a miRNA expression analysis using NanoString technology was performed on BRCA-mutated and sporadic, BRCAwt breast tumor tissues. Eight miRNAs (miR-539, miR-627, miR-99b, miR-24, miR-663a, miR-331, miR-362, and miR-145) were differentially expressed in hereditary breast cancers (73). Let-7a and miR-335 are tumor suppressor miRNAs that can impair both tumorigenesis and metastasis. Let-7a and miR-335 expression levels were significantly downregulated in cancers of patients with BRCA mutations in comparison with tumors of BRCAwt subjects (29, 76).

BRCA-mutated tumors are *in vitro* and *in vivo* sensitive to DNA-damaging agents (such as platinum salts) and to poly-ADP ribose polymerase (PARP) inhibitors (PARPi) (8, 79). Up to 29% of patients with TNBC harbor somatic mutations or epigenetic downregulation of *BRCA1* and *BRCA2* genes, thus being sensitive to platinum salts and PARPi (79). MiR-146a, miR-146b-5p and miR-182 downregulate BRCA1 protein expression and some reports have shown that different expression of miR-182 in breast cancer cell lines affects their sensitivity to PARPi (26, 60). Furthermore, other studies have reported that the over-expression of miR-493-5p restores genomic stability of *BRCA2*-mutated/depleted leading to acquired resistance to PARPi/platinum salts (77).

TABLE 3 | miRNAs and germinal BRCA mutations.

miRNA role/function	Identified miRNAs	miRNA detection tissue	Reference
Upregulated by wild-type BRCA1	miR-182; miR-146-5p; miR-15a; miR-16; miR-638; miR-17	Tumor/normal tissue/serum	(19)
Downregulated by wild-type BRCA1	miR-148; miR-152; miR-205; miR-99; miR-146a	Tumor/normal tissue/serum	(19, 72)
Combined expressions of miRNAs distinguish between BRCA-mutated and BRCA wild-type BC	miR-142-3p; miR-505; miR-1248; miR-181a-2; miR-25; miR-340	Tumor/normal tissue	(72)
	miR-627; miR-99b; miR-539; miR-24; miR-331; miR-663a; miR-362; miR-145	Tumor/normal tissue	(73)
Downregulated in BRCA-mutated BC	miR-155; let-7a; miR-335	Tumor tissue	(27, 29, 61)
	miR-200c	Cell culture	(74)
Overexpressed in BRCA-mutated BC	miR-3665; miR-3960; miR-4417; miR-4498; let-7	Tumor tissue	(75)
Regulate BRCA1 expression in BC	miR-342; miR-182; miR-335; miR-146a, miR-146b-5p; miR-182	Tumor tissue	(26, 28, 60, 76)
Restores HRR and genomic stability in *BRCA2*-mutated cancers	miR-493-5p	Tumor tissue	(77)

miRNA, microRNA; BC, breast cancer; HRR, Homologous Recombination Repair.

NUTRIEPIGENOMICS AND BREAST CANCER RISK

Nutriepigenomics is the study of nutrients and their effects on human health through epigenetic modifications. Numerous studies have suggested that body mass index (BMI), components of food and lifestyle may interfere with miRNA expression, thus affecting tumors' initiation and progression (72) (29) (**Table 4**).

Obesity causes changes in the physiological function of adipose tissue, leading to adipocyte differentiation, insulin resistance, abnormal secretion of adipokines, and altered expression of hormones, growth factors, and inflammatory cytokines. All these factors are involved in the occurrence of several diseases, such as type 2 diabetes mellitus, cardiovascular disease, and various types of cancers (83). Obesity is one of the major risk factors for breast cancer, especially in post-menopausal women. Recent studies have proposed the association between obesity, cancer and up- or down-regulation of some miRNAs (85).

Obesity has been found to reduce expression of miR-10b in primary tumors compared to normal tissue, thus suggesting that the metabolic state of the organism can alter the molecular composition of a tumor (48, 80, 81, 84). Expression levels of miR-191_5p, miR-122-5p and miR-17_5p, which are involved in tumorigenic processes, have been inversely associated with BMI (82). Levels of miR-191-5p significantly increased during a six-month weight-loss intervention (Lifestyle, Exercise, and Nutrition; LEAN trial) in 100 BC survivors (82). Furthermore, the related family member miR-106b_5p, which is up-regulated in breast cancer patients (49, 81), has been found to significantly decrease in response to exercise intervention and diet (82, 85). The nuclear peroxisome proliferator-activated receptor gamma (PPARγ) plays a critical role in the modulation of cellular differentiation, glucose and lipid homeostasis (84). It has been associated with anti-inflammatory activities, and differentiation of preadipocytes into mature adipocytes together with members of the CCAAT/enhancer-binding family protein (C/EBP) family (82). PPARγ is implicated in the pathology of numerous diseases involving cancer and obesity, and altered expressions of miRNAs, such as let-7, miR-27, and miR-143 have been found to regulate the expression and activity of *PPARγ* (81). Interestingly, miR-31 has been found to impair both BC cell

TABLE 4 | miRNAs, body mass index and lifestyle changes.

miRNA role/function	Identified miRNAs	miRNA detection tissue	Reference
Downregulated in BC of obese patients vs. lean subjects	miR-10b	Tumor/normal tissue	(48)
Inversely correlated with BMI in BC survivors	miR-191-5p; miR17- 5p	Serum	(80–82)
Directly correlated with BMI in BC survivors	miR-122-5p		(82)
Overexpressed during diet and exercise intervention in BC survivors	miR-191-5p; miR-122-5p; let-7b-5p; miR-24-3p	Serum	(82)
Underexpressed during diet and exercise intervention in BC survivors	miR-106b; miR-106b_5p; miR-27a-3p; miR-92a-3p	Serum	(49, 81, 82)
Upregulated post lifestyle intervention in responders vs. baseline and vs. nonresponders postintervention.	miR-10a-5p; miR-211-5p; miR-10a-5p	Serum	(81)
Regulate the expression and activity of PPARγ and C/EBP proteins involved in tumor carcinogenesis, adipocyte differentiation and obesity	Let-7b; miR-27; miR-143; miR-31	Tumor/normal tissue	(77, 82–84)

BC, breast cancer; BMI, body mass index; CRP, C-reactive Protein; IL6, interleukin-6; DFS, disease free survival; OS, overall survival; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBP, CCAAT/enhancer-binding family of proteins.

proliferation and adipogenesis by directly targeting C/EBP proteins (81).

Dietary elements, seem to have a key role in regulating miRNAs (84, 86, 87) (**Table 5**).

Polyphenols are a large family of natural compounds widely distributed in plant foods and have been shown to modulate, both *in vitro* and *in vivo*, the activity of several enzymes involved in the DNA metabolism (i.e. DNA methyltransferases, and histone deacetylases) (96). In this group, curcumin and curcuminoids have been assiduously studied as anti-inflammatory and anticancer agents (86, 87).

Curcumin (dyferuloylmethane) plays an onco-suppressor role by inhibiting several oncogenic pathways (88). *In vitro*, curcumin shows an anti-proliferative effect on cancer cell lines even through the modulation of expression of several miRNAs. For example, *in vitro*, curcumin induces the overexpression of the tumor suppressors miR-15a and miR-16, thus inhibiting some anti-apoptotic proteins (89). In MDA-MB-231 cells, curcumin upregulates miR-181b, interfering with the capacity of invasion and with the inflammation related to chemokines (90). In addition, it reduces the expression levels of genes involved in epithelial-mesenchymal transition (EMT) and invasion by controlling miR-34a expression in MCF-10F and MDA-MB-231 lines (91). Despite several studies suggest the anticancer activity of curcumin, its potential use is limited by peculiar pharmacodynamic properties: it has poor absorption, low serum levels, rapid hepatic metabolism, limited tissue distribution and short half-life (96).

Flavonoids (genistein, glabridin, glyceollins) and stilbenes (resveratrol) are polyphenols that affect the epigenetic regulation of genes involved in BC progression and drug-resistance. In TNBC cell lines, such as Hs578t, genistein suppresses miR-155 expression and up-regulates the expression of miR-23b, that has a pro-apoptotic and antiproliferative role (97).

Resveratrol treatment inhibits the proliferation on MCF-7 BC cells by upregulating miR-663, miR-141, miR-774, thus leading to the inhibition of elongation factor 1A2 (EF1A2) (85). In MDA-MB-231 cell lines, resveratrol exhibits strong anti-oxidant activity and induces apoptosis by increasing the levels of tumor-suppressive miRNAs, in particular miR-200c (87, 92).

The active metabolite of the vitamin D (1,25-Dihydroxyvitamin D3, Calcitriol) binds to the vitamin D receptor (VDR) and influences various signaling pathways involved in cell differentiation, cell cycle arrest and apoptosis (93). Furthermore, it regulates miR-182 expression, leading to protection of breast epithelial cells against cellular stress (81). Calcitriol also reduces the level of miR-489, which is an estrogen regulated miRNA promoting tumor cell growth induced by sexual hormones (98).

Other dietary components modulating miRNA expression in breast cancer cells are fatty acids (FAs) (81, 86) and indole alkaloids such as indole 3-carbinol (94, 95).

Several studies describe the role of physical activity and healthy diet in reducing breast cancer risk, also for subjects carrying *BRCA1/2* mutations (94, 99–101). In this context, a nutriepigenetic pilot study is being conducted to evaluate how a personalized nutritional and lifestyle intervention (NLI) can modulate expression of blood and salivary miRNAs associated with breast cancer risk in unaffected young women (<40 years) with *BRCA1/2* mutations (19, 102). This study also aims to evaluate whether NLI as primary prevention strategy may help deal with emotional distress occurring in women at high risk for hereditary breast and ovarian cancer who are involved in intensive screening programs.

CONCLUSIONS

In this review, we have reported most of the published data evaluating the role of miRNAs in influencing BRCA-related

TABLE 5 | Dietary elements and expression/regulation of miRNAs.

Elements	On-target effect	Effect on miRNAs	Outcome	Reference
Curcumin (dyferuloylmethane)	Inhibition of Bcl2 protein	Overexpression of miR-15a and miR-16	Inhibition of anti-apoptotic activity in MCF-7 BC cells	(88)
Curcumin (dyferuloylmethane)	Inhibition of MMPs. Reduction of CXCL1/2 protein levels	Overexpression of miR-181b	Reduction of cancer cells invasivity	(89)
Curcumin (dyferuloylmethane)	Reduction of Axl, Slug, CD24 and Rho-A protein levels	Overexpression of miR-34a	Inhibition of EMT in MCF-10F and MDA-MB-231 BC cell lines	(90)
Genistein	Upregulation of PTEN/FOXO3/AKT axis	Underexpression of miR-155; overexpression of miR-23b	Anti-proliferative and pro-apoptotic effects in Hs578t and MDA_MB-435 BC lines	(91)
Rasveratrol	Downregulation of EF1A2 gene expression	Overexpression of miR-663, miR-141, miR-774 and miR-200c	Antiproliferative effect in MCF-7 BC cells; inhibition of CSC phenotype transition	(87, 92)
1,25-D	p53-mediated regulation of PCNA	Overexpression of miR-182	Reduction of cellular stress	(81)
1,25-D	Erα upregulation	Underexpression of miR-489	Antiproliferative effect in ER-positive BC cell lines	(93)
SCFAs acetate (butyrate, propionate)	Activation of FFARs	Overexpression of miR-31	Induction of cellular senescence	(81, 86)
Omega-3 fatty acids (EPA and DHA)	PTEN-mediated CSF1R inhibition	Underexpression of miR-21	Anti-proliferative and pro-apoptotic effects in BC cell lines	(86)
Indole 3 carbinol	AHR-mediated CD4+ T helper activation	Overepression of miR-212 and miR-132	Enhancement of anti-cancer immune response	(94, 95)

Bcl2 protein, B cell lymphoma 2 protein; BC, breast cancer; MMPs, matrix metalloproteinases; EMT, epithelial-mesenchymal transition; PTEN, phosphatases and tensin homolog; FOXO3, forkhead box 3 protein; EF1A2, elongation factor 1A2; CSC, cancer stem-like cell; PCNA, proliferating-cell nuclear antigen; 1,25D, 1,25-dihydroxycholecalciferol vitamin D; ERα, estrogen receptor-α; SCFAs, short-chain fatty acids; FFARs, free fatty acid receptors; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; CSF1R, colony stimulating factor 1 receptor; AHR, aryl-hydrocarbon receptor.

breast cancer risk and diagnosis. Prospective validation of the reported results and standardization of miRNA isolation methods are, however, still awaited before their use in routine clinical practice. Interestingly, numerous preclinical and clinical studies have showed that *BRCA1/2* genes may interfere with and be silenced by several miRNAs. Furthermore, emerging evidences suggest the role of nutritional and lifestyle interventions in preventing breast cancer development, even through the modulation of breast cancer-related miRNAs. Prospective clinical trials evaluating the association between penetrance of BRCA mutations, NLI strategies for primary prevention and miRNA signatures are ongoing.

AUTHOR CONTRIBUTIONS

CT and BP contributed equally to the writing of the manuscript and tables. AM and DB were involved in planning and supervised the study. ASi, MMi, VU, BB, FB, PM, SP, ASq, MV, DC, GM, and MMe reviewed this work. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a research grant from the Lega Italiana per la Lotta contro i Tumori (LILT) – bando di ricerca sanitaria 2017 (cinque per mille anno 2015) and by the Gruppo Oncologico Italiano di Ricerca Clinica (GOIRC).

ACKNOWLEDGMENTS

BP was supported by ESMO with a Clinical Translational Fellowship aid supported by Roche.

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