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Non-coding RNAs in breast cancer, volume II

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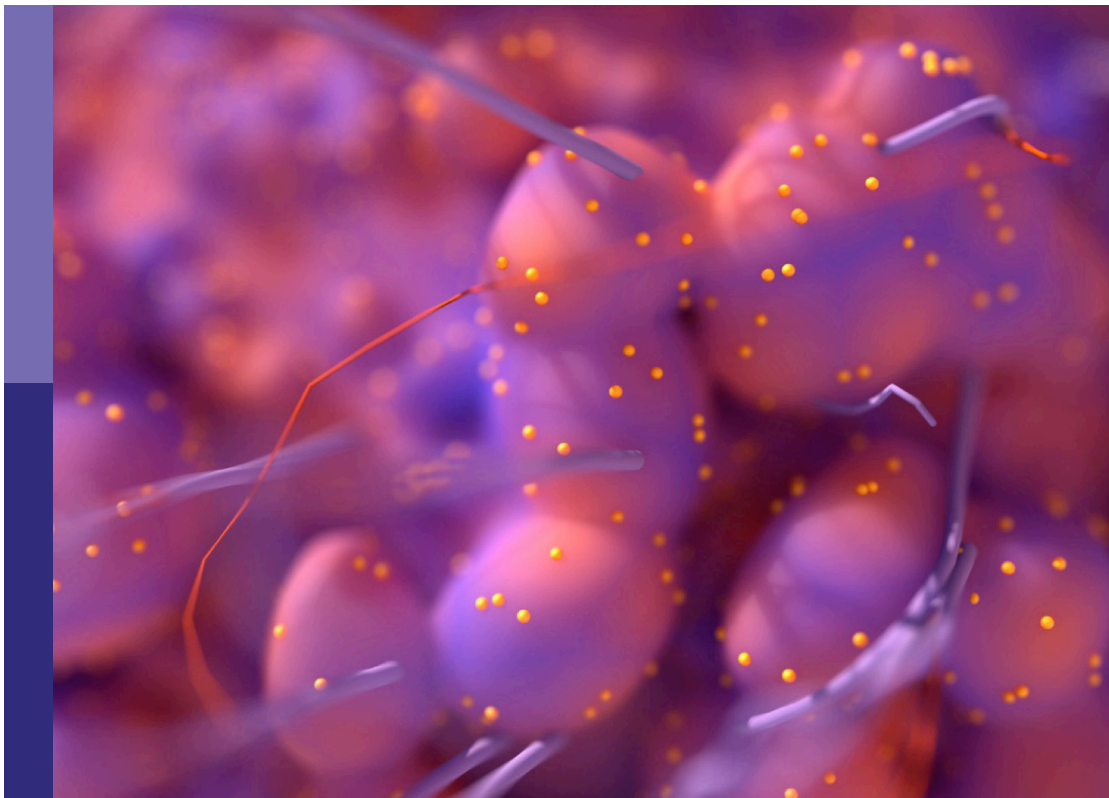

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Non-coding RNAs in breast cancer, volume II

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Editorial: Non-coding RNAs in breast cancer, volume II

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Editorial on the Research Topic
Non-coding RNAs in breast cancer, volume II

Non-coding RNAs (ncRNAs) represent a category of RNA that do not possess protein-coding capabilities, encompassing microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs). Despite their inability to encode proteins, ncRNAs play a crucial role in modulating the expression of various molecular targets, thereby influencing specific cellular biological processes and outcomes. To date, numerous ncRNAs have been identified and shown to be dysregulated across various cancer types, including breast cancer (1). Furthermore, the potential of ncRNAs as diagnostic and prognostic biomarkers and therapeutic targets has been thoroughly investigated in breast cancer (1, 2). This Research Topic collected 12 articles (four original research studies and eight reviews) that concentrated on novel discoveries or assessed recent advancements of ncRNAs in breast cancer.

Firstly, a comprehensive review by Li et al. highlights the promise of two significant classes of non-coding RNAs, namely lncRNAs and miRNAs, as both diagnostic and prognostic biomarkers for breast cancer. It also highlights them as potential targets for innovative therapeutic approaches. Chen et al. conducted a comprehensive review on the substantial impact of lncRNAs on the progression, diagnosis, and effectiveness of neoadjuvant chemotherapy in triple-negative breast cancer (TNBC), highlighting the varied expression profiles of dysregulated lncRNAs. They provided a summary of the precise mechanisms through which lncRNAs modulate gene expression in both the nucleus and cytoplasm, influencing post-transcriptional processes that affect mRNA stability and translation efficiency and thereby regulating tumor cell growth, proliferation, and metastasis.

ncRNAs have been reported to be involved in the regulation of signaling pathways for a variety of molecules or genes (3). 2-Methoxyestradiol (2ME2) is the primary endogenous metabolite of 17β-estradiol. It exhibits a diminished affinity for the estrogen receptor compared to 17β-estradiol and its derivatives, and its mechanism of action operates independently of the cellular response to estrogen (4). Subramani et al. found 2ME2 modifies the expression of pertinent genes by influencing the miRNome in TNBC cells, subsequently inhibiting TNBC through its impact on critical processes such as cell proliferation, apoptosis, and metastasis.

Pyruvate kinase M2 (PKM2) serves as a crucial metabolic enzyme within the glycolytic pathway (5). Jemal et al. have conducted a comprehensive review of recent developments regarding the interactions of PKM2 with various transcription factors and proteins that influence the onset and progression of breast cancer. Additionally, they provide a summary of how natural compounds and non-coding RNAs modulate diverse biological processes in breast cancer cells by regulating the non-metabolic functions of PKM2. Moreover, Song et al. reviewed the localization, structural characteristics, and functional roles of various long non-coding RNAs (lncRNAs) linked to p53 pathway mechanisms or acting as transcriptional targets of p53, thereby enhancing our understanding of the interplay between lncRNAs and p53 in breast cancer. Zhao et al. reviewed the structural characteristics and mechanisms of action of lncRNA LINC00511, subsequently investigating its expression patterns and associated regulatory mechanisms in breast cancer. Furthermore, they examined the biological roles and prospective clinical implications of LINC00511 in breast cancer.

ncRNAs are also involved in the epigenetic regulation and drug resistance of breast cancer (6, 7). Yan et al. provided a comprehensive overview of the pivotal functions and intricate molecular pathways of lncRNAs in the emergence of tamoxifen resistance in breast cancer. Additionally, they evaluated the prospective clinical implications of lncRNAs as novel therapeutic targets and prognostic indicators in breast cancer. Wang et al. constructed an arginine methylation-associated lncRNA model and obtained an arginine methylation-associated lncRNA: lncRNA z68871.1. They demonstrated that the characterized lncRNA z68871.1 had a significant effect on the proliferation and invasion of breast cancer cells.

Single nucleotide polymorphisms (SNPs) are the predominant type of genetic variation. Investigating SNPs elucidates the variations in individual predisposition to diseases, disparities in pharmacological responses, and differences in reactions to environmental stimuli (8). The findings of Qi et al. confirmed that specific LncRNA H19 gene polymorphisms are linked to an increased susceptibility to breast cancer, and that the expression levels of associated genetic markers can profoundly influence the prognosis and therapeutic response in patients with breast cancer.

circRNAs are extensively investigated for their role as molecular sponges for miRNAs, which compete to bind to miRNA-targeted messenger RNAs (9). This interaction contributes to the establishment of a sophisticated post-transcriptional regulatory framework known as the competitive endogenous RNA (ceRNA) network (9). In this Research Topic, Liu et al. found EIF4A3 could enhance the expression of circ_0022382, and elevated levels of circ_0022382 may activate the PI3K/AKT/mTOR signaling pathway and SLC7A11 by sequestering let-7a-5p. Conversely, the silencing of circ_0022382 can impede the proliferation and migration of breast cancer cells, while also promoting the onset of disulfidptosis in breast cancer.

Exosomes represent a category of extracellular vesicles encased in a lipid bilayer, lacking intracellular organelles yet encompassing all known molecular components found within a cell. Tumor cells release exosomes approximately ten times greater than that of normal cells (10). These tumor-derived exosomes play a crucial role in mediating intercellular communication and are implicated in various phases of cancer progression (10). Primary breast tumor cells have been shown to sensitize brain microenvironmental cells, facilitating the development of breast cancer brain metastasis (BCBM) via the secretion of extracellular vesicle-associated miRNAs (11). miRNAs originating from breast tumors can also enhance the invasion of breast cancer cells across the blood-brain barrier by compromising the integrity of brain microvascular endothelial cells (11). Khan et al. provided a comprehensive review of the current literature on miRNAs derived from breast cancer that promote BCBM, detailing their roles in the intricate processes of BCBM, their interactions with microenvironmental cells within the brain metastatic niche, and discussing their potential therapeutic applications in the treatment of BCBM. Additionally, Blancas-Zugarazo et al. reviewed the insights into the cellular mechanisms modulated by exosomal lncRNAs that are critical for the development of chemoresistance and metastasis in breast cancer. They assessed the implications of utilizing exosomal lncRNAs as biomarkers for breast cancer in clinical settings, aiming to facilitate personalized patient management.

Collectively, all these studies in this Research Topic provide new perspectives on the function of ncRNAs in the development and advancement of breast cancer. Nevertheless, the potential of ncRNAs as therapeutic targets for breast cancer warrants further investigation in future studies. We anticipate that ncRNA-based therapies will soon emerge as feasible treatment alternatives for breast cancer patients, either as monotherapy or in combination with current therapeutic modalities.

Author contributions

WZ: Funding acquisition, Writing – original draft. NK: Funding acquisition, Writing – review & editing. XG: Writing – review & editing.

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Conflict of interest

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

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Exosomal lncRNAs as regulators of breast cancer chemoresistance and metastasis and their potential use as biomarkers

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Breast cancer is the most common cancer in women and the leading cause of female deaths by cancer in the world worldwide. Hence, understanding the molecular mechanisms associated with breast cancer development and progression, including drug resistance and breast cancer metastasis, is essential for achieving the best management of breast cancer patients. Cancer-related long noncoding RNAs have been shown to be involved in the regulation of each stage of breast cancer progression. Additionally, exosomes are extracellular microvesicles that are central to intercellular communication and play an important role in tumorigenesis. Exosomes can be released from primary tumor cells into the bloodstream and transmit cellular signals to distant body sites. In this work, we review the findings regarding the cellular mechanisms regulated by exosomal lncRNAs that are essentials to chemoresistance development and metastasis of breast cancer. Likewise, we evaluate the outcomes of the potential clinical use of exosomal lncRNAs as breast cancer biomarkers to achieve personalized management of the patients. This finding highlights the importance of transcriptomic analysis of exosomal lncRNAs to understand the breast cancer tumorigenesis as well as to improve the clinical tests available for this disease.

KEYWORDS

breast cancer, lncRNA, exosomes, metastasis, chemoresistance, cancer biomarkers

1 Introduction

1.1 Breast cancer

Breast cancer (BC) represents a worldwide public health challenge due to its high associated mortality and morbidity rates. The 5-year survival rate of patients with metastatic BC after treatment with adjuvant therapy is less than 30% (1). According to GLOBOCAN 2020 statistics, the number of new cases of BC worldwide was 2.3 million (11.5%), and the number of deaths was 666 103 (6.8%) (2). In women, the BC is the most diagnosed cancer in 159 of 185 countries and is the leading cause of cancer death in 110 of 185 countries (3). BC incidence is highly correlated with human development (4). Thus, incidence rates are 88% higher in developed than in developing countries (55.9 and 29.7 per 100,000, respectively). However, women living in developing countries have 17% higher mortality rates compared with women in developed countries (15.0 and 12.8 per 100,000, respectively) (3). The elevated incidence rates in higher Human Development Index (HDI) countries are associated with BC risk factors (4). The HDI promote a longstanding higher prevalence of reproductive and hormonal risk factors and lifestyle risk factors, as well as increased detection through organized or opportunistic mammographic screening (3).

According with the data, the average age of women deaths associated to BC is lower in developing countries than in countries with high in-come. That is associated to several factor including the late diagnostic, the poor health services and lack of treatments. Thus, in developing countries, more than half of BC is in women under 50, shortening life expectancy in those countries by a decade (4). Almost two-thirds of the deaths in 2020 were recorded in less-developed regions. In developed countries, over 80% of BC patients present an overall survival of 5-year, in contrast to developing regions with 5 years survival less than 50% (3, 4). The observed BC survival profile is associated with later diagnostics that are common in less developed regions of the world, with over half of breast cancers being locally advanced or metastatic at diagnostic (4). Thereby, establishing primary prevention programs for BC remains as a challenge and all approaches to screening tests and early diagnoses are essential, mainly in countries with developing economies (3).

BC usually refers to a group of diseases with biological subtypes that reflect different molecular profiles and clinicopathological characteristics. In addition to histological subtypes, immunohistochemical classification has divided BC into 5 main molecular subtypes: luminal A (estrogen receptor [ER] +, progesterone receptor [PR] +, human epidermal growth factor receptor 2 [HER2] –, Ki-67 low); luminal B HER2 – (ER+, PR+, HER2-, Ki-67 high); luminal B HER2+ (ER+, PR+, HER2+, Ki-67 high); HER2 (ER-, PR-, HER2+); and basal (triple negative [TNBC], ER-, PR-, HER2-), which are related to clinical outcome (5). Additionally, gene expression profiling and molecular diagnosis have significantly impacted the management of BC. Groups of genes have been identified as biomarkers that help predict disease prognosis and estimate the risk of metastasis, tumor recurrence, response to therapy, and clinical decision making. Furthermore, they have helped guide clinical test development for patient follow-up (5, 6).

1.2 Exosome biology and cancer

Exosomes are a class of lipid bilayer-enclosed extracellular vesicles (EVs) that are devoid of intracellular organelles but contain all known molecular constituents within a cell (7, 8). The exosome size ranges from 30 nm to 150 nm (9–11), and exosomes are constantly released by most eukaryotic cells, including platelets, mast cells, dendritic cells, astrocytes, B and T cells, and cancer cells. *In vivo*, exosomes are broadly observed in numerous body fluids, such as blood, serum, saliva, amniotic fluid, and breast milk (12). In particular, exosomes enclose a wide range of molecules, including proteins, lipids, and other metabolites (7, 13). Furthermore, they also contain single-stranded (14) and double-stranded DNA (15) and different kinds of RNA, such as mRNAs, tRNAs, rRNAs, miRNAs, siRNAs, circRNAs (16), lncRNAs (17), snoRNAs, snRNAs (18) and piRNAs (19). In addition, genomic, mitochondrial, and plasmid DNA have all been identified within exosomes (20–22).

Exosomes are important mediators of intercellular communication and are involved in several physiological and pathological processes (10). Intriguingly, tumor cells secrete approximately 10-fold more exosomes than do normal cells (23), and tumor-derived exosomes (TDEs) play important roles in different stages of cancer progression (24, 25). TDEs can enhance angiogenesis, invasion, and migration, promote premetastatic niche establishment and confer chemoresistance (26–29). The high heterogeneity of TDEs likely reflects the phenotypic state of tumor cells that generate exosomes (30, 31). Thus, TDE analysis provides a robust method for monitoring cancer progression and further guiding clinical decisions and treatment strategies (32). The stability of exosomes in most body fluids and the diversity of their cargo, which reflects the status of the parental cells, make them promising candidates for developing new approaches for cancer diagnosis (33, 34). An advantage of TDE analysis is that their lipid bilayers stabilize and protect macromolecules against enzymatic activity existing in biofluids, unlike other biomarker assays requiring fresh biofluid (35, 36). This allows them to be stored for an extended period (35, 36), thereby greatly increasing their clinical applications while reducing the cost of short-term sample storage (12, 37). TDEs provide a promising platform for cancer prognosis, diagnosis, and treatment follow-up in precision and personalized medicine (12). EVs can be purified, and their isolated materials can be further analyzed using next-generation sequencing (NGS), real-time PCR, digital PCR, and bioinformatics for screening and early detection of cancer (38). The previous describe the advantages that exosomes offer in the development of new clinical tests for the diagnosis and monitoring of breast cancer, however it cannot be ignored that there is still a long way to go in the sense of reaching standardized methods for the purification, characterization, and analysis of exosomes with adequate quality for use in the clinic.

1.3 LncRNAs biology and cancer: perspectives in BC diagnostic

Noncoding RNAs (ncRNAs) are classified by their size, the longest of which are long noncoding RNAs (lncRNAs) with more than 200 nucleotides. LncRNAs are transcribed by RNA polymerase II, and similar to mRNAs, they have a 5'-cap and 3' poly-A tail (39, 40). According to their chromosomal positions, lncRNAs are classified as antisense, intronic, divergent, intergenic, promoter associated, transcription start site associated, or enhancer (39). LncRNAs have different functions according with their cellular localization. In the nucleus, they are involved in epigenetic and transcriptional regulation, whereas in the cytoplasm, they are associated with posttranscriptional regulation, including mRNA stability and protein translation, and can act as competitive endogenous RNAs (ceRNAs) (39).

It is widely known that lncRNAs play pivotal roles in each state and process of cancer development (39, 40). Since the discovery of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in 2003 (41), many other cancer-associated lncRNAs have been identified, and their expression can be deregulated. Among the most characterized lncRNAs associated with cancer development and drug resistance are H19 (42), X-inactive-specific transcript (XIST) (43), and homeobox (HOX) transcript antisense RNA (HOTAIR) (44). The role of lncRNAs in cancer development has been established for a number of these molecules, and four main mechanisms of action have been proposed, including acting as signals for transcriptional regulation, acting as decoys that recruit binding partners away from their other targets, acting as scaffolds bringing together multiple biomolecules, and acting as guides directing the targeting of molecular complexes (39, 40).

In this context, due to the lncRNAs are regulators of diverse oncogenic processes, they could be cancer biomarkers. Particularly in BC, the current screening and diagnostic methods for BC are mammography, ultrasound, MRI, and biopsy. However, the prognostic potential of these methods to predict the BC course, including metastasis, is still very limited (45, 46). Likewise, it is important to identify screening biomarkers to measure the BC risk before the tumor onset (45). BC conventional serum biomarkers are carcinoembryonic antigen (CEA) and cancer antigen 15–3 (CA15–3), however their clinical use is limited by their low sensitivity and specificity (45). In this context, aberrant expression of lncRNAs has been observed in many diseases including cancer and lncRNAs possess a high degree of specificity for tissue type and disease, becoming ideal candidates for cancer diagnosis (45). There are some approaches that analyze the use of levels of lncRNAs in circulation as biomarkers of BC. Furthermore, they analyze the potential of measuring the levels of exosomal lncRNAs. In many of these studies, the certainty of using exosome lncRNAs has been compared versus the use of CEA and CA15–3, with promising results that indicate greater sensitivity and specificity when the 3 biomarkers are used (47). However, the current studies investigating the use of EV ncRNA as biomarkers in BC have been focused on discovery and initial technical validation (46) and the clinical implementation for the use of exosomal lncRNAs needs more research to develop standardized methods that reduce the variability between results. It is necessary to resolve issues such as pre-analytical variables (sample type, storage methods, environmental factors, etc); the methods to be used for exosomal RNA isolation and measurements; and standardization of methodologies to normalize the procedures and reduce inter-laboratory and inter-user variability (45).

2 Advantages of tumor-derived exosomes for use in genomic and transcriptomic analyses for biomarker identification

RNA-seq technology has revealed that all forms of RNA can be detected in exosomes and can be useful for cancer analysis (48, 49). Using high-throughput sequencing technologies, researchers have found that exosomes contain different RNA populations, including circRNAs (34), lncRNAs (35, 50), mRNAs (51, 52), miRNAs (53, 54), mRNA fragments (55), piRNAs, and fragments of numerous noncoding RNAs, including tRNAs and rRNAs (35, 56, 57). Specifically, several exosomal miRNAs were recently described as being diagnostic for lung cancer (58, 59). Additionally, increasing evidence has shown that circRNAs are highly enriched and stable in exosomes (49). Compared with circulating tumor DNA (ctDNA) assays, circulating nucleic acids from exosomal sources could increase the number of mutant copies accessible for sampling (34, 60). This suggests that exosomal RNA may increase the potential for detecting mutations in blood samples, particularly when very few copies of ctDNA are available during the early stages of disease (34, 60). For clinically reliable and suitable NGS analyses in the future, standardization and clinical verification are necessary. Currently, exploring biomarkers in TDEs has shown great potential for the diagnosis, monitoring, and treatment of cancer patients but still has obvious limitations (32). However, the development of standardized methods for the isolation and identification of TDEs, as well as the use of NGS techniques, could be an excellent tool for diagnosing, monitoring, and guiding cancer therapy.

There are several examples in which RNA-seq technology has been used to identify and describe the role of exosomal ncRNAs associated with BC. Jenjaroenpun and collaborators (2013) were the first to analyze the transcriptomes of exosomes derived from the human BC cell lines MDA-MB-231 and MDA-MB-436 (61). Exosomes contain many classes of RNA, the most common of which is fragmented rRNA. Importantly, the analysis of exosomal RNA reflected the RNA of the donor cells, and several noncoding transcripts were unique to the MDA-MB-231 and MDA-MB-436 cells. In this case, RNA-seq analysis was able to distinguish exosomal RNA delivered by highly metastatic cells (MDA-MB-231) from that delivered by less metastatic cells (MDA-MB-436) (61). In a subsequent exploratory study, RNA sequencing analysis was carried out on serum exosomes derived from one healthy female and two BC patients, followed by comparative analysis with reference data, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Based on these methodologies, they identified five upregulated and six

downregulated exosomal lncRNAs as potential biomarkers (62). They proposed the lncRNAs VIM-AS1 (upregulated; with 35 predicted target miRNAs), SNHG8 (downregulated; with 12 predicted target miRNAs), and ELDR (downregulated; with 24 predicted target miRNAs) (62) as possible diagnostic biomarkers. In a more recent study, with the analysis of the Cancer Genome Atlas (TCGA) database and the use of RNA-seq technology, the expression profiles of lncRNAs in EVs from BC patient plasma were analyzed. In this work, the authors identified five lncRNAs in tissue and plasma EVs that could be developed as biomarkers for BC. Four of these lncRNAs (C15orf54, AL157935.1, LINC01117, and SNHG3) were proposed as diagnostic markers for BC lesions, although the plasma EVs from patients were not significantly different. The last lncRNA, AL355974.2, was proposed to be an independent protective prognostic factor after survival analysis (63). These three studies previously described are excellent approaches in the use of NGS techniques and bioinformatic analysis for the massive search of new exosomal biomarkers for BC. However, the results of these studies require further validation with a significant number of exosome samples from BC patients. The study of Jenjaroenpun and cols. was carried out with BC cell lines (61). In the second study, only the exosomal samples of serum from two patients were sequenced (62) and in the third, the results of the levels in EVs of the molecules under study, did not show significant differences between BC patients and healthy ones (63), so they proposed the lncRNAs founded as diagnostic biomarkers of BC only for tissue.

Similarly, there have been several initiatives to develop exosome-based databases for biomarker discovery. These include ExoBCD (64) and exoRBase (65). Using exoRBase, the exosomal sequencing data of BC patients and normal controls were analyzed to identify a ceRNA regulatory network with differential expression profiles from exosomes. Overall, 42 mRNAs, 43 circRNAs, and 26 lncRNAs were found to be differentially expressed (65). In another study using the ExoBCD database, the authors analyzed exosomal lncRNAs and microenvironment interactions in BC. They identified 15 exosome-related differentially expressed lncRNAs that correlated with BC prognosis, and further bioinformatic analysis allowed them to construct a risk model to predict survival outcome. This exosome-related lncRNA risk model could provide a tool to estimate prognosis and immune cell infiltration in BC patients, providing important information for immunotherapeutic decisions (66). The last is an excellent example of the potential of using data stored in platforms such as ExoBCD and exoRBase, so it is necessary to increase the data from these platforms and thus increase their potential in the bioinformatic analysis of the content of exosomes, their function and their possible use as diagnostic and monitoring markers for BC.

3 Exosomal lncRNAs mediate chemoresistance in breast cancer

One of the main obstacles in cancer management is the development of drug resistance during chemotherapy treatment. Basically, there are two types of drug resistance: intrinsic or natural resistance and acquired resistance, in which therapeutic effectiveness is attenuated over time (67). However, Vasan and colleagues (2019) explained that many tumors are resistant or become resistant due to combinations of each type of resistance (68). Specifically, in BC, the use of different chemotherapies for each of the molecular types of tumors results in drug resistance in a high percentage of BCs (69). Approximately 30%-40% of BC patients are resistant to endocrine therapies and develop metastatic conditions. In addition, modifications of HER receptor signaling have been reported to play a substantial role in the development of BC drug resistance (69). In this context, the role of exosomal ncRNAs in the development of drug resistance has already been reviewed (69); here, we focus only on exosomal lncRNA functions in chemoresistance, including the most current data and summarized in the Table 1.

3.1 Tamoxifen resistance

Tamoxifen is a nonsteroidal synthetic selective estrogen receptor modulator (SERM) that inhibits estrogen receptor (ER) activity in the breast and is a widely used therapeutic agent for BC patients with ER-positive tumors (79). However, endocrine therapy resistance occurs in a significant number of patients. The oncogenic function of the lncRNA urothelial carcinoma-associated 1 (UCA1) in BC has been described previously (80, 81). In an *in vitro* study in the ER-positive human BC cell line MCF-7 and a tamoxifen-resistant derivative of these cells (LCC2), Xu and collaborators (2016) explored the role of UCA1 in tamoxifen resistance (70). They showed that UCA1 is highly expressed not only in LCC2 cells but also in exosomes released from LCC2 cells compared with exosomes from tamoxifen-sensitive MCF-7 cells. Moreover, incubation with exosomes derived from LCC2 cells increased the viability of MCF-7 cells treated with tamoxifen, reducing apoptosis by decreasing cleaved caspase-3 activity (Figure 1). However, the capacity of LCC2 exosomes to induce tamoxifen resistance was inhibited when their cargo contained impaired UCA1. Thus, UCA1 transfer mediated by exosomes can significantly increase tamoxifen resistance in ER-positive MCF-7 cells (70).

As mentioned above, HOTAIR is a widely studied lncRNA due to its pivotal role in many types of malignant tumors (82–84), including BC, where HOTAIR is highly expressed (85, 86). Additionally, studies have shown that there are greater levels of HOTAIR in exosomes from patients with bladder (87) and cervical (88) cancer than in those from healthy individuals. Moreover, exosomal HOTAIR has been suggested to be a good prognostic and diagnostic biomarker in laryngeal squamous cell carcinoma (89). Tang and coworkers (2019) (71) carried out a study on BC patients who were followed for approximately 6 years to evaluate the diagnostic and prognostic value of serum exosomal HOTAIR. Serum samples from 15 healthy individuals, 15 BC patients treated surgically, 25 patients who received neoadjuvant chemotherapy (anthracycline + taxane + cyclophosphamide regimen) before surgery, and another 25 patients who received tamoxifen treatment after surgery were analyzed. Additionally, 20 BC tissue samples were collected. They found that BC patients had high serum exosomal HOTAIR levels, which decreased 3 months after

TABLE 1 Exosomal lncRNAs associated to chemoresistance in BCC and BC patients.

Drug resistance	LncRNA	Exosome source	Molecular subtype of BC or BCC	Level of exosomal lncRNA	Reference
Tamoxifen	UCA1	MCF-7 cells	ER+	High	Xu et al. (2016) (70)
	HOTAIR	Serum	ER+	High	Tang et al. (2019) (71)
Trastuzumab	AGAP2-AS1	SKBR-3 and BT474 cells	HER2+	High	Zheng et al. (2019) (72)
	OIP5-AS1	SKBR-3 and BT474 cells	HER2+	High	Yu et al. (2021) (73)
	OIP5-AS1	Serum	HER2+	High	Yu et al. (2021) (73)
Doxorubicin	H19	MCF-7 and MDA-MB231 cells	ER+ and TNBC cells	High	Wang et al. (2020) (74)
		Serum	No specify	High	Wang et al. (2020) (74)
	MALAT1	MCF-7 cells	ER+	High	Tao et al. (2022) (75)
	SNHG14	MCF-7 cells	ER+	High	Wan et al. (2023) (76)
Docetaxel	LINC00667	MDA-MB-231 cells	TNBC	High	Li et al. (2022) (77)
Paclitaxel	NEAT1	SKBR3 cells	HER2+	High	Wei et al. (2023) (78)

BCC, Breast cancer cells; BC, Breast cancer.

surgery; thus, exosomal HOTAIR is produced by primary tumor cells and released by exosomes into the bloodstream (71). Furthermore, exosomal HOTAIR levels increased with culture time in the human BC cell lines MDA-MB-231 (triple negative) and MCF-7. In a xenograft assay in which nude mice were injected with BC cells, the expression of serum exosomal HOTAIR in the mice was notably greater than that in the mock control group. The authors also explored the diagnostic and prognostic value of serum exosomal HOTAIR and found that high expression of exosomal HOTAIR led to worse disease-free survival and overall survival, indicating that exosomal HOTAIR could be a good diagnostic and prognostic biomarker. When they analyzed the response to chemotherapy, they observed a correlation between poor response and the overexpression of exosomal HOTAIR. In the HOTAIR high-expression group (n=14), 6 patients achieved a partial response (PR), and 8 achieved stable disease (SD). Conversely, in the low-expression group (n=11), 9 patients achieved a PR, and 2 achieved SD. Similarly, there was a better response to tamoxifen in

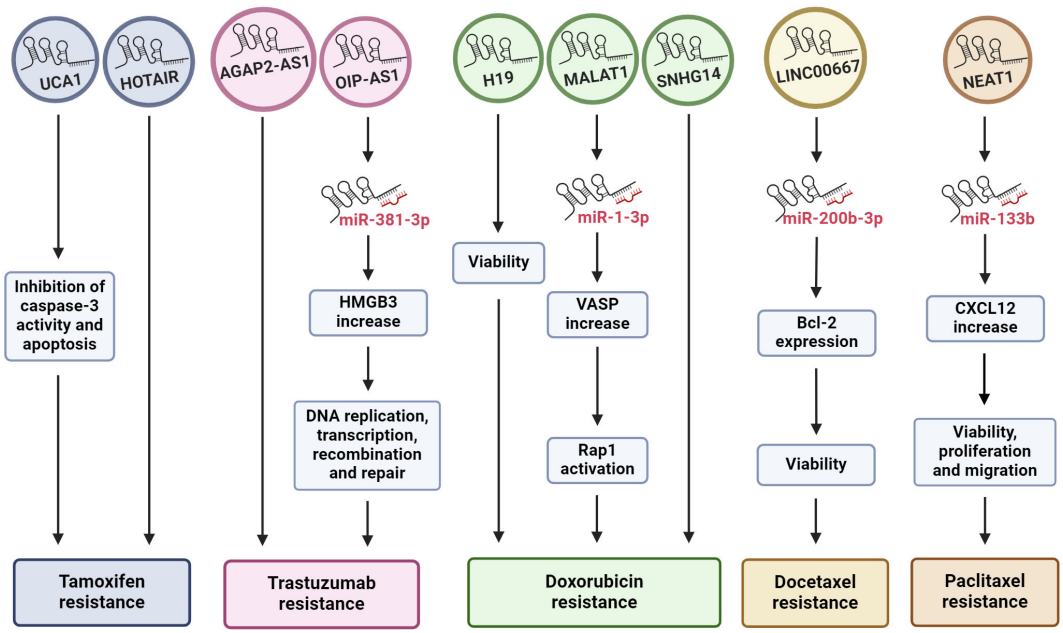

FIGURE 1
Exosomal lncRNAs regulate drug resistance. The diagram shows how the effect of some lncRNAs released by BC cells, mediated by exosomes, can promote drug resistance through different cell pathways, including in many cases the sponging of miRNAs. The chemoresistance to each specific drug can be modulated by more than one exosomal lncRNA. HMGB3, high mobility group box 3; VASP, vasodilator-stimulated phosphoprotein; Rap 1, Ras-related protein 1; CXCL12, C-X-C motif chemokine ligand 12.

the low exosomal HOTAIR expression group, as only 1 patient (of 11) experienced BC recurrence, while in the high-expression group, 6 (of 13) experienced recurrence (71). This finding implies that high HOTAIR expression is associated with a poor response to endocrine therapy with tamoxifen and that this effect could be modulated by increased levels of exosomal HOTAIR in serum derived from primary BC tumors and released into the bloodstream (Figure 1). Therefore, the authors suggest that serum exosomal HOTAIR is a prognostic and diagnostic biomarker for BC patients and may be useful for making therapeutic decisions in terms of endocrine therapy (71). Furthermore, the expression of exosomal HOTAIR in plasma was positively correlated with the HER2 status of BC patients, supporting the possible use of HOTAIR as a BC prognostic biomarker (90).

3.2 Trastuzumab resistance

Trastuzumab is an antibody against HER2 that is commonly used as a treatment for HER-2-positive BC. Trastuzumab treatment improves the clinical prognosis of patients, prolonging overall survival in adjuvant and metastatic settings (91). Recently, the role of AGAP2 antisense RNA 1 (AGAP2-AS1) in trastuzumab resistance *in vitro* was explored. The lncRNA AGAP2-AS1 has an oncogenic function in human non-small cell lung cancer (92, 93) and gastric cancer (94). Zheng and colleagues (2019) (72) induced trastuzumab resistance in the HER2-positive BC cell lines SKBR-3 and BT474. AGAP2-AS1 is upregulated in trastuzumab-resistant SKBR-3 and BT474 cells (SKBR3-TR and BT474-TR, respectively), but silencing AGAP2-AS1 reversed trastuzumab resistance. The authors demonstrated that trastuzumab-resistant cell lines release exosomes loaded with AGAP2-AS1 in a manner dependent on RNA-binding hnRNPA2B1, which mediates the packaging of RNAs into exosomes. Moreover, these exosomes can induce trastuzumab resistance in sensitive cells, an effect that is dependent on exosomal AGAP2-AS1 (Figure 1). The authors suggested that knockdown of AGAP2-AS1 may be helpful for improving the clinical outcome of HER2-positive BC patients and could serve as a therapeutic target (72).

Moreover, the lncRNA OPA-interacting protein 5 antisense transcript 1 (OIP5-AS1) plays many oncogenic roles in multiple cancers (95), including BC (96). In an *in vitro* assay using the same trastuzumab-resistant cells, SKBR3-TR and BT474-TR, OIP5-AS1 was elevated, and resistance was dependent on OIP5-AS1 (73). Like AGAP2-AS1, OIP5-AS1 is released from resistant cells via exosomes, and these exosomes can be absorbed by trastuzumab-sensitive cells and induce cellular resistance. Mechanistically, OIP-AS1 acts as a sponge for miR-381–3p. miR-381–3p targets high mobility group box 3 (HMGB3), which can regulate DNA replication, transcription, recombination, and repair (Figure 1) (97). Therefore, HMGB3 silencing can inhibit cell growth and progression in BC (98). The ability of OIP5-AS1 to induce trastuzumab resistance was established in a murine xenograft model in which the transfer of exosomal OIP5-AS1 induced trastuzumab resistance *in vivo*. Moreover, exosomal OIP5-AS1 was dysregulated in the serum of BC patients and might be a promising diagnostic biomarker for trastuzumab resistance (73).

3.3 Doxorubicin resistance

LncRNA H19 functions as an oncogene in numerous cancer types, including gastric (99), colorectal (100), pancreatic (101) and BC, to the extent that plasma H19 has been proposed as a diagnostic and prognostic biomarker for BC (47, 102). The role of H19 in the development of doxorubicin resistance in BC cells has recently been explored. Doxorubicin (DOX) is an anthracycline used as a broad-spectrum anti-neoplastic drug and is included in first-line adjuvant BC treatment (103). As mentioned above, the human BC cell lines MCF-7 and MDA-MB-231 are molecularly different. MCF-7 is a hormone-responsive human invasive breast adenocarcinoma that represents a luminal A subtype, while MDA-MB-231 is a basal subtype that does not express hormone receptors and contains the mutant p53 protein. Wang and collaborators (2020) (74) induced doxorubicin resistance in both cell types (MCF-7/DOX and MDA-MB231/DOX). They observed an increase in H19 expression in DOX-resistant BC cells compared with the corresponding parental cells. Additionally, they established that DOX resistance was an H19-dependent event since H19 suppression significantly lowered DOX resistance by decreasing cell viability and inducing apoptosis. Furthermore, DOX resistance is induced in sensitive cells by exosomal H19 (Figure 1). In BC patients, serum exosomal H19 levels are elevated in DOX-resistant patients (46 of 82 total patients). The sensitivity and specificity of exosomal H19 were 75% and 65.2%, respectively, for the prediction of BC resistance to therapy (74). Moreover, doxorubicin resistance occurred in both the basal and hormone receptor-positive subtypes.

Small nucleolar RNA host gene 14 (SNHG14) plays a pivotal role in the carcinogenesis of several malignant tumors, such as BC, by regulating cell proliferation, migration, invasion, and chemoresistance (104). Using RNAseq technology, the differential expression profiles of lncRNAs in DOX-resistant MCF7 cells were determined. These results and the analysis of gene expression omnibus (GEO) datasets allowed researchers to identify the altered expression of SNHG14, which was increased in BC tissues and in MCF7/DOX cells (76). In addition, SNHG14 levels were greater in purified exosomes from MCF7/DOX cells than in those from parental cells. Additionally, exosomal SNHG14 could be transmitted from drug-resistant cells to drug-sensitive cells after coincubation of DOX-sensitive cells with purified MCF7/DOX exosomes (Figure 1). However, the role of SNHG14 in cellular DOX resistance must be further explored in detail (76).

Interestingly, although the MCF-7 and MDA-MB-231 cell lines have distinct molecular profiles, they show similar tamoxifen and doxorubicin resistance responses. In both cell lines, tamoxifen resistance is modulated by HOTAIR, and doxorubicin resistance is modulated by H19. These data imply a molecular mechanism of chemoresistance that is dependent on the treatment and not the molecular profile of BC cells, at least in terms of the effects associated with HOTAIR and H19.

3.4 Docetaxel and paclitaxel resistance

Docetaxel and paclitaxel are two taxanes widely used in clinical cancer treatment. Both drugs bind to the β-subunit of tubulin, stabilizing microtubules and interfering with natural cytoskeleton dynamics, thereby inhibiting mitosis. Paclitaxel acts at the M/A-phase transition, whereas docetaxel is primarily active in G2/M-phase. Therefore, these drugs inhibit proliferation and cell viability, induce apoptosis, and have antiangiogenic effects (105). Docetaxel has been used for treating various cancers, including triple-negative breast cancer (TNBC). Although docetaxel arrests the cell cycle in cancer cells, acquired resistance is an important obstacle for the treatment of TNBC (105, 106). Docetaxel resistance appears to be regulated by lncRNAs, such as LINC00667. Studies have shown that LINC00667 expression is elevated in BC tissues (107) and that this lncRNA promotes the proliferation and migration of BC cells (108). LINC00667 expression is elevated in exosomes derived from MDA-MB-231 cells resistant to docetaxel compared to exosomes from docetaxel-sensitive cells. In addition, LINC00667 can be transmitted via exosomes from docetaxel-resistant to docetaxel-sensitive cells. Furthermore, exosomal LINC00667 induced docetaxel resistance in sensitive cells through the upregulation of antiapoptotic Bcl-2. In this regard, LINC00667 seems to function as a ceRNA to sponge miR-200b-3p, resulting in increased expression of Bcl-2 (Figure 1) (77).

Similarly, high expression of the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) in SKBR3 BC cells promotes migration, proliferation, and paclitaxel resistance (78). NEAT1 downregulation decreased paclitaxel resistance, cell migration and cell proliferation. This effect is mediated by the release of miR-133b, which in turn downregulates C-X-C motif chemokine ligand 12 (CXCL12), a well-known inducer of cell survival, proliferation, migration, and drug resistance (Figure 1) (109). Moreover, paclitaxel-resistant SKBR3 cells produce exosomes loaded with high levels of NEAT1, and these exosomes are able to induce paclitaxel resistance, cell migration and growth in paclitaxel-sensitive BC cells. In a xenograft mouse model, knockdown of NEAT1 decreased cancer progression and improved the response to paclitaxel (78).

4 Exosomal lncRNAs promote metastasis

The most common sites of BC metastasis are the lung, bone, lymph nodes, liver, and pleura, with the highest incidence in the lung (110). Therefore, Feng and collaborators (2019) (111) used high-throughput sequencing to analyze lncRNA expression profiles in lung fibroblasts treated with exosomes derived from MDA-MB-231 BC cells compared with exosomes from normal epithelial MCF-10A cells. Exosomes from BC cells increase the proliferation and migration of lung fibroblasts (the WI-38 and HFL1 cell lines). They identified many lncRNA expression abnormalities that could be associated with alterations in the lung microenvironment and metastasis, including 141 upregulated lncRNAs and 98 downregulated lncRNAs in both lung fibroblast lines (111).

More than 30% of patients with metastatic BC develop brain metastasis (112). Additionally, BC is the second most common cause of brain metastases, following lung cancer, with the highest incidence in BC patients with HER2-positive and TNBC subtypes (113). In this context, the lncRNA expression profile associated with brain metastasis from BC patients showed that XIST was downregulated in brain tumors (114). Moreover, XIST is downregulated in the BC cell lines MDA-MB-231-BrM2a and SKBrM3 (derived from MDA-MB-231 and SKBR3, respectively), which preferentially metastasize to the brain. Thus, XIST plays a protective role in brain metastasis development. This finding was supported in a mouse xenograft model in which XIST silencing promoted brain metastasis. Specific knockout of XIST in mouse mammary glands accelerated primary tumor growth as well as metastasis to the brain. Additionally, XIST downregulation stimulated epithelial–mesenchymal transition (EMT) and activated the c-Met pathway by upregulating the MSN gene. Loss of XIST also augmented the secretion of exosomal miRNA-503, leading to reprogramming of microglia in the brain and triggering M1–M2 polarization of microglia. M1-M2 macrophage conversion upregulated the expression of immunosuppressive cytokines in microglia that suppressed T-cell proliferation. The authors searched an FDA-approved drug library and showed that fludarabine selectively inhibits the growth of XIST-low tumor cells. Additionally, they observed that fludarabine blocked brain metastasis in a mouse model. Furthermore, the loss of XIST promoted brain metastasis in BC by altering the tumor cell microenvironment and stimulating EMT. This suggested that fludarabine could be a good therapeutic agent for the prevention and treatment of brain metastasis (114).

Another central element for brain metastasis is the integrity of the blood-brain barrier (BBB). Lu and collaborators (2020) (115) determined that exosomes from BC cells disrupt the BBB system, promoting brain metastasis. They established a highly brain metastatic BC cell line (MDABR3, derived from MDA-MB-231 cells) by *in vivo* and *in vitro* selection after 3 rounds of intracardiac injection in mice and subsequent recovery of brain tumors. MDABR3 cells exhibited high levels of migration and invasion *in vitro* and metastatic capacity *in vivo*. Exosomes derived from MDABR3 cells can be taken up by brain microvascular endothelial cells (BMECs), reducing transepithelial/transendothelial electrical resistance (TEER) and increasing permeability in a BBB model, thus promoting invasion of BC cells in the BBB model (Figure 2). Using the GEO dataset, the authors selected exosomal lncRNAs associated with the BBB and verified that GS1–600G8.5 was highly expressed in MDABR3 cells and their exosomes than in samples with reduced metastatic behavior. GS1–600G8.5 silencing decreased the BBB permeability induced by MDABR3 exosomes and the infiltration of cancer cells through the BBB. In addition, BMECs treated with GS1–600G8.5-derived exosomes expressed higher levels of tight junction proteins than those treated with exosomes containing GS1–600G8.5 (Figure 2) (115).

Angiogenesis is essential for the development of metastasis. Therefore, angiogenesis during cancer progression is another widely studied mechanism. In a recent study, the lncRNA AC073352.1 was identified from a microarray and TCGA database analysis as a novel

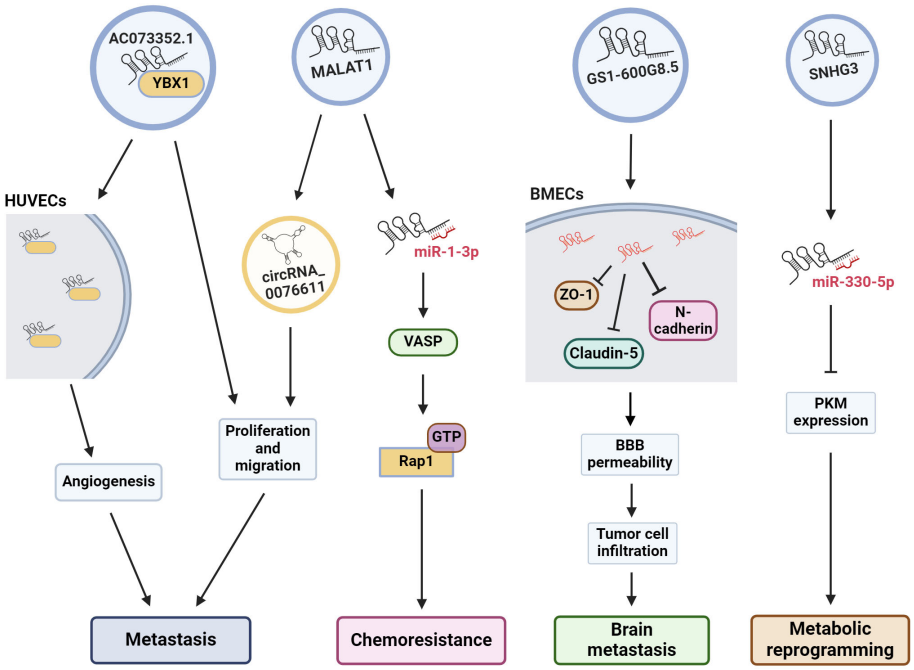

FIGURE 2
Exosomal lncRNAs are associated to many oncogenic pathways. The illustration shows the effect of some exosomal lncRNAs in the regulation of several cell pathways to induce angiogenesis, metastasis, drug resistance, etc. HUVECs, human umbilical vein endothelial cells; VASP, vasodilator-stimulated phosphoprotein; Rap 1, Ras-related protein 1; BMECs, brain microvascular endothelial cells; BBB, blood-brain barrier; PKM, pyruvate kinase muscle M1/M2.

lncRNA involved in BC metastasis (116). In this study, the authors determined that AC073352.1 was upregulated in BC tissue and was associated with advanced tumor node metastasis (TNM) stage and poor prognosis in BC patients. The overexpression of this lncRNA in MDA-MB-231 and BT549 BC cells (both cell lines with low AC073352.1 expression levels) increased *in vitro* migration and invasion and augmented *in vivo* metastasis in a mouse model. However, AC073352.1 suppression in MCF-7 and MDA-MB-468 cell lines (both with high endogenous AC073352.1 levels) reduced their migration and invasion capabilities (116). They also showed that AC073352.1 promoted BC metastasis and angiogenesis through its binding to YBX1, a transcriptional activator and well-known promoter of metastasis (117). The AC073352.1-YBX1 interaction increases YBX1 stability, and in turn, YBX1 promotes the exosomal internalization of AC073352.1. Moreover, exosomes purified from MDA-MB-231 cells overexpressing AC073352.1 promoted angiogenesis in an *in vitro* tube-formation model using endothelial human umbilical vein endothelial cells (HUVECs) (Figure 2) (116).

MALAT1 is another lncRNA found to be important for BC metastasis and doxorubicin resistance (75). Recently, MALAT1 was shown to be highly expressed in MCF-7 cells and their exosomes. Exosomal MALAT1 increases the malignant properties and chemoresistance of BC cells. This effect is mediated by the downregulation of miR-1–3p, which in turn upregulates vasodilator-stimulated phosphoprotein (VASP), resulting in the activation of the Ras-related protein 1 (Rap 1, a member of the RAS oncogene family) signaling pathway (Figures 1, 2) (75), which is well known for promoting invasion, metastasis and chemoresistance (118, 119). MALAT1 is highly expressed in the tissues and serum of BC patients, and higher MALAT1 expression is positively associated with metastasis and TNM stage but negatively associated with overall patient survival (120). Furthermore, MALAT1 promoted the proliferation of MDA-MB-231 and ZR-75–1 BC cells and promoted tumor growth in a xenograft mouse model. Additionally, exosomal MALAT1 from BC cells was able to induce cell proliferation *in vitro* (120). Moreover, MALAT1 induces the expression of circRNA_0076611 and promotes the release of circRNA_0076611 from exosomes of MDA-MB-231 cells (Figure 2) (121). CircRNA_0076611 is associated with cell proliferation and migration, and BC patients have been shown to have high levels of this circRNA in serum (121). Another lncRNA involved in metastasis is SNHG3, which has been associated with bone metastasis in BC patients (122). Exosomes from MDA-MB-231 cells containing SNHG3 positively regulate BMP3 expression and bone marrow mesenchymal stem cells (BMSCs), thereby regulating osteogenic differentiation in bone metastasis. Thus, the overexpression of SNHG3 in BC cells may be important for the regulation of osteolytic metastasis (122). All the data described above and summarized in Table 2, shows us that lncRNA present several cellular effects that can be both protective and oncogenic in the regulation of BC. The protective effect of XIST expression in various cancers is already well recognized, as well the oncogenic effect of MALAT1. In this way, exosomes are the vehicle for lncRNAs to reach and modulate molecular mechanisms and cellular changes in in distant places.

TABLE 2 Exosomal lncRNAs with metastatic activity in BC.

LncRNA	Status of exosomal lncRNA	Experimental model	Metastatic effect	Reference
XIST	Downregulated	BCC	Induces brain metastasis by stimulating epithelial–mesenchymal transition	Xu et al. (2016) (114)
GS1–600G8.5	Overexpressed	BCC and *in vivo* selection of brain metastatic BCC; BBB *in vitro* model	Induces brain metastasis by increasing bloodbrain-barrier permeability	Lu et al. (2020) (115)
AC073352.1	Overexpressed	BCC and metastatic mice model; *in vitro* tube-formation model using endothelial HUVECs	Induces *in vitro* and *in vivo* migration and invasion and promotes angiogenesis	Kong et al. (2021) (116)

BCC, Breast cancer cells; BC, Breast cancer; BBB, blood-brain barrier; HUVECs, human umbilical vein endothelial cells.

5 Other functions of exosomal lncRNAs in breast cancer development: metabolic reprogramming

Previously, we mentioned the many roles of lncRNAs and TDEs in cancer. Several actions of exosomal lncRNAs have been described, particularly in BC. In addition to their role in chemoresistance and metastasis, they can also modulate other cellular processes associated with cellular transformation, including metabolic changes. In this context, MCF-7 and MD-MBA-453 BC cells were treated with exosomes secreted from cultured cancer-associated fibroblasts (CAFs) derived from BC patients. In these assays, CAF exosomes induced the reprogramming of metabolic pathways in BC cells in culture, promoting cell proliferation, decreasing mitochondrial function, and increasing glycolysis, thus modulating metabolic pathways in BC cells (123). This effect is regulated by the lncRNA SNHG3, which is expressed by CAFs and released in exosomes. SNHG3 acts as a molecular sponge for miR-330–5p in cultured BC cells. The suppression of SNHG3 decreases glycolysis and cell proliferation by increasing miR-330–5p, which, in turn, inhibits pyruvate kinase muscle M1/M2 (PKM) expression in cancer cells (Figure 2). Accordingly, the inhibition of miR-330–5p by SNHG3 promotes PKM expression, resulting in the inhibition of mitochondrial oxidative phosphorylation and increased glycolytic carboxylation (123). Thus, SNHG3 has been shown to be an important regulator of metastasis, not only by promoting bone metastasis but also by mediating the metabolic transformation of tumor cells. Importantly, the SNHG3 that is released into exosomes from BC cells and CAFs can have cellular effects on numerous types of cells.

6 Exosomal lncRNAs as biomarkers for breast cancer patients

In cancer research, there is a constant search for biomarkers that will help develop screening tests to achieve more timely diagnoses and better follow-up of BC patients to achieve personalized precision medicine, we summarized the assays made in BC patients in the search for exosomal lncRNAs as biomarkers of BC in the Table 3 and we outline the use of exosomal lncRNAs as BC biomarkers (Figure 3). In this context, the possible use of lncRNA H19 has been studied as a diagnostic biomarker of BC (47). Serum exosomal H19 levels were measured in BC patients, and higher levels were detected in BC patients than in benign breast disease (BBD) patients and healthy controls. Furthermore, when serum exosomal H19 levels were compared between BC patients before and 7 days after surgical removal of the tumor mass, H19 levels were significantly lower in postoperative patients than in preoperative patients. In this manner, the analysis of exosomal H19 levels in serum was shown to be more sensitive and specific than the analysis of cancer antigen 15.3 (CA15.3) and carcinoembryonic antigen (CEA) levels in blood. Moreover, exosomal H19 expression levels are positively associated with lymph node metastasis, distant metastasis, TNM stage, ER status, PR status, and HER2 status (47).

Na-Er and collaborators (2021) (124) examined the prognostic significance of the serum exosomal lncRNA small ubiquitin-like modifier 1 pseudogene 3 (SUMO1P3) in TNBC. The authors analyzed SUMOP3 levels in BC tissue using the TCGA database. SUMO1P3 was upregulated in BC and correlated with poor survival. Moreover, exosomal SUMO1P3 levels were greater in TNBC patients than in non-TNBC patients, patients with benign breast disease, and healthy people. High exosomal SUMO1P3 levels were correlated with lymphovascular invasion, lymph node metastasis, histological grade and chemoresistance. In addition, patients with high levels of exosomal SUMO1P3 had worse overall survival than patients with low levels of exosomal SUMO1P3. Thus, the authors suggest that exosomal SUMO1P3 might be a prognostic biomarker for TNBC (124). Similarly, Lan and collaborators (2021) (125) proposed exosomal XIST as a biomarker for possible recurrence in TNBC patients. They observed that XIST levels in tumor tissue and exosomal XIST in serum were greater in TNBC patients than in healthy controls. Like those of other lncRNAs, the exosomal levels of XIST decreased after surgical removal of the tumor mass. Furthermore, 5-year follow-up studies revealed greater levels of exosomal XIST in recurrent TNBC patients than in nonrecurrent patients. There was an association between high exosomal XIST levels and poor overall survival, but there was no association with other clinicopathological parameters, such as Karnofsky Performance Status (an assessment tool for functional impairment), sex or age (125).

The lncRNA differentiation antagonizing nonprotein coding RNA (DANCR or ANCR) has been postulated to be a good prognostic biomarker for BC. DANCR has been shown to be involved in the initiation and progression of BC (127, 128). The levels of exosomal DANCR were greater in BC patients than in

TABLE 3 Exosomal lncRNAs identified as possible biomarkers for BC.

LncRNA	Molecular subtype of BC	Liquid biopsy	Level of exosomal lncRNA	Parameters correlated with levels of exosomal lncRNA	Biomarker type	Reference
HOTAIR	No correlation with the status of any receptor	Serum	High	Correlated with poor neoadjuvant chemotherapy and tamoxifen resistance	Diagnostic and prognostic biomarker for recurrence with poor DFS and OS at 6-year follow-up	Tang et al. (2019) (71)
	HER2+	Plasma	High	Correlation with HER2 status	Prognostic biomarker for HER2 status	Wang at el. (2019) (90)
H19	ER status, PR status, and HER2 status	Serum	High	Correlated with lymph node metastasis, distant metastasis, TNM stage, ER status, PR status, and HER2 status	Diagnostic and metastatic biomarker with better results in combination with CA15–3 y CEA than any of them alone	Zhong at el. (2020) (47)
	Not specified	Serum	High	Predicted BC resistance with a sensitivity and specificity of 75% and 65.2%	Biomarker for doxorubicin resistance	Wang et al. (2020) (74)
SUMO1P3	TNBC status	Serum	High	Correlated with lymphovascular invasion, lymph node metastasis, histological grade and chemoresistance	Prognostic biomarker for TNBC for poor overall survival	Na-Er et al. (2021) (124)
XIST	TNBC	Serum	High	Correlated with poor OS	Prognostic biomarker for recurrence in TNBC at 5-year follow-up	Lan et al. (2021) (125)
DANCR	ER status and HER2 status	Serum	High	Correlated with clinicopathological parameters, including lymph node metastasis, ER status, HER2 status, and TNM stage	Prognostic biomarker for poor OS at 5-year follow-up, with better results in combination with CA15–3 y CEA than any of them alone	Shi et al. (2022) (126)

BC, Breast cancer; TNM, tumor node metastasis; DFS, Disease-free survival; OS, overall survival; CA15–3, cancer antigen 15–3; CEA, Carcinoembryonic antigen.

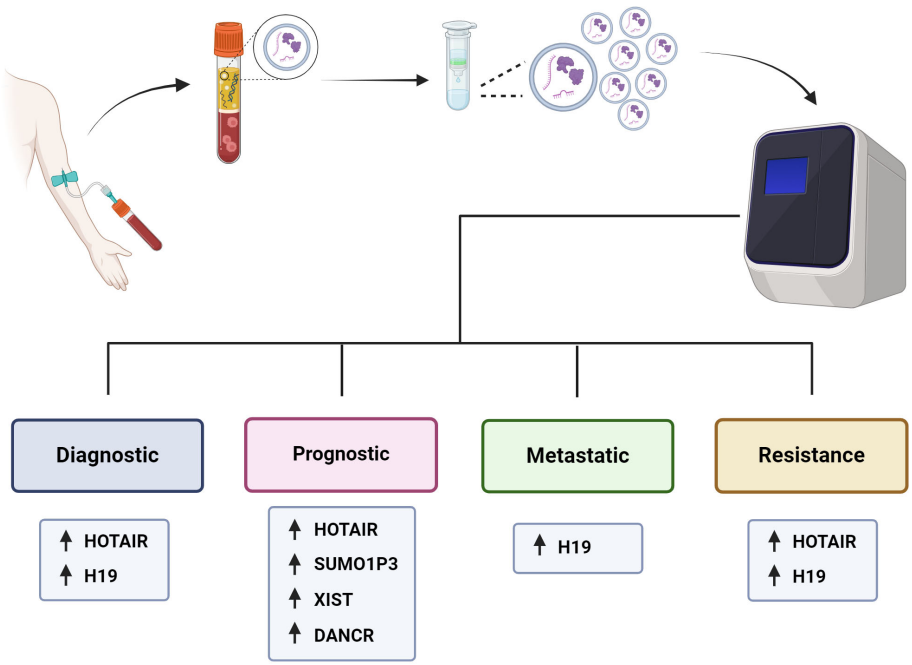

FIGURE 3
Utility of exosomal lncRNAs as biomarkers of BC. It is outlined how patients with BC would have a peripheral blood sample taken, from which the plasma would be isolated, and the exosomes purified to be analyzed and measure the levels of their components, such as proteins or nucleic acids, including lncRNAs. The levels of exosomal lncRNAs can be measured and used as diagnostic, prognostic, metastatic and drug resistance biomarkers for BC patients.

benign breast disease patients or normal controls, and as expected, exosomal DANCR levels were markedly downregulated in postoperative samples. However, although the diagnostic performance of exosomal DANCR was good, the diagnostic accuracy for BC was better when a combination of exosomal DANCR, CA15.3 and CEA was assessed. In this manner, high exosomal DANCR levels were associated with clinicopathological parameters, including lymph node metastasis, ER status, HER2 status, and TNM stage. Patients with high exosomal DANCR levels had shorter 5-year overall survival, and multivariate analysis of exosomal DANCR levels revealed that this parameter was an independent risk factor for BC (126).

7 Conclusion and perspectives

In this review, we describe the importance of exosomal lncRNAs in intercellular communication associated with BC development. The role of lncRNAs in cancer development is well established and the significance of exosomes as mediators of intercellular communication in tumorigenesis has been demonstrated. Particularly, exosomal lncRNAs promote processes such as angiogenesis, invasion, migration, metastasis and chemoresistance, etc, in numerous cancer types, including BC. In this way, the transcriptomic analysis of exosomal lncRNAs is a useful tool in the search for lncRNAs associated with BC tumorigenesis as well in the identification of biomarkers with potential clinical use for the disease.

We have described the significance of exosomal lncRNAs in promoting resistance to drugs such as tamoxifen, trastuzumab, doxorubicin, docetaxel and paclitaxel. Interestingly, it has been observed that resistance to a specific drug can be regulated by more than one exosomal lncRNA, and apparently through different molecular pathways, as we can see in the development of doxorubicin resistance. However, in many cases the intracellular pathways by which some of the lncRNAs induce chemoresistance are not fully described, as in the case of SNHG14. Curiously, the evidence about the chemoresistance induction in sensitive BC cells suggests that the mechanism of drug resistance is dependent on the treatment and not on the molecular profile of the BC cells. For example, the development of chemoresistance to tamoxifen and doxorubicin, in the MCF-7 and MDA-MB-231 cell lines, representatives of different BC subtypes, both cell lines develop resistance to tamoxifen mediated by exosomal HOTAIR and doxorubicin resistance modulated by exosomal H19. Likewise, exosomal lncRNAs not only participate in the development of chemoresistance, but they also modulate metabolic changes in cancer cells as well as several processes associated with metastasis such as proliferation, invasion and angiogenesis. However, the information available regarding the importance of exosomal lncRNAs in the development and progression of BC is still insufficient. There are still many lncRNAs for which the molecular pathways they promote have not been described, and there are surely many more that have not even been identified. Finally, another important aspect is the identification of exosomal lncRNAs and their potential clinical use both as therapeutic targets and as biomarkers of the disease. In this way, there are already approaches and some exosomal lncRNAs have been proposed as diagnostic, prognostic, metastatic and chemoresistance biomarkers. The transfer of lncRNAs mediated by exosomes provides advantages compared with that mediated by cell-free circulating RNA since exosomes provide a more stable environment for measuring macromolecules that reflect tumor characteristics, and these advantages can be exploited to measure different biomarkers that could guide and improve the management of breast cancer. However, exosomal lncRNAs still must be validated for use in clinical trials. Likewise, the most appropriate method for the isolation and analysis of exosomes remains to be validated.

Author contributions

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Conflict of interest

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

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Crosstalk between breast cancer-derived microRNAs and brain microenvironmental cells in breast cancer brain metastasis

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Breast cancer is the most frequent malignancy in women, constituting 15.2% of all new cancers diagnosed in the United States. Distant breast cancer metastasis accounts for the majority of breast cancer-related deaths; brain metastasis is the third most common site for metastatic breast cancer but is associated with worst prognosis of approximately eight months of survival. Current treatment options for breast cancer brain metastasis (BCBM) are limited and ineffective. To help identify new and effective therapies for BCBM, it is important to investigate the mechanisms by which breast cancer cells metastasize to the brain and thrive in the brain microenvironment. To this end, studies have reported that primary breast tumor cells can prime brain microenvironmental cells, including, astrocytes and microglia, to promote the formation of BCBM through the release of extracellular vesicle-microRNAs (miRNAs). Breast tumor-derived miRNAs can also promote breast cancer cell invasion through the blood-brain barrier by disrupting the integrity of the brain microvascular endothelial cells. In this review, we summarize current literature on breast cancer-derived BCBM-promoting miRNAs, cover their roles in the complex steps of BCBM particularly their interactions with microenvironmental cells within the brain metastatic niche, and finally discuss their therapeutic applications in the management of BCBM.

KEYWORDS

breast cancer, microRNA, brain microenvironment, blood-brain barrier, brain metastasis

1 Introduction

According to the latest statistics by the American Cancer Society, the estimated number of new breast cancer cases in 2024 is 310,720, accounting for 32% of all cancers diagnosed in women (1). Breast cancer patients have the second highest rate of brain metastasis behind lung cancer (2). Furthermore, subtype analysis of breast cancer brain metastasis (BCBM) patients showed that HER2-enriched breast cancer and triple-negative breast cancer (TNBC) subtypes have a higher potential to develop brain metastasis, underscoring the particular importance of studying BCBM in these two subtypes of breast cancer (3).

In 1993, Ambros and his team made the groundbreaking discovery of a microRNA (miRNA) in *Caenorhabditis elegans* (4). The miRNA, transcribed from the lin-4 gene, exhibits complementarity to and consequently regulates the expression of the lin-14 protein (4). miRNAs are part of the small non-coding RNA family that are endogenous, single-stranded RNAs with an average length of 20–22 nucleotides, known to regulate gene expression in many physiological processes (5, 6). Studies have shown that miRNAs play a pleiotropic role acting as tumor suppressors and/or oncogenes in different cancers (7, 8). Numerous studies have now implicated miRNAs in every step of brain metastasis beginning from epithelial-mesenchymal transition to colonization in the brain parenchyma (9–29).

Brain organotropism in breast cancer is influenced by several factors: breast cancer subtype, molecular features of circulating tumor cells, extracellular vesicle-derived miRNA expression profile, tumor microenvironment, and the ability of breast cancer cells to penetrate the blood-brain barrier (BBB) (30). BBB is a specialized neurovascular unit adjoining blood capillaries with brain parenchyma, comprising of brain microvascular endothelial cells (BMECs), astrocytic end-feet, and pericytes (31). BMECs line the luminal and abluminal membranes and are held together by tight and adherens junctions (32). They tightly regulate the transport of cells and molecules from blood to the brain parenchyma. BMECs lack leukocyte adhesion molecules and have a higher concentration of mitochondria, which limits the influx of immune cells from blood into central nervous system (CNS) and indicates the prevalence of high-energy requiring role of BMECs respectively (33). In response to pathological changes in the CNS, astrocytes undergo molecular, functional, and morphological transformation and are termed as “reactive astrocytes”. Studies have shown the reactive astrocytes stimulate BMECs through secretion of *SERPINA3* by activating NF-κB/STAT3 signaling axis (34). Pericytes play a few roles in the maintenance of BBB integrity including regulating microvascular stability, angioarchitecture, and clearance of foreign proteins and tissue debris (35).

The interactions between tumor cells and brain microenvironmental cells, primarily astrocytes and microglia, facilitate various stages of metastasis. Reactive astrocytes through the secretion of inflammatory chemokines such as interferon-α (IFNα) and Ciliary Neurotrophic Factor promote tumor growth by activating transcriptional and cell survival pathways (36–38). Microglial cells, known as the resident macrophages of the CNS, are often polarized from M1 to M2 microglia to secrete immunosuppressive chemokines (39). These modulations in the environment play a key role in promoting the growth of brain metastasis. Through regulating the brain-metastatic microenvironmental cells and their interaction with breast cancer cells, miRNAs can influence breast cancer metastasis to the brain and progression within the brain.

2 MicroRNA biogenesis and mechanism of action

The biogenesis of miRNAs is classified into canonical and non-canonical pathways. The canonical pathway begins with the transcription of a hairpin-containing primary miRNA (pri-miRNA) in the nucleus (8). The pri-miRNA transcript is then cleaved by the microprocessor complex containing DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha to form the precursor miRNA (pre-miRNA). DGCR8 is an RNA-binding protein that recognizes and binds N6-methyladenosine GGAC motif in the pri-miRNA, and the RNase III enzyme, Drosha, recognizes and cleaves the base of the hairpin structure (40, 41). The pre-miRNA is transported from the nucleus to the cytoplasm through an exportin5/RanGTP complex where RNase III endonuclease Dicer cleaves the hairpin loop structure and leads to the formation of mature double-stranded miRNA (40, 42, 43).

There are two non-canonical pathways: Drosha/DGCR8-independent and Dicer-independent pathways. In the former pathway, (mirtrons) RNAs are exported to the cytoplasm through exportin 1 without undergoing Drosha processing; and in most cases, the 3p strand is loaded onto the AGO protein due to the presence of a 7-methylguanosine cap at the 5’ end (44). In the Dicer independent pathway, shRNA transcripts are processed by Drosha/DGCR8 complex and exported to the cytoplasm by exportin5/RanGTP where they are loaded onto AGO2 and processed (45, 46).

miRNAs binding to a specific seed sequence at either the 3’ or 5’ untranslated region of the target mRNA can lead to mRNA degradation or translational repression, leading to gene silencing (47–50). miRNAs can also bind at the promoter region of target mRNAs leading to transcriptional activation (51). miRNAs can regulate multiple biological pathways such as cell proliferation, cell death, immune evasion, invasion, metastasis, and angiogenesis. miRNAs are classified as tumor suppressors or oncogenes depending on their target gene and cell type (8).

3 Development of brain metastases

For the initiation of metastasis to occur, cancer cells undergo epithelial-mesenchymal transition (EMT) demonstrated by an increase in self-renewing stem cells, anoikis resistance, and dissemination (52–56). TWIST1, SNAIL1, and SLUG are some of the most heavily studied transcription factors in the context of breast cancer metastasis (57). Under regulation of pathways like the Notch signaling pathway, these transformed cells can penetrate the vascular endothelium where endothelial cells promote membrane

remodeling and cancer cells enter blood vasculature (58–61). An important part of metastasis is the development of a tumor-supportive environment in distant target organs. Cancer cells prime a secondary site by secreting tumor-promoting extracellular vesicles and inflammatory chemokines, forming the premetastatic niche (62, 63). Extravasation into the brain requires tampering with the BBB permeability. The cross-talk between cancer cells and BMEC is stimulated by the expression of cellular adhesion molecules (E-selectin, VCAM-1) on cancer cells and degradation of the BBB by matrix metalloproteinases (64–66). Extravasation is followed by mesenchymal-epithelial transition (MET) or partial MET lending a higher aggressive phenotype to the cancer cell (67–69). Reactive astrocytes play a dichotomous role by initially inhibiting brain metastases and switching to a pro-metastatic role in later stages (37, 62, 70–72). Glial cells such as tumor-associated microglia/macrophages lend a supportive hand to cancer cells by stimulation of TGF-β1 signaling pathway (73).

4 miRNAs implicated in the cross-talk between breast cancer cells and brain cells

The cross-talk between breast cancer cells and astrocytes/microglia at any stage of brain metastasis leads to microenvironmental modulation that subsequently facilitates the progression of brain metastasis. miRNAs involved in these interactions are listed in Table 1 and depicted in Figure 1.

miR-122 is upregulated in the conditioned media of breast cancer cells (80). Uptake of breast cancer-derived miR-122 by astrocytes led to a reprogramming of glucose uptake, notably through the downregulation of PKM1/2 and GLUT1, resulting in decreased uptake of 2-NBDG (a fluorescent glucose analogue). The authors reported reduced glucose uptake by tumor cells led to inhibition of tumor cell proliferation in primary tumors, while simultaneously supporting metastatic tumor cell colonization in the pre-metastatic niche (80).

miR-194 and miR-802 were downregulated in plasma samples collected from brain metastasis model of a 4T1-injected mice (83). *MEF2C* was validated as a target gene using an *in vivo* model through immunofluorescence staining. *MEF2C* was found to be highly expressed in established metastatic cells in the brain parenchyma and peritumoral astrocytes. These findings were further validated in human TNBC brain metastases samples (83). Moreover, miR-802 inhibits FoxM1 decreasing the proliferation of breast cancer cells and miR-194 inhibits proliferation and migration of breast cancer cells, supporting the tumor-suppressive roles of these miRNAs (91, 92).

Breast cancer-derived miR-345 expression is upregulated due to increased astrocytic secretion of CCL2 and CXCL12 (87). miR-345 downregulates KISS1 which in turn leads to localization of breast cancer cells in the brain microenvironment (87). Another study reports that miR-345-mediated KISS1 transcriptional inhibition plays a role in promoting autophagy and invasiveness of breast cancer cells (87, 93).

miR-1290 is upregulated in the sera of breast cancer patients (36). miR-1290 activates astrocytes and promotes cancer stemness factors, subsequently aiding the progression of BCBM. miR-1290 binds to FOXA2 and induces transcriptional repression, leading to upregulation of CNTF expression in astrocytes. Astrocytes activated by miR-1290 promoted intracranial growth of co-implanted breast cancer cells in the brain *in vivo* (36).

miR-199a, miR-150, and miR-155 are downregulated, whereas miR-132-3p is upregulated in tissue obtained from BCBM patients (81). The authors report an increase in miR-132-3p correlated with improved brain metastasis-free survival (BMFS) and overall survival (OS) whereas miR-199a, miR-150 and miR-155 correlated with poorer BMFS and OS. *MET* was identified as a target oncogene and reported to be overexpressed in microglial cells responsible for tumor angiogenesis and colonization of breast cancer cells (81). miR-503 is upregulated in the sera of BCBM patients and is reported to promote the M1 to M2 polarization of microglia, demonstrated by increased phosphorylation of STAT3 along with decreased phosphorylation of NF-κB (26). Taken together, these findings suggest an important role for miRNAs in regulating the brain microenvironmental cells.

5 miRNAs implicated in the cross-talk between breast cancer cells and BBB cells

A pivotal aspect of brain metastasis involves the disruption of the BBB integrity. Thus, studying the interactions between breast cancer cells and BBB cells is of utmost importance. miRNAs involved in mediating cross-talk between breast cancer cells and BBB cells are listed in Table 1.

miR-101-3p is decreased in BCBM leading to increased expression of COX-2 and stimulation of COX-2/MMP-1 signaling pathway, promoting trans-endothelial migration of breast cancer cells and extravasation across the BBB (78). miR-105 downregulates ZO-1 expression in endothelial cells hindering the integrity of endothelial and epithelial tight junctions leading to trans-endothelial invasion of breast cancer cells. Disruption of the endothelial barriers and increased vascular permeability promoted distant metastases formation in lung and brain (79). miR-181c is increased in sera of brain metastasis patients compared to non-brain metastasis patients. The study reports that miR-181c regulates the expression of PDPK1 in BMECs, where PDPK1 plays a role in localization of N-cadherin and actin filaments in BMECs. Delocalization of actin in the BMECs plays a role in BBB dysregulation (82).

miR-202-3p is decreased in brain-tropic breast cancer cell lines. The authors report restoration of miR-202-3p leading to MMP-1 suppression results in inhibited extravasation of BCBM cells (85). MMP-1 has previously been reported to promote trans-endothelial migration of breast cancer cells by degrading endothelial junctions in BMECs and permeabilizing the endothelial barrier. One study reported the upregulation of miR-205 and miR-181a-1-3p along with downregulation of miR-194 in co-culture models. The authors report overexpression of miR-181a-1-3p results from the

TABLE 1 miRNAs mediate dysregulation of various cells and stages of BCBM.

miRNA	Activity	Validated Target(s)	*In-vitro* Models	Additional Model(s)	Reference
let-7d	Promote brain metastatic colonization	Pdgfb/PGDFA	4T1, D2A1, MDA-MB-231, MDA-MB-231-BrM2	Mouse	(74)
miR-7	Suppress brain metastases formation by inhibiting proliferation, invasion, and transmigration of CSCs	KLF4	MDA-MB-231, 231BoM-1833, 231BrM-2a, CN34, CN34-BoM2d, CN34-BrM2c, MCF7, MCF7-BoM2d	Mouse	(75)
miR-10b	Promote invasion of BC cells	No specific target	MDA-MB-231, MDA-MB-468	Patient samples	(76)
miR-20b	Promote colony formation and invasion of BC cells	No specific target	MCF-7, MDA-MB-231, MDA-MB-231-brain and bone derivatives	Patient samples	(77)
miR-101-3p	Promote extravasation and trans-endothelial migration of BC cells	COX2	MCF-7, MDA-MB-231, MDA-MB-231-BrM2, MDA-MB-231-TGL	N/A	(78)
miR-105	Promote tumor cell invasion and disrupt vascular endothelium	ZO-1	MDA-MB-231, MCF-10A, MCFDCIS, MDA-231-HM, Primary HMVECs	Patient samples	(79)
miR-122	Inhibit glucose uptake by astrocytes	PKM	MCF10A, MDA-MB-231	Mouse	(80)
miR-132-3p, miR-199a, miR-150 and miR-155	Promote tumor angiogenesis and colonization of BC cells	MET		Patient samples	(81)
miR-181c	Downregulate PDPK1 promoting BBB destruction	PDPK1	MDA-MB-231-luc-D3H1, MDA-MB-231-luc-D3H2LN, BMD2a, BMD2b	Mouse	(82)
miR-194 and miR-802	Upregulate MEF2C highly expressed in peritumoral astrocytes promoting cross-talk	MEF2C	4T1	Mouse, patient samples	(83)
miR-194, miR-181a-1-3p, miR-205,	Mediate cross-talk between BC cells and BMECs	No specific target	b.End5, 4T1	Mouse	(84)
miR-200 family (miR-141)	Induce MET and brain metastatic colonization	E-cadherin	SUM149, SUM159, MDA-IBC3, MDA-231, MCF7	Mouse, patient samples	(18)
miR-202-3p	Induce MMP-1 and promote extravasation of BC cells	MMP-1	MCF-7, MDA-MB-231-TGL, MDA-MB-231-BrM2, hCMEC/D3	Mouse	(85)
miR-211	Promote early colonization and BBB adherence through regulation of SOX11/NGN2 axis	SOX11/NGN2	MDA-MB-231, HCC1806, LM2-4175, BrM-831	Mouse	(86)
miR-345	Induce cancer progression through cross-talk and niche formation	KISS1, E-cadherin	CN34TGL, MDA-MB-231, CN34Br, MDA231Br	Mouse	(87)
miR-503	Induce M1 to M2 polarization of microglia	STAT3/NF-κB pathways	MCF7, ZR75-1, SKBR3, MDA-MB-231, SKBrM3, MDA-MB-231BrM2a, SIM-A9	Mouse, patient samples	(26)
miR-509	Suppress RhoC and TNFα mediated transmigration and invasion of BC cells	RhoC, TNF-α	MDA-MB-231, MDA-MB231BrM-2a, CN34, CN34-BrM2c, MCF7, MDA-MB-231-HM, 293TN, mBrEC	Mouse, patient samples	(88)
miR-623	Promote extravasation and trans-endothelial migration of BC cells	MMP-1	MDA-MB-231-BrM2, MDA-MB-231-TGL, hCMEC/D3	N/A	(89)
miR-637	Promote autophagy and invasion of TNBC cells	STAT3	MCF10A, BT549, MDA-MB-361, MDA-MB-453, MDA-MB-, MDA-MB-468, SUM-159, HCC-1806	Mouse, patient samples	(90)
miR-1258	Inhibit Heparanase to promote invasion	HPSE	MDA-MB-231BR1, MDA-MB-231BR3, SUM-225, SUM-149, HMEC, MCF-10A	Mouse, patient samples	(25)
miR-1290	Activate astrocytes in the brain metastatic microenvironment via the FOXA2→CNTF axis	FOXA2	MDA-MB-231, MDA-231-BRM, SKBR3, CN34, SKBRM-tGLI1, immortalized human astrocytes	Mouse	(36)

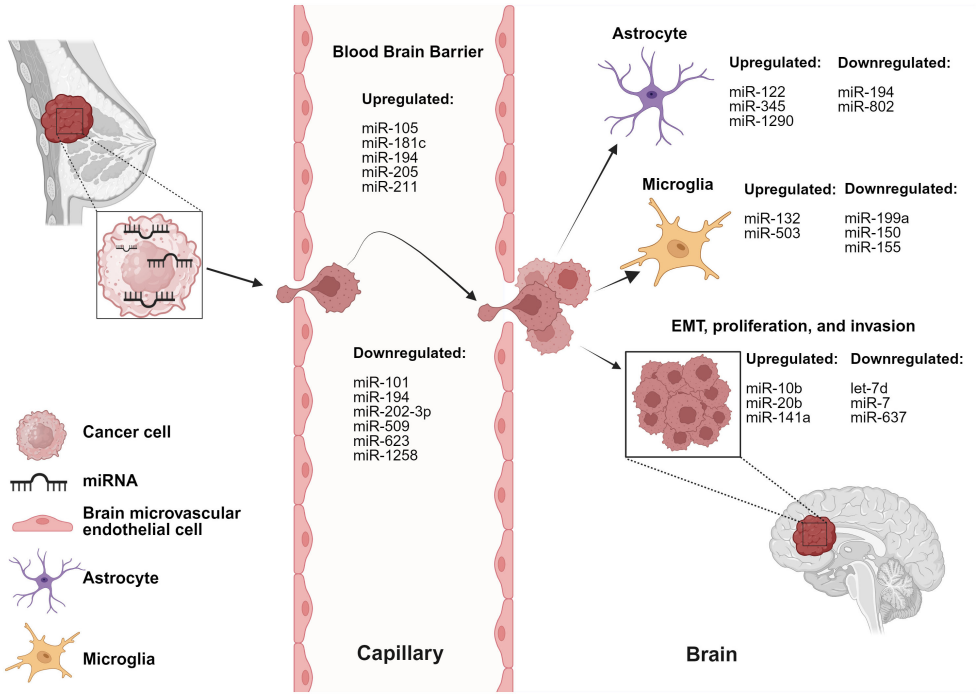

FIGURE 1
miRNAs are involved in dysregulation of various cells and stages of BCBM. BC-derived miRNAs alter functionality of brain and BBB microenvironmental cells. Created with BioRender.com.

interaction between breast cancer cells and BMECs, and that BMECs contribute to the downregulation of miR-194, while breast cancer cells upregulate the expression of miR-205 (84).

miR-211 is upregulated in the brain-tropic TNBC cells and human breast cancer tissue from TNBC and non-TNBC patients. miR-211 overexpressing breast cancer cells promote brain metastases *in vivo*; inhibition of miR-211 with anti-miR-211 treatment suppresses brain metastases *in vivo*. Increased expression of miR-211 promoted migration and invasion of TNBC cells and enhanced adherence of cancer cells to the BBB through downregulation of SOX11/NGN2 axis (86).

miR-509 is downregulated in brain metastases compared to primary breast tumors and targets RhoC, a critical mediator of metastasis and invasion. Additionally, miR-509 suppressed the trans-endothelial migration of breast cancer cells and contributed to the suppression of MMP9 via modulation of RhoC (94). miR-509 also indirectly represses TNFα leading to decreased BBB permeability (88). miR-623 is downregulated in brain metastatic lesions in comparison to primary breast tumors. MMP-1 is known to play a significant role in promoting extravasation of TNBC cells into the brain endothelium and is suppressed by miR-623. This study reports the restoration of miR-623 inhibits trans-endothelial migration of brain-tropic TNBC cells, thereby suppressing BCBM (89). Downregulation of miR-1258 was associated with an increase in HPSE levels in BCBM cell lines, paired primary breast tissue, and BCBM tissue. miR-1258 expression leads to a decrease in HPSE levels and HPSE-related proteins: p-Akt, p-EGFR, MMP-9, COX2 consequently, leading to inhibition of brain metastasis by limiting breast cancer cell invasion (25). These studies are further proof of the crucial role of miRNAs in maintenance of BBB integrity.

6 miRNAs implicated in EMT, invasion, and colonization of breast cancer cells in BCBM

EMT, invasion, and colonization serve an essential role in metastasis of cancer cells. miRNAs involved in these processes are briefly described in Table 1.

miR-7 is downregulated in mammospheres and brain-tropic breast cancer cell lines compared to parental cells (75). *KLF4* is a miR-7 target gene and *in vivo* studies report miR-7 inhibits the expression of *KLF4* downregulating the proliferation, invasion, and transmigration of brain-tropic cancer stem cells (CSCs). The miR-7 and *KLF4* correlation was further validated in human samples from primary breast tumor and brain metastatic lesions, and the authors suggest interaction between CSCs and brain cells promotes formation of a pre-metastatic niche (75).

miR-10b is significantly upregulated in tumor samples of BCBM patients when compared to primary breast tumors without brain metastasis (76). It was reported that higher levels of miR-10b were correlated with increased invasiveness of breast cancer cells (76). miR-20b is increased in brain metastatic lesions of BCBM patients compared to breast cancer patients without brain metastasis (77). miR-20b is also upregulated in brain-tropic breast cancer cells compared to bone-tropic breast cancer cells highlighting brain-tropism of miR-20b. miR-20b overexpression

resulted in increased colony formation and invasiveness of breast cancer cells (77).

let-7d is downregulated in brain metastatic breast cancer cells and regulates PGDFA expression (74). PGDFA inhibition leads to decreased brain metastases formation in mice models due to the loss of autocrine proliferation loop activity which promotes metastatic colonization. The authors also reported HIF1 activity is negatively regulated by let-7d (74). miR-141 expression is upregulated in sera from BCBM patients (18). Upregulation of miR-141 was correlated with increased E-cadherin expression, which suggests miR-141 plays a role in EMT. The role of miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429) in EMT has been well established in various metastatic models and the authors propose a possible role in breast cancer brain metastatic colonization (18). Moreover, circKIF4A sponges miR-637 to suppress its expression in brain metastatic lesions compared to primary breast tumors. miR-637 inhibits STAT3, therefore, miR-637 inhibition increases STAT3 protein levels and promotes the brain metastatic properties of TNBC cells through autophagic activation (90). These studies suggest that miRNAs play key roles in regulating multiple stages of BCBM; however, further investigations into these mechanisms are necessary.

7 Application of miRNAs in cancer therapeutics

The dismal overall survival of patients with BCBM is partly due to the lack of early biomarkers and targeted BBB-penetrant therapies (95–97), and miRNAs could possibly be used to address both deficiencies. The ubiquitous presence of miRNAs in peripheral blood, urine, and saliva makes it a highly valuable and non-invasive biomarker of disease burden, progression, treatment response, and resistance (98–100). Currently, there are several clinical trials assessing the potential application of miRNAs as biomarkers. Project CADENCE (NCT05633342) is a cohort study aimed at investigating miRNA expression along with other biomarkers and ultimately developing *in-vitro* diagnostic assays for screening nine highly prevalent cancers: breast, colorectal, lung, prostate, liver, pancreatic, gastric, ovarian and esophageal (101). Oncoliq US (NCT06439940) is actively recruiting for a prospective cohort study to identify early diagnosis markers for breast cancer utilizing liquid biopsies and miRNAs (102). Another prospective cohort study (NCT05417048) at Peking University is currently investigating the performance of a blood-based assay utilizing miRNAs to differentiate between benign and malignant breast disease (103). MiraKind is currently running a prospective cohort study (NCT02253251) to validate the role of mutations at miRNA binding sites in breast and ovarian cancer patients (104). A randomized diagnostic clinical trial (NCT04516330) is investigating the role of an 84-miRNA panel in predicting multicentricity in breast cancer (105). City of Hope Medical Center conducted a cohort study (NCT01231386) to perform miRNA profiling in patients undergoing treatment for locally advanced or inflammatory breast cancer (106).

miRNA profiling has also been used as a non-surgical tool to differentiate between medulloblastoma, glioblastoma, BCBM, and lung cancer brain metastasis (107). High expression of miR-200 family in the cerebrospinal fluid of brain metastasis patients helps differentiate between cases of brain metastasis and glioblastoma (108).

Therapeutic miRNA development is being explored given the significant roles that miRNAs play in dysregulation of multiple genes leading to tumor initiation, progression, and metastasis (95–97). MRG-106, an inhibitor of miR-155, was investigated for the treatment of cutaneous T-cell lymphoma, mycosis fungoides subtype. The Phase I study reported tolerability and reduction in the Composite Assessment of Index Lesion Severity score and modified Severity Weighted Assessment Tool used to measure skin lesions/disease (109). However, the study by Miragen Therapeutics was discontinued during Phase II (NCT03713320) due to business reasons. Miragen Therapeutics also completed a Phase I study (NCT03603431) with MRG-110, a miR-92a inhibitor, which was investigated in healthy volunteers and was reported to augment wound healing and angiogenesis (110). TransCode Therapeutics recently entered a Phase I/II dose-escalation study (NCT06260774) with TTX-MC138, a miR-10b inhibitor, which has previously been implicated in metastatic lesions arising from advanced solid tumors (111, 112). MRX34, miR-34a mimic, was investigated in patients with unresectable primary liver cancer, hematological malignancies and advanced solid tumors (NCT01829971). MiRNA therapeutics reported treatment with MRX34 demonstrated some clinical activity, however, treatment-associated severe adverse events led to termination of the study (113). CDR132L, a selective miR-132-3p inhibitor, is currently in Phase II clinical trial (NCT05350969) for patients with reduced Left Ventricular Ejection fraction post-myocardial infarction (114). In 2019, Regulus Therapeutics announced pre-clinical success of RGLS5579, an anti-miR-10b, in combination with temozolomide (TMZ) in glioblastoma animal models (115). Regulus Therapeutics reported the median survival rate of glioblastoma-bearing mice models treated with anti-miR-10b, anti-miR-10b in combination with TMZ, and TMZ alone increased by 18%, >120%, and 27% respectively. Combination of tumor suppressive miRNAs with conventional chemotherapy is another promising avenue. Tumor suppressive miR-770 inhibited doxorubicin resistance in TNBC and promoted sensitivity to trastuzumab in HER2-positive breast cancer (116, 117). Overexpression of miR-298 sensitizes doxorubicin-resistant breast cancer cells to treatment by targeting MDR1 (118). Other studies have also reported targeting of ABCG2 overexpression of miR-181a or miR-328 in breast cancer cells led to increased sensitivity to mitoxantrone (119, 120). miRNAs may be the key to improving targeted therapeutics for cancer patients; however, it is worth noting that many Phase I and II clinical trials have been halted in the past due to severe adverse effects (96, 121, 122). Nonetheless, these studies highlight the importance of further

investigations into the role of miRNAs as biomarkers and for the advancement of miRNA incorporation into cancer therapeutics.

8 Discussion

There has been a rise in incidence of BCBM, due to significantly advanced and effective therapies that prolong patient survival. This extended survival period allows latent metastatic cells greater opportunity to penetrate the BBB and colonize in the brain parenchyma. Given the rise in frequency and limited treatment opportunities of brain metastasis, there is an urgent need for new predictive, diagnostic, and prognostic biomarkers to assess brain metastasis. miRNAs are small but mighty in the regulation of every step of brain metastasis starting from the cancer stemness genes, genes responsible for intravasation and extravasation into a foreign site, organ tropism, and colonization-related genes. The specificity of dysregulated miRNAs in brain metastasis from various primary tumors can be utilized to differentiate tumor types and identify the origin of primary tumors in unknown cases. Certainly, further research aimed at identifying novel miRNAs, elucidating their biological functions, and uncovering their target genes will significantly enhance our understanding of the role miRNAs play in metastases formation and progression. This will, in turn, lay the groundwork for the advancement of miRNA-related approaches for cancer prognosis, diagnosis, and treatment.

Author contributions

MK: Writing – original draft, Conceptualization. GW: Writing – review & editing. CZ: Writing – review & editing. MN: Writing – review & editing, Visualization. H-WL: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Conflict of interest

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

Publisher's note

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The emerging roles of LINC00511 in breast cancer development and therapy

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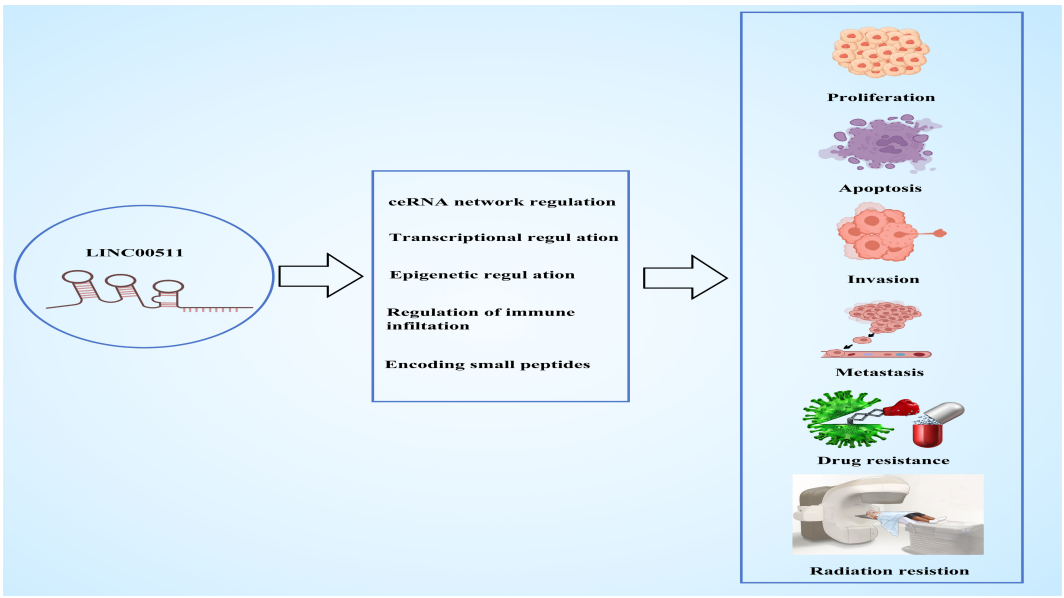
Breast cancer (BC) is associated with malignant tumors in women worldwide with persistently high incidence and mortality rates. The traditional therapies including surgery, chemotherapy, radiotherapy and targeted therapy have certain therapeutic effects on BC patients, but acquired drug resistance can lead to tumor recurrence and metastasis. This remains a clinical challenge that is difficult to solve during treatment. Therefore, continued research is needed to identify effective targets and treatment methods, to ultimately implement personalized treatment strategies. Several studies have implicated that the long non-coding RNA LINC00511 is closely linked to the occurrence, development and drug resistance of BC. Here we will review the structure and the mechanisms of action of lnc RNA LINC00511 in various cancers, and then explore its expression and its related regulatory mechanisms during BC. In addition, we will discuss the biological functions and the potential clinical applications of LINC00511 in BC.

KEYWORDS

lncRNAs, LINC00511, breast cancer, mechanisms, biological functions



GRAPHICAL ABSTRACT

1 Introduction

Although governments and health organizations around the world have taken a series of measures and made great efforts with regards to cancer prevention and treatment, the incidence and mortality rates for breast cancer (BC) have not been effectively controlled. These continue to show an increasing trend (1). Cancer data statistics show that in 2020, there were about 2.3 million newly diagnosed cases of BC. As a result, BC has surpassed lung cancer (LC) for the first time, and has become the leading cancer among women (2). Currently, BC remains one of the main causes of death in women worldwide, posing a serious threat to the health and lives (3). There are currently several ways to classify BC. Based on genetic and epigenetic characteristics, breast cancer cell lines can be classified as luminal A, luminal B, HER2 positive, triple negative A and triple negative B (4) Clinically, BC can be classified into estrogen receptor (ER)-positive and ER-negative as well as HER2 (human epidermal growth factor receptor 2)-positive and HER2-negative subtypes. This classification is based on the levels of ER, progesterone receptor (PR) and HER2 present (5, 6).

Although medical professionals have conducted extensive and long-term research on the diagnosis and treatment of BC, as yet no significant breakthroughs have been achieved. Therefore, the therapeutic effects for BC patients have not improved significantly, and the survival times have not been significantly extended. To date, the conventional treatment methods for BC are primarily surgery and targeted therapies using drugs, radiography, hormones and antibodies (7). Although the emerging targeted therapies have brought hope to BC patients in recent years, cellular drug resistance leading to tumor recurrence and metastasis has posed a severe challenge to its treatment. Therefore, continued research to identify effective targets and treatment methods are in demand from BC patients as well as health professionals. In this context, the role of long non-coding RNAs (lncRNAs) in cancers has been the subject of intense research from scholars, worldwide.

LncRNAs are transcripts that contain more than 200 nucleotides. Due to their lack of long open reading frames, these molecules were previously assumed to have no protein-coding ability. However, with the continuous development of medical testing techniques, there is now a substantial body of evidence to show that some lncRNAs have these abilities (8–11). The discovery that lncRNAs can encode proteins has drawn widespread attention to their biological roles and have made them a research hotspot in recent years.

Studies have shown that lncRNAs can regulate the expression and function of various genes through multiple mechanisms. These include regulation of chromatin remodeling, splicing, mRNA transcription, DNA methylation, mRNA stability and translation and post-transcriptional regulation (12–17). In addition, mature lncRNAs can also bind to RNA/DNA binding proteins, transcription factors (TFs), chromatin modification complexes, RNA transcripts, mature mRNAs, microRNAs, DNA and chromatin) to form supramolecular structures (18). This can lead to regulation of the expression and function of target genes. Numerous studies implicate lncRNAs in the regulation of essential biological and cellular processes. These include proliferation, differentiation, invasion, migration, angiogenesis, stemness, epithelial-mesenchymal transition, cell apoptosis, immune responses and tumor treatment resistance in malignant tumors (19–21).

Therefore, dysregulation of either their expression or function can lead to pathological and physiological changes in the human body. These can result in the development of abnormalities and malignancy resulting in diseases. In recent years, the functional roles of lncRNAs in malignant tumors have gradually been revealed. Several studies have shown that lncRNAs are abnormally expressed in malignant tumors such as renal cell carcinoma, gastric cancer, liver cancer, non-small cell LC, colorectal cancer, glioblastoma, osteosarcoma and ovarian cancer (22–31). They have been shown to be implicated in the regulation and development of cancers.

Long intergenic non-coding RNAs (lincRNAs) can be considered to be lncRNAs as they share similarities of structure and function. Although lincRNAs do not participate in encoding specific proteins, they can act as regulatory factors to regulate the expression of target genes and thus play roles in various cellular and biological processes (32). Existing studies have shown that dysregulation of lincRNA expression and function can also lead to the occurrence and progression of tumors (33–36).

LINC00511 is a newly discovered lncRNA and it is known to be associated with LC (37, 38). It has also been reported to be abnormally expressed in many types of malignant tumors where it can accelerate tumor progression. It inhibits malignant cell apoptosis and promotes the proliferation, migration, invasion, metastasis and chemotherapy resistance of tumor cells (39–41). Therefore, LINC00511 is a potential cancer biomarker and a promising therapeutic target. Currently, there have been multiple reports on the link between LINC00511 and BC, but the existing research directions are scattered and there is a lack systematic summarization. Therefore, we reviewed the expression, structural characteristics, mechanisms and functional roles of LINC00511 in BC with a view to clarifying its clinical significance and therapeutic potential.

2 Structural characteristics of LINC00511

Cabanski et al. first reported LINC00511, which is also known as onco-LncRNA-12, in 2015 after a pan-cancer transcriptome analysis (42). It is located on the negative strand of 17q24.3 region, 72,290,091-72,640,472 (GRCh38/hg38), and it has been allocated the transcript number, ENST00000453722.6 (Figure 1A). It is 1716nt in length and consists of 5 exons (43). Studies have identified 107 alternative splice variants of LINC00511. Except for the two splicing variants, LINC00511-279 and LINC00511-278, which retain the introns, the others belong to the lncRNA transcriptome (https://asia.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000227036;r=17:72290091-72640472). The NIH Genotype-Tissue Expression (GTEx) project (https://commonfund.nih.gov/GTEx, accessed on April 9, 2024) suggested that LINC00511 is widely expressed in normal human tissues (Figure 1B). However, its expression is highest in the skin, vagina and esophagus, suggesting that its presence in these organs has biological significance.

3 Functional roles of LINC00511

Studies have shown that the biochemical functions and regulatory mechanisms of lncRNAs are closely related to their genomic locations and subcellular localizations (44). For example, lncRNAs that are located in the cell nuclei are mainly associated with regulation in epigenetic modifications and transcriptional processes. However, cytoplasmic lncRNAs typically exert their

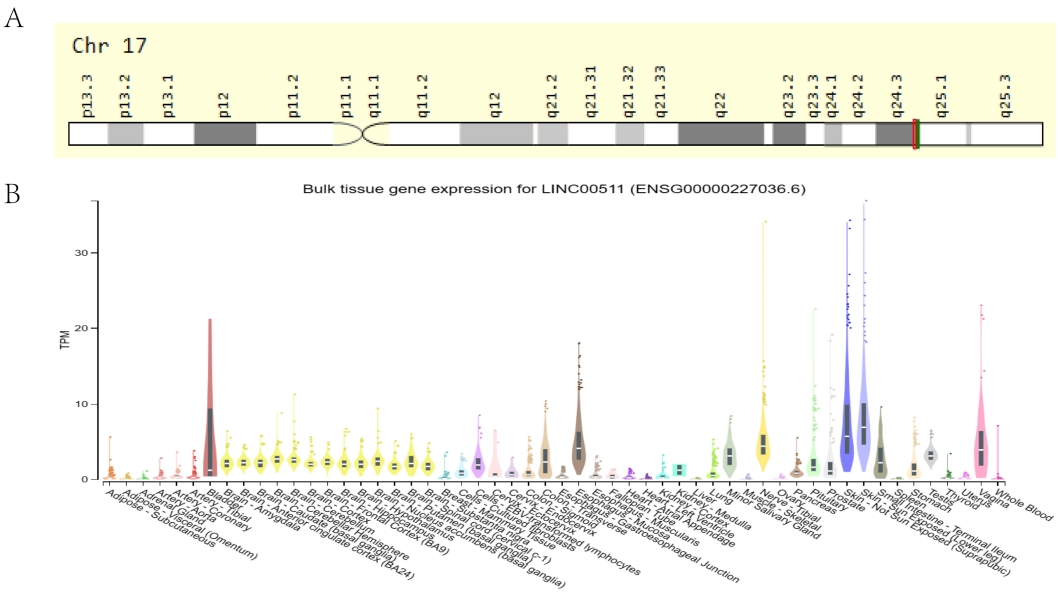


FIGURE 1
(A) Localization of LINC00511 in the human chromosome. **(B)** The expression of LINC00511 in normal human tissues from information obtained from the GTEx database.

regulatory effects through post-transcriptional mechanisms such as the regulation of mRNA stability, protein translation and competition with endogenous RNA (ceRNA) networks (9, 45, 46) (Figure 2).

With respect to LINC00511, studies have shown that its intracellular localization varies in different cancers. Wu et al. used subcellular fractionation and FISH assays and they localized LINC00511 primarily in the cytoplasm in lung squamous cell carcinoma (46). However, in ovarian cancer it was localized in the nuclei of ovarian cancer cells (47). Zhao et al. found that LINC00511 was abundant in both the cytoplasm and nuclei of pancreatic cancer cells by using FISH (48). The widespread subcellular localization of LINC00511 differs in different types of tumor cells and this is likely to reflect on its biological functions and regulatory mechanisms (Table 1).

Although it has a relatively complex role in malignant tumors, the regulatory effects LINC00511 can be exerted in three main ways. Firstly, LINC00511 can act as a ceRNA, where it regulates the expression of target genes by interacting with microRNAs (miRNAs) and thereby affecting malignant tumor cells. Secondly, LINC00511 can regulate its own or target gene expression via epigenetic modifications thereby promoting tumor occurrence. Thirdly, LINC00511 can exert effects at the transcriptional level, such as the regulation of target genes and related signaling pathways, thereby influencing tumor progression.

4 Expression of LINC00511 in BC

LINC00511 is known to be dysregulated in various human malignant tumors. In addition, its expression levels is known to be associated with the age patients and their tumor size, stage and subtype as well as their lymph node status. These factors can affect the diagnosis and prognosis of tumor patients, which suggests that

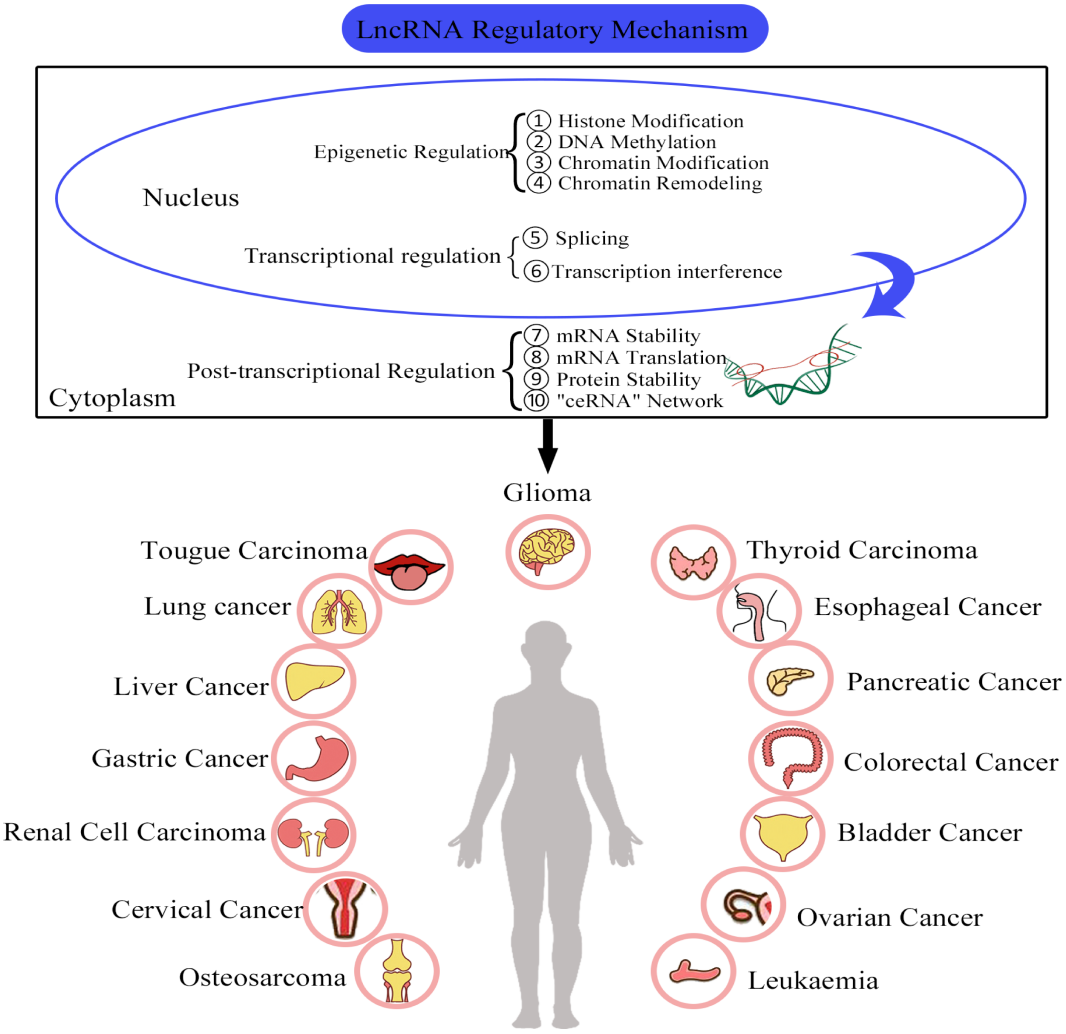


FIGURE 2
The functional roles and regulatory mechanisms of lncRNAs in various cancers. ① LncRNAs are involved in the regulation of histone modification. ② LncRNAs are involved in DNA methylation. ③ LncRNAs can regulate chromatin modification. ④ LncRNAs can bind to chromatin re-modelling factors and regulate gene expression. ⑤ LncRNAs can interact with splicing factors. ⑥ LncRNAs can cause transcription factors to interfere with gene transcription. ⑦ LncRNAs can regulate RNA stability. ⑧ LncRNAs can participate in the regulation of RNA translation. ⑨ LncRNAs are involved in the regulation protein stability. ⑩ LncRNAs can act as miRNA sponges.

TABLE 1 The functions and regulatory mechanisms associated with LINC00511 in various malignant tumors.

Tumor type	Molecular function	Mechanism of action	Biological function	References
Glioma	ceRNA network regulation	Sponge effect of miR-126-5p and activation of Wnt/β-catenin	Promotion of glioma cell resistance to temozolomide	(49)
	ceRNA network regulation	Sponge effect of miR-15a-5p and activation of AEBP1	Regulation of glioma cell proliferation and migration	(50)
	ceRNA network regulation	Sponge effect of miR-524-5p and activation of YB1/ZEB1	Promotion of glioblastoma cell proliferation, migration and ETM	(51)
	ceRNA network regulation	Sponge effect of miR-124-3p and activation of CCND2	Accelerated proliferation and invasion of glioma cells	(52)
Non-small cell lung cancer (NSCLC) Non-small cell lung cancer (NSCLC)	Epigenetic regulation	Inhibition of LATS2 and KLF2 expression by binding EZH2 and LSD1	Promotion of NSCLC cell proliferation and invasion	(53)
	Epigenetic regulation	Inhibition of P57 expression by binding to EZH2	Promotion of NSCLC cell proliferation and invasion	(54)
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of PKM2	Promotion of lung adenocarcinoma (LA) cell proliferation, invasion and migration	(55)
	ceRNA network regulation	Sponge effect of miR-195-5p and activation of GCNT3	Promotion of LA cell proliferation, invasion and migration	(56)
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of GSPT1	Promotion of LA cell proliferation, invasion and migration	(57)
	ceRNA network regulation	Sponge effect of miR-150-5p and activation of TADA1	Promotion of lung squamous cell carcinoma progression	(46)
	ceRNA network regulation	LINC00511 silencing promotes miR-497-5p expression to inhibit SMAD3	Increased radio-sensitivity of LA cells	(58)
	ceRNA network regulation	Sponge effects of miR-126-5p and miR-218-5p, upregulation of COL1A1 and activation of PI3K/AKT	Promotion of LA cell proliferation and metastasis	(59)
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of PKM2	Promotion of LA cell proliferation and metastasis	(55)
	ceRNA network regulation	Silence of LINC00511, promotion of miR-182-3p expression and inhibition of BIRC5	Inhibition of cisplatin resistance in LA cells	(60)
Gastric cancer (GC)	ceRNA network regulation	Sponge effect of miR-515-5p	Promotion of GC cell proliferation, migration, stemness and inhibition of apoptosis	(61)
	ceRNA network regulation	Sponge effect of miR-124-3p and activation of PDK4	Promotion of GC cell growth	(62)
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of NFIX	Promotion of GC cell growth	(63)
	Epigenetic regulation	Activation of SOX4, inhibition of PTEN and activation of PI3K/AKT	Promotion of GC cell growth and migration	(64)
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of STAT3	Promotion of GC cell proliferation and migration	(65)
	ceRNA network regulation	Sponge effect of MiR-124-3p and activation of EZH2	Promotion of GC cell proliferation and invasion	(39)
Colorectal cancer (CRC)	ceRNA network regulation	Sponge effect of miR-153-5p and activation of HIF-1α	Promotion of CRC cell proliferation	(66)
	Epigenetic regulation	HNF4α mediation of the LINC00511/EZH2 axis and inhibition of IL-24	Promotion of CRC cell proliferation and migration	(67)
	ceRNA network regulation	Sponge effect of miR-29c-3p and activation of NFIA	Promotion of CRC cell proliferation, migration and stemness	(68)

(Continued)

TABLE 1 Continued

Tumor type	Molecular function	Mechanism of action	Biological function	References
	ceRNA network regulation	Sponge effect of miR-625-5p and activation of WEE1	Promotion of CRC cell growth	(69)
Liver cancer (LC) Liver cancer (LC)	ceRNA network regulation	Sponge effect of miR-424	Promote liver cancer cell proliferation and metastasis	(70)
	ceRNA network regulation	Sponge effect of miR-195 and activation of EYA1 expression	Promotion of LC cell proliferation and invasion	(41)
	ceRNA network regulation	Sponge effect of miR-29c	Promotion of LC cell proliferation and migration	(71)
	Induction function	Induction of invasive pseudopod formation and exosome secretion	Promotion of LC invasion	(72)
Osteosarcoma (OS)	ceRNA network regulation	Sponge effect of miR-618 and activation of MAEL	Promotion of OS cell proliferation and migration	(73)
	ceRNA network regulation	Sponge effect of miR-765 and activation of APE1	Promotion of OS cell proliferation and migration	(74)
	–	–	Inhibition of OS cell proliferation and migration	(75)
Pancreatic cancer (PC)	ceRNA network regulation	Sponge effect of miR-29b-3p and activation of VEGFA	Promotion of PC cell proliferation, migration and invasion	(48)
	ceRNA network regulation	Sponge effect of miR-193a-3p and activation of PLAU	Induction of PC cell invasion and migration	(76)
Cervical cancer (CC)	transcriptional regulation	Increased RXRA and upregulation of PLD1	Promotion of CC cell growth	(77)
	transcriptional regulation	Regulation of drug resistance and apoptosis-related genes	Promotion of CC cell apoptosis and reduction resistance to paclitaxel by inhibition of LINC00511	(40)
	ceRNA network regulation	Sponge effect of miR-324-5p and activation of DRAM1	Promotion of CC cell proliferation and invasion	(78)
	ceRNA network regulation	Sponge effect of miR-497-5p and activation of MAPK1	Promotion of CC cell proliferation and invasion	(79)
T-cell acute lymphoblastic leukaemia (TCALL)	ceRNA network regulation	Sponge effect of miR-195-5p and activation of LRRK1	Promotion of TCALL cell migration and proliferation	(80)
Thyroid cancer (TC)	transcriptional regulation	Activation of CDK	Promotion of TC cell proliferation	(81)
	transcriptional regulation	Bound to TAF1 and regulation of JAK2/STAT3	Altered radio-sensitivity of TC cells	(82)
Renal cell carcinoma (RCC)	ceRNA network regulation	Sponge effect of miR-625 and activation of CCND1	Promotion of RCC cell proliferation and migration	(83)
Bladder cancer (BC)	ceRNA network regulation	Sponge effect of miR-143-3p and activation of PCMT1 expression	Promotion of BC cell proliferation and migration	(84)
	transcriptional regulation	Regulation of Wnt/β-cathepsin	Proliferation and apoptosis of BC cell	(85)
Tongue cancer (TC)	ceRNA network regulation	Sponge effect of miR-765 and activation of LAMC2	Promotion of squamous TC cell proliferation and invasion	(86)
Ovarian cancer (OC)	ceRNA network regulation	Sponge effects of miR-424-5p and miR-370-5p	Promotion of OC cell proliferation and migration	(87)
	ceRNA network regulation	Bound to EZH2 and inhibition of P21	Promotion of OC cell proliferation and migration	(47)
Esophageal cancer (EC)	ceRNA network regulation	Sponge effect of miR-150-5p	Promotion of EC cell proliferation, migration and invasion	(88)

(Continued)

TABLE 1 Continued

Tumor type	Molecular function	Mechanism of action	Biological function	References
Melanoma	ceRNA network regulation	Sponge effect of miR-150-5p and activation of ADAM19	Promotion of melanoma cells migration	(89)

it has a role as a potential molecular biomarker (90). By using the publicly available online databases, GEPIA (91), UALCAN (92) and ENCORI (93), LINC00511 was found to be upregulated in BC tissues. The expression levels of this lncRNA were found to be closely correlated to the clinical stage, subtype and subclass of BC (Figure 3).

A subsequent study of BC tissue samples confirmed the results obtained from the above online databases. Lu et al. measured LINC00511 expression levels in BC and non-cancerous tissues by qRT-PCR and found that LINC00511 was significantly upregulated in the cancer samples (94). This was confirmed by Tan et al. These authors showed that LINC00511 expression was closely associated with tumor size, clinical stage and lymph node status by using *in vitro* cell cultures (95). Interestingly, another study using serum samples from BC patients also showed that LINC00511 was overexpressed in BC patients where it was found that this lncRNA was significantly associated with the patients' PR status, ER status, HER2 status, tumor stage, tumor size as well as lymph node status (43).

In summary, several lines of evidence obtained from bioinformatics, blood, tissue and cell experiments, would suggest that there is an overexpression of LINC00511 in BC. Therefore, further research on the regulatory mechanisms of LINC00511 dysregulation in BC would be beneficial for revealing the molecular nature this cancer and this exercise may point to potential new therapeutic targets to combat this disease.

5 Related regulatory mechanisms of LINC00511 dysregulation in BC

Currently, the specific regulatory mechanisms underlying the upregulation of LINC00511 expression in BC remain unclear. Studies have shown that the abnormal levels of LINC00511 may affect regulatory mechanisms such as epigenetic modifications. DNA methylation is an epigenetic mechanism that has been proven to play a crucial role in the occurrence and development of BC (96). Liu et al. also confirmed that DNA hypo-methylation in the CpG island region of the gene promoter region was positively and significantly correlated with the levels of LINC00511 expression (97).

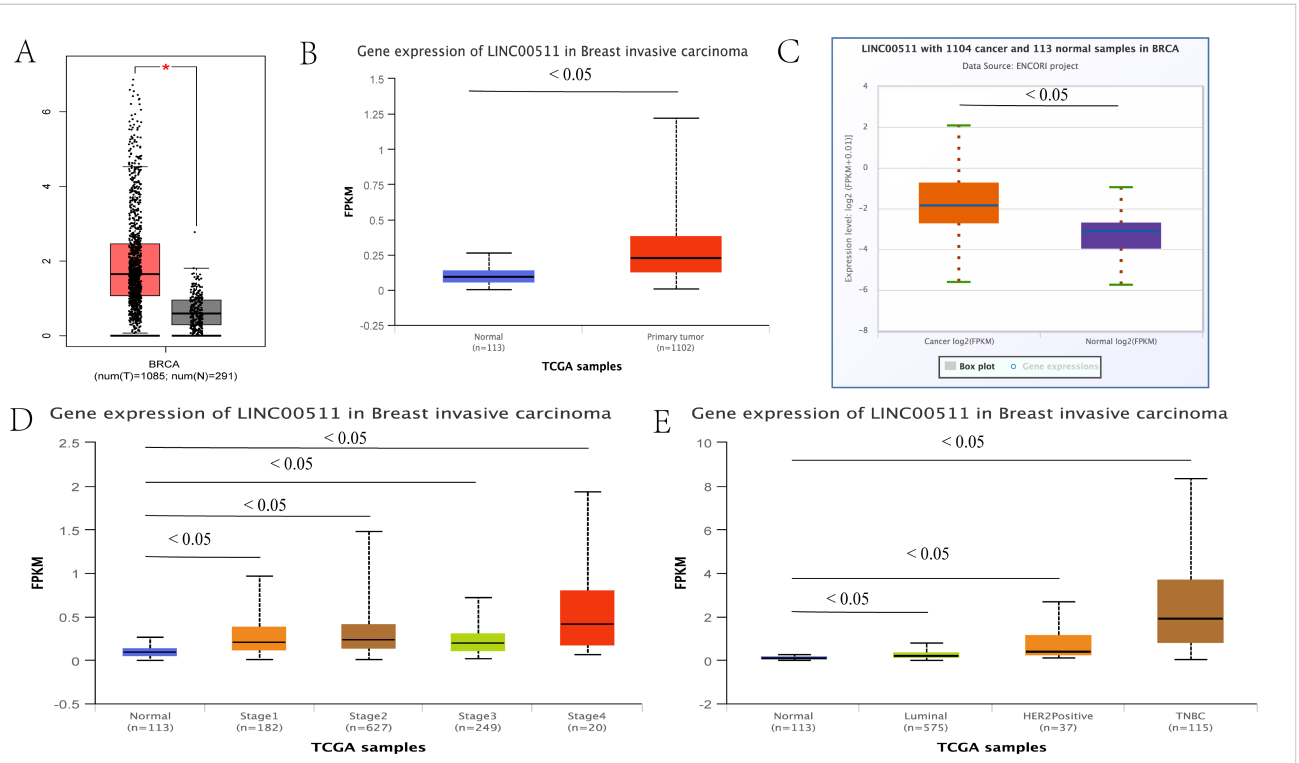


FIGURE 3
The relationship between the expression levels of LINC00511 in breast cancer and tumor stage and the breast cancer subtype. **(A)** GEPIA, **(B)** UALCAN, **(C)** ENCORI and other online websites were used to show the LINC00511 expression levels in breast cancer. **(D)** The UALCAN online website showed that LINC00511 expression levels were closely related to tumor stage of cancer progression.**(E)** The UALCAN online website showed that LINC00511 expression levels were closely related to the breast cancer subtype.

The estrogen-signaling pathway is another factor that can affect LINC00511 expression. Zhang et al. explored the relationship between LINC00511 and ER-negative status in a study on ER-negative BC and found that silencing ER expression in cancer cells could induce its expression. Surprisingly, after stimulating BC cells with tamoxifen, an anti-estrogen drug, LINC00511 expression was significantly upregulated. Further studies revealed that estrogen deficiency could directly activate the TF, AP-2 (TFAP-2), thereby upregulating LINC00511 expression in BC cells (98). Currently, only DNA methylation and the estrogen-signaling pathway have been reported to be associated with the regulation of LINC00511 expression in BC. Further research is needed to elucidate the precise mechanisms of action of LINC00511 in BC.

6 Molecular regulatory mechanisms of LINC00511

6.1 CeRNA networks

LncRNAs often exert their biological functions as ceRNAs. LINC00511 is a typical ceRNA molecule that is capable of base pairing in a complementary fashion with various miRNAs, by competitively binding to them. This prevents the miRNAs from binding to and degrading target genes, thereby regulating target gene expression. Among them, the LINC00511/miR-185-3p/E2F1 axis is a classic ceRNA interaction network. Lu et al. showed that LINC00511 can bind to miR-185-3p through molecular complementarity. This prevents miR-185-3p from binding to its target gene, E2F1, leading to upregulation of E2F1 protein expression and activation of the expression of the downstream TF, Nanog. These interactions promotes the stem-like state and malignant phenotype of BC cells (94). Additionally, LINC00511 can also promote tumor cell proliferation by alleviating the inhibitory effect of miR-150 on matrix metalloproteinase 13 (MMP13) (99). Such typical ceRNA networks can play important roles in the oncogenic functions of LINC00511.

6.2 Involvement in transcriptional regulation

Numerous studies have shown that LINC00511 may either directly or indirectly affect the transcription and expression of downstream genes through interactions with TFs, regulatory factors as well as other molecules. Blasiak et al. found that LINC00511 could interact with the vitamin D receptor (VDR), thereby affecting the transcriptional activation process and interfering with the expression of genes related to the vitamin D signaling pathway. This can lead to an anti-BC effect (100). Additionally, Liu et al.'s study also confirmed that LINC00511 could alter the radio-sensitivity of BC cells by regulating the expression levels of STXBP4 (101). This suggests that LINC00511 achieves its biological functions at multiple levels, including gene expression regulation.

6.3 Encoding small peptides to regulate signaling pathways

In addition to acting as a classic lncRNA, recent studies have found that LINC00511 may also encode a 133-amino acid small peptide (LINC00511-133aa) resulting in unique biological functions. Tan et al. found that the small peptide encoded by LINC00511 is capable of activating the Wnt/β-catenin signaling pathway. This can promote the invasive capacity and the stem cell state of BC cells (95). This finding provides a novel molecular mechanism for the oncogenic effects of LINC00511 and suggest additional roles for lncRNAs in tumor progression.

6.4 Mediating target gene regulation of the immune microenvironment

Recently, it was shown that there is a connection between LINC00511 and tumor immunogenicity and the tumor microenvironment. Sun et al. reported that LINC00511 could regulate the activation of inflammasomes through the LINC00511/miR-573/GSDMC axis, thereby affecting immune cell infiltration and tumor immunogenicity (102). Lian et al. also provided evidence through bioinformatics analysis combined with tissue and cell experiments that LINC00511 participated in regulating immune cell infiltration by modulating the related signaling pathways. They found that LINC00511 could target miR-29-3p, thereby promoting the upregulation of SLC31A1 expression. This molecule then promoted BC progression by regulating tumor immune infiltration (103). These findings suggest that LINC00511 may be involved in remodeling of the immune microenvironment leading to tumor evasion by immune system. This may provide another potential target for tumor immunotherapy.

7 Biological functions of LINC00511 in BC

7.1 Mediation of tumor cell apoptosis, proliferation, migration and invasion

In several studies, LINC00511 has been shown to significantly promote the proliferation, migration and invasion capabilities of BC cells. Liu et al. found that DNA hypo-methylation could lead to upregulation of LINC00511, thereby promoting BC cell proliferation, invasion and migration (97). LINC00511 could also inhibit cell apoptosis and promote cell proliferation in some *in vivo* experiments where it was found that knocking down LINC00511 could inhibit tumor growth (98). Another study revealed that LINC00511 could positively regulate the expression of MMP13,

thereby promoting the migration and invasion of BC cells (99). Therefore, LINC00511 can promote the progression of malignancy in BC by regulating various downstream molecular targets.

7.2 Maintenance the tumor stem cell state

Cancer stem cells belong to a small subgroup of tumor cells, which possess self-renewal and tumor-initiating abilities, playing a crucial role in various stages of tumorigenesis, drug resistance, recurrence as well as metastasis. Studies have shown that LINC00511 could positively regulate Nanog expression, thereby maintaining the tumor stem cell-like state and characteristics. Knocking down LINC00511 can significantly downregulate Nanog expression and its downstream genes, inhibiting the formation and self-renewal capacity of tumor stem cells. There is also evidence that LINC00511 may encode a 133-amino acid small peptide (LINC00511-133aa), which can activate the Wnt/β-catenin signaling pathway, and thus maintaining the stem-like state of BC cells (95). Hence, LINC00511 has the ability to regulate the existence and activity of tumor stem cells.

7.3 Involvement of LINC00511 in tumor chemotherapy and radiotherapy resistance

Tumor cell resistance to chemotherapeutic drugs and radiation can lead to failure in cancer treatment, recurrence and metastasis. Multiple studies have found that LINC00511 can cause resistance in BC patients. Liu et al. confirmed that LINC00511 could regulate miR-185 expression, thereby affecting the levels of its target gene, STXBP4, ultimately leading to enhanced radio-resistance in BC cells (101). Zhang et al. showed that LINC00511 could enhance cellular resistance to paclitaxel by upregulating the expression levels of CDK6, while knocking down LINC00511 could significantly increase tumor cell sensitivity to this chemotherapeutic drug (104). Wu et al. found that nanomaterial-mediated LINC00511 siRNA delivery technology could significantly improve the chemosensitivity of triple negative BC (TNBC) cell lines to cisplatin (105). These findings reveal a key regulatory role of LINC00511 in processes such as chemotherapeutic drug- and radiation-induced tumor cell apoptosis and DNA damage repair.

8 Potential clinical applications of LINC00511 in BC

8.1 LINC00511 as a molecular biomarker for diagnosis and prognosis of BC

Extensive preclinical and retrospective clinical data have shown that LINC00511 may be used as a potential molecular biomarker for the diagnosis and prognosis of BC. Currently, the main blood biomarkers used in the clinical screening of BC are CEA and CA15-3. However, these have limited diagnostic efficacy for early-stage BC. To find new blood biomarkers in order to improve the diagnostic efficacy of BC, Mahmoud et al. conducted relevant studies

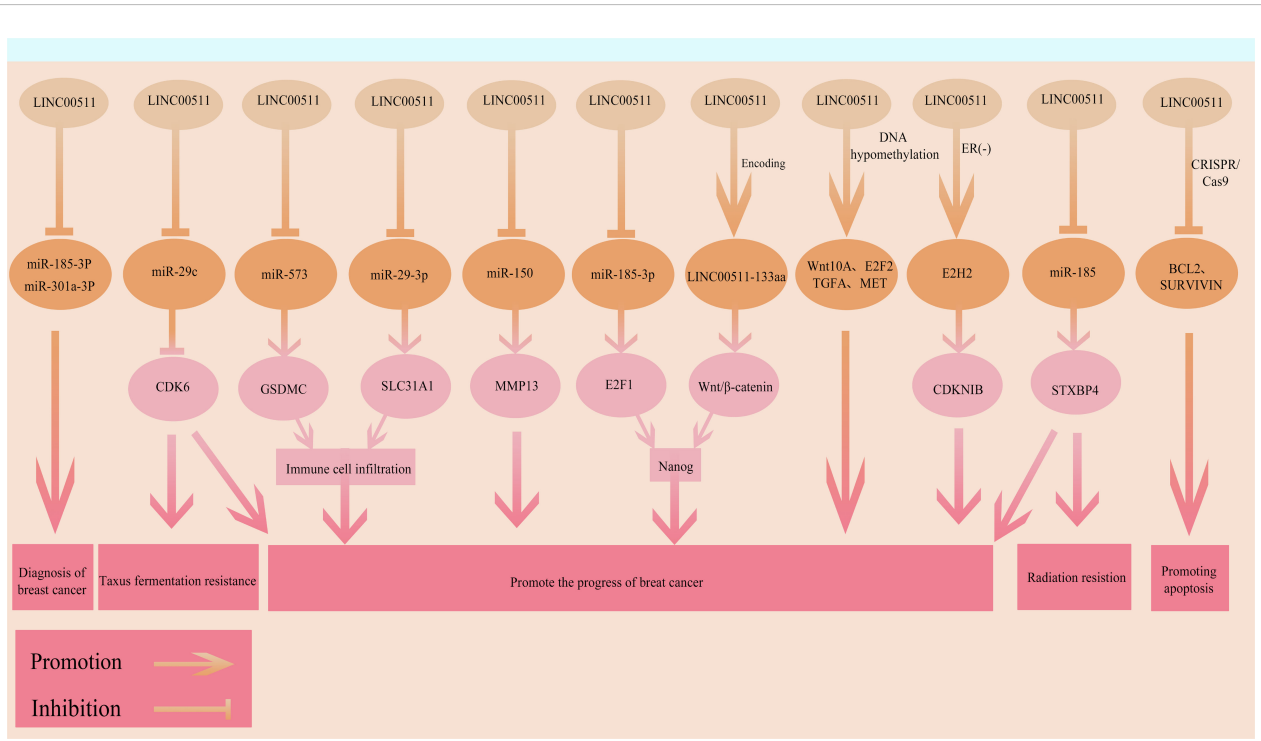


FIGURE 4
The functional roles and regulatory mechanisms of LINC00511 in BRCA. The arrow and blocking symbols represent promotion and inhibition of molecules/events, respectively.

on the sera of Egyptian female BC patients (43). They found that, compared to using CEA and CA15-3 alone for diagnosis, a combination screening with either LINC00511/CA15-3 or LINC00511/CEA had higher diagnostic efficacy in distinguishing BC patients from healthy individuals. In addition, LINC00511 also had a certain diagnostic value in the staging and metastasis of BC patients. This study found that the expression pattern of LINC00511 also differed among different molecular subtypes of BC, with a relatively higher expression in TNBC, suggesting its intrinsic association with the occurrence and development of specific subtypes.

LINC00511 has also shown to have a potential value in predicting the survival of BC patients. A systematic review and meta-analysis of LINC00511 in BC patients showed that those with high LINC00511 expression had a shorter overall survival (106). Chen et al. also reached a similar conclusion (107). These findings suggest that LINC00511 may be a potentially valuable molecular marker for BC. Further in-depth studies on LINC00511 will help to optimize the molecular subtyping, prognostic assessment and personalized treatment strategies for BC.

8.2 LINC00511 as a therapeutic target for BC

High expression levels of LINC00511 in BC patients precedes a worse survival prognosis with the lncRNA acting as an oncogene in BC. Therefore, knocking out LINC00511 expression in order to inhibit its effects on oncogenes and their related signaling pathways may be an effective new strategy for treatment of BC. Currently, the main targeting strategies for lncRNAs include antisense oligonucleotides, siRNAs (99, 104) and shRNAs (94, 101), which can act as small inhibitor molecules. SiRNA silencing studies by Yuan et al. have involved the construction of a carrier by combining siRNA with cationic nano-bubbles (CNBs). This significantly improved the silencing efficiency of LINC00511 by employing ultrasound-mediated nano-bubble destruction and forming siRNA-CNBs, which were ideal carriers for treating BC (107). Additionally, Wu et al. constructed a novel type of therapeutic diagnostic agent, by using a complex of low-frequency ultrasound irradiation and nano-bubbles, which appeared to be an efficient and safe siRNA transfection strategy (105).

Another method that could be used to inhibit the effects of LINC00511 on oncogenes, involved CRISPR-Cas9-mediated gene editing (108). Azadbakht et al. showed that CRISPR/Cas9 was another potential method for knocking out LINC00511, and subsequent studies have shown that this technique could specifically knock out this lncRNA gene (109). These studies indicate that LINC00511 may a potential novel therapeutic target for BC, and many scholars have proposed and implemented various methods for targeting LINC00511 (38, 110). However, up to now, these practices have only yielded results in cell and animal models, and clinical application studies have not produced satisfactory results. Therefore, further in-depth understanding and elucidation of the functional mechanisms of LINC00511 are needed, followed by the development of targeted drugs, to bring new treatment options for BC patients.

9 Prospects and challenges

LINC00511 is a functionally diverse and broad acting oncogenic lncRNA in BC, participating in multiple key biological processes in these cells (Figure 4). Its mechanisms of action involve ceRNA networks, transcriptional regulation, signaling pathway activation as well as small peptide encoding at multiple levels. New functions and regulatory mechanisms of LINC00511 in BC are being discovered. However, there are currently several gaps in our understanding of the overall mechanism of action of LINC00511 in tumor occurrence and development, as well as its associated networks with other molecular events. Additionally, the precise mechanisms by which LINC00511 regulates downstream targets and signaling pathways, and its differential roles at different time points in BC remain to be further explored. With respect to its clinical applications, LINC00511 may be used to improve prognostic assessment for BC, although further studies are urgently needed.

In conclusion, in-depth elucidation of the functions and mechanisms of action LINC00511 will not only expand our understanding of the molecular networks underlying tumor occurrence and development, but will also provide new insights for early diagnosis, molecular subtyping and clinical treatment of BC. The study of lncRNAs and in particular, LINC00511 and its regulatory networks, has the potential to deliver novel breakthroughs in the precise and personalize diagnosis and treatment of all cancers.

Author contributions

LZ: Data curation, Visualization, Writing – original draft, Writing – review & editing. SB: Conceptualization, Writing – original draft, Writing – review & editing. YL: Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. SS: Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Influence of H19 polymorphisms on breast cancer: risk assessment and prognostic implications via LincRNA H19/miR-675 and downstream pathways

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Introduction: Breast cancer, as the most prevalent malignancy among women globally, continues to exhibit rising incidence rates, particularly in China. The disease predominantly affects women aged 40 to 60 and is influenced by both genetic and environmental factors. This study focuses on the role of H19 gene polymorphisms, investigating their impact on breast cancer susceptibility, clinical outcomes, and response to treatment.

Methods: We engaged 581 breast cancer patients and 558 healthy controls, using TaqMan assays and DNA sequencing to determine genotypes at specific loci (rs11042167, rs2071095, rs2251375). We employed *in situ* hybridization and immunohistochemistry to measure the expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in formalin-fixed, paraffin-embedded samples. Statistical analyses included chi-squared tests, logistic regression, and Kaplan-Meier survival curves to evaluate associations between genetic variations, gene expression, and clinical outcomes.

Results: Genotypes AG at rs11042167, GT at rs2071095, and AC at rs2251375 were significantly associated with increased risk of breast cancer. Notably, the AA genotype at rs11042167 and TT genotype at rs2071095 were linked to favorable prognosis. High expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in cancer tissues correlated with advanced disease stages and poorer survival rates. Spearman correlation analysis revealed significant positive correlations between the expression of LincRNA H19 and miR-675 and specific genotypes, highlighting their potential regulatory roles in tumor progression.

Discussion: The study underscores the critical roles of LincRNA H19 and miR-675 as prognostic biomarkers in breast cancer, with their overexpression associated with disease progression and adverse outcomes. The H19/LincRNA H19/miR-675/MRP3-HOXA1-MMP16 axis offers promising targets for new therapeutic strategies, reflecting the complex interplay between genetic markers and breast cancer pathology.

Conclusion: The findings confirm that certain H19 SNPs are associated with heightened breast cancer risk and that the expression profiles of related genetic markers can significantly influence prognosis and treatment response. These biomarkers hold potential as targets for personalized therapy and early detection strategies in breast cancer, underscoring the importance of genetic research in understanding and managing this disease.

KEYWORDS

H19, Mir675, breast cancer, genetic polymorphism, susceptibility, prognosis

Introduction

Breast cancer is a common malignant tumor in women, with 2.26 million women diagnosed and 685,000 deaths in 2020 (1). Statistics from multiple cities in China also show that breast cancer is the most common malignant tumor in Chinese women, and its incidence is increasing yearly (2). Epidemiological studies have shown that breast cancer mostly occurs in women aged between 40 and 60, especially before and after menopause (3). The occurrence and development of breast cancer is thought to be the result of the combined effects of environmental and genetic factors (4). Improving breast cancer screening in high-risk groups, reducing the incidence of breast cancer and improving the cure rate are challenges that need to be addressed (5). The risk of breast cancer has been confirmed to be closely associated with mutations and expression changes in genes such as BRCA1, BRCA2, P53, epidermal growth factor receptor (EGFR), and Ki-67 (6–8).

Human genes have two copies (alleles), one inherited from the father and the other from the mother. For most genes, the two copies are equally expressed. However, at some loci, expression is determined by parental origin, and only the copy from one parent is expressed. This parent-of-origin-dependent differential expression phenomenon is called genomic imprinting. Genes that are differentially expressed under imprinting regulation are called imprinted genes. The human H19 gene is located in the imprinted gene cluster of chromosome 11p15.5. The paternal allele is highly methylated and not expressed, whereas the maternal allele is unmethylated and expressed (9). The gene contains five exons and four introns, and the transcription products of the H19 gene are a 2.3 kb RNA molecule and a small molecule, miR-675, both of which lack open reading frames and do not encode proteins. Hence, they are called noncoding RNAs (10, 11). Noncoding RNAs generally play regulatory roles in genes at the transcriptional, posttranscriptional, and epigenetic levels (12). Although H19 RNA molecules can be detected in both the cytoplasm and the nucleus, they exist mainly in the cytoplasm, where they function as regulatory RNAs or riboregulators (13). New studies have found that H19, an imprinted gene associated with the occurrence and development of various tumors, may also be one of the risk factors for breast cancer (14). Genetic studies have shown that the promoter and intron regions of the H19 gene are involved in gene expression regulation. Key site mutations in these regions can directly affect the expression of targeted genes, thereby participating in the occurrence, development, and drug treatment efficacy of diseases (15).

This suggests that finding and verifying the H19 polymorphisms that are closely related to breast cancer is of great significance. Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation, accounting for more than 90% of known polymorphisms; approximately one SNP is present in every 1,000 base pairs (16). The study of SNPs helps to explain differences in individual susceptibility to diseases (17), differences in drug tolerance (18), and differences in reactions to environmental factors (19). The study of polymorphisms in the H19 gene may help to uncover the relationship between breast cancer and H19 (9). Additionally, LincRNA H19 has been confirmed to endogenously generate miR-675 (20). Studies indicate that the aberrant expression of LncRNA H19 and its derivative miR-675 is closely associated with the occurrence, development, and clinical prognosis of various tumors (21–24). In tumor tissues, variations in LincRNA H19 expression correlate positively with changes in miR-675 expression. This interaction orchestrates the regulation of downstream target genes, leading to the emergence of resistance, invasion, and metastasis in cancer cells (25–27).

This study explored the role of H19 polymorphisms in breast cancer, assessing their impact on risk, clinical outcomes, treatment responses, and prognosis via SNP analysis. We posited that increased levels of LincRNA H19 and miR-675 in breast cancer biopsies could serve as effective prognostic biomarkers. Using *in situ* hybridization and immunohistochemistry, we measured the expression of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in formalin-fixed, paraffin-embedded samples. Our findings indicate that LincRNA H19 and miR-675 are reliable indicators of patient outcomes. The expression profiles suggest that the H19/LincRNA H19/miR-675/MRP3-HOXA1-MMP16 axis contributes to the initiation and progression of breast cancer, highlighting potential targets for therapeutic strategies (refer to Figure 1).

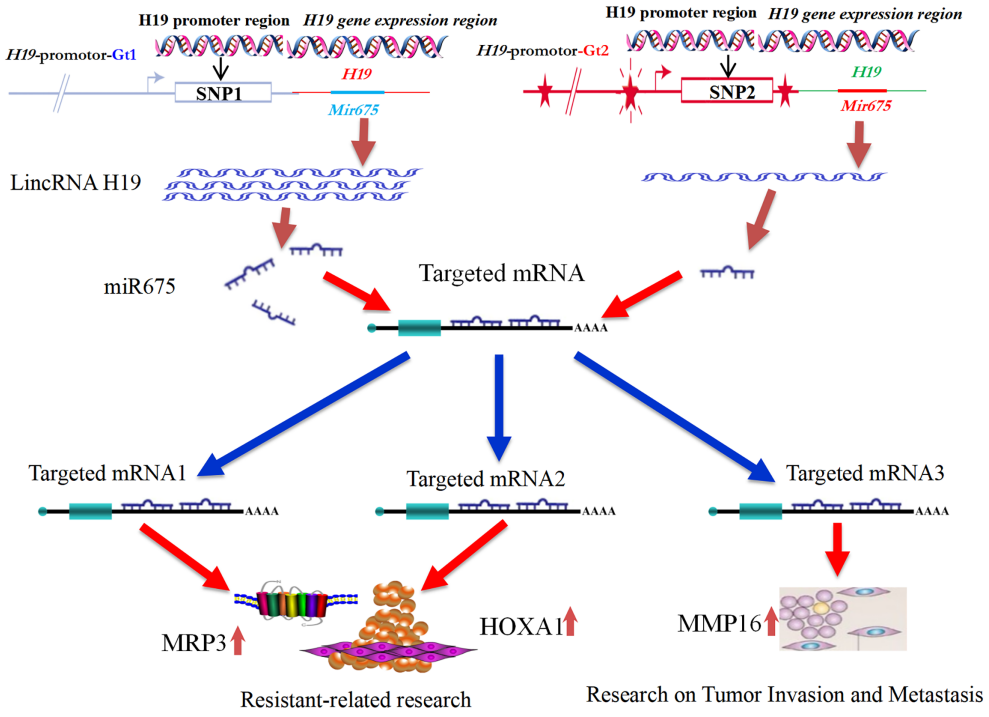

FIGURE 1
Diagram of the LincRNA H19/miR-675 and MRP3-HOXA1-MMP16 Pathways Influencing the Pathogenesis and Progression of Breast Cancer.

Materials and methods

Study subjects and specimen collection

This study involved 581 breast cancer patients and 558 healthy controls, all Chinese women. The Ethics Committee of Shengjing Hospital at China Medical University approved the study protocol (IRB2018PS132J). Written informed consent was waived as data were extracted from clinical records. Patients were recruited from the Oncology and Breast Surgery Departments at The First Hospital of China Medical University and the Breast Surgery Department at Shengjing Hospital, between September 2003 and July 2013. All participants were newly diagnosed with tumors verified by histopathology, had not undergone any anticancer treatments prior to joining the study, and had comprehensive medical records ensuring the reliability of clinical data.

The healthy control group comprised individuals from routine health examinations at the Health Examination Center of The First Hospital of China Medical University between September 2003 and July 2013. This group included unrelated, healthy female volunteers without tumors or genetic diseases. Criteria for inclusion were: absence of any tumors (benign or malignant), age comparable to the breast cancer patient group, and no significant medical conditions as determined by examination results.

After obtaining informed consent, 2 mL of fasting peripheral venous blood was drawn from both breast cancer patients and healthy volunteers into vacuum blood collection tubes with EDTA-$2Na^+$ anticoagulants. The samples were then stored at −80°C for subsequent analysis. Breast cancer tissue microarray chips were prepared from previously diagnosed patients, using adjacent non-cancerous tissue as controls.

Experimental methods

SNP site selection for the TaqMan® assay

We employed dual-labeled TaqMan probes, one with FAM and the other with HEX, to distinguish between genotypes at specific SNP loci using a multiplex PCR assay. Selection of SNPs was guided by criteria from the NCBI dbSNP database, specifically choosing SNPs with a heterozygosity rate above 8% in the Chinese population and those validated for TaqMan assays, as per the Life Technologies website. Consequently, we targeted rs11042167, rs2071095, and rs2251375 SNPs in the H19 gene promoter for this study.

TaqMan fluorescent probe SNP genotyping method

We extracted genomic DNA from peripheral blood leukocytes using the KI method and performed SNP genotyping via the TaqMan assay. Specific TaqMan probes used for allelic discrimination included: rs11042167 labeled with HEX for the A allele and FAM for the G allele; rs2071095 labeled with HEX for the G allele and FAM for the T allele; and rs2251375 labeled with HEX for the C allele and FAM for the G allele. Detailed probe sequences are provided in the Supplementary Material. PCR was conducted on an Applied Biosystems MX3000p Real-time PCR system, starting with a 30-second denaturation at 95°C, followed by 45 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C for 20

seconds, with fluorescence measurements taken post each cycle. Components of the PCR mix are detailed in Table 1. The PCR products were sequenced by Sangon Biotech, Shanghai.

In Situ hybridization experiments and interpretation standards

The *in situ* hybridization kit, purchased from Exiqon, Denmark, involves a standard procedure starting with routine deparaffinization of the tissue microarrays. The samples are then digested at 37°C with 3% freshly diluted citric acid pepsin for 15 minutes, followed by a gradient ethanol dehydration. Probes for LincRNA H19 or miR675, labeled with digoxigenin, are pre-hybridized at 55°C for 2 hours. After removing the excess liquid without washing, the probes are hybridized overnight. Post-hybridization washing is done with SSC, followed by blocking solution at 37°C for 30 minutes without subsequent washing. Biotinylated mouse anti-digoxigenin antibodies are incubated at room temperature for 120 minutes. This is followed by the addition of SABC for 30 minutes at room temperature and three PBST washes for 5 minutes each. Biotinylated peroxidase is added and incubated at room temperature for 30 minutes, followed by four PBS washes for 5 minutes each. The samples are then stained with DAB, counterstained with hematoxylin, thoroughly washed with water, dehydrated with alcohol, cleared in xylene, and finally coverslipped. Omission of the probe serves as a negative control. The results are determined by the brown-yellow granular positive signals of H19 and miR675 located in the cytoplasm and nuclei of breast ductal epithelium or cancer cells. The signal intensity for H19 and miR675 is scored from 0 to 4, with 0–1 indicating no signal, >1–2 a weak signal, >2–3 a moderate signal, and >3–4 a strong signal. Under high magnification (×400), 100 cells per field are counted in 10 random fields per slide to calculate the percentage of positive cells. The positivity rates are classified as 0, <25%, 25%–50%, 51%–75%, >75%, corresponding to scores of 0, 1, 2, 3, and 4, respectively. A product score of 0–1 indicates negative expression (–), >1–6 indicates positive expression (+), and >6 indicates strong positive expression (++). The tissue microarray results are independently reviewed and scored by three pathologists.

In this study, we employed *in situ* hybridization (ISH) to analyze the expression of miR-675 in breast cancer tissues and adjacent non-tumor tissues. We used probes targeting the overall sequence of miR-675, without specifically distinguishing between its mature isoforms, miR-675-5p and miR-675-3p. This decision was based on our primary objective to assess the role of miR-675 as a whole in the pathological processes, rather than to differentiate the specific contributions of its isoforms. The ISH procedure included the use of DIG-labeled LNA probes for hybridization. The probe design was specific to the mature sequence of miR-675 but did not differentiate between the 5p and 3p isoforms. Following hybridization, anti-DIG antibodies were used for signal detection. The final results were assessed under a microscope based on the intensity and distribution of the staining.

TABLE 1 Taqman real-time PCR reaction system.

reagent	1×Enzyme digestion reaction system/μL
Taqman Assay	1.25
2×Premix Ex Taq MIX	2.5
DNA	1.0
deionized water	0.5
total volume	5.0

Immunohistochemistry testing and interpretation standards

After formaldehyde fixation and paraffin embedding, tissue sections are cut thickly. The procedure includes routine deparaffinization and rehydration, followed by peroxidase blocking with hydrogen peroxide in deionized water. Antigen retrieval is performed using EDTA at high temperatures, then cooled naturally and rinsed with PBS. Each section is treated with 100μl of primary antibodies: mouse anti-human MRP3 monoclonal, rabbit anti-human HOXA1 polyclonal, and mouse anti-human MMP-16 monoclonal, all diluted 1:100. After applying secondary antibodies, the sections are developed with DAB, counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted with neutral resin. PBS replaces the primary antibody in the negative control. Positive results are semi-quantitatively determined using brown-colored HMGA2 particles as a positive marker. Staining intensity is graded on a scale from 0 (no color) to 3 (dark brown). Under high magnification (×400), 100 cells per field are counted across 10 random fields per slide to calculate the percentage of positive cells. Cell positivity rates are scored from 0 (no positive cells) to 4 (>75% positive cells). Total scores range from 0–1 points indicating negative expression (–), >1–6 points indicating positive expression (+), and >6 points indicating strong positive expression (++). The tissue microarray results are independently reviewed and scored by three pathologists.

Establishment of a database and statistical analysis

We developed databases using Excel to manage genotype frequencies for rs11042167, rs2071095, and rs2251375, and expression data for LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 for both case and control groups. Additional data on demographic and clinical characteristics including age, menopausal status, family history, pathology type, clinical stage, ER/PR/HER2 status, P53/BRCA1/BRCA2 status, lymph node metastasis, survival status, and treatment modalities were also cataloged.

Data were formatted for compatibility with various analytical software. We employed chi-squared tests to assess differences in sex and age between groups and to compare genotype frequencies and histological data. Hardy-Weinberg equilibrium for control group genotype frequencies was tested using Arlequin software, considering distributions in equilibrium at $P > 0.05$. Relative risks were quantified using a nonconditioned logistic regression model to generate odds ratios (ORs) and 95% confidence intervals (CIs).

Kaplan-Meier survival analysis was used to estimate survival outcomes of breast cancer patients. All statistical analyses were performed using SPSS 29.0 and Python 3.10.

Results

Basic characteristics of the study subjects

This study involved 581 female breast cancer patients and 558 healthy female controls, matched by age and sex. The average age of the breast cancer group was 50 years (range: 22–85; median: 50), while the controls averaged 49 years (range: 23–70; median: 49). An independent sample t-test confirmed no significant age differences between the groups ($P > 0.05$). Detailed demographic data, risk factors, and clinical variables for both groups are presented in Table 2.

TABLE 2 Clinical characteristics of breast cancer patients (n=581) and healthy female subjects (n=558).

Characteristic	Cases		Controls	
	n	%	n	%
Total number	581	100	558	100
Mean age (range, yrs)	50 (22~85)		49 (23–70)	
Age, yrs				
<50	308	53.0	297	53.2
≥50	273	42.0	261	46.8
Menopausal status				
Premenopausal	315	54.2	305	54.7
Postmenopausal	266	45.8	253	45.3
Tumor size				
≤ 2.0 cm	251	43.2		
2.1-5.0 cm	236	40.6		
>5.0 cm	94	16.2		
Histology				
IDC	499	85.9		
ILC	22	3.8		
Others[b]	60	10.3		
Clinical stages				
I or II	472	81.2		
III or IV	109	18.8		
Lymph node metastasis				
Node-negative	326	56.1		
Node-positive	255	43.9		
ER status				
Negative	247	42.5		
Positive	326	56.1		
Undetermined	8	1.4		
PR status				
Negative	233	40.2		
Positive	339	58.3		
Undetermined	9	1.5		
HER2 status				
Negative	301	51.8		
Positive	265	45.6		
Undetermined	15	2.6		
TNBC status				
Yes	88	15.1		
No	485	83.5		

(Continued)

TaqMan genotyping

Genotypes were identified using TaqMan real-time PCR based on changes in fluorescence intensity. The homozygous genotypes AA/GG/CC (rs11042167/rs2071095/rs2251375) displayed a significant increase in fluorescence in the FAM channel with no corresponding increase in the VIC (HEX) channel. Conversely, the homozygous genotypes GG/TT/GG (rs11042167/rs2071095/rs2251375) exhibited a significant increase in the VIC (HEX) channel without an increase in the FAM channel. The heterozygous genotypes AG/GT/CG (rs11042167/rs2071095/rs2251375) showed increased fluorescence in both FAM and VIC (HEX) channels. These findings are illustrated in Figure 2.

Sequencing results for the amplification products of the H19 rs11042167, rs2071095, and rs2251375 SNPs

Figure 3 presents the sequencing results for specific genotypes at three SNPs: GG/GA at rs11042167, GG/GT at rs2071095, and AA/AC at rs2251375. The sequences, shown from the complementary strand in a 5′ to 3′ direction, illustrate that the homozygous genotypes produce a single peak at the nucleotide position, while heterozygous genotypes result in a double peak.

Correlation between the H19 rs11042167, rs2071095, and rs2251375 SNPs and susceptibility to breast cancer

Table 3 illustrates the genotype distributions for SNPs rs11042167, rs2071095, and rs2251375 in both breast cancer cases and controls. For rs11042167, the case group comprised 40 (6.9%) AA, 310 (53.3%) AG, and 231 (39.8%) GG genotypes, while the control group showed 54 (9.7%) AA, 209 (37.4%) AG, and 295 (52.9%) GG genotypes. Chi-squared testing confirmed Hardy-

TABLE 2 Continued

Characteristic	Cases		Controls	
	n	%	n	%
TNBC status				
Undetermined	8	1.4		
p53 status				
Negative	254	43.7		
Positive	306	52.7		
Undetermined	21	3.6		
BRCA1 status				
Negative	111	19.1		
Positive	439	75.6		
Undetermined	31	5.3		
BRCA2 status				
Negative	219	37.7		
Positive	317	54.6		
Undetermined	45	7.7		
Therapeutic regimens				
Anthracycline-based chemotherapy[c]	355	61.1		
Paclitaxel-based chemotherapy[d]	47	8.1		
Anthracycline+paclitaxel-based chemotherapy[e]	47	8.1		
Other chemotherapies or treatments[f]	132	22.7		

IDC, Ivasive ductal carcinoma; ILC, Invasive lobular carcinoma; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; p53,Tumor suppressor protein 53; BRCA1, Breast carcinoma type 1 susceptibility protein; BRCA2, Breast carcinoma type 2 susceptibility protein.

[b]Others contains: invasive cribriform carcinoma, medullary carcinoma, and invasive papillary carcinoma.

[c]Chemotherapy of anthracycline-based contains: CE (Cyclophosphamide, Epirubicin); CA (Cyclophosphamide, Adriamycin); CEF (Cyclophosphamide, Epirubicin and 5-Fluorouracil); CAF(Cyclophosphamide, Adriamycin and 5-Fluorouracil).

[d]Chemotherapy of paclitaxel-based contains: Docetaxel or Paclitaxel and/or Capecitabine (T or TC regimens).

[e]Chemotherapy of anthracycline plus paclitaxel-based contains: CE or CA plus T regimens; CEF or CAF plus T regimens.

[f]Other chemotherapies or treatments included: CMF (Cyclophosphamide, Methotrexate and 5-Fluorouracil); C or CP, P or GP (Cyclophosphamide or Cyclophosphamide and Platinum, Platinum or Gemcitabine and cisplatin); NP, NX, or X (Navelbine and Platinum; Navelbine and Xeloda, Xeloda alone); 5-FU (5-Fluorouracil); Surgery only; radiation therapy and biological treatments or Chinese traditional treatment.

Weinberg equilibrium in the control group for rs11042167 (P > 0.05).For the rs2071095 SNP, the case group exhibited a genotype distribution of 231 cases (39.8%) with homozygous GG, 310 cases (53.3%) with heterozygous GT, and 40 cases (6.9%) with homozygous TT. In comparison, the control group showed 286 cases (54.6%) with homozygous GG, 227 cases (43.3%) with heterozygous GT, and 63 cases (12.0%) with homozygous TT. Chi-squared analysis confirmed that the genotype distribution in the control group was in Hardy-Weinberg equilibrium (P > 0.05). For SNP rs2251375, the case group presented 92 cases (15.8%) with homozygous AA, 366 cases (63.0%) with heterozygous AC, and 123 cases (21.2%) with homozygous CC. In the control group, there were 114 cases (20.4%) with homozygous AA, 271 cases (48.6%) with heterozygous AC, and 173 cases (31.0%) with homozygous CC. Chi-squared testing confirmed that the genotype distribution for rs2251375 in the control group was in Hardy-Weinberg equilibrium (P > 0.05).

Table 3 also presents the age-adjusted odds ratios (ORs) for genotypes at SNPs rs11042167, rs2071095, and rs2251375. For rs11042167, the OR for the AG genotype is 2.002 (95% CI: 1.283–3.124, P = 0.002), indicating a higher disease risk compared to the AA genotype. The GG genotype does not show an increased risk (OR = 1.057, 95% CI: 0.678–1.647, P = 0.806). At rs2071095, individuals with the GT genotype have an increased risk (OR = 1.584, 95% CI: 1.239–2.025, P < 0.001) compared to those with the GG genotype, while the TT genotype shows no increase (OR = 0.737, 95% CI: 0.477–1.136, P = 0.166). For rs2251375, the AC genotype is associated with a higher risk (OR = 1.674, 95% CI: 1.220–2.296, P = 0.001) relative to the AA genotype. Conversely, the CC genotype does not confer an increased risk (OR = 0.881, 95% CI: 0.615–1.262, P = 0.489).

Correlation analysis between rs11042167, rs2071095, and rs2251375 SNPs and clinical pathological parameters of breast cancer patients

Pearson's χ^2 test and corrective measures (age, menopausal status, and family history) were utilized, alongside unconditional logistic regression analysis, to explore the relationship between the genotypes of the H19 SNPs and clinical pathological parameters of breast cancer patients. The data are presented in Table 4. Statistical analysis did not demonstrate any noteworthy frequency distribution differences between different genotypes of the three H19 SNPs and patient age, menopausal status, tumor size, family history, clinical staging, lymph node metastasis, ER status, PR status, HER2 status, triple-negative breast cancer status, P53 status, and BRCA1 and BRCA2 status (P > 0.05).

Correlation analysis between the rs11042167, rs2071095, and rs2251375 SNPs and prognosis of breast cancer patients

Kaplan–Meier survival analysis was utilized to predict the prognosis of breast cancer patients (n = 581). Progression-Free Survival (PFS) is typically defined as the time from the initial diagnosis of breast cancer to the first occurrence of disease progression or death from any cause. Patients with the AA genotype at rs11042167 SNP had a median PFS of 168 months (95% CI: 146.845–189.283), significantly longer than those with the AG or GG genotypes, who had a median PFS of 122 months (log-rank P = 0.029). At rs2071095, patients with the TT genotype showed a median PFS of 138 months (95% CI: 120.123–155.877), significantly longer than those with the GT or GG genotypes, who had a median PFS of 113 months (log-rank P = 0.036). However, at

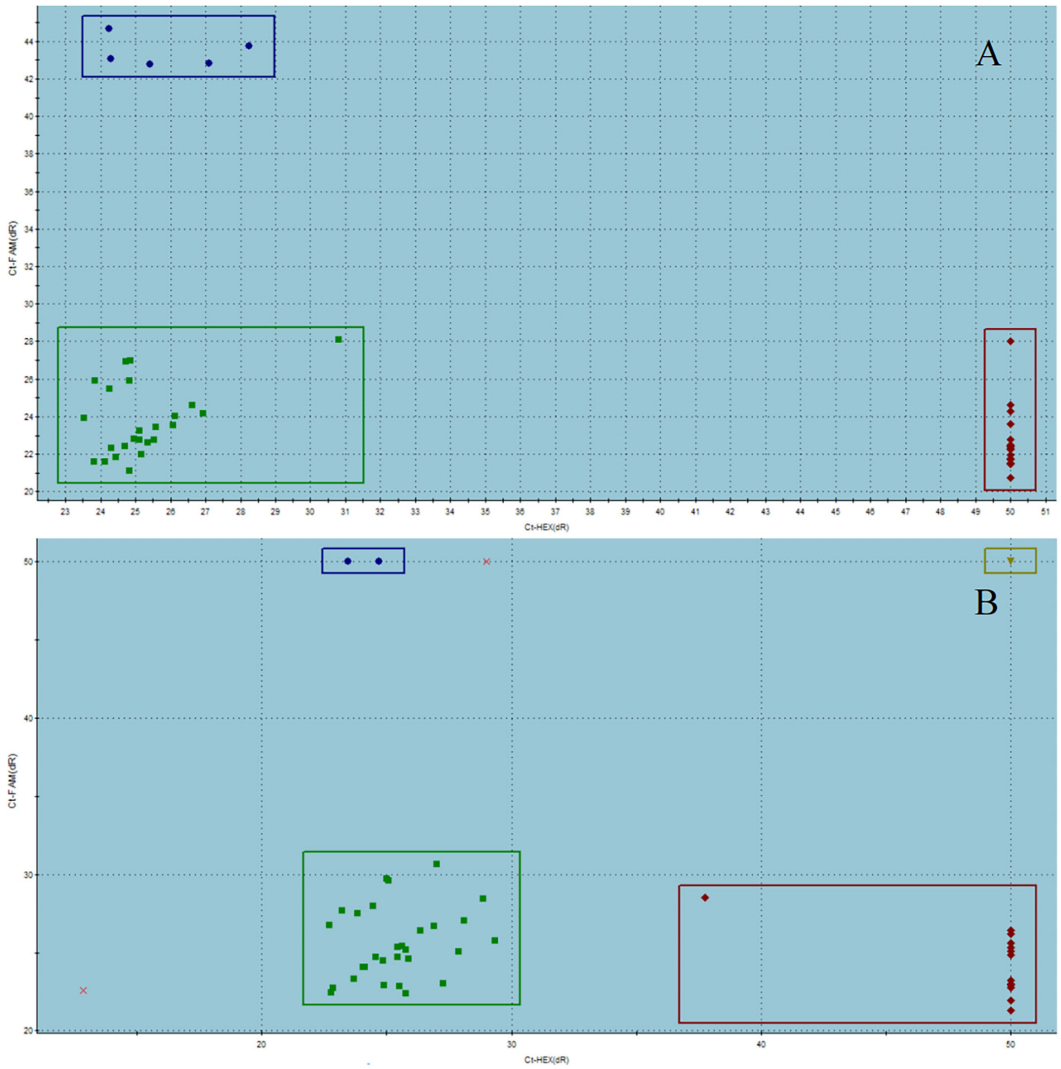

FIGURE 2
Fluorescence signal characteristics of genotypes in the control group **(A)** and the case group **(B)**.

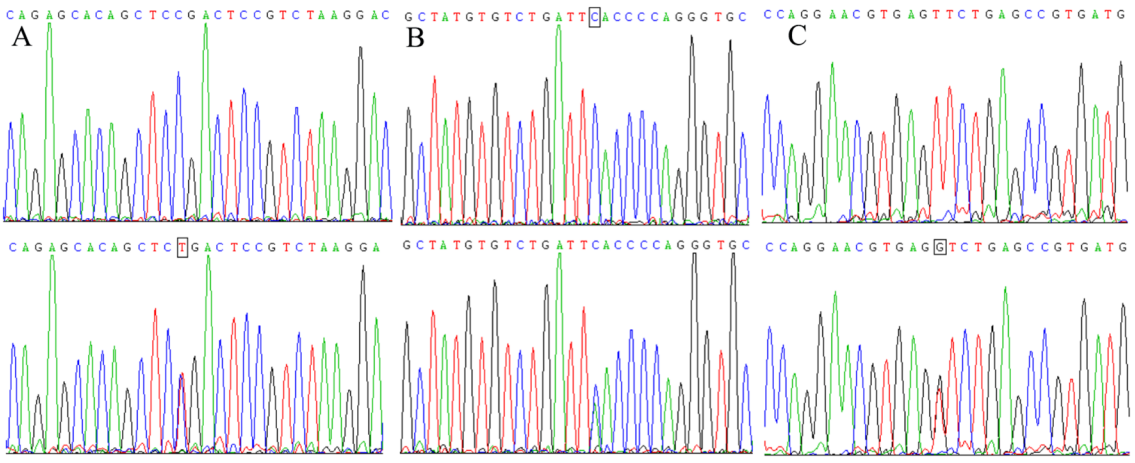

FIGURE 3
Genotyping sequencing results for rs11042167 **(A)**, rs2071095 **(B)**, and rs2251375 **(C)** SNPs.

TABLE 3 Frequency distribution of *H19* genotypes and their associations with the risk of developing breast cancer.

Genotypes	Controls † n (%)	Cases n (%)	*P* ‡	Adjusted OR (95% CI) §
All patients	558 (100)	581 (100)		
*rs*11042167 (A→G)				
AA	54 (9.7)	40 (6.9)		1 (Reference)
AG	209 (37.4)	310 (53.3)	**0.002**	2.002 (1.283-3.124)
GG	295 (52.9)	231 (39.8)	0.806	1.057 (0.678-1.647)
AG/GG	469 (90.3)	541 (93.1)	0.087	1.449 (0.946-2.220)
A	320 (28.7)	390 (33.6)		1 (Reference)
G	728 (65.3)	772 (66.4)	**0.008**	**0.785 (0.657-0.939)**
rs2071095(G→T)				
GG	286 (54.6)	231 (39.8)		1 (Reference)
GT	227 (43.3)	310 (53.3)	**<0.001**	**1.584 (1.239-2.025)**
TT	63 (12.0)	40 (6.9)	0.166	0.737 (0.477-1.136)
GT/TT	290 (55.3)	350 (60.2)	**0.005**	**1.400 (1.107-1.771)**
G	799 (76.2)	772 (66.4)		1 (Reference)
T	353 (33.7)	390 (33.6)	0.325	1.092 (0.916-1.301)
rs2251375(A→C)				
AA	114 (20.4)	92 (15.8)		1 (Reference)
AC	271 (48.6)	366 (63.0)	**0.001**	**1.674 (1.220-2.296)**
CC	173 (31.0)	123 (21.2)	0.489	0.881 (0.615-1.262)
AC/CC	444 (79.6)	489 (84.2)	**0.044**	**1.365 (1.008-1.848)**
A	499 (44.7)	550 (47.3)		1 (Reference)
C	619 (55.3)	612 (52.7)	0.209	0.900 (0.763-1.061)

H19, H19 gene; H19,, Odds ratio; CI, Confidence interval.
The significance levels are P< 0.05 for all the bold values.
†The observed genotype frequency among individuals in the control group was in agreement with Hardy-Weinberg equilibrium.
‡P values were calculated from 2-sided chi-square tests for either genotype distribution or allele frequency.
§Adjusted OR and 95% CI values were calculated by unconditional logistic regression adjusted for age, menopausal state.

rs2251375, there was no significant difference in median PFS between patients with the AA genotype (116 months) and those with the AC or CC genotypes (129 months) (log-rank P = 0.795). Please refer to Figures 4A, E.

Overall Survival (OS) is generally defined as the time from randomization in a study until death from any cause. We analyzed the correlation between the rs11042167, rs2071095, and rs2251375 SNPs and OS in breast cancer patients. We found that in patients with the AA genotype at rs11042167, the median time to death was 189 months (95% CI = 172.833–205.168), while in patients with the AG or GG genotype, the time to death was 136 months, and there was a statistically significant difference between the two groups (log-rank P = 0.038). Similarly, we analyzed rs2071095 and found that there was a statistically significant difference in median time to death between patients with the TT genotype 132 months(95% CI = 114.357–149.643) and those with the GT or GG genotype 108 months (log-rank; P = 0.029).We also analyzed rs2251375 and found that there was no statistically significant difference in median time to death between patients with the AA genotype (132 months) and those with the AC or CC genotype (129 months) (log-rank P = 0.780). Please refer to Figures 4B, D.

Recurrence-Free Survival (RFS) is typically defined as the time from treatment initiation or randomization until disease recurrence or death from any cause. We analyzed the correlation between three SNPs, rs11042167, rs2071095, and rs2251375, and RFS in breast cancer patients. We found that in patients with the AA genotype at rs11042167, the median time to recurrence was 176 months (95% CI = 160.245–191.755), whereas in those with the AG or GG genotypes, the median time to recurrence was 130 months, showing a statistically significant difference (log-rank P = 0.046). Similarly, we analyzed rs2071095 and found that in patients with the TT genotype, the median time to recurrence was 125 months (95% CI = 109.624–140.376), compared to 98 months in those with the GT or GG genotypes, which also demonstrated a statistically significant difference (log-rank P = 0.033). We also analyzed rs2251375 and found that in patients with the AA genotype, the median time to recurrence was 128 months, compared to 124 months in those with the AC or CC genotypes, with no statistically significant difference between the groups (log-rank P = 0.879). For the results that exhibited statistical significance, please refer to Figures 4C, F.

We further investigated the relationship between the rs11042167, rs2071095, and rs2251375 SNPs and the survival outcomes, specifically Progression-Free Survival (PFS), Overall Survival (OS), and Recurrence-Free Survival (RFS), across various pathological parameters in breast cancer patients. Our stratified analysis, which accounted for factors such as age, menopausal status, tumor size, family history, clinical stage, lymph node metastasis, estrogen receptor (ER) status, progesterone receptor (PR) status, HER2 status, triple-negative breast cancer prevalence, P53 status, and BRCA1/2 status, revealed no significant correlation between these SNPs and PFS in breast cancer patients (P > 0.05).

The correlation analysis between SNPs rs11042167, rs2071095, and rs2251375 and the expression of LincRNA H19 and miR-675 in breast cancer tissues

Through Spearman correlation heatmap analysis, it was found that the AG genotype of rs11042167 [A/G] is positively correlated with the expression of LincRNA H19 and miR-675 (P < 0.001). For rs2071095 [G/T], the GT genotype shows a positive correlation with the expression of LincRNA H19 and miR-675 (P < 0.001), while the other genotypes are negatively correlated. For rs2251375 [A/C], Spearman correlation heatmap analysis reveals no significant correlation with the expression levels of LincRNA H19 and miR-675. Additionally, the genotypes rs11042167 [A/G]_AA and rs2071095 [G/T]_TT exhibit a complete positive correlation (P < 0.001), as do the genotypes rs2071095 [G/T]_GT and rs11042167 [A/G]_AG (P < 0.001). See Figure 5.

TABLE 4 Correlations of H19 pomorphisms with clinicopathological parameters in patients with breast cancer.

Characteristic	*rs*11042167 (A→G)				rs2071095 (G→T)				rs2251375 (A→C)			
	AA n (%)	AG/GG n (%)	*P*†,‡	Adjusted OR (95%CI) §	GG n (%)	GT/TT n (%)	*P*†,‡	Adjusted OR (95%CI) §	AA n (%)	AC/CC n (%)	*P*†,‡	Adjusted OR (95%CI) §
Age, yrs												
<50	21 (6.8)	287 (93.2)	0.946	1 (Reference)	133 (43.2)	175 (56.8)	0.073	1 (Reference)	48 (15.6)	260 (84.4)	0.861	1 (Reference)
≥50	19 (7.0)	254 (93.0)	0.784	0.86 (0.313-2.402)	98 (35.9)	175 (64.1)	0.093	1.581 (0.926-2,700)	44 (16.1)	229 (83.9)	0.540	0.802 (0.396-1.624)
Menopausal status												
Premenopausal	22 (7.0)	293 (93.0)	0.918	1 (Reference)	131 (41.6)	184 (58.4)	0.327	1 (Reference)	51 (16.1)	264 (83.8)	0.798	1 (Reference)
Postmenopausal	18 (6.8)	248 (93.2)	0.797	1.143 (0.412-4.169)	100 (37.6)	166 (62.4)	0.508	0.835 (0.489-1.426)	41 (15.4)	225 (84.6)	0.534	1.251 (0.618-2.531)
Tumor size (cm)												
≤ 2.0	19 (7.6)	232 (92.4)	0.761	1 (Reference)	97 (38.6)	154 (61.4)	0.695	1 (Reference)	39 (15.5)	212 (84.5)	0.577	1 (Reference)
2.1-5.0	16 (6.8)	220 (93.2)	0.446	1.485 (0.537-4.103)	93 (39.4)	143 (60.6)	0.385	0.807 (0.497-1.309)	41 (17.4)	195 (82.6)	0.514	1.261 (0.629-2.531)
>5.0	5 (5.3)	89 (94.7)	0.603	1.316 (0.467-3.708)	41 (43.6)	53 (56.4)	0.476	1.133 (0.515-1.363)	12 (12.8)	82 (87.2)	0.293	1.451 (0.725-2.905)
Histology												
IDC	35 (7.0)	464 (93.0)	0.774	1 (Reference)	201 (40.3)	289 (59.7)	0.457	1 (Reference)	77 (15.4)	422 (84.6)	0.324	1 (Reference)
ILC	2 (9.1)	20 (90.9)	0.533	1.471 (0.437-4.946)	6 (27.3)	16 (72.7)	0.961	0.986 (0.569-1.709)	6 (27.3)	16 (72.7)	0.908	1.045 (0.494-2.213)
Others	3 (5.0)	57 (95.0)	0.474	1.980 (0.306-12.823)	24 (40.0)	36 (60.0)	0.231	0.516 (0.175-1.521)	9 (15.0)	51 (85.0)	0.202	2.157 (0.662-7.029)
Clinical stages												
I or II	35 (7.4)	437 (92.6)	0.293	1 (Reference)	191 (40.5)	281 (59.5)	0.469	1 (Reference)	70 (14.8)	402 (85.2)	0.168	1 (Reference)
III or IV	5 (4.6)	104 (95.6)	0.257	1.748 (0.666-4.590)	40 (36.7)	69 (63.3)	0.582	1.130(0.732-1.745)	22 (20.8)	87 (79.8)	0.183	0.695 (0.407-1.187)
Lymph node metastasis status												
Node-negative	28 (8.6)	298 (91.4)	0.067	1 (Reference)	125 (38.3)	201 (61.7)	0.431	1 (Reference)	60 (18.4)	266 (81.6)	0.055	1 (Reference)
Node-positive	12 (4.7)	243 (95.3)	0.074	1.895 (0.941-3.815)	106 (41.6)	149 (58.4)	0.406	0.867 (0.619-1.214)	32 (12.5)	223 (87.5)	0.060	1.564 (0.981-2.493)
ER status												
Negative	17 (6.9)	230 (93.1)	0.936	1 (Reference)	101 (40.9)	146 (59.1)	0.488	1 (Reference)	38 (15.4)	209 (84.6)	0.777	1 (Reference)
Positive	23 (7.1)	303 (92.9)	0.903	0.960 (0.499-1.847)	124 (38.0)	202 (62.0)	0.417	1.152 (0.819-1.620)	53 (16.3)	273 (83.7)	0.731	0.923 (0.585-1.457)

(Continued)

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TABLE 4 Continued

Characteristic	*rs*11042167 (A→G)				rs2071095 (G→T)				rs2251375 (A→C)			
	AA n (%)	AG/GG n (%)	*P*[†,‡]	Adjusted OR (95%CI) [§]	GG n (%)	GT/TT n (%)	*P*[†,‡]	Adjusted OR (95%CI) [§]	AA n (%)	AC/CC n (%)	*P*[†,‡]	Adjusted OR (95%CI) [§]
PR status												
Negative	14 (6.0)	219 (94.0)	0.444	1 (Reference)	92 (39.5)	141 (60.5)	0.952	1 (Reference)	33 (14.2)	200 (85.8)	0.344	1 (Reference)
Positive	26 (7.7)	313 (92.3)	0.431	0.762 (0.388-1.498)	133 (39.2)	206 (60.8)	0.798	1.046 (0.741-1.476)	58 (17.1)	281 (82.9)	0.342	0.798 (0.500-1.272)
HER2 status												
Negative	19 (6.3)	282 (93.7)	0.563	1 (Reference)	121 (40.2)	180 (59.8)	0.678	1 (Reference)	51 (16.9)	250 (83.1)	0.470	1 (Reference)
Positive	20 (7.5)	245 (92.5)	0.564	0.825 (0.429-1.586)	102 (38.5)	163 (61.5)	0.779	1.050 (0.747-1.477)	39 (14.7)	226 (85.3)	0.491	1.174 (0.744-1.852)
ER/PR/HER2 status												
TNBC	5 (5.7)	83 (94.3)	0.603	1 (Reference)	34 (38.6)	54 (61.4)	0.895	1 (Reference)	8 (9.1)	80 (90.9)	0.058	1 (Reference)
Non-TNBC	35 (7.2)	450 (92.7)	0.579	1.316 (0.499-3.470)	191 (39.4)	294 (60.6)	0.989	1.003 (0.627-1.604)	83 (17.1)	402 (82.9)	0.058	2.095 (0.974-4.507)
p53 status												
Negative	15 (5.9)	239 (94.1)	0.300	1 (Reference)	97 (38.2)	157 (61.8)	0.522	1 (Reference)	43 (16.9)	211 (83.1)	0.541	1 (Reference)
Positive	25 (8.2)	281 (91.8)	0.314	0.711 (0.366-1.380)	125 (40.8)	181 (59.2)	0.519	0.849 (0.635-1.258)	46 (15.0)	260 (85.0)	0.535	1.154 (0.733-1.818)
BRCA1 status												
Negative	8 (7.2)	103 (92.8)	0.975	1 (Reference)	40 (36.0)	71 (64.0)	0.340	1 (Reference)	18 (16.2)	93 (83.8)	0.945	1 (Reference)
Positive	31 (7.1)	408 (92.9)	0.923	1.041 (0.464-2.335)	180 (41.0)	259 (59.0)	0.316	0.801 (0.519-1.236)	70 (15.9)	369 (84.1)	0.950	1.018 (0.578-1.794)
BRCA2 status												
Negative	10 (4.6)	209 (95.4)	0.058	1 (Reference)	83 (37.9)	136 (62.1)	0.347	1 (Reference)	40 (18.3)	179 (81.7)	0.170	1 (Reference)
Positive	28 (8.8)	289 (91.2)	0.066	0.497 (0.236-1.047)	133 (42.0)	184 (58.0)	0.304	0.830 (0.582-1.183)	44 (13.9)	273 (86.1)	0.167	1.392 (0.871-2.225)

H19, H19 gene; OR, Odds ratio; CI, Confidence interval; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; TNBC, Triple-Negative Breast Cancer; p53, Tumor suppressor protein 53; BRCA1, Breast carcinoma type 1 susceptibility protein; BRCA2, Breast carcinoma type 2 susceptibility protein.

[†]P values were calculated from 2-sided chi-square tests or Fisher's Exact Test.

[‡]P values were calculated by unconditional logistic regression adjusted for age, menopausal status.

[§]OR and 95% CI values were calculated by unconditional logistic regression adjusted for age, menopausal status.

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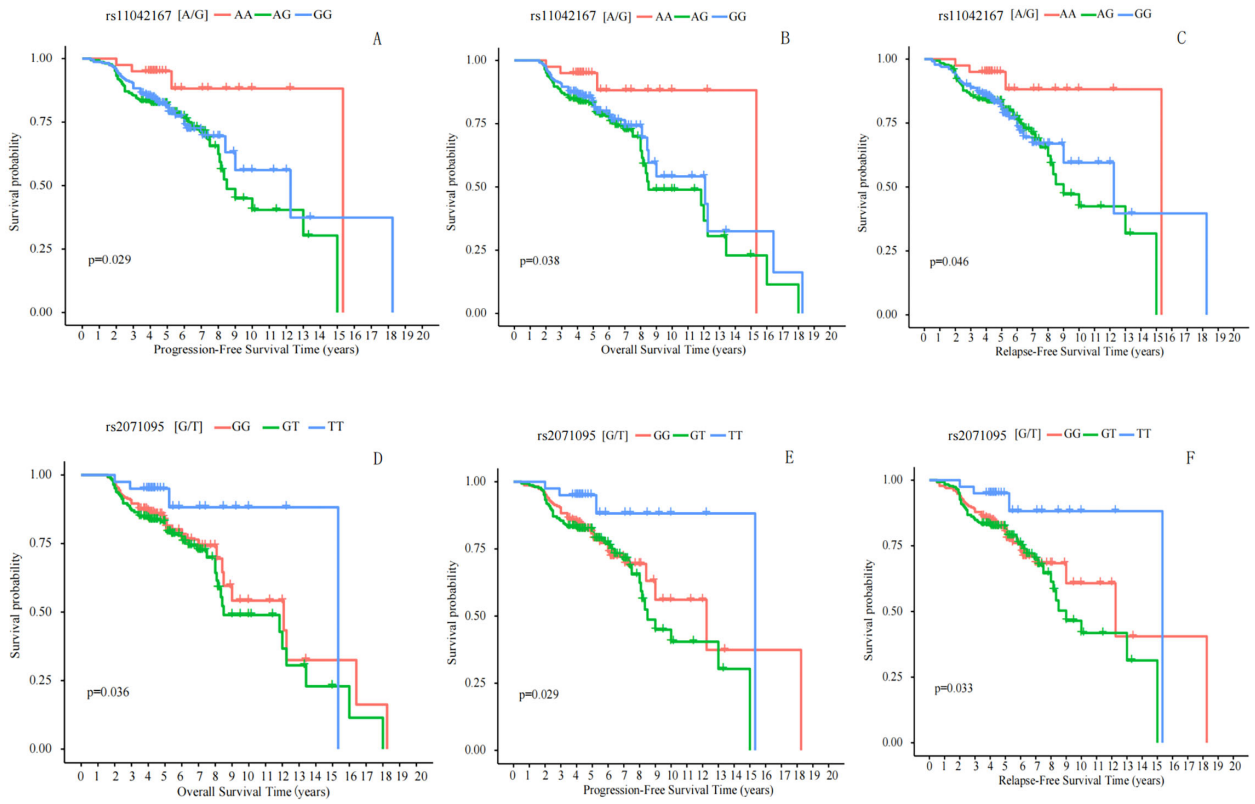

FIGURE 4
Correlation Analysis of rs11042167, rs2071095, and rs2251375 SNPs with Breast Cancer Prognosis. **(A, E)** Progression-Free Survival: Significant differences in PFS were observed for rs11042167 (A) and rs2071095 (E) SNPs, with patients carrying the AA and TT genotypes, respectively, showing longer survival times. (B, D) Overall Survival: Kaplan - Meier curves for rs11042167 **(B)** and rs2071095 **(D)** reveal that the AA and TT genotypes are associated with improved OS compared to other genotypes. **(C, F)** Recurrence-Free Survival: Significant differences in RFS were found for rs11042167 **(C)** and rs2071095 **(F)**, where AA and TT genotypes were linked to longer recurrence-free periods.

Expression profiles and correlation analysis of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in breast cancer tissues

In situ hybridization results revealed significant expression differences of LincRNA H19 in breast cancer tissues and adjacent non-tumor tissues. Out of 581 breast cancer samples, 563 showed high expression of LincRNA H19 and 18 exhibited low expression. In contrast, in the adjacent non-tumor tissues (n = 581), 35 samples displayed high expression and 546 showed low expression, demonstrating statistical significance ($P < 0.01$). Similarly, miR-675 was highly expressed in 552 of the breast cancer tissues and lowly expressed in 29, while in the adjacent non-tumor tissues, high expression was noted in only 16 samples and low expression in 565 samples, also with significant differences ($P < 0.01$).

Immunohistochemistry results were consistent with these findings. High expression of MRP3 was observed in 563 out of 581 breast cancer tissues, with low expression in 18, whereas among the adjacent tissues, only 35 showed high expression compared to 546 with low expression, indicating significant differences ($P < 0.01$). For HOXA1, high expression was noted in 552 cancerous samples and low expression in 29, with the adjacent tissues showing high expression in only 16 samples and low in 565 samples, which was statistically significant ($P < 0.01$). A similar pattern was observed for MMP16, with high expression in 552 breast cancer tissues and low expression in 29, while in the adjacent tissues, high expression was noted in 16 samples and low in 565 samples, indicating significant differences ($P < 0.01$).

Furthermore, Spearman correlation heatmap analysis revealed a strong positive correlation among the expressions of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in breast cancer tissues. The detailed results of the *in situ* hybridization and immunohistochemistry are depicted in Figure 6, with the correlation heatmap shown in Figure 7. (The numerical values on the image that are closer to 1 indicate a stronger correlation, See Table 5 for more details).

The association between the expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 and clinical pathological parameters in breast cancer

The expression of LincRNA H19 is associated with age, clinical stages, histology, ER status, HER2 status, and p53 status ($P < 0.05$), but not with other clinical pathological parameters. miR-675 expression correlates with age, menopausal status, tumor size,

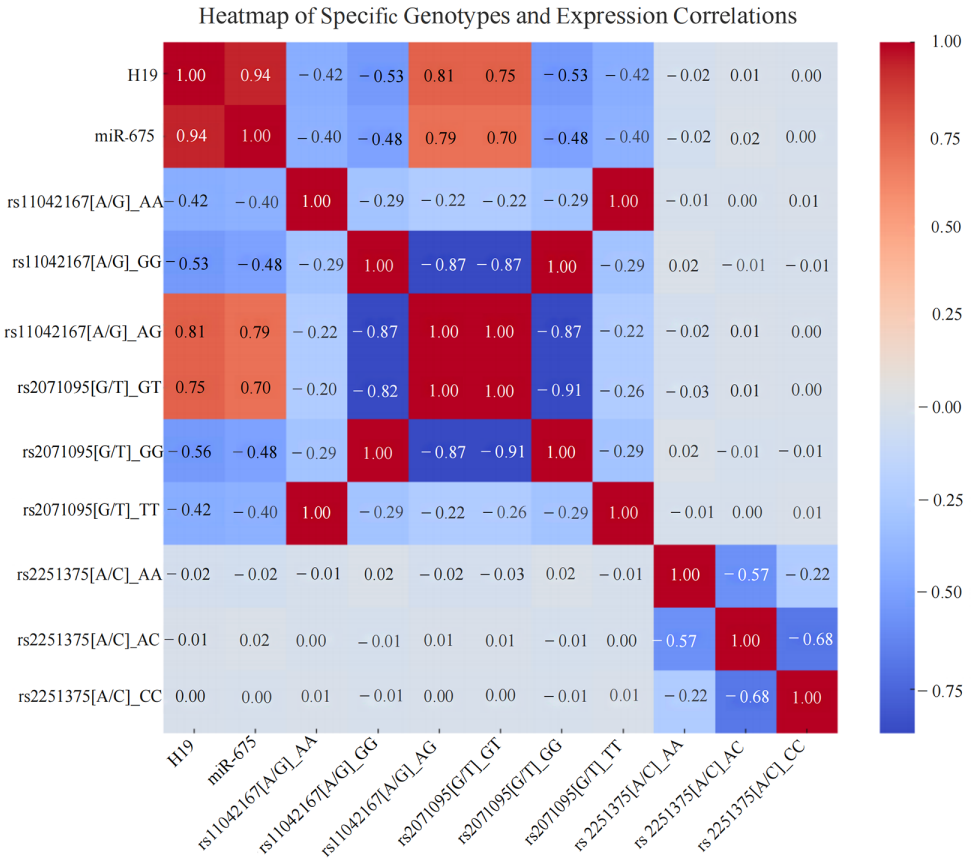

FIGURE 5
Heatmap of the Correlation Between Different Breast Cancer Genotypes and the Expression of LincRNA H19, miR-675 in Breast Cancer Tissues.

histology, clinical stages, ER status, HER2 status, non-TNBC, BRCA1 status, and BRCA2 status (P<0.05), but not with other clinical pathological parameters. MRP3 expression is associated with menopausal status, tumor size, histology, clinical stages, lymph node metastasis status, PR status, HER2 status, p53 status, and BRCA2 status ($P < 0.05$), but not with other clinical pathological parameters. HOXA1 expression correlates with histology, clinical stages, BRCA1 status, and BRCA2 status ($P < 0.05$), but not with other clinical pathological parameters. MMP16 expression is associated with age, tumor size, histology, clinical stages, lymph node metastasis status, PR status, HER2 status, non-TNBC, and BRCA1 status ($P < 0.05$), but not with other clinical pathological parameters. These associations are detailed in Table 6.

The association between the expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 and the prognosis in breast cancer patients

Kaplan-Meier analysis revealed that breast cancer patients with high MRP3 expression have shorter overall survival (OS) and

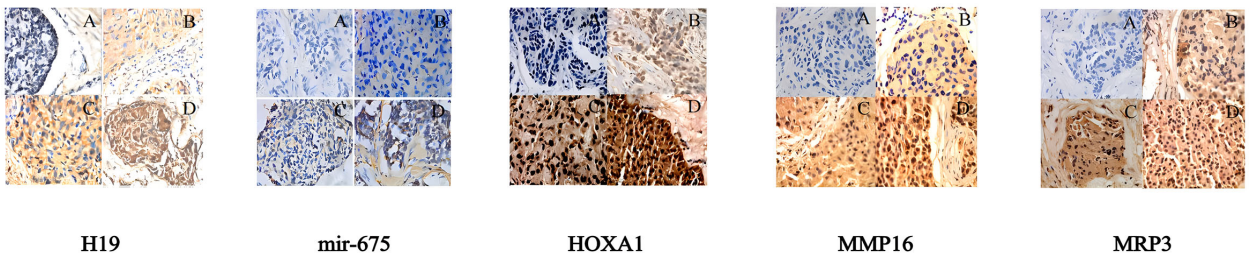

FIGURE 6
Pathological test results for LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 (400x magnification): **(A)** Negative expression in breast cancer tissues; **(B)** Low expression in breast cancer tissues; **(C)** Moderate expression in breast cancer tissues; **(D)** High expression in breast cancer tissues.

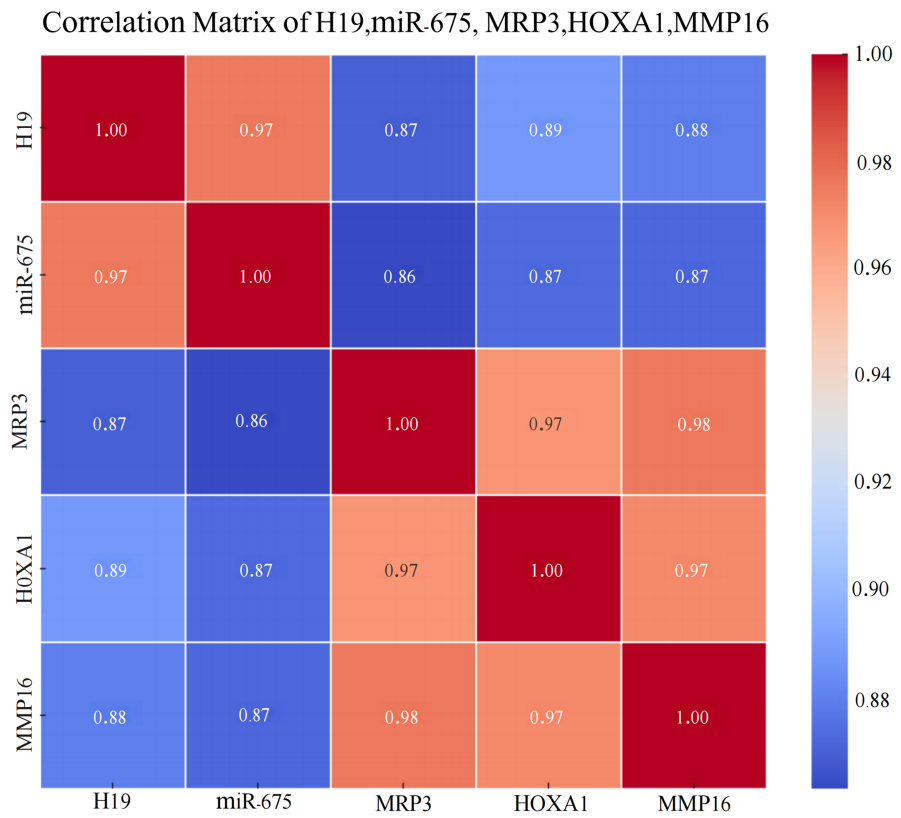

FIGURE 7
Heatmap of the Correlation Among LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in Breast Cancer Tissues.

TABLE 5 Positive expression rates of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 across various tissues.

Biomarkers	Breast cancer tissue n (%)	Adjacent non-cancerous tissue n (%)	$\chi 2$	P†
LincRNA H19			487.9	<0.0001
+	563 (94.1)	35 (5.9)		
–	18 (3.1)	546 (96.9)		
miR-675			672.2	<0.0001
+	552 (97.1)	16 (2.9)		
–	29 (4.8)	565 (95.2)		
MRP3			145.6	<0.0001
+	513 (94.8)	28 (5.2)		
–	68 (10.9)	553 (89.1)		
HOXA1			908.8	<0.0001
+	566 (96.2)	22 (3.8)		
–	15 (2.6)	559 (97.4)		
MMP16			179.7	<0.0001
+	538 (93.5)	37 (6.5)		
–	43 (7.3)	544 (92.7)		

†P values were calculated from 2-sided chi-square tests or Fisher's Exact Test.

TABLE 6 Correlations of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 with clinicopathological parameters in patients with breast cancer.

Characteristic	*LincRNA H19*			miR-675			MRP3			HOXA1			MMP16		
	+	-	*P*†	+	-	*P*†	+	-	*P*†	+	-	*P*†	+	-	*P*†
Age, yrs			**0.0326**			**0.0018**			>0.9999			0.4329			**0.0384**
<50	303 (98.3)	5 (1.7)		301 (97.7)	7 (2.3)		272 (88.3)	36 (11.7)		302 (98.0)	6 (2.0)		292 (94.8)	16 (5.2)	
≥50	260 (95.3)	13 (4.7)		251 (91.9)	22 (8.1)		241 (88.2)	32 (11.8)		264 (96.7)	9 (3.3)		246 (90.1)	27 (9.9)	
Menopausal status			0.8117			**0.0207**			**<0.0001**			0.0632			0.6358
Premenopausal	306 (97.1)	9 (2.9)		299 (97.0)	9 (3.0)		263 (83.4)	52 (16.6)		303 (96.1)	12 (3.9)		290 (92.0)	25 (8.0)	
Postmenopausal	257 (96.6)	9 (3.4)		253 (92.6)	20 (7.4)		289 (94.7)	16 (5.3)		263 (98.8)	3 (1.2)		248 (93.2)	18 (6.8)	
Tumor size (cm)			0.6624			**<0.0001**			**<0.0001**			>0.9999			**<0.0001**
≤ 2.0	245 (97.6)	6 (2.4)		249 (99.2)	2 (0.8)		244 (97.2)	7 (2.8)		247 (98.4)	4 (1.6)		248 (98.8)	3 (1.2)	
2.1-5.0	227 (96.1)	9 (3.9)		230 (97.4)	6 (2.6)		214 (90.6)	22 (9.4)		228 (96.6)	8 (3.4)		221 (93.6)	15 (6.4)	
>5.0	91(96.8)	3(3.2)		73 (77.6)	21 (22.4)		55 (58.5)	39 (41.5)		91 (96.8)	3 (3.2)		69 (73.4)	25 (26.6)	
Histology			**<0.0001**			**<0.0001**			**<0.0001**			**<0.0001**			**<0.0001**
IDC	491 (98.3)	8 (1.7)		490 (98.1)	9 (1.9)		481 (96.3)	18 (3.7)		481 (98.3)	8 (1.7)		471 (94.3)	28 (5.7)	
ILC	15 (68.1)	7 (31.9)		4 (18.1)	18 (81.9)		7 (31.8)	15 (68.2)		16 (72.7)	6 (28.3)		11 (50.0)	11 (50.0)	
Others	57 (95.0)	3 (5.0)		58 (96.6)	2 (3.4)		25 (41.6)	35 (58.4)		59 (98.3)	1 (1.7)		56 (93.3)	4 (6.7)	
Clinical stages			**0.0128**			**0.0008**			**<0.0001**			**<0.0001**			**<0.0001**
I or II	481 (97.8)	11 (2.2)		456 (96.6)	16 (3.4)		445 (94.2)	27 (5.8)		469 (99.3)	3 (0.7)		454 (96.1)	18 (3.9)	
III or IV	82 (92.1)	7 (7.9)		96 (88.0)	13 (12.0)		68 (62.3)	41 (37.7)		97 (88.9)	12 (11.1)		84 (77.0)	25 (33.0)	
Lymph node metastasis status			>0.9999			0.1809			**<0.0001**			0.5993			**<0.0001**
Node-negative	316 (96.9)	10 (3.1)		306 (93.8)	20 (6.2)		308 (94.4)	18 (5.6)		321 (96.9)	10 (3.1)		290 (88.9)	36 (11.1)	
Node-positive	247 (96.8)	8 (3.2)		246 (96.4)	9 (3.6)		205 (80.3)	50 (19.7)		245 (98.0)	5 (2.0)		248 (97.2)	7 (2.8)	
ER status			**0.0146**			**0.0008**			0.5156			0.1965			>0.9999
Negative	234 (94.7)	13 (5.3)		243 (98.3)	4 (1.7)		215 (87.0)	32 (13.0)		238 (96.3)	9 (3.7)		229 (92.7)	18 (7.3)	
Positive	321 (98.4)	5 (1.6)		301 (92.3)	25 (7.7)		290 (88.9)	36 (11.1)		320 (98.1)	6 (1.9)		301 (92.3)	25 (7.7)	
PR status			0.3326			0.1751			**0.0025**			0.6067			**<0.0001**
Negative	228 (97.8)	5 (2.2)		225 (96.5)	8 (3.5)		211 (90.5)	22 (9.5)		228 (97.8)	5 (2.2)		201 (86.2)	32 (13.8)	
Positive	326 (96.1)	13(3.9)		318 (93.8)	21 (6.2)		193 (80.7)	46 (19.3)		329 (97.0)	10 (3.0)		315 (96.6)	11 (3.4)	

(Continued)

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TABLE 6 Continued

Characteristic	*LincRNA H19*			miR-675			MRP3			HOXA1			MMP16		
	+	-	*P*†	+	-	*P*†	+	-	*P*†	+	-	*P*†	+	-	*P*†
HER2 status			**0.0028**			**0.0004**			**0.0197**			>0.9999			**0.0379**
Negative	298 (99.0)	3 (1.0)		295 (98.0)	6 (2.0)		274 (91.0)	27 (9.0)		293 (97.3)	8 (2.7)		285 (94.6)	16 (5.4)	
Positive	250 (94.3)	15(5.7)		242 (91.3)	23 (8.7)		224 (84.5)	41 (15.5)		258 (97.3)	7 (2.7)		238 (89.8)	27 (10.2)	
ER/PR/HER2 status			0.5010			**<0.0001**			0.2489			>0.9999			**<0.0001**
TNBC	84 (95.4)	4 (4.6)		73 (82.9)	15 (17.1)		76 (86.3)	12 (13.7)		86 (97.7)	2 (2.3)		66 (75.0)	22 (25.0)	
Non-TNBC	471 (97.1)	14 (2.9)		471 (97.1)	14 (2.9)		439 (90.5)	46 (9.5)		472 (97.3)	13 (2.7)		464 (95.6)	21 (4.4)	
p53 status			**0.0001**			0.1797			**0.0131**			0.7953			>0.9999
Negative	238 (93.7)	16 (6.3)		237 (93.3)	17 (6.7)		233 (91.7)	21 (8.3)		248 (97.6)	6 (2.4)		235 (92.5)	19 (7.5)	
Positive	304 (99.3)	2 (0.7)		294 (96.0)	12 (4.0)		259 (84.6)	47 (15.4)		297 (97.0)	9 (3.0)		282 (92.1)	24 (7.9)	
BRCA1 status			>0.9999			**<0.0001**			0.1362			**0.0040**			**<0.0001**
Negative	108 (97.2)	3 (2.8)		91 (81.9)	20 (19.1)		98 (88.2)	13 (11.8)		103 (92.7)	8 (7.3)		79 (71.1)	32 (28.9)	
Positive	424 (96.5)	15 (3.5)		430 (97.9)	9 (2.1)		251 (82.0)	55 (18.0)		432 (98.4)	7 (1.6)		306 (96.5)	11 (3.5)	
BRCA2 status			0.4693			**0.0311**			**0.0244**			**0.0322**			0.5180
Negative	210 (95.8)	9 (4.2)		213 (97.2)	6 (2.8)		200 (91.3)	19 (8.7)		217 (99.0)	2 (1.0)		199 (90.8)	20 (9.2)	
Positive	308 (97.1)	9 (2.9)		294 (92.7)	23 (7.3)		268 (84.5)	49 (15.5)		304 (95.8)	13 (4.2)		294 (92.7)	23 (7.3)	

H19, H19 gene; OR, Odds ratio; CI, Confidence interval; IDC, Invasive ductal carcinoma; ILC, Invasive lobular carcinoma; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; TNBC,Triple-Negative Breast Cancer; p53, Tumor suppressor protein 53; BRCA1, Breast carcinoma type 1 susceptibility protein; BRCA2, Breast carcinoma type 2 susceptibility protein.

†Bold values indicate statistically significant results ($P < 0.05$) from 2-sided chi-square tests or Fisher's Exact Test, highlighting important findings in the correlation of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 with clinicopathological parameters in breast cancer patients.

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progression-free survival (PFS) compared to those with low MRP3 expression, a difference that is statistically significant ($P < 0.05$). See Figure 8. Surprisingly, the expression levels of other markers were not associated with the prognosis of breast cancer patients ($P > 0.05$).

Discussion

In recent years, the "common disease, common variant" hypothesis has frequently been mentioned in research on disease diagnosis and treatment (28–31). This hypothesis suggests that susceptibility to certain diseases is caused by variations at genetic loci, particularly variations in gene regulatory or coding regions. Comparisons of SNP sites between a malignant tumor population and a normal control population allow correlations between SNP sites and the risk of tumor incidence to be determined and applied to the study of genetic susceptibility to tumors. With the successful completion of the Human Genome Project, the focus of life science research is rapidly shifting toward understanding the function of each gene, the interactions between multiple genes and their products (32–36), and the interactions between the genome and the environment (37–39). The study of SNPs within the sequence of the human genome has become the focus of a new round of genome science research (40–44).

Polymorphisms in the H19 gene have been suggested to play very important roles in the occurrence and development of tumors (45). Ayesh et al. found that lncRNA H19 upregulates many genes that are closely related to the invasion, migration, and angiogenesis of tumor cells (46). Tanos et al. found that overexpression of H19 is important for the growth of esophageal and colorectal cancer cells (47). Berteaux et al. also found that H19 overexpression in breast cancer tissue promotes cell proliferation (48). A few studies have shown that H19 may act as a tumor suppressor gene and participate in mediating human growth and development (49–51). However, the appearance of genetic variations may cause abnormal expression of H19, which leads to the disruption of gene regulation mechanisms and increases the risk of tumor occurrence (52–54).

In this study, the rs11042167, rs2071095, and rs2251375 SNPs were found to be associated with breast cancer risk in both the case and control groups. At the rs11042167 SNP, individuals with the AG heterozygous genotype had an increased risk of disease compared to those with the AA genotype. At the rs2071095 SNP, individuals with the GT heterozygous genotype had an increased risk of disease when compared to those with the GG genotype. At the rs2251375 SNP, individuals with the AC heterozygous genotype had an increased risk of disease when compared to those with the AA genotype. This suggested that heterozygous genotypes at the rs11042167, rs2071095, and rs2251375 SNPs may be risk factors for breast cancer in the Chinese population. However, these SNPs did not show any correlation to breast cancer with different pathological parameters. In a survival analysis, it was found that patients carrying the AA genotype at the rs11042167 SNP had an increased PFS and OS, which indicated a better prognosis. In contrast, rs2071095 and rs2251375 did not show any correlation with survival in the overall breast cancer patient population. The rs2071095 and rs2251375 SNPs did not show statistically significant differences in PFS and OS in breast cancer patients stratified by pathological status.

In this study, the expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 were consistently higher in 581 breast cancer tissues compared to adjacent non-tumor tissues. This significant upregulation suggests a pivotal role for LincRNA H19 as an oncogene in the progression and prognosis of breast cancer. Additionally, it indicates the potential of LincRNA H19 and miR-675 as diagnostic and prognostic markers and therapeutic targets. The study also found that high expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 were more common in patients with early-stage breast cancer (TNM stages I or II) and those diagnosed with invasive ductal carcinoma (IDC), suggesting their utility as early diagnostic markers to facilitate the early detection and treatment of IDC.

Furthermore, Spearman correlation heatmap analysis showed a positive correlation between the AG genotype of rs11042167 [A/G] and the expression of LincRNA H19 and miR-675 ($P < 0.001$), and between the GT genotype of rs2071095 [G/T] and the expression of LincRNA H19 and miR-675 ($P < 0.001$), with other genotypes showing negative correlations. However, no significant correlation was observed for rs2251375 [A/C], likely due to the complexity of gene regulation, genetic background variability, environmental factors, and the biological heterogeneity of cancer.

Moreover, the study explored the relationship between the expression of these markers and survival in breast cancer tissues,

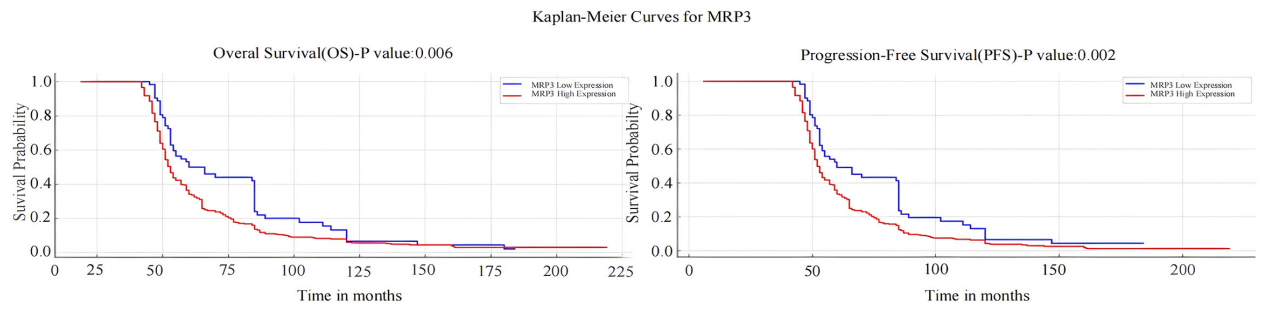

FIGURE 8
Kaplan-Meier Survival Analysis of MRP3 Expression with Overall Survival (OS) and Progression-Free Survival (PFS) in Breast Cancer Patients.

assessing their prognostic value. Results indicated that higher MRP3 expression correlates with shorter overall survival (OS) and disease-free survival (DFS), suggesting poorer prognosis for patients with high expression levels, and underscoring their relevance in prognosis assessment.

Genetic correlation analysis identified complete positive correlations (correlation coefficient of 1) among several genotypes, indicating complete linkage disequilibrium. Specifically, the AA genotype of rs11042167 [A/G] correlated perfectly with the TT genotype of rs2071095 [G/T], and similarly, the GG genotypes of both rs11042167 and rs2071095 showed a correlation coefficient of 1. Additionally, the GT genotype of rs2071095 and the AG genotype of rs11042167 were also perfectly correlated ($P < 0.001$). This strong linkage suggests that these loci may co-regulate H19 expression, potentially influencing transcription factor binding. Given the association of H19 with the development of various cancers, these variants are potentially crucial for studying disease mechanisms and clinical diagnostics, warranting further investigation into their precise functional roles and their impact on disease progression.

In addition to the findings on SNPs and LincRNA H19, this study also investigated the expression of MRP3, HOXA1, and MMP16 in breast cancer tissues. MRP3 expression did not vary significantly with age ($P>0.9999$) but showed significant differences with menopausal status, tumor size, histology, clinical stage, lymph node metastasis, PR status, and BRCA2 status. Specifically, MRP3 expression was lower in premenopausal compared to postmenopausal patients ($P<0.0001$), higher in tumors ≤2.0 cm ($P<0.0001$), and higher in IDC compared to ILC ($P<0.0001$). MRP3 was also more frequently expressed in early-stage (I/II) than late-stage (III/IV) cancers ($P<0.0001$) and in lymph node-negative patients ($P<0.0001$). PR-positive patients showed higher MRP3 expression ($P=0.0025$), and BRCA2-positive patients had higher MRP3 levels ($P=0.0244$).

For HOXA1, no significant age-related differences were observed ($P=0.4329$), but significant differences were found in tumor size, histology, clinical stage, ER status, and BRCA1 status. HOXA1 was more frequently expressed in smaller tumors (≤2.0 cm), particularly in IDC ($P<0.0001$), and in early-stage cancers ($P<0.0001$). Its expression was higher in BRCA1-positive patients ($P=0.0040$), indicating its potential role in breast cancer progression.

MMP16 expression varied with age, tumor size, histology, clinical stage, lymph node metastasis, and PR status. It was higher in younger patients ($P=0.0384$), smaller tumors (≤2.0 cm, $P<0.0001$), IDC ($P<0.0001$), early-stage cancers ($P<0.0001$), lymph node-positive patients ($P<0.0001$), and PR-positive patients ($P<0.0001$), suggesting its involvement in tumor invasiveness and metastasis. These findings reveal the potential roles of MRP3, HOXA1, and MMP16 in breast cancer, highlighting their significance as prognostic markers and potential therapeutic targets.

Conclusions

In summary, the heterozygous AG genotype at rs11042167, the GT genotype at rs2071095, and the AC genotype at rs2251375 were associated with an increased susceptibility to breast cancer. Additionally, the AA genotype at rs11042167 and the TT genotype at rs2071095 were correlated with a favorable prognosis for breast cancer patients. The expression levels of LincRNA H19, miR-675, MRP3, HOXA1, and MMP16 in breast cancer tissues also suggest a strong link with the early onset and types of breast cancer, with MRP3 showing high prognostic value. Furthermore, the AG genotype at rs11042167 and the GT genotype at rs2071095 were positively correlated with the expression of LincRNA H19 and miR-675 ($P < 0.001$), supporting the potential of the H19/LincRNA H19/miR-675/MRP3-HOXA1-MMP16 axis as a new direction for targeted therapy in breast cancer. LincRNA H19 and miR-675 are also promising as new diagnostic markers for breast cancer.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Shengjing Hospital of China Medical University (IRB2018PS132J). The studies were conducted in accordance with the local legislation and institutional requirements. The patients/participants provided written informed consent to participate in this study.

Author contributions

YQ: Methodology, Funding acquisition, Writing – original draft, Writing – review & editing. PZ: Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1436874/full#supplementary-material

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Deciphering the code: the pivotal role of lncRNAs in advancing TNBC therapy

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Triple-negative breast cancer (TNBC) represents the most formidable subtype of breast cancer, characterized by a notable dearth in targeted therapeutic options. Deciphering the underlying molecular mechanisms of TNBC is pivotal for improving patient outcomes. Recent scientific advancements have spotlighted long non-coding RNAs (lncRNAs) as key players in the genesis, progression, and metastasis of cancers. This review delineates the significant influence of lncRNAs on the advancement, detection, and neoadjuvant chemotherapy efficacy in TNBC, detailing the diverse expression patterns of aberrant lncRNAs. The paper explores the specific mechanisms by which lncRNAs regulate gene expression in both the nucleus and cytoplasm, with a special focus on their involvement in TNBC's post-transcriptional landscape. Thorough investigations into TNBC-associated lncRNAs not only forge new avenues for early diagnosis and potent treatment strategies but also highlight these molecules as promising therapeutic targets, heralding an era of personalized and precision medicine in TNBC management.

KEYWORDS

triple-negative breast cancer (TNBC), long non-coding RNAs (lncRNAs), therapeutic targets, gene expression regulation, precision medicine

1 Introduction

Triple-negative breast cancer (TNBC), as a distinct subtype of breast cancer, accounts for approximately 15% of all breast cancer cases (1). Its unique biological characteristics have made it a focal point of research in the field of breast cancer. TNBC is particularly notable because it lacks the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2), rendering traditional hormone therapies and HER2-targeted therapies ineffective (2). TNBC exhibits aggressive biological behavior, characterized by rapid proliferation, high histological grade, and strong metastatic potential (3). This malignancy often leads to late-stage diagnosis, presenting significant treatment challenges and reducing patient survival expectations (4). Although some progress has been made in the treatment of TNBC in recent years, overall, the therapeutic options remain

relatively limited. With a five-year survival rate of only 77%, TNBC is one of the leading threats to women's health (5). To improve the prognosis of patients with TNBC, researchers continue to explore new treatment strategies and biomarkers.

Leveraging data from The Cancer Genome Atlas (TCGA), recent studies have revealed the molecular heterogeneity of TNBC through epigenetic analysis, identifying six molecular subtypes with unique biological characteristics and clinical prognoses (6). Among these, the basal-like BL1 subtype is characterized by high expression of Ki-67, NRAS, Myc, ATR, and BRCA genes; the BL2 subtype mainly exhibits high expression of MET, TP53, EGFR, and EPHA2 genes; the immunomodulatory subtype involves overexpression of genes related to immune cell signaling; the mesenchymal and mesenchymal stem-like subtypes are closely associated with chemoresistance; and the luminal androgen receptor (LAR) subtype is associated with better overall survival (OS) rates. The discovery of these subtypes provides a theoretical basis for the precision treatment of TNBC.

To enhance the survival rates of patients with TNBC, it is necessary to further identify biomarkers that can predict the risk of metastasis, treatment response, or even aid in the development of new therapies. In this field, research on long non-coding RNAs (lncRNAs) has emerged, showing that abnormal expression of lncRNAs is closely related to the development of TNBC (7–12). lncRNAs play important regulatory roles in eukaryotic cells, and their dysfunction is key in tumor formation. Research not only focuses on mutations or epigenetic modifications at the level of gene expression regulation but has also discovered that lncRNAs finely regulate post-transcriptionally, affecting mRNA stability and translation efficiency, thus regulating tumor cell growth, proliferation, and metastasis. The lncRNA expression profiles of tumors have become an important tool for distinguishing different types of cancer, providing new insights for the precise diagnosis and treatment of TNBC (Table 1).

2 The biological functions of lncRNAs

LncRNAs are a unique class of RNA molecules exceeding 200 nucleotides in length, lacking the capacity to encode proteins (13). They exhibit distinctive expression patterns in specific stages of tissue differentiation or certain types of cancer, making them key molecules in the study of cellular biology and disease mechanisms.

LncRNAs reside in the nucleus or cytoplasm (14), overlapping with coding and non-coding RNA transcripts. Based on their genomic proximity to adjacent genes, lncRNAs are categorized into five main types (15): sense lncRNAs, overlapping with one or more exons of a protein-coding gene on the same strand; antisense lncRNAs, overlapping with one or more exons of a protein-coding gene on the opposite strand; bidirectional lncRNAs, initiating expression less than 1000 base pairs away from a neighboring coding transcript on the opposite strand; intronic lncRNAs, originating from an intron of another transcript; and intergenic lncRNAs, existing independently within the genomic interval between two genes.

In the realm of pathology, particularly within the scope of cancer diagnostics, the exploration of lncRNAs holds paramount significance. These molecular entities are not mere harbingers of prognostic insights but also pivotal in steering therapeutic decisions. As sentinels of cellular dynamics and architects of gene regulatory frameworks, lncRNAs orchestrate gene expression through a spectrum of modalities, encompassing chromatin reconfiguration, transcriptional governance, and post-transcriptional modulation (16).

Empirical evidence underscores the integral role of lncRNAs in modulating a myriad of biological functions, including cellular proliferation, programmed cell death, cell cycle regulation, tissue invasion, metastatic spread, cellular differentiation, chromatin architecture, and intracellular transport mechanisms (17, 18). Notably, the unique expression patterns of lncRNAs in specific

TABLE 1 Important lncRNAs associated with triple-negative breast cancer.

LncRNAs	Expression	Biological function	Potential Targets	Reference
LINC00096	Up	Promote cell proliferation and invasion	miR-383-5p/RBM3	(19)
LUCAT1	Up	Enhances cellular proliferation, advances cell cycle progression, and promotes metastasis	miR-5702	(20)
LINC01096	Up	Promote cell proliferation, migration, and invasion	miR-3130-3p	(21)
CCAT1	Up	Promote cell proliferation, migration, and invasion	miR-218/ZFX	(22)
HOTAIR	Up	Promote cell proliferation, migration, and invasion;activating the Wnt/β-catenin signaling pathway	miR-34a	(23–25)
MALAT1	Up	Promote cell proliferation, cell cycle arrest, and invasion	miR-129-5p; miR-1/Slug; miR-448/KDM5B	(26–28, 60)
DANCR	Up	Promote cell proliferation, migration, and invasion	miR-216a-5p; RXRA; CD44, ABCG2	(32–34)
H19	Up	Bolster tumor cell proliferation and survival	Akt	(41–44)
GAS5	Down	Inhibit cell proliferation and invasion; Promote cell apoptosis	miR-378a-5p, miR-196a-5p	(47–50)
PVT1	Up	Promote cell proliferation and migration;aerobic glycolysis	miR-145-5p; miR-497	(52–55)

types of cancer highlight their potential as diagnostic markers. For instance, the upregulation of certain lncRNAs in breast cancer has been linked to tumor aggressiveness and poor prognosis, making them valuable for early detection and risk stratification.

Furthermore, the ability of lncRNAs to regulate gene expression at multiple levels positions them as promising therapeutic targets. Targeting specific lncRNAs could disrupt cancer-promoting pathways, thereby offering new avenues for cancer treatment. For example, inhibiting the expression of lncRNAs that promote angiogenesis in glioblastoma could potentially slow tumor growth and improve patient outcomes.

Consequently, profound investigations into lncRNAs transcend the mere elucidation of disease pathogenesis. They unveil novel diagnostic markers and therapeutic targets, heralding innovative trajectories in the management and treatment of malignancies. The discovery of lncRNAs as potential biomarkers and therapeutic targets could revolutionize personalized medicine, enabling more precise and effective treatment strategies tailored to individual patient needs.

3 Roles of lncRNAs in TNBC

3.1 Regulating the growth of breast cancer cells

In the intricate landscape of TNBC, lncRNAs are emerging as pivotal elements. LINC00096 is known to markedly enhance TNBC cell proliferation, likely through its interplay with miR-383-5p and the modulation of RBM3, an RNA-binding protein (19). While the direct effects of LINC00096 on the cell cycle are yet to be fully elucidated, its influence on cell proliferation intimates a potential indirect role in cell cycle dynamics.

LUCAT1 (lung cancer associated transcript 1) propels the division and growth of TNBC cells by orchestrating gene expression and cell cycle processes via miR-5702 (20). Its elevated expression levels enable tumor cells to circumvent programmed cell death, thereby fostering tumor expansion and contributing to invasion and metastasis. Thus, LUCAT1 stands as a critical factor in TNBC progression, offering itself as a promising therapeutic target and a significant prognostic marker.

The distinct lncRNA LINC01096 is implicated in the proliferation, migration, and invasion of TNBC cells, while concurrently impeding apoptosis, potentially through the regulatory pathways involving miR-3130-3p (21). The expression of LINC01096 bears a strong correlation with TNBC patients' resistance to certain treatments, hinting at a possible association with chemotherapy resistance. Consequently, LINC01096 serves as a valuable biomarker for diagnosis, prognosis, and treatment responsiveness, as well as a prospective avenue for novel therapeutic approaches.

CCAT1 (Colon Cancer Associated Transcript 1) assumes a notably critical role in TNBC, with its expression intricately linked to the migratory and invasive traits of the cells (22). By modulating the epithelial-mesenchymal transition (EMT) via the miR-218/ZFX signaling axis, CCAT1 may amplify tumor virulence and the propensity for metastasis (22). The expression levels of CCAT1 are also tied to treatment resistance in TNBC patients, positioning it as a potential biomarker for disease diagnosis, prognostic evaluation, or treatment response, and as a target for emerging therapeutic strategies.

3.2 Regulating the invasion and metastasis of breast cancer cells

In the intricate tapestry of TNBC, specific lncRNAs such as HOTAIR and MALAT1 are emerging as critical players. HOTAIR is intricately linked to TNBC's invasiveness and metastatic potential, orchestrating chromatin remodeling and activating the Wnt/β-catenin signaling pathway (23, 24). Its elevated expression correlates with a grim prognosis, marked by advanced tumor grade, lymph node metastasis, and distant spread. Furthermore, HOTAIR modulates the epithelial-mesenchymal transition (EMT) through the miRNA sponge mechanism, a pivotal process for tumor invasion and metastasis (25). It is also implicated in therapeutic resistance in TNBC, potentially inducing chemotherapy drug resistance by altering drug metabolism and cell apoptosis pathways. Thus, HOTAIR serves as a potential biomarker for TNBC diagnosis, prognosis assessment, and treatment response monitoring, as well as a strategic target for novel therapeutic interventions.

Similarly, MALAT1's increased expression augments tumor cell migration and invasion, engaging key signaling pathways like PI3K/AKT/mTOR and Wnt/β-catenin (26, 27). MALAT1 also facilitates TNBC progression by modulating intercellular interactions within the tumor microenvironment. Its strong association with TNBC's invasiveness and metastatic potential positions MALAT1 as a potential prognostic biomarker. At the molecular level, MALAT1 functions as a 'sponge' for miRNAs, such as miR-129-5p, influencing miRNA-mediated gene regulation (28).

Other lncRNAs, including MIR503HG, sONE, and ZEB2-AS1, also significantly contribute to TNBC's pathogenesis. MIR503HG effectively curtails TNBC cell migration and invasion via the miR-103/OLFM4 axis (29). sONE hampers these processes by affecting signaling pathways triggered by eNOS and NO production (30). ZEB2-AS1 is suspected of promoting EMT through the PI3K/Akt/GSK3β/ZEB2 signaling cascade (31). The expression levels of these lncRNAs are intimately connected to TNBC's invasiveness and metastatic capacity, highlighting their potential as biomarkers for diagnosis and treatment response.

The lncRNA DANCR is associated with heightened proliferation and invasion of tumor cells, potentially through its influence on specific signaling pathways or miRNA interactions, such as with miR-216a-5p (32). DANCR also engages the PI3K/Akt pathway by binding to the retinoid X receptor alpha (RXRA), a key player in TNBC cell proliferation and invasion (33). Moreover, DANCR fosters TNBC stem cell-like traits by regulating tumor stem cell marker expression, including CD44 and ABCG2 (34). Its upregulation is also linked to chemotherapy resistance, possibly due

to its involvement in cell cycle and apoptosis regulation. Therefore, DANCR is not merely a potential biomarker for TNBC diagnosis and treatment monitoring but also a vital target for innovative therapeutic strategies.

3.3 Regulating the tumor microenvironment

Within the tumor microenvironment, select lncRNAs assume a critical role, engaging in dynamic interactions with cellular components, the extracellular matrix, immune cells, and vasculature. These interactions are instrumental in modulating tumor growth, tissue invasion, metastatic spread, and resistance to therapies. Gaining a comprehensive understanding of the operational mechanisms of these lncRNAs is essential for the innovation of novel therapeutic approaches. Their study not only illuminates the complex interplay within the tumor milieu but also provides a foundation for targeted treatment strategies that could revolutionize cancer care.

NEAT1 (nuclear paraspeckle assembly transcript 1), a lncRNA distinguished by its nuclear enrichment, has emerged as a focal point of interest due to its multifaceted influence within the tumor microenvironment. Research indicates that NEAT1 modulates tumor evolution through a plethora of mechanisms (35–40): it orchestrates immunomodulation by impacting immune cells like T cells, dendritic cells, and NK cells, thereby fine-tuning anti-tumor immune responses; it fosters interactions between tumor and stromal cells, intensifying invasion and metastasis; it contributes to angiogenesis by guiding the proliferation and migration of endothelial cells, facilitating the development of the tumor's vascular network; it remodels the extracellular matrix (ECM), reshaping the tumor microenvironment's physical attributes; it partakes in metabolic reprogramming, influencing the metabolic circuits of both tumor and stromal cells; it governs pivotal signaling pathways, including PI3K/Akt and MAPK/ERK, essential for tumor cell proliferation, survival, and invasion; it modulates cancer stem cell traits, impacting tumor recurrence and drug resistance; it is implicated in chemotherapy resistance, potentially through the modulation of gene expression linked to drug metabolism and apoptosis; it advances EMT, bolstering the invasive and metastatic capacities of tumor cells; and it acts as a competitive endogenous RNA (ceRNA), regulating miRNA activity, thus altering gene expression within the tumor microenvironment. NEAT1's actions weave a complex regulatory web, pivotal to the tumor's ecological dynamics.

H19, an lncRNA aberrantly expressed in various malignancies, notably TNBC, orchestrates a complex array of mechanisms within the tumor microenvironment. It modulates pivotal signaling pathways, including PI3K/Akt, to bolster tumor cell proliferation and survival (41). H19 is intimately linked to cancer stem cell traits, potentially influencing self-renewal and differentiation through gene expression regulation (42). It also shapes the behavior of immune cells, affecting their infiltration, activation, and function, thereby contributing to tumor immune evasion (43). In the realm of angiogenesis, H19 facilitates the proliferation and migration of endothelial cells, supplying the tumor with essential oxygen and nutrients (44). It governs genes implicated in cell invasion and metastasis, such as those involved in the EMT process, enhancing the invasive and metastatic potential of tumor cells. The expression level of H19 is associated with tumor cell resistance to chemotherapy, possibly by modulating genes related to drug metabolism and apoptosis (41). Furthermore, H19 partakes in the remodeling of the ECM, altering the tumor microenvironment's physical landscape to favor tumor cell invasion and metastasis (45). As a ceRNA, it regulates miRNA activity, thereby influencing gene expression within the tumor milieu. H19 may also interact with other non-coding RNAs, proteins, or metabolites, maintaining the homeostasis of the tumor microenvironment (18, 46). Consequently, the expression level of H19 serves as a biomarker for cancer diagnosis, prognosis evaluation, and monitoring treatment response in the tumor microenvironment.

GAS5 (growth arrest specific 5), a lncRNA, has garnered significant attention in oncology, especially in the context of TNBC. Within the tumor microenvironment, GAS5 exerts multifaceted roles: it curtails tumor cell proliferation and fosters apoptosis, mediated by interactions with miRNAs or modulation of genes governing the cell cycle and apoptosis. GAS5 engages with specific miRNAs, such as miR-196a-5p and miR-378a-5p, to influence the expression of genes related to the cell cycle, thereby impacting tumor cell proliferation (47, 48). It plays a role in enhancing chemosensitivity, with its expression levels correlating with tumor cell responsiveness to chemotherapy agents, potentially heightening their susceptibility by regulating genes linked to drug resistance (49). In the sphere of immunoregulation, GAS5 influences immune-related genes or miRNAs, thereby affecting immune cells like T cells and NK cells, which is crucial for modulating tumor immune evasion. It also impedes tumor angiogenesis by altering the expression of angiogenesis-related genes, thus inhibiting the formation of the tumor vascular network (50). GAS5 regulates genes associated with tumor metastasis, including those involved in the EMT process, influencing the invasiveness and metastatic capabilities of tumor cells (51). Functioning as a ceRNA, it partakes in the regulation of miRNA activity, affecting their regulatory impact on target genes via miRNA interactions (50). The expression level of GAS5 serves as a biomarker for cancer diagnosis and prognosis, mirroring the biological attributes of the tumor and patient clinical outcomes (50). Additionally, GAS5 is implicated in reversing chemotherapy resistance by modulating specific signaling pathways or miRNAs and plays a role in regulating metabolic processes within the tumor microenvironment, affecting the energy metabolism and biosynthesis of tumor cells (50).

3.4 Regulating aerobic glycolysis in tumor cells

Certain lncRNAs exert a profound influence on the energy metabolism of TNBC cells. They achieve this objective through a variety of mechanisms, particularly by directly or indirectly regulating key enzymes and transport proteins within the glycolytic pathway. An

in-depth exploration of these lncRNAs' roles in energy metabolism not only sheds light on the adaptive metabolic strategies of TNBC cells but also holds the potential to unlock novel avenues for therapeutic intervention in cancer treatment.

PVT1, a long non-coding RNA frequently upregulated in diverse cancers, is intimately linked with the proliferative, invasive, and metastatic propensities of tumor cells. Within the context of aerobic glycolysis, known as the Warburg effect, PVT1 assumes a critical role, potentially orchestrating the expression and functionality of essential enzymes in the glycolytic pathway to fulfill the heightened energy demands of rapidly dividing tumor cells (52). Moreover, PVT1 modulates the metabolic landscape of tumor cells by adjusting key metabolites in the tumor microenvironment, such as lactate and pyruvate, which in turn serve as signaling entities influencing tumor cell conduct (53). Functioning as a ceRNA, PVT1 engages with miRNAs, alleviating the repression of crucial glycolytic genes, thus fostering aerobic glycolysis (54). It may further amplify this process via interactions with the HIF-1α (Hypoxia-Inducible Factor-1α) signaling pathway, a pivotal regulator of glycolytic gene expression (55). Most significantly, PVT1's impact on metabolic reprogramming entails a reconfiguration of cellular metabolic routes, bolstering tumor growth and viability (54). This metabolic flexibility enables tumor cells to endure metabolic stress, with PVT1 playing an indispensable role in this adaptive survival strategy.

UCA1 (Urothelial Carcinoma Associated 1), a lncRNA aberrantly expressed in a spectrum of cancers, is intricately associated with the proliferative, invasive, metastatic, and metabolic attributes of tumor cells. UCA1 assumes a multifaceted role in glycolysis, potentially steering the expression or functionality of critical enzymes, thereby directly influencing tumor cell glycolysis (56). Concurrently, it modifies metabolites within the tumor microenvironment, impacting both glycolysis and the metabolic state of tumor cells (57). As a ceRNA, UCA1 engages with miRNAs, alleviating the repression of essential glycolytic genes, thus further propelling glycolysis (58). It may also indirectly foster glycolysis via interactions with the HIF-1α signaling pathway (59). By dictating the metabolic reprogramming of tumor cells and reshaping cellular metabolic pathways, UCA1 underpins tumor growth and sustenance (56). This metabolic versatility permits tumor cells to withstand metabolic stress, with UCA1 playing a pivotal role. Ultimately, by catalyzing glycolysis, UCA1 supplies the requisite energy and biosynthetic precursors for tumor cells, bolstering their invasive and metastatic prowess.

3.5 Regulating the apoptosis of breast cancer cells

LncRNAs are crucial in regulating apoptosis, a vital process for maintaining tissue homeostasis and preventing tumor development. In the context of TNBC, specific lncRNAs influence apoptosis through various mechanisms, significantly impacting tumor progression and metastasis.

MALAT1 has been identified as a key player in modulating apoptosis in TNBC. It is suggested that MALAT1 can regulate apoptosis by interacting with miRNAs, such as miR-1 (60). Research indicates that Slug, a transcription factor essential for cell migration, invasion, and apoptosis, is a direct target of miR-1 and may be regulated by MALAT1. Downregulation of MALAT1 leads to an increase in miR-1 expression, which can promote apoptosis by targeting specific mRNAs. By suppressing apoptosis, MALAT1 potentially enhances the invasiveness and metastatic potential of tumor cells, increasing the malignancy of the tumor.

Furthermore, NEAT1, another nuclear-enriched lncRNA, is implicated in TNBC development through its influence on apoptosis-related signaling pathways. NEAT1 may regulate the expression of proteins that either inhibit or promote apoptosis via interactions with miRNAs (40). Additionally, it may indirectly preserve genomic stability by affecting DNA damage response and repair mechanisms, thus impacting apoptosis. As a ceRNA, NEAT1 can modulate the expression of miRNA target genes involved in apoptosis regulation, such as cyclin E1 and cyclin D1 (40).

The intricate and multifaceted regulation of apoptosis by lncRNAs in TNBC underscores the complexity of the disease's molecular landscape. By dissecting the interactions between these lncRNAs, miRNAs, and their target genes, we can gain a deeper understanding of TNBC's molecular underpinnings. This knowledge is invaluable for identifying potential therapeutic targets and developing novel strategies to combat this aggressive form of cancer.

3.6 Regulating the angiogenesis of breast cancer cells

Angiogenesis, the intricate process of new blood vessel formation, is a pivotal mechanism underpinning the growth and metastatic cascade of breast cancer. The orchestration of angiogenesis within breast cancer cells results from a complex interplay among numerous regulatory factors. Within this landscape, lncRNAs have emerged as key modulators with significant implications for cancer progression.

HOTAIR, an lncRNA exhibiting aberrant expression across various cancers, has been particularly implicated in TNBC due to its high expression levels. These levels correlate closely with the tumor's invasive edge, metastatic propensity, and angiogenic activity. HOTAIR's influence on angiogenesis is multifaceted; it is postulated to reshape the tumor microenvironment by orchestrating the delicate balance of cytokines and growth factors, including VEGF and FGF (61). This modulation paves the way for new blood vessel formation, thereby nurturing a conducive milieu for tumor cells and amplifying their invasive and metastatic prowess. Moreover, HOTAIR's reach extends to the immunological realm of the tumor, potentially skewing the polarization of tumor-associated macrophages (TAMs) and implicating itself in the dual narratives of angiogenesis and immune evasion within the tumor (61).

ANRIL, another lncRNA with disrupted expression in various cancers, mirrors HOTAIR's impact on TNBC's angiogenesis. Current understanding suggests that ANRIL's role is mediated through the regulation of genes and signaling pathways pivotal to angiogenesis, such as the VEGF and Notch pathways (62). As a

ceRNA, ANRIL adds layers of complexity to its regulatory influence by acting as a sponge for specific miRNAs, thereby modulating the expression of a spectrum of target genes involved in angiogenic processes (63). Its ripple effects on the tumor microenvironment, touching upon immune and stromal cells, further highlight ANRIL's therapeutic potential in TNBC.

The discovery of lncRNAs like HOTAIR and ANRIL as conductors of angiogenesis unveils fresh avenues for targeted therapeutics in TNBC. Targeting these lncRNAs to inhibit their function or expression may critically disrupt the angiogenic machinery of tumors, effectively starving them of the vital blood supply imperative for growth and metastasis. As we stand on the precipice of new discoveries, future research must diligently unravel the intricate mechanisms through which these lncRNAs operate, identify their synergistic or antagonistic interplays with other regulatory molecules, and rigorously assess the safety and efficacy of lncRNA-targeted therapies in the preclinical and clinical spectrum. This pursuit will not only deepen our comprehension of the molecular underpinnings of TNBC but also illuminate the path toward innovative and efficacious treatment modalities.

3.7 Regulating the stemness of breast cancer cells

Cancer stem cells (CSCs) possess remarkable abilities of self-renewal and multi-lineage differentiation, which are pivotal in the genesis, progression, metastasis, and resistance to therapy of tumors. Their stem-like characteristics enable these cells to foster the recurrence and relentless growth of tumors, complicating the landscape of cancer treatment. In breast cancer, especially the aggressive TNBC, the presence of CSCs is identified as a principal factor contributing to a grim prognosis. Thus, deciphering and targeting the molecular underpinnings that govern the properties of CSCs is essential for the formulation of efficacious therapeutic approaches.

In TNBC, lincRNA-ROR emerges as a pivotal lncRNA in modulating the characteristics of cancer stem cells. Evidence indicates that lincRNA-ROR is markedly overexpressed in TNBC tissues, in stark contrast to non-cancerous tissues, and it facilitates the initiation and spread of cancer by orchestrating the EMT (64). The suppression of lincRNA-ROR is observed to curb the EMT phenotype in TNBC cells. Advanced deep sequencing has unveiled that the diminished expression of miR-145 is indicative of metastatic potential, a process intricately linked to regulation by lincRNA-ROR (65). lincRNA-ROR is hypothesized to function as a ceRNA to miR-145, thereby constraining miR-145 levels in TNBC. Moreover, both miR-145 and lincRNA-ROR are implicated in diverse stages of embryonic and adult stem cell development. lincRNA-ROR is shown to modulate the expression of a suite of stemness factors, such as SOX2, OCT4, and NANOG, by sequestering miR-145 (66).

NEAT1, another lncRNA significantly overexpressed in TNBC, assumes a critical role in the regulation of tumor stemness. NEAT1 propels the advancement of cancer by spurring cell proliferation, EMT, invasion, and metastasis, and it instigates resistance to chemotherapy in TNBC cells. At the molecular level, NEAT1 is found to foster breast cancer growth by modulating miRNAs, including miR-548, miR-448, and ZEB1 (40, 67). The overabundance of NEAT1 is linked to the suppression of miR-448 expression, thereby liberating ZEB1 to manifest its influence. The inhibition of NEAT1 has been shown to deplete the reservoir of cancer stem cells endowed with robust self-renewal and multi-lineage differentiation capabilities, underscoring NEAT1's cardinal role in the regulation of tumor stemness.

lncRNAs such as lincRNA-ROR and NEAT1 play indispensable roles in the modulation of TNBC stem cell properties. Through a tapestry of intricate molecular mechanisms—ranging from acting as ceRNAs to influence miRNA expression to impacting the functionality of stemness factors, either directly or indirectly—they synergize to propel the malignant evolution of TNBC. These revelations not only amplify our comprehension of the regulatory dynamics of TNBC cancer stem cells but also chart new horizons and potential therapeutic targets for the development of precision medicine aimed at cancer stem cells.

3.8 Regulating the drug resistance of breast cancer cells

The challenge of drug resistance in TNBC significantly undermines the effectiveness of chemotherapy. The emergence of resistance mechanisms frequently culminates in treatment failure and disease recurrence. LncRNAs have emerged as pivotal regulators in the intricate dynamics of drug resistance, with their misregulation being linked to altered chemosensitivity in TNBC cells.

The lncRNA FTH1P3 epitomizes the complex interplay between lncRNAs and drug resistance. Research has illuminated its role in modulating paclitaxel resistance in TNBC via the miR-206/ABCB1 axis, with ABCB1 being a quintessential chemotherapy resistance protein found in multidrug-resistant cancers (68). In paclitaxel-resistant cells, such as the MDA-MB-231/PTX line, the overexpression of lncRNA FTH1P3 and its subsequent suppression have been shown to enhance paclitaxel sensitivity by 50%, underscoring the lncRNA's pivotal role in chemotherapy resistance mechanisms. Moreover, *in vivo* studies using transplants of paclitaxel-resistant cells have demonstrated that silencing FTH1P3 leads to diminished ABCB1 protein levels and curtailed tumor growth, highlighting its direct influence on the miR-206/ABCB1 signaling pathway.

Similarly, the lncRNA H19 has been recognized as an overexpressed entity in breast cancer that confers resistance to chemotherapy (41). Its elevated expression in paclitaxel-resistant TNBC cells and the reversion to chemosensitivity following H19 knockdown underscore its contribution to the resistant phenotype (41). The Akt signaling pathway, often dysregulated in breast cancer, is implicated in treatment resistance. Knockdown of H19 initiates apoptosis mediated by the Akt pathway, thereby sensitizing TNBC cells to paclitaxel (41). Clinical trials corroborate the relevance of targeting the Akt pathway, as the combination of Ipatasertib with paclitaxel has been shown to markedly enhance progression-free survival in patients with metastatic TNBC (69).

Investigating lncRNAs such as FTH1P3 and H19 in the context of TNBC chemotherapy resistance offers profound insights into the molecular underpinnings of drug resistance. These lncRNAs not only serve as biomarkers for chemotherapy resistance but also represent promising therapeutic targets. By modulating the expression of proteins critical to drug efflux and apoptosis pathways, these lncRNAs offer a means to devise strategies aimed at overcoming TNBC chemotherapy resistance. Future research should focus on delineating the comprehensive regulatory networks of these lncRNAs and identifying effective combination therapies that can target these lncRNAs, thereby augmenting TNBC's responsiveness to chemotherapy.

4 lncRNAs as potential therapeutic targets for TNBC treatment

In the realm of TNBC research, lncRNAs have risen to prominence for their pivotal role in cellular biology and oncogenesis (70, 71). The dysregulation of lncRNAs across a spectrum of cancers underscores the potential for lncRNA-centric therapeutic strategies. Contemporary studies have enriched our comprehension of lncRNA functions in TNBC, especially concerning their influence on drug resistance and cancer progression. The pursuit of lncRNA-targeted drugs stands at the forefront of oncological innovation, heralding a new era in cancer therapeutics. Given the aberrant expression of lncRNAs in malignancies and their regulatory capacity over tumor growth, invasion, metastasis, and treatment resistance, they are increasingly recognized as auspicious targets for therapeutic intervention.

Antisense oligonucleotides (ASOs) are synthetic nucleic acid polymers designed to bind specifically to complementary sequences of lncRNAs, effectively silencing their activity (72). The strategic deployment of ASOs against targeted lncRNAs can substantially diminish their presence in tumor cells, thereby modulating cellular behavior. ASOs act as precision molecular instruments, influencing RNA processing and subsequent protein synthesis through a variety of pathways. Advances in ASO architecture and chemical refinement have markedly amplified their therapeutic promise and efficacy (73). A multitude of ASOs are currently advancing through clinical trials, showcasing the breadth and ingenuity of ASO-driven therapeutic modalities (74). In the context of TNBC, ASOs have been employed to downregulate lncRNA targets implicated in oncogenesis. For instance, the lncRNA TROJAN, known to facilitate TNBC proliferation and metastasis, correlates with diminished patient survival (75). Treatment with TROJAN-targeted ASOs has led to a notable decrease in metastatic lung nodules in murine models, following direct transfection into TNBC cells and systemic administration in xenograft scenarios, all while maintaining a minimal toxicity profile (75). These observations herald the potential of ASO-mediated TROJAN regulation as an innovative therapeutic avenue for TNBC. Additionally, ASOs aimed at lncRNA NRAD1 have demonstrated an ability to decelerate tumor progression and suppress TNBC stem cell-like properties (76). Research also reveals that targeting lncRNA MALAT1 with ASOs can lower its RNA abundance, culminating in retarded TNBC growth, diminished cellular proliferation, and heightened apoptosis (77). Experimental models have further indicated that MALAT1 suppression reshapes the tumor microenvironment, characterized by a decrease in immunosuppressive elements such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), coupled with an upsurge in cytotoxic CD8+T cells (77). In preclinical settings, the MALAT1-targeting ASO, when combined with chemotherapy or immune checkpoint blockade (ICB), has shown promise in augmenting therapeutic outcomes (77).

RNA nanotechnology plays a crucial role in the study of lncRNAs. This technology utilizes nanoscale tools and methods to delve into the role of lncRNAs in cellular functions and genomic stability (78). With RNA nanotechnology, scientists can more precisely reveal the structure and function of lncRNAs and study their impact on gene expression and disease formation (78). By designing nanoparticles that can specifically target and regulate the activity of lncRNAs, RNA nanotechnology not only can control gene expression but also achieve refined gene regulation (78). Additionally, RNA nanoparticles can serve as drug delivery carriers, transporting therapeutic molecules directly to specific cells or tissues. In the treatment of TNBC, the application prospects of RNA nanotechnology are particularly broad. For example, the expression level of lncRNA DANCR is abnormally elevated in TNBC and closely related to tumor development (34). Scientists have successfully developed a new type of nanoparticle—RGD-PEG-ECO/siDANCR, which is self-assembled from the multifunctional amino lipid ECO, cyclic RGD peptide-PEG, and siDANCR, and is specifically designed for systemic delivery to TNBC (79). In MDA-MB-231 and BT549 cell lines, after treatment with RGD-PEG-ECO/siDANCR nanoparticles, the expression of DANCR decreased by 80-90% within 7 days, demonstrating efficient siRNA delivery capability and sustained gene silencing effect (79). Moreover, these nanoparticles exhibited outstanding therapeutic effects *in vitro*, significantly reducing the invasion, migration, survival, spheroid formation, and proliferation capabilities of TNBC cells. At the molecular level, the effective inhibition of DANCR dynamically regulated the oncogenic network, by reducing the levels of PRC2-mediated H3K27me3 and inhibiting the Wnt/EMT signaling pathway, while also altering the phosphorylation patterns of various kinases in TNBC cells (79). In animal models, systemic administration of RGD-PEG-ECO/siDANCR nanoparticles at a siRNA dose of 1 mg/kg to nude mice with TNBC xenografts showed significant inhibitory effects on TNBC progression, without observable severe toxic side effects, highlighting the therapeutic potential and safety of these nanoparticles (79). Additionally, LINC00511, as a target gene related to drug resistance, has also attracted attention for its potential role in TNBC treatment. Researchers have constructed a new type of theranostic agent—a nanobubble complex carrying LINC00511-siRNA, for siRNA delivery, and evaluated its impact on drug sensitivity in TNBC models (80). The study results indicate that the nanobubble complex is an efficient and safe siRNA transfection tool. These findings provide a new perspective for RNA nanoparticle-based targeted treatment strategies, with the potential to bring breakthrough progress in the treatment of TNBC and other cancers through specific targeting and inhibition of lncRNAs.

The therapeutic promise of lncRNA-based strategies, including ASOs and RNA nanotechnology, is considerable. However, several hurdles must be surmounted to achieve their full clinical potential. A paramount challenge is the refinement of delivery systems. Ensuring the precise delivery of ASOs and RNA nanoparticles to tumor sites, while circumventing off-target effects and minimizing toxicity, is essential. The quest for targeted delivery systems that leverage tumor-homing peptides or specific ligands is a vibrant field of research (81). Moreover, the biocompatibility and biodegradability of these systems are crucial for ensuring patient safety.

Identifying the most efficacious lncRNA targets is a sophisticated endeavor, given the multitude of lncRNAs and their intricate roles in TNBC. A rigorous vetting process involving bioinformatics, experimental studies, and clinical data is imperative to guarantee the specificity and potency of therapeutic interventions. The ongoing quest to optimize ASO and nanoparticle design to bolster binding affinity, stability, and cellular uptake is a testament to the dynamic nature of this field (77, 82). The journey from bench to bedside is not without its obstacles, including the scalability of production, navigating regulatory landscapes, and the considerable financial investment required for personalized medicine. Tackling these issues necessitates a synergistic approach, with researchers, clinicians, and industry stakeholders collaborating to forge standardized protocols and economically viable manufacturing processes.

Looking ahead, future research endeavors should concentrate on deepening our comprehension of the molecular underpinnings of lncRNAs in TNBC pathogenesis, with a focus on the intricate interplay between lncRNAs and other cellular constituents, such as proteins and miRNAs, which may modulate treatment efficacy. The exploration of synergistic combination therapies that marry lncRNA-targeting approaches with traditional treatments like chemotherapy, radiotherapy, and immunotherapy could amplify therapeutic impact and potentially surmount resistance. The advancement of personalized medicine, facilitated by the discovery of predictive biomarkers for patient stratification and treatment response monitoring, will enable the tailoring of lncRNA-targeted therapies to individual patient profiles, enhancing the likelihood of favorable outcomes.

lncRNA-targeted therapeutics for TNBC, encompassing ASOs and RNA nanotechnology, stand at the vanguard of oncological innovation. Nonetheless, the route to clinical application is replete with challenges that demand persistent investigation and advancement. By surmounting current limitations, honing delivery systems, pinpointing and refining therapeutic targets, and charting a course for future research, we can illuminate a path toward treatments that are safer, more efficacious, and exquisitely tailored to the unique needs of TNBC patients.

5 Conclusion

In summary, lncRNAs play a multifaceted role in the progression and treatment of TNBC, ranging from modulating tumor cell proliferation and metastasis to serving as potential therapeutic targets. As our understanding of lncRNA functions and regulatory mechanisms deepens, lncRNA-based therapeutic strategies are emerging at the forefront of TNBC treatment. The advancement of technologies such as antisense oligonucleotides, locked nucleic acids, and RNA nanoparticles opens up new possibilities for the diagnosis and treatment of TNBC. These modalities have demonstrated potential not only *in vitro* but also in animal models, showcasing their safety and efficacy. Looking ahead, these studies hold the promise of delivering more personalized and precise treatment options for patients with TNBC.

Author contributions

SZ: Writing – review & editing, Writing – original draft, Funding acquisition. WC: Writing – original draft, Investigation. ZP: Writing – original draft, Investigation. ZF: Writing – original draft, Investigation. XW: Writing – original draft, Investigation.

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Conflict of interest

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

Publisher's note

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2-methoxyestradiol inhibits the malignant behavior of triple negative breast cancer cells by altering their miRNome

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Background: Triple-negative breast cancer (TNBC) is a subtype of breast cancer with no effective targeted treatment currently available. Estrogen and its metabolites influence the growth of mammary cancer. Previously, we demonstrated the anti-cancer effects of 2-methoxyestradiol (2ME2) on mammary carcinogenesis.

Materials and methods: In the present study, we investigated the effects of 2ME2 on TNBC cells. TNBC (MDA-MB-231 and MDA-MB-468) and non-tumorigenic breast (MCF10A) cell lines were used to determine the effects of 2ME2 on cell proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS assay), cell cycle (flow cytometric assay), migration (transwell migration assay), invasion (matrigel invasion assay), apoptosis (annexin V/propidium iodide assay), colony formation (soft agar assay), and miRNome (human miRNA profiling array). The miRNome data were analyzed using the c-BioPortal and Xena platforms. Moreover, Kyoto Encyclopedia of Genes and Genomes, Gene Ontology, and reactome pathway analyses were performed.

Results: We found that 2ME2 effectively inhibited cell proliferation and induced apoptosis. Furthermore, 2ME2 treatment arrested TNBC cells in the S-phase of the cell cycle. Treatment with 2ME2 also significantly decreased the aggressiveness of TNBC cells by inhibiting their migration and invasion. In addition, 2ME2 altered the miRNA expression in these cells. In silico analysis of the miRNome profile of 2ME2-treated MDA-MB-468 cells revealed that miRNAs altered the target genes involved in many different cancer hallmarks.

Conclusion: 2ME2 inhibits triple negative breast cancer by impacting major cellular processes like proliferation, apoptosis, metastasis, etc. It further modifies gene expression by altering the miRNome of triple negative breast cancer cells. Overall, our findings suggest 2ME2 as a potent anti-cancer drug for the treatment of TNBC.

KEYWORDS

2-methoxyestradiol, microRNA, cell cycle, invasion, migration, proliferation, apoptosis, triple negative breast cancer

1 Introduction

Triple-negative breast cancer (TNBC) is an aggressive and highly metastatic disease with a poor prognosis. This lethal breast cancer subtype is histologically classified as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 [HER2]-negative (1, 2) and accounts for approximately 20% of all diagnosed breast cancer cases (3). TNBC has worse prognosis than other breast cancer subtypes (3). It is commonly diagnosed in younger women (4) and poses a high risk of recurrence (4). Metastasis is the main cause of breast-cancer-associated mortality. Treatment options are limited because of the lack of specific targets for TNBC. The two major subtypes of TNBC cell lines are basal A and basal B (5). These cell line subtypes have clear distinguishing features between them in the expression of cytokeratins, integrins, claudins, mesenchymal and stem cell markers (6–9). Currently, chemotherapy is the only approved systemic treatment for TNBC that shows limited benefits in terms of overall survival and toxicity (10, 11). Extensive efforts have been made to develop effective drugs for the treatment of high-risk TNBC. However, non-toxic and effective therapies are urgently required to treat advanced-stage TNBC.

2-Methoxyestradiol (2ME2) is a primary endogenous metabolite of 17β-estradiol (12). Its affinity for ERs is weak compared to that of its parent compound and 17β-estradiol and its other derivatives (12). Its effect is independent of the estrogen responsiveness of the cells (13). Several studies have demonstrated the impact of 2ME2 on hypoxia, angiogenesis, vascularization, and drug resistance (14). The compound 2ME2 has demonstrated anti-cancer effects across various cancer types, including breast cancer (15–17), ovarian cancer (18), prostate cancer (19), osteosarcoma (20), leukemia (21), cervical adenocarcinoma (22), melanoma (23), and sarcoma (24). These effects are attributed to its capacity to induce apoptosis, cause cell cycle arrest, instigate nitro oxidative damage, promote autophagy, and inhibit invasion, angiogenesis, and overall tumor growth. Notably, oral administration of 2ME2 does not cause any significant side effects in xenograft and metastatic disease models (25, 26). Over the last decade, 2ME2 has gained attention for its notable anti-cancer activity and potential cardiovascular benefits (27). Phase I and II clinical trials have been conducted to evaluate the antiproliferative and antiangiogenic effects of 2ME2 on multiple myeloma, glioblastoma multiforme, and carcinoid, prostate, and breast tumors (13, 28).

2ME2 has been shown to inhibit the proliferation of cancer cells by inducing apoptosis (15, 29), through upregulation of death receptor 5 (30) targeting cells in the G2/M phase of cell cycle (31, 32), inhibiting microtubule assembly (25) and also inhibit angiogenesis (33). However, the exact molecular mechanisms by which 2ME2 exerts its pleiotropic effects on breast cancer cells remain unclear. Therefore, in this study, we aimed to elucidate the signaling pathways regulated by miRNAs that are associated with the anti-cancer effects of 2ME2 on TNBC cells. MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate the expression of several genes in humans, plants, and microbes via RNA silencing and post-translational regulation (34). In cancer, miRNAs function as either tumor suppressors or oncogenes, ultimately affecting their target gene expression (35). Differential expression of miRNAs regulates different pathways such as apoptosis, cancer-related, and cell proliferation pathways. It has been demonstrated that TNBC subtypes A and B cells had a distinct microRNA profiles resulting in differential metastatic potentials (5, 6). In this study, we observed a change in the global human miRNome profile of 2ME2-treated TNBC cells. Furthermore, the altered miRNome influenced various cancer pathways, including the proliferation, metastasis, cell cycle, apoptosis, mammalian target of rapamycin (mTOR), focal adhesion, p53, carbohydrate digestion and absorption, insulin, cell cycle, Janus kinase (JAK)-signal transducer and activator of transcription (STAT), ErbB, adherens, neurotrophin, T cell receptor, and endocytosis pathways. Our findings provide insights into the effects of 2ME2 on TNBC, suggesting its potential as a drug for the treatment of this aggressive type of breast cancer.

2 Materials and methods

2.1 Cell lines

The non-malignant breast epithelial cell line, MCF-10A (Cat# CRL-10317), and triple-negative metastatic breast cancer cell lines

MDA-MB-231 (Cat # HTB-26) and MDA-MB-468 (Cat # HTB-132) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-10A cells were grown in a mammary epithelial cell basal medium supplemented with 5% horse serum, 0.5 mL epidermal growth factor, 0.5 mL insulin, 0.5 mL hydrocortisone, 2 mL bovine pituitary extract (all from Lonza, Clonetics, MEGM SingleQuots kit), 10 U/mL penicillin, and 10 mg/mL streptomycin (Gibco, Life Technologies). MDA-MB-231 and MDA-MB-468 cells were maintained in the Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 U/mL penicillin, and 10 mg/mL streptomycin (Gibco, Life Technologies). Phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped serum (Equitech-Bio, Kerrville, TX, USA) was utilized for all cell lines in this study. This medium was applied 48 hours before 2ME2 treatment to prevent estrogen interference and ensure the accuracy of the observed results. Mycoplasma contamination was tested using the MycoSEQ Plus Mycoplasma Detection Kits (ThermoFisher Scientific Cat # A55124). All cells were maintained at 37°C in a 5% CO_2 atmosphere.

2.2 Cell proliferation assay

An *in vitro* cell viability assay was performed as previously described (36). Briefly, MDA-MB-231, MDA-MB-468, and MCF-10A cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and incubated at 37°C. After 24 h, the cells were treated with different concentrations of 2ME2 (0, 1, 2, 5, 7.5, 10, and 20 µM) and incubated for different time periods from 24 to 72 h at 37°C. After incubation, twenty microliters of the Cell Titer 96 Aqueous One Solution Reagent (Promega) was added to each well of the 96-well assay plate containing the samples in 100 µL of the culture medium. The plates were incubated at 37°C for 4 h in a humidified 5% CO_2 atmosphere. Cell viability was determined by measuring the absorbance at 490 nm using a 96-well microplate reader (CLARIOstar; BMG LABTECH, Cary, NC, USA).

2.3 Immunoblotting

Western blotting was performed to quantify the expression of various proteins. Mammalian protein extraction reagent (Cat. no. 78501; Thermo Scientific, Rockford, IL, USA) was used to extract the total protein from whole cell lysates. Proteins were separated using SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF; cat. no. IPVH00010; EMD Millipore) membranes. The membranes were blocked for 1 h with 5% bovine serum albumin (Cat. no. A7906; Sigma-Aldrich, St Louis, MO, USA) prepared in Tris-buffered saline with 0.1% Tween-20. Then, the blots were incubated with primary antibodies (pAKT, AKT, pmTOR, mTOR, pP70S6K, P70S6K, E-cadherin, N-cadherin, vimentin, slug, zinc finger E-box-binding homeobox 1 [Zeb1], cyclin-dependent kinase [CDK]4, CDK6, cyclin D1, p21, cleaved poly [ADP ribose] polymerase [PARP], cleaved caspase 3, Bcl-associated killer (BAK), Bcl-2, and β-actin) overnight at 4°C. Details regarding antibodies and their dilutions are provided in the Supplementary Materials as Table 1. After washing thrice with TBST, the blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies to detect the primary antibodies and visualized using enhanced chemiluminescence.

Image Studio software was used to quantify protein expression from digitally imaged Western blots. An equal dimension rectangle tool from the analysis ribbon was used to draw lanes around the entire region where bands were present, allowing for measurement

TABLE 1 List of antibodies and dilutions.

Antibody	Company	Dilution	Molecular weight (kDa)
pAKT S473	Cell Signaling 4060	1:1000	60
AKT1	Santa cruz 5298	1;500	62
pmTOR S2448	Cell Signaling 5536	1:500	289
mTOR S2448	Cell Signaling 4517	1:500	289
pP70S6K	Cell Signaling 9206	1:500	70-85
P70S6K	Cell Signaling 9202	1:500	70-85
E-cadherin	Cell Signaling 3195	1:500	135
N-cadherin	Cell Signaling 13116	1:500	140
Vimentin	Cell Signaling 5741	1:500	57
Slug	Cell Signaling 9585	1:500	30
Zeb1	Cell Signaling 3396	1:500	200
CDK4	BD Biosciences 610147	1:1000	33
CDK6	Cell Signaling 30483	1:500	36
Cyclin D1	Santa cruz 717	1:1000	37
P21	Abcam 7960	1:100	21
Cleaved PARP/PARP (Asp 214)	Cell Signaling 5625	1:1000	89-116
BAK	Cell Signaling 3814	1:1000	25
Bcl2	Santa cruz 783	1:500	26
Cleaved Caspase 3	Cell Signaling 9661	1:500	17-19
β-Actin	Sigma A1978	1:1000	42

of all protein signals. Background subtraction was applied to each band within a lane to eliminate background noise from the calculations. The intensity values of the bands corresponding to the protein of interest were normalized to their respective β-actin intensity values. The data are represented as the percentage of protein expression normalized to β-actin.

2.4 Cell cycle analysis

MDA-MB-468 cells in logarithmic growth phase were harvested and seeded in a 6-well plate at a density of 5.0×10^5 cells/well. After overnight culture, the cells were treated with 5 μM 2ME2 for 24 h, harvested, and stained with propidium iodide. The percentage of cells in each phase of the cell cycle was analyzed via flow cytometry using FACS Accuri C6 flow cytometer (BD Biosciences San Jose, CA, USA).

2.5 Apoptosis analysis

MDA-MB-468 cells were seeded in a 6-well plate at a density of 2.0×10^5 cells/well and treated with 5 μM 2ME2 for 24, 48, and 72 h. Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit I and analyzed using the FACS Accuri C6 flow cytometer (BD Biosciences San Jose, CA, USA), according to the manufacturers' instructions.

2.6 Migration assay

The migration assay was performed using 2ME2 (2 and 5 μM)-treated MDA-MB-468 cells. Cells (5×10^3 cells/well) were seeded in a serum free medium in the top chamber of the transwell with a non-coated polycarbonate membrane (6.5 mm diameter insert, 8.0 μm pore size; Corning Incorporated). In the lower chamber, RPMI-1640 medium with 10% FBS was added. The cells were incubated for 48 h at 37°C and 5% CO_2. Migrating cells on the lower surface of the membrane were fixed with 5% formalin and stained with 0.2% crystal violet. Non-migrating cells on the upper side of the insert were wiped off using a cotton swab. Images of the migrated cells were captured and counted in a blinded manner in five different microscopic fields at 20× magnification.

2.7 Invasion assay

Invasive ability of MDA-MB-468 breast cancer cells was further assessed after treatment with 2ME2 (5 μM) using a matrigel-based invasion assay. Cells were seeded in a serum-free medium at a density of 0.5×10^5 cells/well in the upper chamber of a 6.5-mm transwell with 8.0 mm pore polycarbonate membrane inserts (Corning) coated with matrigel (Cat. no. 354234; BD Biosciences). The lower chamber was filled with the medium containing 10% FBS and incubated for 48 h at 37°C. After incubation, matrigel from the upper chamber was gently scraped using Q-tips. The cells invaded on the other side of the membrane facing the lower chamber were rinsed with 1× PBS and the cells were fixed with 4% paraformaldehyde. The invaded cells were then stained with 0.1% crystal violet for 2 h. Then, the insert wells were washed with tap water and imaged under an inverted phase contrast microscope (Eclipse Ti; Nikon).

2.8 Colony formation assay

Colony formation assay was performed to assess the clonogenic ability of single cells under anchorage-independent growth conditions. MDA-MB-468 cells (2×10^4) were seeded in 60-mm dishes containing a top layer of 0.7% agarose and bottom layer of 1% agar. The cells were treated with 2 and 5 μM 2ME2 for 24 h and incubated at 37°C for four weeks, and stained with 0.2% crystal violet. Stained colonies were imaged using an inverted microscope. Colonies containing more than 50 cells were manually counted.

2.9 Human miRNome profiling

miRNA profiling was performed using the Qiagen Human miRNome miScript miRNA PCR Array (Cat. no. MIHS-216Z). First, RNA was extracted from MDA-MB-468 cells using TRIzol (Life Technologies) after treatment with 2ME2 (5 μM) for 24 h. Then, whole human miRNome profiling PCR array was performed using the miScript SYBR Green PCR Kit (Qiagen). miRNA expression data were assessed using miRNet (http://www.mirnet.ca/).

2.10 RTqPCR analysis

Total RNA was isolated from MDA-MB-468 and MDA-MB-231 cells after treatment with 2ME2 (5 μM) for 24 h using the Trizol reagent (Life Technologies). The top 5 up-regulated and down-regulated miRNAs and its target genes were validated using RTqPCR. The appropriate primers used for each miRNA and its target genes is provided in the Supplementary Materials section as Tables 2 and 3. The analyses were performed based on the comparative Ct method (2−ΔΔCt).

2.11 Kyoto encyclopedia of genes and genomes, gene ontology and reactome pathway analyses

KEGG pathway analysis comprises pathway maps that help our understanding of molecular interactions, reactions, and relational networks in various categories, including Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, Human Diseases, Drug Development, etc. Altered pathways were studied using KEGG and GO after 2ME2 treatment. Enriched pathway analysis was performed via hierarchical genome-wide visualization using the Reactome Analysis Tool (http://reactome.org). Significantly

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TABLE 2 List of miRNA primers.

Top 5 up regulated miRNAs by 2ME2	Forward	Reverse
hsa-miR-4326	TGTTCCTCTGTCTCCCAG	GAACATGTCTGCGTATCTC
hsa-miR-646	5'-ACACTCCAGCTGGGAAGCAGCTGCCTC-3'	5'-
hsa-miR-600	CCACCTCTACGCATCATTCA	CCAAGCTCGTCTGGTTCTC
hsa-miR-2117	TGTTCTCTTTGCCAAGGAC	GAACATGTCTGCGTATCTC
hsa-miR-3200-5p	AATCTGAGAAGGCGCACAAG	GAACATGTCTGCGTATCTC
Top 5 downregulated miRNAs by 2ME2	Forward	Reverse
hsa-miR-19a-3p	GCGTGTGCAAATCTATGCAA	AGTGCAGGGTCCGAGGTATT
hsa-miR-15a-5p	5′-TAGCAGCACATAATGGTTTGTG-3′	5′-GAACATGTCTGCGTATCTCAC-3′
hsa-miR-21-3p	GACCCAACACCAGTCGATG	TCCTCCTCTCCTTCCTTCTC
hsa-miR-19b-3p	GTGCAAATCCATGCAAAACTGA	GTGCAGGGTCCGAGGTGCT
hsa-miR-96-5p	ATGCTTTCTCAACTTGTTGG	TCACCG CTCTTGGCCGTCACA

enriched pathways are shown in yellow, whereas less significantly enriched pathways are shown in gray. Then, miRNA target genes were uploaded to the Reactome database and significantly enriched pathways had a false discovery rate < 0.05.

2.12 In silico analysis of 2ME2-induced genetic alterations

Using the miRNA target genes, we explored the possible genetic alterations, including truncation, in-frame mutations, missense mutations, gene amplification (overexpression), and deletions, caused by 2ME2 treatment. Our differentially expressed gene dataset was uploaded to the cBioPortal (http://cbioportal.org/) database. In the cBioPortal database, we validated genes using The Cancer Genome Atlas (TCGA) and analyzed the overall survival of patients with either high or low expression of miRNA target genes after 2ME2 treatment. Kaplan–Meier plots were generated using breast carcinoma samples from TCGA database using Kmplot.com and UALCAN. Genetic alterations were visualized using Oncoprint diagrams of breast carcinoma samples. Using the Xena platform (http://xenabrowser.net), we further determined the changes in the gene expression profiles of TCGA breast cancer samples after 2ME2 treatment.

TABLE 3 List of miRNA target genes primers.

Genes	Forward	Reverse
PIK3R1	TGGACGGCGAAGTAAAGCATT	AGTGTGACATTGAGGGAGTCG
HSP90AA1	CAGAGGCGGACAAGAACGACAAG	GATCCTGTTGGCGTGCGTCTG
ERBB2	GGAAGTACACGATGCGGAGACT	ACCTTCCTCAGCTCCGTCTCTT
CCND1	TCTACACCGACAACTCCATCCG	TCTGGCATTTTGGAGAGGAAGTG
PTPRF	ATGTCATCGCCTACGACCACTC	GTGGCGATGTAGGCATTCTGCT
CDC42	TGACAGATTACGACCGCTGAGTT	GGAGTCTTTGGACAGTGGTGAG
HSP90B1	GGAGAGTCGTGAAGCAGTTGAG	CCACCAAAGCACACGGAGATTC
HSPA8	ACTCCAAGCTATGTCGCCTTT	TGGCATCAAAAACTGTGTTGGT
PRKAR1A	GCAGGCGAGCTATTAGTTTA	CATCCATCTCCTATCCCCTTT
AKT1	TGGACTACCTGCACTCGGAGAA	GTGCCGCAAAAGGTCTTCATGG
RAB5B	GGAGACTTCAGCCAAGACAGCT	ACACTGGCTCTTGTTCTGCTGG
FASN	TTCTACGGCTCCACGCTCTTCC	GAAGAGTCTTCGTCAGCCAGGA
GRB2	GAAATGCTTAGCAAACAGCGGCA	TCCATCTCGGAGCACCTTGAAG
GAPDH	GATGCTGGCGCTGAGTACG	GCTAAGCAGTTGGTGGTGC

2.13 Statistical analyses

All data were analyzed using GraphPad Prism 9 (Dotmatics). All ***in vitro*** experiments were repeated at least three times and had 3–6 replicates. Student's *t*-test was performed to compare between two groups and one-way ANOVA and Tukey ***post hoc*** test were used for analysis when more than two groups were compared, and statistical significance was set at $P < 0.05$.

3 Results

3.1 2ME2 inhibits TNBC cell growth and proliferation

To determine the efficacy of 2ME2 for TNBC treatment, we evaluated its effects on the growth and proliferation of TNBC cells. TNBC (MDA-MB-231 and MDA-MB-468) and non-malignant breast epithelial (MCF-10A) cell lines were treated with various concentrations of 2ME2 (0, 1, 2, 5, 7.5, 10, and 20 µM). High doses of 2ME2 increased the death of TNBC cells but did not exert any significant effects on MCF10A cells. After 24-h treatment, none of the tested doses induced 50% cell death in the cell lines. Treatment with 2 and 5 µM of 2ME2 for 48 h inhibited the proliferation of MDA-MB-468 cells by approximately 50% (Figure 1A). Even though 2 µM of 2ME2 was effective 5 µM dose was closest to the IC_{50} value, so we chose to use this dose for our experiments. In contrast, even high doses of 2ME2 (20 µM) did not have a major impact on MCF-10a cell proliferation. MDA-MB-231 cell proliferation was inhibited by 2ME2 in a dose-dependent manner but higher doses of 2ME2 were required (Figure 1A). Therefore, MDA-MB-468 cells were used for subsequent analyses. AKT/mTOR signaling pathway is hyperactive in cancer cells, facilitating cancer cell growth and proliferation (37, 38). Here, treatment with 5 µM 2ME2 for 24 h decreased the phosphorylation of AKT and mTOR (Figure 1B and Supplementary Figure 1A). We also observed decreased phosphorylation of AKT in MDA-MB-231 cells treated with 2ME2 (Supplementary Figures 1B, C). Next, we focused on the ribosomal protein kinase, P70S6K, which acts downstream of mTOR and regulates cell growth via G1 phase cell cycle progression (37, 39). Here, the active form of P70S6K was minimally expressed in 2ME2-treated cells (Figure 1C and Supplementary Figure 1D). 2ME2 effectively inhibited cell proliferation by reducing the activation of AKT, mTOR and P70s6Kinase. However, further analysis is needed to better understand the involvement of the PI3K/AKT signaling pathway by 2ME2 as a crucial mechanism in controlling TNBC proliferation.

3.2 2ME2 inhibits the metastasis of TNBC cells

Metastatic characteristics of cancer cells are defined by their ability to migrate, invade, and form a niche (colony) in other tissues/organs (40). This makes cancer more aggressive and

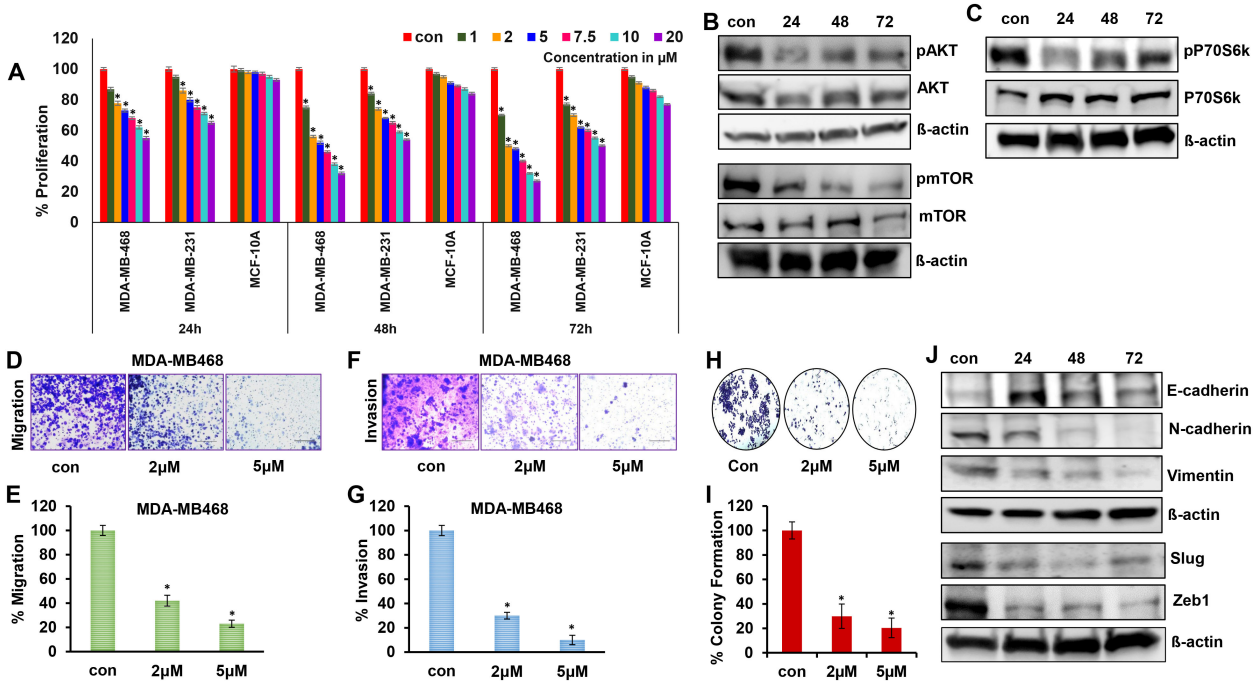

FIGURE 1
2ME2 inhibits breast cancer cell proliferation and metastasis. **(A)** Cell viability of MDA-MB-468, MDA-MB-231 and MCF-10A cells treated with 1-20µM of 2ME2 for 24, 48 and 72h. **(B, C)** Expression level of proliferative markers in MDA-MB-468 cells at different time points of treatment with 5µM of 2ME2. **(D, E)** Effect of 2ME2 on migratory capacity of MDA-MB-468 cells. **(F, G)** Effect of 2ME2 on invasive ability of MDA-MB-468 cells. **(H, I)** Colony formation ability of MDA-MB-468 cells with 2ME2 treatment. **(J)** Expression level of EMT markers in 2ME2 treated MDA-MB-468 cells at different time points. *$P < 0.05$.

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difficult to treat. In this study, the metastatic characteristics of TNBC cells were assessed in the presence of 2ME2. We found that TNBC cell migration was inhibited by 58 and 77% after treatment with 2 and 5 µM 2ME2, respectively (Figures 1D, E). Invasion and colony formation were reduced in 2ME2-treated MDA-MB-468 cells than in the untreated cells (Figures 1F–I). Similarly, MDA-MB-231 cells treated with 2ME2 showed a significant reduction in migration and invasion capabilities (Supplementary Figures 1E, F) respectively. Change in cellular morphology from epithelial to mesenchymal, known as the epithelial–mesenchymal transition, is a prerequisite for metastasis (41). Treatment with 2ME2 decreased the levels of mesenchymal markers, such as N-cadherin, vimentin, slug, and Zeb1, but increased the levels of the epithelial marker, E-cadherin (Figure 1J and Supplementary Figure 1G). MDA-MB-231 cells also responded similarly to 2ME2 in terms of E-cadherin and N-cadherin regulation (Supplementary Figures 1H–J) respectively. These data demonstrate the potent roles of 2ME2 in inhibiting the aggressiveness and metastatic characteristics of TNBC cells.

3.3 2ME2 inhibits the cell cycle and induces apoptosis

Ribosomal protein P70S6K is active during the G1 phase of cell cycle and regulates the cellular machinery via protein synthesis required for cell division (42). P70S6K phosphorylates and regulates the functions of the 40s ribosomal protein (S6), eukaryotic initiation factor 4B, and eukaryotic elongation factor 2 kinase that are required for protein synthesis (43–45). The potent role of P70S6K in the cell cycle and its low activation in 2ME2-treated cells lead us to further analyze the cell cycle in 2ME2-treated MDA-MB-468 cell lines. We analyzed the effect of 2ME2 on the cell cycle using flow cytometry. Studies on many cancer types, including breast cancer, have reported that 2ME2 accumulates and arrests cells in almost all phases of the cell cycle, including the G0/G1, G2/M, M phases (46–49). However, its exact roles during specific phases of the cell cycle remain unknown. Our analysis of the cell cycle in 2ME2-treated MDA-MB-468 cells revealed minute changes in the cell population in S-phase (6.46% more cell population was arrested in the S-phase in 2ME2-treated cells compared to that in the non-treated cells) (Figures 2A, B). However, analysis of the markers of the G0/G1 or G1/S transition phases revealed a significant difference in the expression patterns of cell cycle regulators (Figures 2C, D). Cyclin D1 is a proto-oncogene that forms a complex with CDK-4 and -6 to regulate cell cycle progression at G1 and G1/S phases (50). The active form of cyclin D1, CDK4–CDK6 complex, phosphorylates and inactivates the tumor suppressor gene, retinoblastoma (*Rb*) (50, 51). Hence, high levels of cyclin D1, CDK4, and CDK6 are often observed in most patients with breast cancer (52, 53). Accordingly, we observed high levels of cyclin D1, CDK4, and CDK6 in MDA-

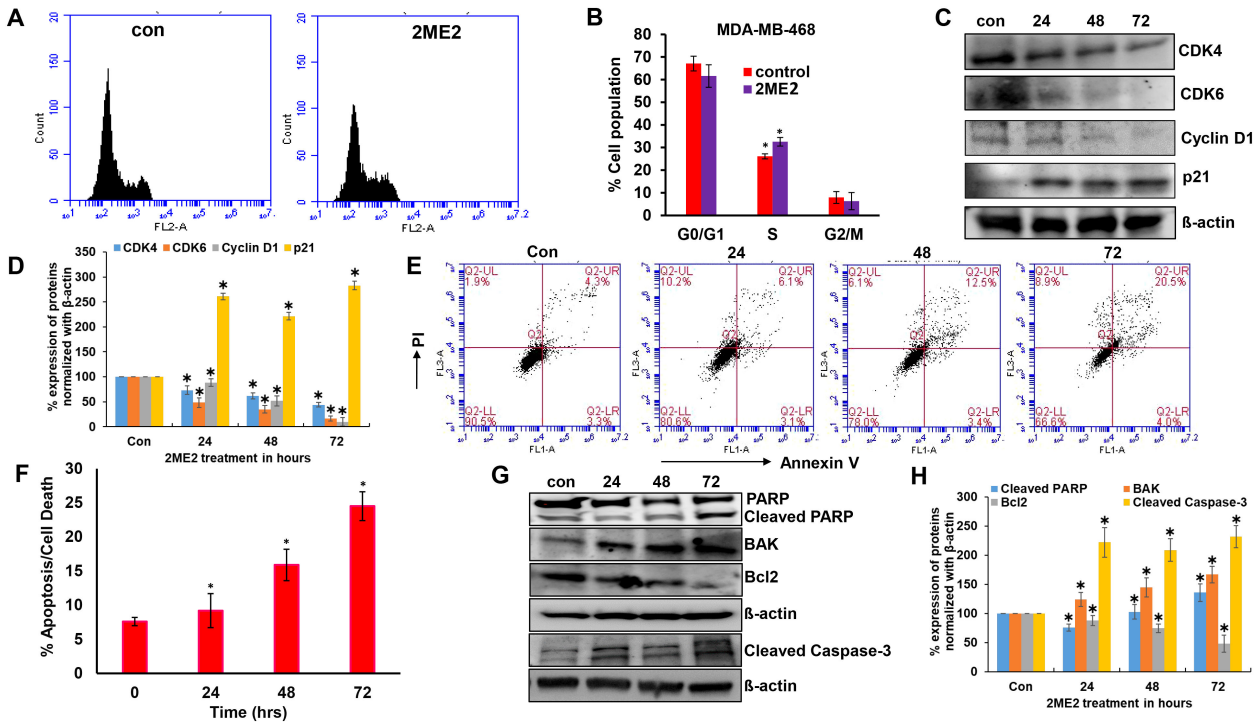

FIGURE 2
2ME2 induces cell cycle arrest and Apoptosis. **(A, B)** Cell cycle analysis of 2ME2 treated MDA-MB-468 cells. **(C, D)** Cell cycle regulators were assessed in 2ME2 treated MDA-MB-468 cells at 24, 48 and 72h using Western blot and densitometric analysis. **(E)** Apoptosis analysis of 2ME2 treated MDA-MB-468 cells at various time points, **(F)** Graphical representation of percentage apoptosis/cell death in 2ME2 treated MDA-MB-468 cells. **(G, H)** Expression levels and densitometric analysis of pro and anti-apoptotic markers in 2ME2 treated MDA-MB-468 cells at different time points. *$P < 0.05$.

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MB-468 cells, which were significantly reduced by 2ME2 treatment in this study (Figures 2C, D). MDA-MB-231 cells also exhibited a similar response to 2ME2 in terms of Cyclin D1 regulation (Supplementary Figures 2A, B). Similarly, we analyzed the levels of the cell cycle inhibitor, p21, which inhibits the activity of CDK4 during the G1 phase of the cell cycle (54). p21 protein levels were higher in 2ME2-treated MDA-MB-468 cells than in the untreated control cells (Figures 2C, D). Our results demonstrate that 2ME2 induced cell cycle arrest in TNBC cells.

Cell death or apoptosis is poorly regulated in cancer cells, resulting in their uncontrolled growth (55). Here, we investigated whether 2ME2 induces apoptosis in MDA-MB-468 breast cancer cells. We used the Annexin V-FITC Apoptosis Detection Kit I with flow cytometry to evaluate apoptosis/cell death in 2ME2-treated TNBC cells. Cells treated with 2ME2 showed 9.2, 15.9, and 24.5% apoptotic death 24, 48, and 72 h after treatment, respectively (Figures 2E, F). To confirm this, we analyzed the regulators of apoptosis based on the expression levels of different apoptotic markers using immunoblotting analysis. Notably, 2ME2 treatment significantly increased the levels of cleaved PARP, cleaved caspase 3 and BAK but decreased the levels of Bcl-2 (Figures 2G, H). Finally, we investigated the effect of 2ME2 on Bak and cleaved caspase-3 expression using MDA-MB-231 cells, which demonstrated the activation of these apoptotic markers at all tested time points (Supplementary Figures 2C, D). This finding indicates that 2ME2 acts as an anti-cancer agent by inducing intrinsic mediated apoptosis in TNBC cells.

3.4 Estrogen metabolite 2ME2 disrupts global miRNome profiling

The specific effects of 2ME2 on TNBC miRNomes remain unknown. Therefore, in this study, we explored the miRNA disruptions. MDA-MB-468 cells treated with 2ME2 (5 µg for 24 h) were subjected to miRNome analysis. Over 1,000 mature miRNAs were studied using the miScript miRNA PCR array (miRNome V16). We identified the top 20 most upregulated and downregulated miRNAs (Figure 3A). We validated the differential expression of top 5 up- and down-regulated miRNAs using RTPCR in 2ME2 treated MDA-MB-468 (Supplementary Figures 3A, B) and MDA-MB-231 cells (Supplementary Figures 3C, D). Here, we aimed to identify the target genes of both the upregulated and downregulated miRNAs. In silico analysis was performed using the miRNet database to identify the miRNA target genes (Figures 3B, C). Moreover, we used the miRNet database to identify the signaling pathways associated with the genes altered by 2ME2 through miRNAs. KEGG pathway analysis demonstrated that the upregulated miRNA target genes were mainly involved in mTOR signaling, cancer-related, and p53 signaling pathways (Figure 3D). Downregulated miRNA target genes were also involved in cancer-related, p53 signaling, mitogen-activated protein kinase (MAPK) signaling, and apoptosis pathways (Figure 3E). Further, the impact of some of the differentially regulated miRNAs on overall survival was assessed using Kmplot and UALCAN, which demonstrated that the miRNAs upregulated (hsa-miR-600, hsa-miR-2117 & hsa-miR-3200)

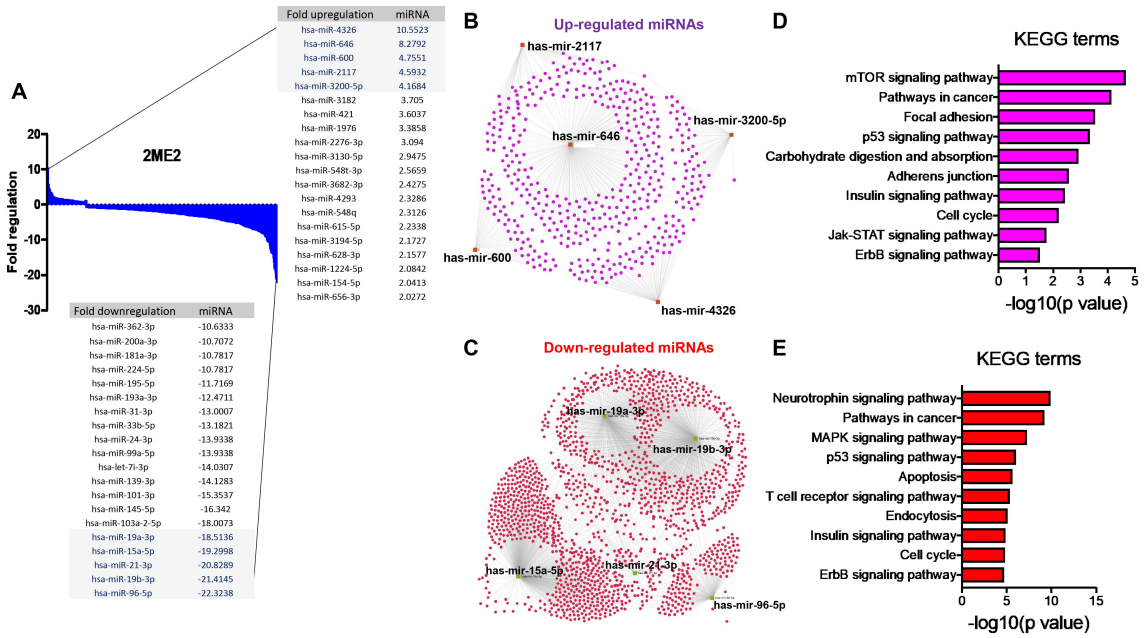

FIGURE 3
2ME2 influences global miRNome profile and network analysis identifies target genes. **(A)** Human miRNome profiling with 2ME2 treated MDA-MB-468 cells identified differentially regulated miRNAs. The 20 most upregulated and downregulated miRNAs were identified all which had a greater than 2 fold difference. **(B)** The top five upregulated miRNAs depicted in red with their target genes highlighted in purple. **(C)** The top five downregulated miRNAs are also depicted in green with their respective target genes highlighted in red. **(D, E)** The top 5 up and down-regulated miRNAs influences different signaling pathways via KEGG pathway analysis.

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and downregulated miRNAs (hsa-miR-96, hsa-miR-19a & hsa-miR-19b-2) by 2ME2 increased survival of triple negative breast cancer patients (Supplementary Figures 4A, B). These data demonstrate that 2ME2 effectively alters miRNome.

3.5 miRNA target genes are upregulated in human breast cancer

After identifying the miRNA target genes using the miRNet database, we uploaded the target genes to the Xena browser to study their expression patterns. Xena browser database enabled the exploration of gene expression directly using the human samples profiled from TCGA database. Then, the expression patterns of miRNA target genes from mTOR signaling, cancer signaling, focal adhesion, p53 signaling, carbohydrate digestion and absorption, insulin signaling, cell cycle, JAK-STAT, ErBb signaling, and adherens junction pathways were plotted (Figures 4A–E and Supplementary Figures 5A–E). Downregulated miRNA target genes were associated with neurotrophin signaling, cancer signaling, p53 signaling, MAPK signaling, apoptosis, T cell receptor signaling, endocytosis, insulin signaling, cell cycle, and ErBb signaling pathways (Figures 5A–D and Supplementary Figures 6A–F). *PIK3R1*, *HSP90AA1*, *ERBB2*, *CCND1*, and *PTPRF* were the target genes of the top five upregulated miRNAs (Figures 6A, B). Meanwhile, *CDC42*, *HSP90B1*, *CCND1*, *HSPA8*, *PRKAR1A*, *AKT1*, *RAB5B*, *FASN*, and *GRB2* were the target genes of the top downregulated miRNAs (Figure 6C). The expression of these genes were validated using RTqPCR. In general the all the target genes of up-regulated miRNAs were down regulated significantly by 2ME2 treatment in both TNBC cell lines. Interestingly, we also observed that some of the target genes of the down-regulated miRNAs were significantly upregulated (Supplementary Figures 7A-D). These genes have been shown to influence overall survival of breast cancer patients. When we analyzed combined effect of these up- and down-regulated genes on overall survival, we observed the expected trends but they were not statistically significant. (Figure 6D).

3.6 Signal transduction pathways altered by *2ME2* were identified using Reactome

Pathway topology analysis of miRNA target genes was performed using the Reactome pathway identifier database. In silico analysis enabled the identification of the differentially expressed genes mapped to different pathways. Interestingly, 2ME2 treatment led to the activation of various pathways, including the development, signal transduction, immune system, and vehicle-mediated transport pathways (Figure 7).

4 Discussion

2ME2 is an endogenous metabolite of estrogen. It is produced when 17 β-estradiol undergoes hydroxylation by cytochrome P450 1A1 to produce 2-hydroxyestradiol, followed by methylation by catechol-O-methyltransferase to produce 2-ME2. Compared with 17 β-estradiol, 2ME2 has a very low affinity (<500 fold) for ERα (56). Here, we demonstrated the anti-cancer effects of 2ME2 against

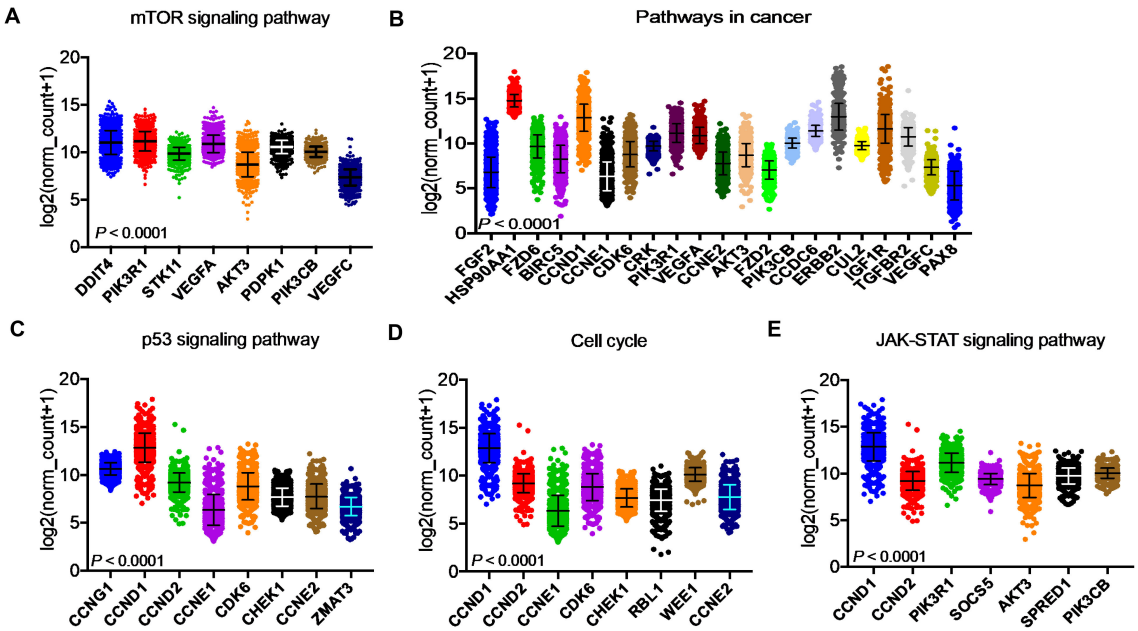

FIGURE 4
Up-regulated miRNA DEG targets. **(A-E)** miRNA target genes upregulated in breast cancers following 2ME2 treatment identified using Network analysis. KEGG analysis identified top miRNA direct target genes that are involved in different pathways. **(A)** mTOR signaling pathway, **(B)** pathways in cancer, **(C)** p53 signaling pathway, **(D)** cell cycle, and **(E)** JAK-STAT signaling pathway.

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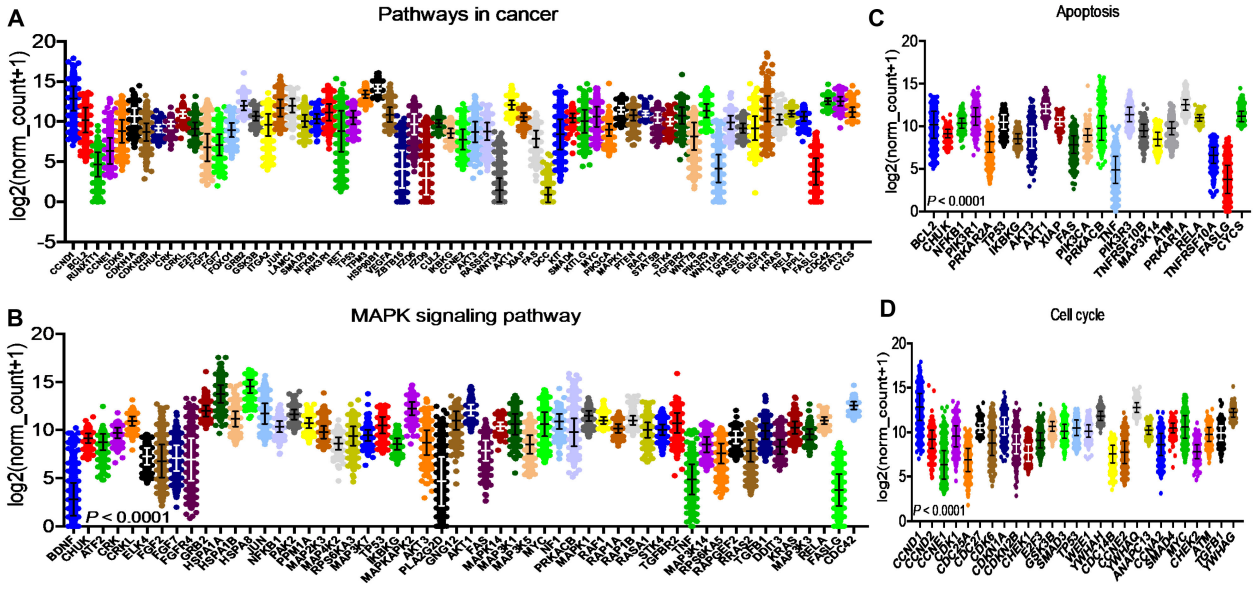

FIGURE 5
Down-regulated miRNA DEG targets. **(A-D)** miRNA target genes down regulated in breast cancers following 2ME2 treatment identified using Network analysis. KEGG analysis identified top miRNA direct target genes that are involved in different pathways. **(A)** pathways in cancer, **(B)** MAPK signaling pathway, **(C)** Apoptosis, and **(D)** cell cycle.

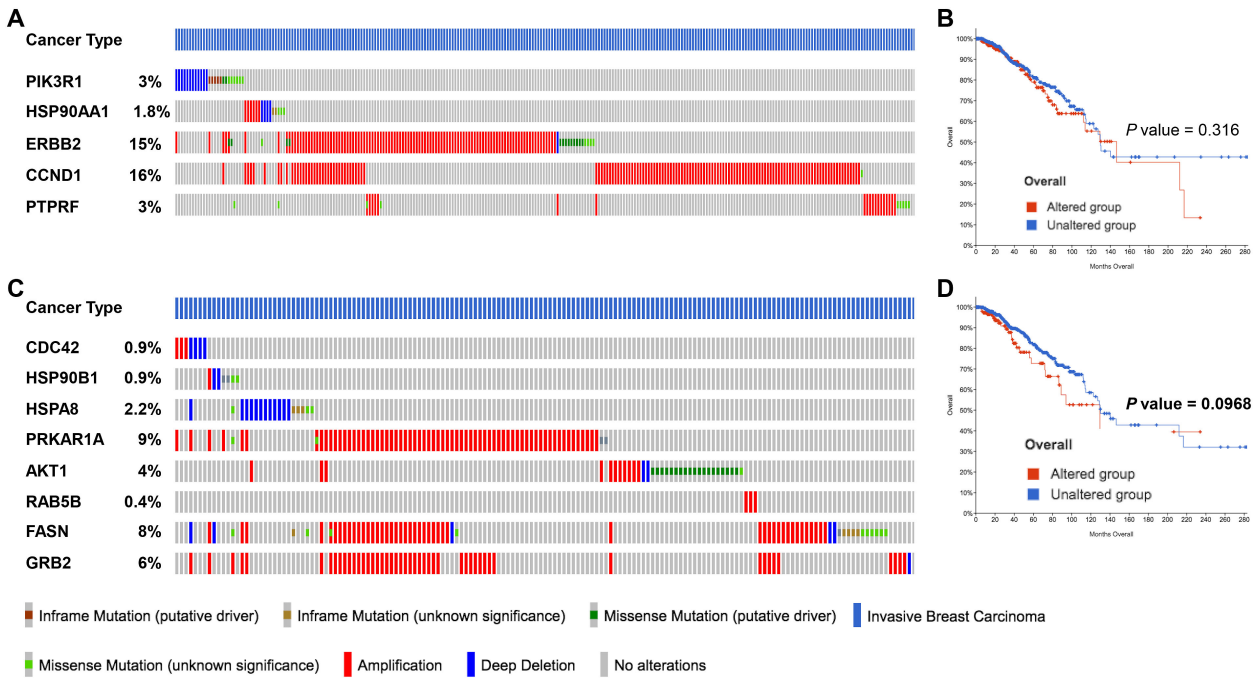

FIGURE 6
miRNA target genes altered by 2ME2 treatment are amplified in breast cancer patients. **(A, B)** Five target genes of up-regulated miRNA in 2ME2 treatment, **(A)** Oncoprint represents genetic alterations such as inframe mutations (putative driver and unknown significance), missense mutation (putative driver and unknown significance), amplification and deep deletion of breast cancer tumor samples. **(B)** KMPlot analysis shows cumulatively breast cancer patient samples that have high expression of miRNA target genes that are associated with overall survival. **(C, D)** Eight target gene of down-regulated miRNA in 2ME2 treatment, **(C)** Oncoprint represents genetic alterations such as inframe mutations (putative driver and unknown significance), missense mutation (putative driver and unknown significance), amplification and deep deletion of breast cancer tumor samples. **(D)** KMPlot analysis shows cumulatively breast cancer patient samples that have altered expression of miRNA target genes that are associated with overall survival. In KMPlot, unaltered means that there were no gene alterations in the queried genes within the selected profile of 2ME2 treated samples. Altered means that there was at least one gene altered in the queried genes within the selected profile of 2ME2 treated samples.

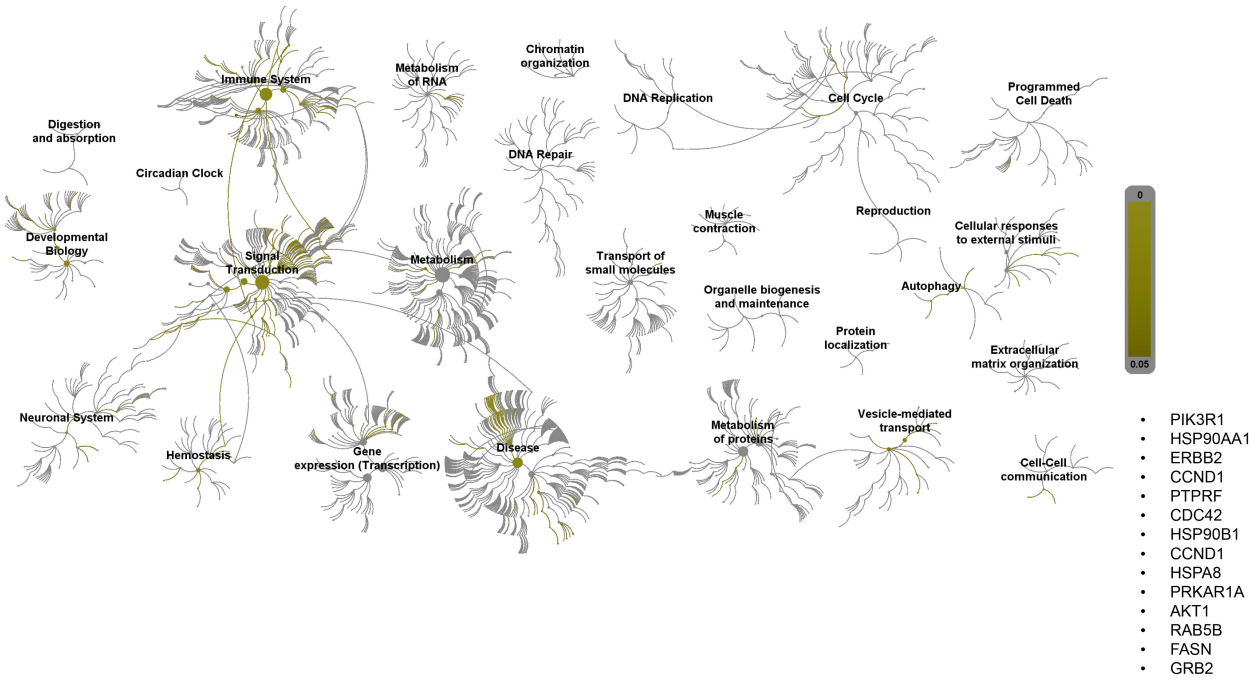

FIGURE 7
Reactome pathway-topology analysis of miRNA target genes. Reactome pathway analysis of the genes identified by KEGG term analysis. Target genes of miRNAs altered by 2ME2 treatment influenced various cellular pathways. Over-represented pathways are highlighted in yellow. All overexpressed pathways are from gene lists of formerly annotated and published signatures.

TNBC. The action of 2ME2 on normal and breast cancer cells is interesting because the production of 2ME2 from 2-hydroxyestradiol via O-methylation is associated with a reduced risk of breast cancer (12, 57). Metabolic pathways involved in the production of 4-hydroxyestradiol and 16α-hydroxyestrone increase the risk of breast and endometrial cancers (58, 59). Interestingly, our data demonstrated that estrogen metabolite 2ME2 exerts mild apoptotic effects on normal mammary cells in culture. In the past few years, many studies have focused on 2ME2 to better understand its anti-cancer properties and unique biological effects. Cellular and molecular action mechanisms of this estrogen metabolite indicate its potential as a chemotherapeutic drug for different types of human cancer (60, 61). Pharmacological concentrations of 2ME2 have been reported to exert strong anti-proliferative, anti-angiogenic, and apoptotic effects in several cancer models, leading to many phase I and II clinical trials (62). 2ME2 acts directly on the vascular endothelial cells, inhibiting their proliferation and migration and decreasing their drug resistance (63). In addition to its anti-angiogenic properties, we found that 2ME2 also exerts anti-proliferative effects and inhibits metastasis by suppressing proliferative markers, such as AKT and mTOR, and by reducing the migration, invasion, and colony formation of TNBC cell lines. Everolimus, an AKT/mTOR inhibitor, can only be used for the treatment of hormone receptor-positive and human epidermal growth factor receptor 2-negative metastatic breast cancer (64); however, 2ME2, which acted as an AKT/mTOR inhibitor in this study, can be used as a promising drug for TNBC treatment. Additionally, 2ME2 acts synergistically with other chemotherapeutic drugs to target breast cancer cells. When combined with doxorubicin, 2ME2 increases the sensitivity of doxorubicin-resistant breast cancer cells to doxorubicin by increasing the apoptosis and cell cycle arrest at the G1 phase in MCF-7 cells (65). When combined with paclitaxel (Taxol), 2ME2 increases centrosome decluttering and kills the MCF-7, MDA-MB-231, and SUM-149 breast cancer cells (66).

Various anti-cancer activities of 2ME2, including cell cycle regulation, have been proposed for different cancers. It acts on pancreatic cancer by prolonging the cell cycle events during the S phase and inducing apoptosis (67). In TNBC cell lines, 2ME2 treatment arrests the cells in the S-phase, in which DNA replication, single strand breaks (SSBs), and repair occurs. PARP, which repairs SSBs (68) is inhibited by 2ME2, thereby increasing the apoptosis and inhibiting the excessive growth of TNBC cell lines. Various mechanisms of action of 2ME2 have been reported for the growth control of different cancers, including breast cancer. To the best of our knowledge, this is the first study to reveal that 2ME2 treatment affects the global miRNA profile in TNBC. miRNAs play key roles in human diseases, particularly cancer. miRNAs are differentially expressed, acting as either oncogenes or tumor suppressors in cancer cells (35). miRNAs regulate the functions of many genes, and a single miRNA can control the functions of different genes. We identified many signaling pathways that were upregulated or downregulated in 2ME2-treated MDA-MB-468 TNBC cell line. Analysis of the gene targets of the upregulated and downregulated miRNAs revealed that these genes were mostly involved in signal transduction and immune pathways. Cyclin D1 is a cell cycle regulator that is overexpressed in breast cancer (69). Here, cyclin D1 and its regulator, CDK6, were found to be the top

target genes of both upregulated and downregulated miRNAs. It is well-known that miRNAs regulate gene expression both negatively and positively by direct inhibition or indirectly through other regulators of gene expression. Our data indicates that the expression of cyclin D1 and CDK6 are tightly regulated by various miRNAs.

Mechanisms underlying the anti-cancer effects of 2ME2 are complex, warranting further investigation. A limitation of the current study is that the specific molecular mechanisms are not identified. In this study, miRNome analysis revealed many pathways are affected by 2ME2 treatment. Our findings also revealed the miRNAs control key pathways in cancer and their target genes. Further, our results are expected to provide the basis to further elucidate the specific mechanisms by which 2ME2 inhibits breast cancer and also could lead to the development of 2ME2 as a promising therapeutic drug for TNBC.

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 for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

RS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, Validation, Visualization. AC: Writing – original draft, Writing – review & editing. DP: Writing – original draft, Writing – review & editing. SP: Writing – original draft, Writing – review & editing. PR: Writing – original draft, Writing – review & editing. JA: Writing – original draft, Writing – review & editing. EP: Writing – original draft, Writing – review & editing. RL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1371792/full#supplementary-material

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Potential therapies for non-coding RNAs in breast cancer

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Breast cancer (BC) is one of the frequent tumors that seriously endanger the physical and mental well-being in women with strong heterogeneity, and its pathogenesis involves multiple risk factors. Depending on the type of BC, hormonal therapy, targeted therapy, and immunotherapy are the current systemic treatment options along with conventional chemotherapy. Despite significant progress in understanding BC pathogenesis and therapeutic options, there is still a need to identify new therapeutic targets and develop more effective treatments. According to recent sequencing and profiling studies, non-coding (nc) RNAs genes are deregulated in human cancers via deletion, amplification, abnormal epigenetic, or transcriptional regulation, and similarly, the expression of many ncRNAs is altered in breast cancer cell lines and tissues. The ability of single ncRNAs to regulate the expression of multiple downstream gene targets and related pathways provides a theoretical basis for studying them for cancer therapeutic drug development and targeted delivery. Therefore, it is far-reaching to explore the role of ncRNAs in tumor development and their potential as therapeutic targets. Here, our review outlines the potential of two major ncRNAs, long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) as diagnostic and prognostic biomarkers as well as targets for new therapeutic strategies in breast cancer.

KEYWORDS

non-coding RNA, breast cancer, ncRNA therapy, long non-coding RNA, RNA interference

1 Introduction

Human Genome Project information shows that > 90% of the genome is transcribed, however, only about 2% of the genome is translated, and non-coding (nc) RNAs make up 98% of the total RNAs. Compared to protein-coding genes, a growing number of studies have shown that ncRNAs play key roles in a variety of biological processes such as transcription, post-transcriptional modification, chromatin remodeling and signal

transduction (1). Abnormal expression levels of many genes involved in breast cancer (BC) development are presumably influenced by ncRNA activity (2). In addition, ncRNAs can be therapeutically targeted and the delivery of ncRNAs can be based on the existing basis of RNAi and oligonucleotide delivery for targeting protein-coding mRNAs (3, 4). Therefore, understanding specific ncRNA signatures can help to understand the complex BC cellular mechanisms and facilitate research advances in the diagnosis and treatment of BC subtypes.

The two main classes of ncRNAs are the well-studied short microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). LncRNA usually has more than 200 nucleotides, while miRNA has 19-28 nucleotides (5). Small RNA species in the usual sense, including miRNAs, piwi-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs), which interact with Argonaute proteins (Ago proteins) to mediate RNA silencing effects (6). Whereas lncRNAs positively or negatively regulate BC cell multiplication, invasion, metastasis and stemness properties by regulating the expression of miRNAs or transcription factors (7). Dysregulation of both types of transcripts has been associated with every cancer studied to date and affects all major cancer hallmarks. A variety of RNA-based therapeutics have been developed, including antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), ASO-anti-microRNAs (antimiRs), miRNA mimics, miRNA sponges, therapeutic circular RNAs (circRNAs), and CRISPR-Cas9-based gene editing, and there are several excellent reviews describing these drugs (8, 9). In breast cancer therapy, ncRNAs can be used as potential therapeutic targets by designing specific siRNAs or miRNAs to inhibit tumor-promoting ncRNAs or by overexpressing certain tumor-suppressing mRNAs to treat breast cancer. The application of nanodrug delivery systems in breast cancer therapy has also shown great potential, for which promising nanodelivery/nanoparticle-based approaches have been developed using multiple molecules for systemic drug delivery and improved targeted delivery of tumor ncRNAs with reduced side effects. Despite the promise of ncRNA therapeutics, challenges such as complexity and diversity, stability issues, delivery systems, specificity, and more in-depth research need to be overcome to achieve clinically applicable applications. One of the greatest challenges in the field today is to illuminate the multiple functions and mechanisms of action of ncRNA, which is critical for determining its clinical relevance and developing its potential use as a biomarker or therapeutic target (10).

2 lncRNA

2.1 Biological functions of lncRNAs

LncRNA is a heterogeneous set of non-protein-coding transcripts greater than 200 nucleotides in length (11). Similar in biogenesis to mRNAs, lncRNAs are transcribed by RNA polymerase II and have conserved secondary structures, many of which are spliced, capped, and polyadenylated. The complexity of these transcripts arises from their multifaceted 3D structures, which change rapidly and give them the ability to perform multiple functions (12). Depending on the relative position of lncRNAs to protein-coding genes in the genome, they can be categorized as positive lncRNAs, antisense lncRNAs, bidirectional lncRNAs, intronic lncRNAs, intergenic lncRNAs, and enhancer lncRNAs (13). LncRNAs have been found to be involved in a variety of physiological and pathological cellular activities, such as adipogenesis, inflammation, cellular differentiation, and tumorigenesis, by interacting with chromatin, proteins, and RNAs in the nucleus or cytoplasm, and through genomic expression regulation in cis or trans, epigenetic modification, and post-transcriptional modulation (14–16). In the nucleus, lncRNAs can modify gene expression by interacting directly with DNA or chromatin regulators such as transcription factors and RNA-binding proteins, acting as enhancers, decoys, scaffolds, or guides. In the cytoplasm, lncRNAs decay mRNAs, regulate mRNA stability or translation, compete with miRNAs for binding mRNAs, and can be processed into miRNAs (17). Some of the potential therapeutic targets of lncRNAs are summarized in Table 1. Growing evidence suggests that lncRNAs play an important role in a variety of cellular processes such as proliferation, apoptosis, treatment resistance and metastasis in human cancers (18–20).

2.2 lncRNA therapy in breast cancer

2.2.1 Targeted therapies

Programmed cell death ligand 1 (PD-L1), an immune checkpoint protein frequently expressed in human cancers, promotes immune escape by binding to PD-1 on activated T cells (21). Some lncRNAs act specifically in cancer cells by regulating antigen presentation or PD-L1 expression. Lin et al. (22) found that lncRNA, a HIF-1α inhibitor at the translation level (HITT), coordinated with the regulator of G protein signal transduction 2 (RGS2), binds to the 5 'UTR of PD-L1 under IFN-γ stimulation, resulting in reduced PD-L1 translation. In human breast cancer, HITT/RGS2 was negatively correlated with PD-L1 expression, suggesting that HITT may suppress PD-L1 expression *in vivo*. The metastasis-associated lung adenocarcinoma transcript (Malat1) is an abundant lncRNA, and many studies have shown that its expression is upregulated in a variety of cancers (23–26). Using the TNBC cell line MDA-MB-231, Samir and colleagues demonstrated that miR-182-5p, in additon to down-regulating the expression of the tumor suppressor gene XIST in the same cells, can act as an oncomiR by promoting the up-regulation of oncogenic PD-L1 and the lncRNA MALAT1 (27). In addition to immunotherapy targeting immune checkpoint molecules, in a study of TNBC, Adewunmi et al. (28) found that Malat1 inhibition resulted in delayed primary tumor growth in macrophage-rich T12 tumor subtypes and neutrophil-rich 2208 L tumors and a significant reduction in tumor volume in both models after 14 days of treatment. By using Malat1 ASO, they found that they were able to knock down Malat1 RNA expression, which delayed primary tumor growth, decreased proliferation, and

TABLE 1 Potential therapeutic targets of lncRNAs in breast cancer.

lncRNA	Cell	Target	Function	Reference
TYMSOS	MCF-7、MDA-MB-231	High expression upregulates CBX3 and promotes CBX3-mediated repression of ULBP3 transcription	Inhibits NK cell cytotoxicity and promotes metastasis and immune escape	(104)
NKILA	MUC1+breast cancer tissues	High expression downregulates NF-κB	Contributes to the AICD sensitivity of tumor-antigen-activated CTLs	(105)
GATA3-AS1	TNBC	High expression and up-regulation of COPS5 promotes PD-L1 deubiquitination	Promotes immune escape of TNBC cells	(106)
TINCR	UACC812、MDA-MB-231	Acts as a molecular sponge for miR-199a-5p and upregulates the stability of USP20 mRNA, thereby promoting PD-L1 expression by inhibiting its ubiquitination	Induction of immune escape and promotion of disease progression in breast cancer	(107)
SNHG16	MCF-7、T-47D、MDA-MB-231、MDA-MB-468	Acts as a molecular sponge for miR-16-5p and enhances the TGF-β1/SMAD5 pathway to upregulate CD73 levels	Enhancement of effective immunosuppression by CD73 +Vδ1 T cells	(108)
lncRNA Xist	MCF-7	Competes as a molecular sponge for miR-101 to regulate C/EBPα and KLF6	Promotion of Xist Expression in M1 Macrophages and Inhibition of miR-101 Expression in M2 Macrophages Inhibit Mammary Proliferation and Migratory Capacity	(109)
lncRNA-p21	4T1	High expression of targeted p53 eliminated MDM2 degradation to p53	Knockdown reverses the phenotype of TAMs and produces TNF-α to kill tumor cells, thus exerting anti-tumor functions	(110)
MALAT1	SK-BR-3	Targeting miR-485-3p to downregulate P-gp and Bcl-2 and upregulate Bax	Oncogenic and tumor-suppressive roles	(111)

increased apoptosis. Also in a breast cancer model, Kumar et al. (29) found that deletion of Malat1 activates T cells and kills early metastatic cells, and its absence inhibits metastatic reactivation and restores dormancy. The use of Malat1 Gapmer locked nucleic acid (LNA) ASOs sufficiently inhibited the expression of Malat1 and Serpinb6b, significantly reducing lung colonization. This effect was associated with increased CD4+ and CD8+ T cell infiltration in micrometastases and decreased recruitment of Ly6G+ neutrophils. Therefore, targeting Malat1 may be a potential therapeutic avenue for the treatment of metastatic breast cancer.

Interferons have recently returned to the forefront of tumor biology research. Tumor cell response to conventional therapy is regulated by activation of the IFN pathway. In addition to cytotoxic drugs, blocking growth signaling pathways (such as EGFR and HER2 pathways) relies on IFN signal transduction. Zhang et al. (30) showed that blockade of IFN receptor 1 (IFNAR1) impaired the therapeutic effect of anti-HER2 monoclonal antibodies. LINC00624 promotes ADAR1 (adenosine deaminase RNA specific 1) RNA editing ability by binding to ADAR1. Expression of ADAR1, an A-to-I RNA editing protein that inhibits innate immune responses and is associated with the regulation of type I IFN responses, further inhibits IFN-induced expression of IFN-stimulated genes (ISGs), which stimulate antigen-presentation pathways, thereby recruiting immune cells and facilitating antiviral responses. Through mouse experiments, they found that xenograft tumors treated with ASO exhibited reduced ADAR expression. In addition, the expression levels of ISGs and innate immune response genes were significantly enhanced in ASO-treated xenograft tumors. Therefore, targeting LINC00624 by ASO significantly inhibited tumor cell proliferation, suppressed ADAR1 activity and promoted type I IFN response. In addition to, a study (31) found that IFN induced the expression of cytoplasmic lncRNA IFN-responsive nuclear factor-κB activator (IRENA) in macrophages, which triggered nuclear factor-κB signaling through dimerization of protein kinase R, followed by an increase in the production of pro-tumor inflammatory cytokines. The specificity of IRENA lncRNA expression in TAMs and its induction under chemotherapy make it a promising therapeutic site for avoiding chemoresistance and inhibiting cancer progression. The immune function of cancer-promoting and cancer-suppressing lncRNAs suggests that lncRNAs can be involved in regulating the crosstalk between tumors and immune cells during cancer onset and progression (Figure 1).

2.2.2 lncRNA therapy in breast cancer chemoresistance

Development of therapeutic resistance and metastasis as major issues in breast cancer treatment (32). LncRNAs are dysregulated in various malignancies and interact with multiple RNAs and proteins to influence drug resistance. LncRNA DIO3OS has been found to be upregulated in breast cancer patients treated with aromatase inhibitors (AI). The mechanism of action of DIO3OS includes its interaction with polypyrimidine bundle-binding protein 1 (PTBP1), which stabilizes mRNA for lactate dehydrogenase A (LDHA), thereby upregulating LDHA expression and promoting glycolytic metabolism. That is, DIO3OS enhances aerobic glycolysis by regulating the splicing switch, thereby conferring a growth advantage to AI-resistant cells. Therefore, inhibition of LDHA activity by exploring DIO3OS knockdown approaches could re-sensitize breast tumor cells to anti-

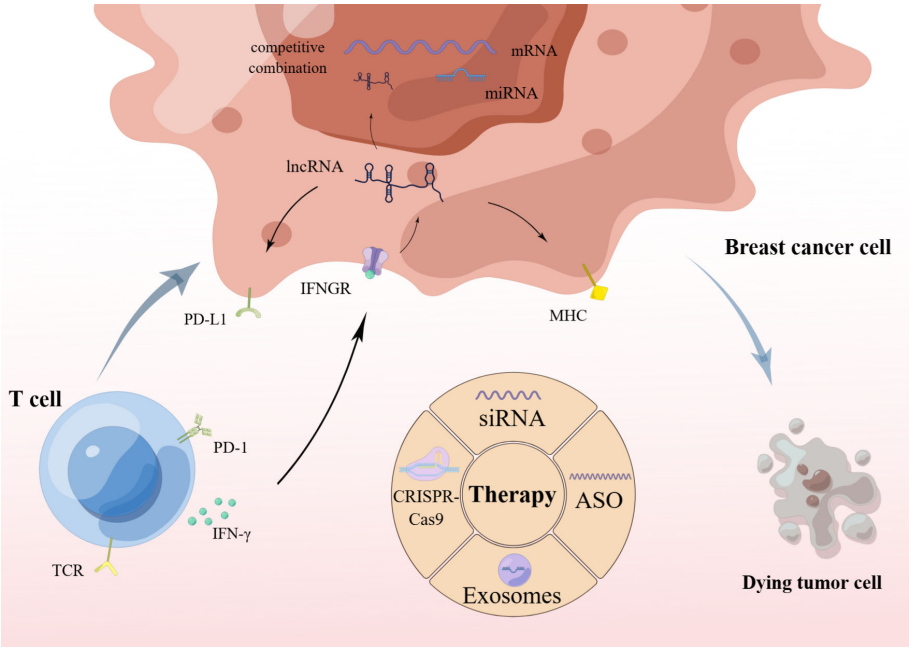

FIGURE 1
lncRNA functions and therapies.

HER2 therapies (trastuzumab) or chemotherapies (paclitaxel) as a breast cancer treatment target (33).

In a study for the treatment of paclitaxel-resistant breast cancer, it was observed that LINC00115 was strongly upregulated in paclitaxel-resistant BCSC, and that LINC00115 acted as an RNA linker recruiting the SETDB1/PLK3 complex to activate the HIF1α signaling pathway (34). SETDB1 is an oncogene in breast cancer and play an important role in the treatment of endocrine therapy resistance (35, 36). Methylation of PLK3 leads to failure of HIF1α phosphorylation, which promotes HIF1α protein stability by inhibiting its ubiquitinated degradation pathway. HIF1 can enhance the stability of LINC00115 in turn, and this feedback loop further enhances BCSC characteristics, thereby promoting chemotherapy resistance and metastasis in breast cancer. Thus, inhibition of LINC00115 in combination with SETDB1 inhibitors significantly improved the efficiency of paclitaxel chemotherapy in an animal xenograft model of breast cancer metastasis.

Chen et al. (37) found that LINC02568 regulates estrogen/estrogen receptor-induced transcriptional activation of target genes in the cytoplasm by competitively binding miR-1233-5p to the estrogen receptor ESR1 mRNA itself, thereby trans-regulating the stability of ESR1 mRNA. LINC02568 is involved in transcriptional activation of neighboring genes CA12 by cis regulation in the nucleus, thereby participating in the maintenance of specific pH inside and outside tumor cells. LINC02568 is involved in the transcriptional activation of the neighboring gene CA12 in the nucleus through cis-regulation, which in turn is involved in the maintenance of specific pH inside and outside the tumor cell. ASO targeting LINC02568 significantly inhibited the growth and tumor formation of estrogen receptor-positive breast cancer cells and restored the sensitivity of tamoxifen-resistant breast cancer cells to tamoxifen. Therefore, the combination of ASO targeting LINC02568 and endocrine drugs or CA12 inhibitors has a synergistic effect on tumor growth inhibition.

LINC00460 was observed to be significantly elevated in doxorubicin-resistant breast cancer cells, and LINC00460, together with FUS, promotes MYC expression by influencing the efficiency of intron removal during mRNA maturation. Conversely, LINC00460 transcription is directly activated by c-MYC and forms a positive feedback loop in breast cancer cells, driving resistance to tamoxifen. The simultaneous depletion of LINC00460 and c-MYC inhibition remarkably re-sensitized ADR cells to Doxorubicin. In this context, Yang et al. (38) further suggested simultaneous antagonism of LINC00460 and c-MYC, which presumably efficiently abrogated the positive feedback loop and may represent a promising novel approach to improve therapeutic outcomes for patients with acquired resistance to Doxorubicin therapies. In another study of adriamycin resistance in breast cancer, Liu et al. (39) found that lncRNA aspartate-trna synthetase-antisense RNA 1 (DARS-AS1) was overexpressed in TNBC, and its silence effectively inhibited tumor growth and liver metastasis. They constructed a TNBC-specific natural nanomedicine delivery system, EXOs-CL4, which was loaded with DARS-AS1 siRNA and DOX (DARS-AS1 siRNA/DOX@EXOs-CL4) that synergistically inhibited tumor growth, metastasis, and anti-apoptotic effects (40). Resistance-causing lncRNAs can be used to develop new targeted and tailored therapies, providing a new approach to introducing promising personalized treatment modalities to overcome chemotherapy resistance in breast cancer patients.

3 miRNA

3.1 Biological functions of miRNAs

MiRNAs were the first to be discovered and analyzed in cancer (41, 42). MiRNA biogenesis is a multistep process: first, miRNAs are transcribed into pri-miRNAs by RNA polymerase II; second, pre-miRNAs are exported into the cytoplasm via exportin 5 (XPO5) after processing by the nuclear ribonuclease Drosha complex and DGCR8; Third, mature double-stranded miRNAs are generated and loaded into the rna-induced silencing complex (RISC) by processing mediated by the RNase III enzyme Dicer and TAR RNA binding protein 2 (TARBP2) (43–45). MiRNA gene expression usually occurs post-transcriptionally, an effect known as gene silencing, which is established primarily through mRNA cutting, translational repression, or DNA methylation. These molecules participate in the post-transcriptional repression of specific gene expression by binding to the 3' untranslated region of the target mRNA, a process that requires the miRNA to bind to Ago proteins, which are the core components of RISC. Once loaded onto Ago proteins, miRNAs can direct RISCs to reach complementary target mRNAs for translational repression or mRNA degradation (46). However, this mechanism is not exclusive; binding of miRNAs to the 5'-UTR is also possible and induces activation or repression of translation (47, 48). MiRNAs play a crucial role in regulating transcription and post-transcriptional gene expression by specifically interacting with target mRNAs. Moreover, miRNAs can target multiple genes through a single pathway. For example, the miR-15-miR-16 cluster down-regulates several anti-apoptotic factors including BCL-2 and MCL1 (49). Thus, therapies using miRNAs or targeting miRNAs also have the potential to improve the efficacy of treatments compared to siRNAs or ASOs that can affect only a single target gene. Clinical trials of drugs based on miRNA-targeting ncRNAs have already begun, whether it is therapy to increase or decrease target miRNA, and they are being used in cancer treatment (50–52). Some miRNA markers in breast cancer are summarized in Figure 2. Although this strategy has shown great utility as an experimental tool, miRNA therapy has not yet been applied in the clinical treatment of breast cancer.

3.2 miRNA-targeted therapy in breast cancer

Circulating extracellular vesicle (EV)-derived miRNAs are now recognized as next-generation cancer "therapeutic diagnostic" tools with strong clinical relevance (53). Exosomes are involved in the transferring of miRNAs from donor cells to adjacent cells, acting as messengers between tumoral and stromal cells (54). Exosomes derived from cancer cells are not only enriched in miRNAs, but also contain a complete miRNA cargo, including pre-miRNAs, proteins involved in miRNA biogenesis and function, such as RISC loading complex (RLC), Dicer, trans-activating response element RNA-binding protein (TRBP), and AGO2, and thus pre-miRNAs can be processed to produce mature miRNAs (55, 56). Exosomes containing miRNA are taken up through receptor-ligand interaction, and subsequently regulate gene expression in recipient cells (57).

As one of the most prevalent and important post-translational modifications, ubiquitination is involved in multiple cancer-related pathways (58). Deubiquitinating enzymes (DUB) are involved in cancer regulatory processes by regulating ubiquitination. It was shown that miR-500a-5p was highly expressed in MDA-MB-231 and MCF7 cells treated with cancer-associated fibroblast (CAF)-derived exosomes. Upregulation of miR-500a-5p was also confirmed in CAF and CAF-derived exosomes. MiR-500a-5p is transferred from CAF to cancer cells and subsequently promotes proliferation and metastasis by binding to ubiquitin specific peptidase 28 (USP28). MiR-500a-5p promotes breast cancer progression and metastasis by sponging USP28 (59).

Yang et al. (60) found that exosomes produced by BC cells after stimulation with DOX or PTX delivered miR-378a-3p and miR-378d to neighboring cells to activate the WNT and NOTCH stemness pathways and induce resistance by targeting Dickkopf 3 (DKK3) and NUMB. In addition, chemotherapy activated the EZH2/STAT3 pathway in tumor cells, resulting in elevated levels of miR-378a-3p and miR-378d in cells and exosomes. More importantly, the combination of chemotherapeutic agents with the EZH2 inhibitor tazemetostat reversed chemotherapy-induced exosome-induced resistance in a tumor xenograft model in nude mice.

In addition to this, exosomes secreted by breast cancer cells deliver miR-148-3p, miR-520b, and miR-138-5p to target macrophages to induce M2 polarization, thereby promoting tumor growth (Figure 3). Thus, we can use exosomes to deliver antagonist tumor suppressor miRNAs for cancer therapy. In addition, removing exosomes from the circulatory system, or preventing the fusion/uptake of exosomes by target cells, can be used as therapeutic strategies to inhibit tumorigenesis. It can also be isolated from the patient's circulatory system, modified, and relocated to the same patient for cancer treatment (61–63).

miRNA antagonists (antagomiRs) are synthetic oligonucleotides that target and antagonize oncogenic miRNAs of similar length. miRNA sponges are synthetic nucleotide structures that act similarly to antagomiRs in that they capture oncogenic miRNAs in the cell and impair their function. Transfection of metastasis-associated miRNA-10b overexpressing MDA-MB-231 cells with miRNA-10b-sponges resulted in decreased cell growth, migration, and invasion. MiRNA-10b overturning by miRNA-10b-sponges has been demonstrated to upregulate HOXD10, thereby inhibiting BC metastasis (64).

MiR-378 was downregulated in tamoxifen-resistant as well as chronically estrogen-deprived MCF7 cells (65). MiR-378 is growth inhibitory in ER-positive breast cancer and Arabkari et al. found that XBP1 (a transcription factor) was able to down-regulate the expression of miR-378 and PARGC1B (the host gene for miR-378) during UPR (a cellular stress response pathway involved in the maintenance of protein homeostasis in the endoplasmic reticulum). As a result their development of ORIN1001, an IRE1 inhibitor that blocks XBP1 production, is being evaluated for efficacy in a phase 1 trial in patients with advanced solid tumors or recurrent refractory metastatic breast cancer (66). A brief summary of potential therapeutic targets of miRNAs in breast cancer were shown in Table 2.

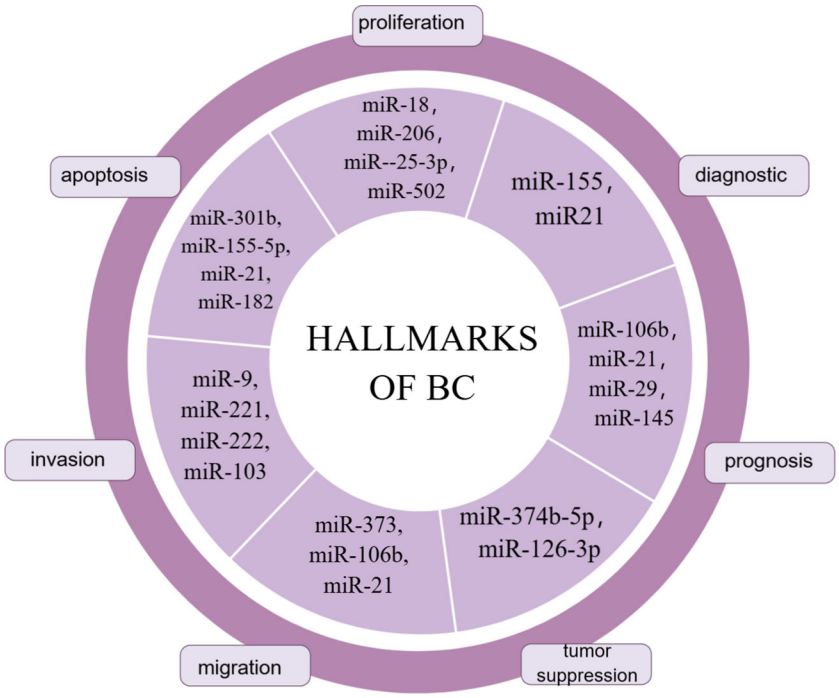

FIGURE 2
miRNA markers in breast cancer.

3.3 Targeted delivery during miRNA therapy

Overexpression of some miRNAs that act as oncogenes in tumors may reduce the ability of tumor cells to undergo EMT, invasion and metastasis. However, delivery in cells remains the most important barrier to the use of miRNAs as therapeutic agents (67, 68). Significant down-regulation of miR-206 levels targeting NOTCH 3 has been reported in breast cancer cells compared to normal breast cells (69, 70). Chaudhari et al. (71) showed decreased expression of NOTCH 3 using up-regulation of miR-206 mimics by gold nanocomplexes, and miR-206 administered via gold nanocomplexes in MCF-7 cells was able to block cell proliferation, induce G0-G1 cell arrest, and alter mitochondrial membrane potential. Garrido et al. (72) used mesoporous silica nanoparticles to deliver miR-200c-3p for breast cancer therapy. MiR-200c-3p is a well-known tumor suppressor miRNA that inhibits tumor progression and metastasis in breast cancer by downregulating ZEB1 and ZEB2. They demonstrate that nanoparticles loaded with miR-200c-3p are a potential strategy for breast cancer therapy and a safe and effective system for tumor-targeted delivery of miRNAs.

In addition to delivering oncogenic miRNA factors, another study found that breast tumor cells induce miRNA (miR-182) expression in macrophages, and miR-182 promotes selective activation of macrophages to drive tumor development. Importantly, they found that loading miR-182 inhibitors using cationic mannan-modified extracellular vesicles and delivering the inhibitors specifically into macrophages effectively inhibited macrophage alternative activation and suppressed breast tumor development (73). Kardani et al. (74) inhibited miR-155 by designing a nanocarrier containing gold nanoparticles, antagomir-155, and a nuclear protein-specific aptamer. they reported a dramatic decrease in miR-155 mRNA levels and an increase in the levels of TP53INP1 mRNA, which is a direct target protein of miR-155.

Another promising delivery system to transport miRNA is the use of exosomes. The use of exosomes as delivery vectors for miRNAs may be effective in overcoming miRNA degradation *in vivo*, as exosomes can efficiently cross biological vectors and maintain communication with target cells. The biogenesis and targeting mechanisms of exosomes suggest that exosomes can optimize the expression of specific endogenous miRNAs and promote the regulation of multiple physiological mechanisms, including apoptosis in cancer cells (75). Nie et al. (76) found that once loaded with microRNA molecules in the exosome carriers, the resulting, miRNA-126 loaded 231-Exo (miRNA-231-Exo) strongly suppressed A549 lung cancer cell proliferation and migration through the interruption of the PTEN/PI3K/AKT signaling pathway. In addition, miRNA-126-loaded exosomes produced a potent lung homing effect in mice after intravenous administration of miRNA-126-loaded exosomes.

4 Potential therapies for other non-coding RNAs in breast cancer

CircRNAs act as miRNA sponges to regulate endocrine resistance (77, 78). Xia et al. (79) found that miR-217 expression was reduced, while G3BP2 was overexpressed in BC tissues. G3BP2 was verified as a direct target of miR-217 by luciferase assay. Inhibition of G3BP2 expression inhibits cell migration of BC

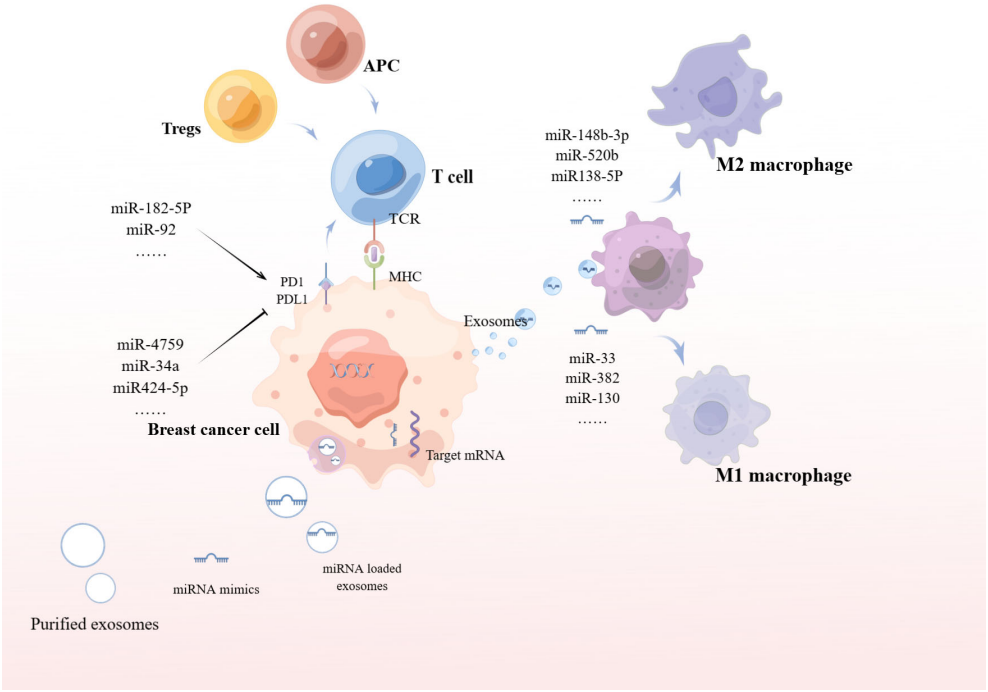

FIGURE 3
miRNA affects tumor cell immune function.

TABLE 2 Potential therapeutic targets of miRNAs in breast cancer.

miRNA	Cell	Target	Function	Reference
miR-500a-5p	CAF (exosome)、MDA-MB-231、MCF7	Acts as a molecular sponge for USP28	Promotes cell proliferation, migration, invasion, and EMT in breast cancer cells	(59)
miR-378a-3p、miR-378d	BC cells stimulated with DOX or PTX	Regulates the WNT/β-catenin and Notch stemness pathways by targeting NUMB and DKK3	Induces chemoresistance	(60)
miR-206	MCF-7	Blocks the G1/S transition by targeting cyclin D2	Inhibites cell growth, migration and invasion and on down regulation causes apoptosis functions as a tumor suppressor	(71)
miR-200c-3p	MDA-MB-231、4T1	Inhibits the expression of *ZEB1* and *ZEB2*	Decreases tumor cell proliferation and induces cell cycle arrest in G2	(72)
miR-182	Py8119、TAM	Directly suppresses *TLR4*, leading to NFκb inactivation and M2 polarization of TAMs	Promotes macrophage alternative activation to drive tumor development	(73)
miR-378	MCF-7	Upregulates the type I interferon signalling pathway	Suppresses cell growth, colony formation, and migration of ER-positive breast cancer cells	(66)
miR-497/195	ER+ breast cancer cell	Reduced expression resulted in increased levels of miR-497/195 target genes (AKT3, BCL2, MAP2K1, RAF1, and CCND1), which activated the of PI3K-AKT signaling	Induces endocrine resistance and tamoxifen resistance	(112)
miR-142-3p	Different grade of breast cancer	High expression downregulates HMGA2 mRNA and protein levels inhibits the ERK/AKT/STAT3 signaling pathways	Induces apoptosis and G2/M cell cycle arrest in breast cancer cells and decreases cell proliferation	(113)
miR-18b	MDA-MB-231、MCF-7	Specifically bind to the 3'UTR of Transcription Elongation Factor A Like 7 (TCEAL7), and activate nuclear factor-kappa B (NF-κB)	Induces epithelial-mesenchymal transition (EMT) and promoting cell invasion and metastasis	(114)

(Continued)

TABLE 2 Continued

miRNA	Cell	Target	Function	Reference
miR-485-5p	MCF-7	Low expression upregulates MUC1 by binding to the 3'-UTR region	Promotes the proliferation, invasion and migration of breast cancer cells	(115)
miR-183	MCF-7、MDA-MB-231	High expression downregulates PTEN thereby regulating PI3K/AKT signaling path	Increases cell viability, accelerates cell cycle progression, and induces further migration of BC cell lines	(116)
miR-375	MDA-MB-231、MCF-7、MCF-7/ADR	Low expression upregulates JAK2 expression and its downstream effector P-STAT3 expression	MiR-375 stable overexpression via lentivirus infection reduces the stemness and decreases adriamycin resistance of breast cancer cells	(117)
miR-361-3p	MDA-MB-231、T47D	High expression decreases the expression of P73 by targeting E2F1	Promotes proliferation and inhibites apoptosis of breast cancer cells	(118)
miR-132-3p	MDA-MB-231、T47D	Low expression directly binds to the 3′UTR of the LAPTM4B gene and functiones at the post-transcriptional level thereby regulating the PI3K-AKT-mTOR signaling pathway	MiR-132-3p significantly represses cell viability, colony formation, migration and invasion of breast cancer cells	(119)

cells. Paclitaxel-induced exosome circBACH1 regulates BC cell stemness and migration by sponging miR-217 to upregulate G3BP expression, which provides a new therapeutic target for paclitaxel resistance and BC progression through the circBACH1/miR-217/G3BP2 axis. Multiple studies have demonstrated that miR-204-5p is down-regulated in breast cancer patients and MCF-7 cells (80–82). Jiang et al. (83) demonstrated that circRHOT1 acts as a sponge for miR-204-5p and promotes breast cancer cell invasion and epithelial-mesenchymal transition (EMT). They found that miR-204-5p targets the protein arginine methyltransferase 5 (PRMT5) and shows an opposite expression pattern, and thus reversed EMT by overexpressing PRMT5 to reverse the effects of circRHOT1 knockdown on cell growth, apoptosis, wound healing, and cell invasion, as well as on the expression of E-calcineurin, N-calcineurin, and poikilodulin.

SiRNA is an RNAi tool with the ability to inhibit target genes. Li et al. (84) developed an endosomal pH-responsive nanoparticle that carried Rac1 siRNA along with cisplatin, which resulted in efficient delivery of Rac1 targeting oligonucleotides and cisplatin in breast tumors and showed promising synergistic antitumor effects. Wu et al. (85) used lipid-coated calcium phosphate nanoparticles to inhibit PD-1 and PD-L1. This allows the siRNA to efficiently enter the MCF-7 BC cell line and subsequently inhibit the PD 1 receptor and ligand.

The clustered regulated interspaced short palindromic repeats (CRISPR)/Cas9 system is emerging as a powerful tool for precision medicine as a revolutionary and viable genome editing tool (86–88). Mao et al. (89) targeted EZH2 with the CRISPR/Cas9 system and inhibited EZH2 mRNA and protein expression in MDA-MB-231 cells, whereas knockdown of EZH2 inhibited the proliferation and migration of MDA-MB-231 *in vitro*. Based on the role of CRISPR/Cas9, many experts considered that some nanoparticles could be designed for efficient targeted delivery of CRISPR/Cas9 plasmids (90–92).

TABLE 3 NcRNA therapies already in clinical trials.

NcRNA Therapies	Cancer	Clinical Trial Number	Reference
siRNA	BC	NCT06357689	(120)
ASO	BC	NCT01563302	(121)
shRNA	Sickle Cell Disease	NCT03282656	(122)
siRNA nanoparticle	Glioblastoma	NCT03020017	(123)
miRNA liposomal formulation mimic	HCC、Colorectal、Leiomyosarcoma	NCT01829971	(50)
EnGeneIC Dream Vectors、mimic microRNA	Malignant pleural mesothelioma	NCT02369198	(52)
Miniature biodegradable polymeric matrix loaded siRNA	Pancreatic ductal adenocarcinoma	NCT01188785	(124)
Exosome delivery vehicles with siRNA	Pancreatic cancer	NCT03608631	(125)
LNA Selective inhibitor	Refractory advanced cancer	NCT04811898	(126)

5 Discussion of breast cancer therapies

Traditional treatments for breast cancer mainly include surgical excision, radiotherapy, chemotherapy, endocrine therapy, targeted therapy and immunotherapy. Among the available treatments for HER2-positive breast cancer, the combination of trastuzumab, patuximab and paclitaxel analogs (THPs) is still the preferred first-line treatment (93). HR-positive/HER2-negative breast cancer is treated primarily with hormone therapy, and intermediate- and high-risk patients may receive concurrent chemotherapy (94). First-line standard therapy for metastatic patients is CDK4/6 inhibitors in combination with hormone therapy (95). Treatment for triple-negative breast cancer then includes the PD-L1 inhibitor Tecentriq and the PD-1 inhibitor Keytruda, as well as the PARP inhibitors Lynparza and

Talzenna (96–98). These methods play an important role in the treatment of breast cancer, but have some limitations. For example, although chemotherapy can kill cancer cells, it may also harm normal cells and bring about side effects; whereas endocrine therapy and targeted therapy require the patient's tumor to have the corresponding receptor expression or gene mutation in order to be effective. ncRNA therapy has a number of potential advantages over conventional treatment. First, ncRNA therapies may be more precise because they can target specific molecular pathways or signaling networks, reducing the impact on normal cells. Second, ncRNA therapies may help overcome the problem of drug resistance to conventional treatments because they can intervene in the biological behavior of tumor cells from a new perspective. In addition, ncRNA therapies may have a better safety profile and fewer side effects because they are based on modulating endogenous molecules rather than introducing foreign chemotherapeutic agents (45). The ncRNA-based diagnostic field has much advanced with numerous diagnostic tools already offered to clinical trials (Table 3).

The clinical application of ncRNAs as potential therapeutic targets for cancer can be manifested in two ways: the use of ncRNAs to "complement" inhibited or missing RNAs (replacement therapy) or to "block" the action of overactive oncogenic RNAs (99). However, the existence of many different ncRNAs associated with BC suggests that the regulation of BC is more complex than we expected. In this aspect, we need to further investigate the possibility that the expression of ncRNAs changes during the course of the disease, just as other oncogenic molecules do in cancer. Therefore, specific targeting of different ncRNAs may be required to effectively combat disease recurrence. In addition, targeting multiple ncRNAs can be rationally supplemented for effective BC inhibition. Determining which ncRNAs to target may depend on the specific expression profile of each patient to realize the idea of personalized medicine (100). Another aspect to consider is the complex interactions between different ncRNAs. As in breast cancer the anti-tumor miR-149-5p is sponged by both CircFAM64A (101) and Circ_0072995 (102) molecules, implying that CircFAM64A and Circ_0072995 may need to be silenced simultaneously. Also, it has been shown that just one miR cluster (miR-15a-16-1 cluster) can affect (through direct and indirect targeting) approximately 14% of the entire transcriptome in leukemia cells (103). Finally, no siRNAs targeting lncRNAs are currently in clinical trials due to a variety of challenges, including "off-target" issues, inefficient delivery to tissues and cells, and non-specific immune responses. "Off-target" issues can lead to unexpected transcription and protein silencing, false-positive hits and cell growth inhibition, as well as competition with endogenous non-coding RNAs. siRNAs are inefficiently delivered primarily due to their tendency to accumulate and be absorbed predominantly by the liver, and the challenge of delivering siRNAs to other tissues remains. Therefore, optimizing anti-ncRNAs therapeutics must also take into account the delivery of therapeutic molecules.

6 Conclusions

BC is one of the most common diseases globally, and its incidence continues to rise despite long-term efforts to reduce its impact on human life. Conventional BC therapy remains inadequate due to the heterogeneity and high chemoresistance of this disease. Currently, most of the studies on ncRNAs regulating tumorigenesis and development are mainly cellular or animal experiments, and clinical research is still in the initial stage, and more clinical trials need to be carried out in the future to find out more safe and effective ncRNAs with universal or tissue specificity and specific ncRNAs for different tumor types. While advances have been made with non-viral delivery systems such as lipid nanoparticles (LNPs), further improvements in their targeting and reduction of immune responses are still necessary. NcRNAs are diverse and functionally distinct, and the complexity of their mechanisms of action in cells adds to the difficulty of understanding their role in disease. NcRNA therapies may also need to be personalized to an individual's genome and disease characteristics. However, sufficient technological advances have been made to synthesize and manufacture most of the ncRNA mimics and inhibitors for use in preclinical studies and eventually in human clinical trials. In conclusion, although the field seems to be in its infancy, we are currently witnessing the growing potential of ncRNAs in cancer therapy. There is a need to further elucidate the development and clinical application of ncRNAs in breast cancer research to provide a theoretical basis for biomarkers and targeted therapies for breast cancer.

Author contributions

RL: Writing – original draft. YJ: Methodology, Writing – review & editing. RY: Methodology, Writing – review & editing. GT: Investigation, Writing – review & editing. WW: Writing – review & editing. CC: Writing – review & editing. QY: Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Non-metabolic enzyme function of pyruvate kinase M2 in breast cancer

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Breast cancer (BC) is a prevalent malignant tumor in women, and its incidence has been steadily increasing in recent years. Compared with other types of cancer, it has the highest mortality and morbidity rates in women. So, it is crucial to investigate the underlying mechanisms of BC development and identify specific therapeutic targets. Pyruvate kinase M2 (PKM2), an important metabolic enzyme in glycolysis, has been found to be highly expressed in BC. It can also move to the nucleus and interact with various transcription factors and proteins, including hypoxia-inducible factor-1α (HIF-1α), signal transducer and activator of transcription 3 (STAT3), β-catenin, cellular-myelocytomatosis oncogene (c-Myc), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), and mammalian sterile 20-like kinase 1 (MST1). This interaction leads to non-metabolic functions that control the cell cycle, proliferation, apoptosis, migration, invasion, angiogenesis, and tumor microenvironment in BC. This review provides an overview of the latest advancements in understanding the interactions between PKM2 and different transcription factors and proteins that influence the initiation and progression of BC. It also examined how natural drugs and noncoding RNAs affect various biological processes in BC cells through the regulation of the non-metabolic enzyme functions of PKM2. The findings provide valuable insights for improving the prognosis and developing targeted therapies for BC in the coming years.

KEYWORDS

breast cancer, non-metabolic enzyme function, pyruvate kinase M2, noncoding RNA, tumorigenesis

Introduction

Breast cancer is the most commonly occurring cancer in women and the most common cancer overall (1). There were more than 2.26 million new cases of BC, and approximately 685 000 women died from the disease in 2020 (2). It can be divided into three main subtypes: luminal, HER2-positive, and triple-negative breast cancer (TNBC) (3). TNBC, in particular, is known for its high invasiveness. Around 30% of individuals with early-stage BC experience metastases, resulting in a 5-year relative survival rate of 25% (4). Nonetheless, the precise molecular mechanisms responsible for BC development across different subtypes remain unclear. Further exploration is needed to identify specific biomarkers that can be targeted to improve the overall prognosis of patients with this disease (5).

The primary distinguishing feature of cancer cells is their metabolic reprogramming. Unlike healthy cells, tumor cells rely on aerobic glycolysis for energy production, even when enough oxygen present in the environment (6, 7). This shift from the typical respiratory pathway to aerobic glycolysis is referred to as the Warburg effect (7, 8). Aerobic glycolysis is a characteristic feature of the Warburg effect and is crucial for the survival of cancer cells (9). Pyruvate kinase (PK) is a key rate-limiting enzyme for glycolysis that catalyzes the phosphorylation of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to produce pyruvate and adenosine triphosphate (ATP), which play critical roles in glycolysis during tumor formation (10). Since PKs are found in various tissues and exhibit distinct catalytic activities, it suggests that there may be different isotypes of this enzyme (11). There are four different subtypes of PKs that are expressed in specific tissues: muscle (M1), liver (L), erythrocyte (R), and ubiquitous (M2) (12). Among these subtypes, PKM2 is frequently overexpressed in cancer cells and has been extensively studied as a subtype specific to tumors (13).

Since Christofk et al. first demonstrated the necessity of PKM2 expression for cancer-specific aerobic glycolysis, known as the Warburg effect, there has been significant interest in its role in cancer development (14). In addition to its role in tumor metabolism, PKM2 plays a role in oncogenic cytokinesis, tumor growth, and metastasis (15–17). Furthermore, PKM2 functions as a protein kinase by phosphorylating its substrates and regulating gene expression (18). Previous studies have highlighted the importance of PKM2 in promoting cancer cell growth and survival (16, 19). Therefore, comprehending the biochemical functions of PKM2 during tumor progression is essential for identifying possible therapeutic targets and developing novel therapies for BC (5). Hence, this review highlights current advancements in understanding how PKM2 interacts with different transcription factors and proteins that influence the initiation and progression of BC. Additionally, it explored the impact of natural products and noncoding RNAs on various biological functions of BC cells by controlling the nonmetabolic functions of PKM2.

Abbreviations: CA, Carpesium abrotanoides Linn.; TNBC, triple-negative breast cancer; PK, pyruvate kinase; PEP, phosphoenolpyruvate; PKM2, pyruvate kinase M2; FBP, fructose 1,6-bisphosphate; ISCD, HIF-1α, hypoxia-inducible factor-1α; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; BC, breast cancer; STAT3, signal transducer and activator of transcription 3; YHC, yuanhuacine; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; NF-κB, nuclear factor-kappa B; CTS, cryptotanshinone; CsA, cyclosporin A; HER2, human epidermal growth factor receptor 2.

Structure and function of PKM2

The PKM gene, which is located in the 15q23 region of the chromosome, has the ability to undergo alternative splicing to produce either PKM1 or PKM2. The PKM gene spans approximately 32 kb and comprises 12 exons and 11 introns. The lengths of exons 9 and 10 are identical, which contributes to the differences in the final transcript. For PKM2, the final messenger ribonucleic acid (mRNA) product includes exon 10 but omits exon 9, a feature unique to PKM1 (20, 21). PKM2 is composed of 531 amino acids and has four domains: A (244 aa), B (102 aa), C (142 aa), and N (43 aa)-terminal domains (22). The catalytic active site is formed by the interface between the A- and B-domains, whereas the C-domain includes the fructose 1,6-bisphosphate (FBP) binding site, which acts as an allosteric activator. Additionally, the C-domain contains a nuclear localization signal sequence (NLS) and an inter-subunit contact domain (ISCD) (Figure 1) (22, 23). Furthermore, the arrangement of the C-domain plays a crucial role in explaining the differences observed in allosteric regulation by FBP among various PKM isoforms (22). The ISCD domain of PKM2 has a protein sequence that differs from that of its alternate splice variant, PKM1, by 23 amino acids, resulting in distinct properties such as allosteric regulation by FBP. These differences also enable PKM2 to interact with specific protein partners, including phosphotyrosine proteins (24).

Unlike other PK isoforms, such as PKL, PKR, and PKM1 which are exclusively tetramers, PKM2 is present in both tetrameric and dimeric forms. The A-domain of individual PKM2 units combines to form a dimer, and two such dimers bind at the interface of the ISCD (the C-domain) to create the full PKM2 tetramer (25). The shift between PKM2 dimers and tetramers is controlled by the structural changes caused by endogenous and exogenous activators and inhibitors (26). Fructose-1,6-bisphosphate (FBP) and serine are both potent allosteric activators of PKM2, which directly bind to PKM2 and stabilize it in the active tetramer configuration (27). In addition, when succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5'-phosphate (SAICAR) binds to PKM2, it can trigger both the pyruvate kinase and the protein kinase activity of PKM2 (28). Furthermore, modifications such as phosphorylation, acetylation, and oxidation of PKM2 at the Tyr-105, Lys-305, and Cys-358 sites can prevent FBP from binding to tetrameric PKM2, thereby maintaining it in dimer form (29). PKM2 in tetrameric form is highly active at physiological concentrations of PEP and has a high affinity for PEP (30). In cases where PKM2 exists mainly in its highly active tetrameric form, as is the case in differentiated tissues and most normal proliferating cells, glucose is converted to pyruvate to produce energy (31). Meanwhile, PKM2 dimer is described by weak attraction to its substrate, PEP, and is virtually

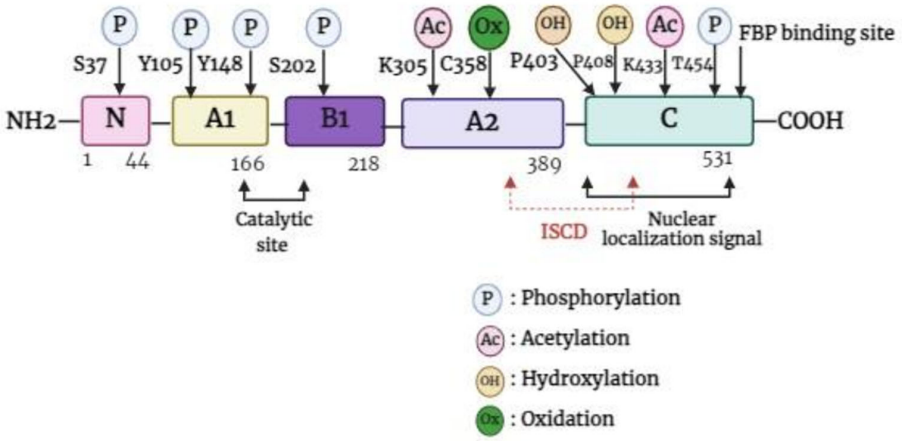

FIGURE 1
Basic structure of PKM2 monomer. FBP, fructose 1,6-bisphosphate; ISCD, inter-subunit contact domain.

inactive at normal concentrations of PEP. In this form, PKM2 generates minimal ATP during the conversion of PEP to pyruvate, resulting in no net production of ATP through glycolysis (31, 32). This scenario occurs primarily in tumor cells, where PKM2 predominantly exists in the less active dimeric state. As a consequence, all glycolytic intermediates beyond PK accumulate and are redirected toward various synthetic processes such as nucleic acid production, phospholipid synthesis, and amino acid synthesis (26, 30, 33). Like tumor cells, cells that undergo rapid proliferation require a large amount of essential components such as nucleic acids, phospholipids, and amino acids (34). Importantly, the dimeric form of PKM2 can enter the nucleus and act as a protein kinase (35). The basic functions of the PKM2 dimer and tetramer are described in Figure 2.

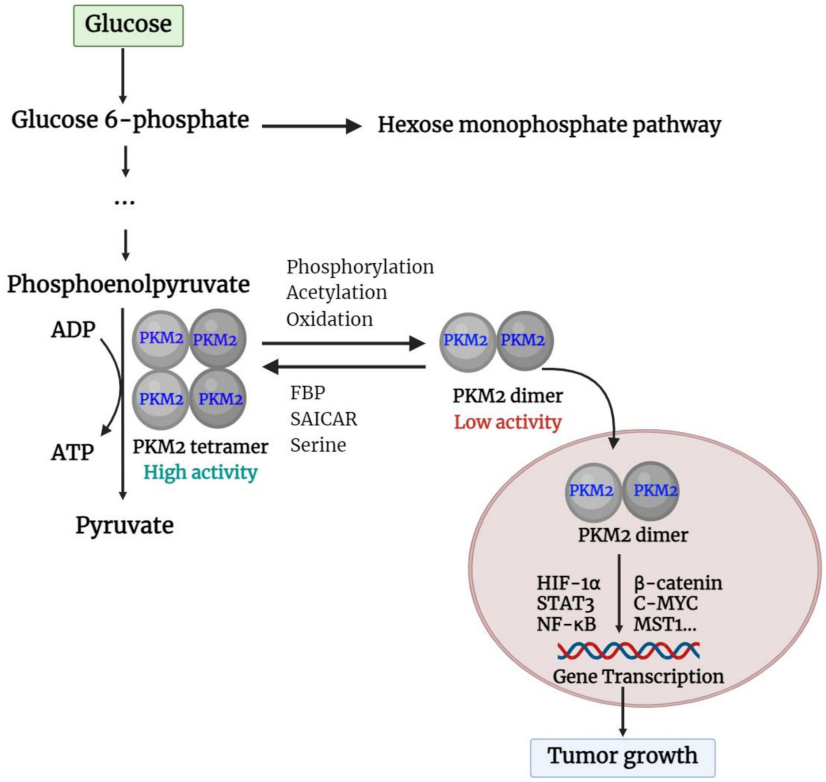

FIGURE 2
Basic function of PKM2 dimer and tetramer. PKM2, pyruvate kinase M2; FBP, fructose 1,6-bisphosphate; SAICAR, succinyl-5-aminoimidazole-4-carboxamide-1-ribose 5'-Phosphate; HIF-1α, hypoxia-inducible factor-1α; STAT3, Signal transducer and activator of transcription 3; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; c-Myc, cellular-myelocytomatosis oncogene; MST1, mammalian sterile 20-like kinase 1.

The effect of PKM2 on breast cancer tumorigenesis and development

A large body of evidence supports the notion that cancers predominantly express PKM2 (14). Immunohistochemical analysis revealed that PKM2 is commonly expressed in BC (5). In BC, the activation of HIF-1α and epidermal growth factor receptor (EGFR) can facilitate the nuclear translocation of PKM2 (36), which is determined by the nuclear localization signal at its C-terminus. The phosphorylation of the PKM2 S37 site by extracellular regulatory protein kinases leads to its transformation from a tetramer to a monomer through the peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1), enabling the nuclear localization signal to enter the nucleus (23). In addition, acetylation of PKM2 at Lys433 decreases FBP binding to PKM2 and the conversion of monomers or dimers to tetramers but increases PKM2 nuclear import and protein kinase activity (37). Furthermore, the Jumonji C domain-containing dioxygenase Jumonji domain-containing protein 5 (JMJD5) interacts directly with PKM2, promoting its movement into the nucleus and HIF-1α-mediated transactivation. The interaction between JMJD5 and PKM2 occurs at the intersubunit interface region of PKM2, preventing its tetramerization and inhibiting its kinase activity (38). Once within the nucleus, PKM2 serves as a transcriptional co-activator and stimulates the activation of various transcription factors, such as HIF-1α, β-catenin, STAT3, C-MYC, NF-κB, etc., which influences the expression of their respective downstream target genes. Overall, Figure 3 shows how nuclear PKM2 regulates gene expression in relation to breast cancer development and progression.

PKM2/HIF-1α

Cancer cells exhibit unregulated cell proliferation, unrestricted cell division, and suppression of autophagy, making them reliant on additional oxygen and nutrients for survival (39). To meet these demands, tumor cells can utilize angiogenesis and metabolic alterations. The transcription factor HIF-1α plays a crucial role in regulating genes related to angiogenesis and the Warburg effect, both of which contribute to tumor formation (40). Previous studies have focused primarily on HIF-1α as a transcription factor that increases the expression of various glycolysis-related genes, such as glucose transporters (GLUT-1 and GLUT3) and enzymes involved in glycolytic pathways [lactate dehydrogenase A, pyruvate dehydrogenase kinase 1, hexokinase 2 (HK2), and PKM2], to promote BC glycolysis and consequently affect the proliferation of BC cells (36). Intriguingly, studies have revealed that PKM2 can influence the expression and activation of HIF-1α (41, 42). The activation of PKM2 in hypoxic BC cells leads to the activation of NF-κB/p65 and HIF-1α, resulting in increased production and secretion of vascular endothelial growth factor (VEGF), which promotes angiogenesis and tumor growth (41). Angiogenesis plays a crucial role in tumor invasion and migration. Notably, studies have shown that vascular endothelial growth factor A (VEGFA) can enhance the self-renewal of cancer stem cells and promote epithelial–mesenchymal transition (EMT), potentially contributing to tumor metastasis in BC cells (43). Furthermore, clinical studies have shown that patients with metastatic BC have increased circulating VEGFA levels (44, 45). Consequently, targeting VEGF through the PKM2/HIF-1α axis in BC is a feasible approach. Chai et al. (46) reported that silencing PKM2 in BC cells resulted in reduced expression of HIF-1α and VEGF, as well as cell migration capabilities. These findings suggest that PKM2 influences the expression of HIF-1α and subsequently affects the onset and progression of various tumors, including BC. Specifically, prolyl hydroxylase domain 3 (PDH3) hydroxylates PKM2 at proline 403/408, leading to its binding to the HIF-1α subunit, which enhances HIF-1α binding to p300. This interaction then recruits p300 to the hypoxia response element, facilitating the transactivation of HIF-1α target genes and promoting PKM2 transcription (47). PKM2 and HIF-1α establish a positive feedback loop. Recent investigations have revealed that in BC cells under hypoxic conditions, nuclear PKM2 recruits HIF-1α and p300, leading to the upregulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), an enzyme involved in glycolysis that is associated with cancer progression and aggressiveness (36). These findings suggest that nuclear PKM2 can induce glycolysis by activating HIF-1α, potentially influencing various biological processes in BC. These results underscore the potential of targeting the PKM2/HIF-1α axis as a promising strategy for anti-BC therapies.

PKM2/β-catenin

β-catenin is the central component of the Wnt signaling pathway and plays a key role in the regulation of cell proliferation, differentiation, and apoptosis (48). The Wnt-signaling pathway is an evolutionarily conserved and complex signaling cascade that plays crucial roles in both development and disease (49). The Wnt signaling pathway plays an important role not only in the development and maintenance of healthy breast and mammary glands but also in BC etiology (50). To validate the regulation of β-catenin by PKM2, Zhao and colleagues investigated the levels of β-catenin in MDA-A-231-shPKM2 and BT20-PKM2 cells. The findings demonstrated that β-catenin expression was suppressed in PKM2-silenced cells, whereas both the protein and RNA levels of β-catenin were increased in PKM2-overexpressing cells (51). In addition, several studies have indicated that PKM2 is translocated into the nucleus in cancer cells, where it acts as a transcription factor and controls β-catenin transactivation (52, 53). Once inside the nucleus, PKM2 can interact with the transcription factor T-cell factor (TCF), leading to an increase in the transcription of the c-Myc and cyclin D1 genes. This process ultimately facilitates BC cell proliferation and EMT (51, 54). Notably, the involvement of β-catenin in various cancer cell activities, such as cell migration, invasion, and angiogenesis, is noteworthy. Findings from a clinical investigation indicated a potential correlation between the expression of β-catenin in TNBC tissue and survival prognosis (52). A recent study revealed that cryptotanshinone significantly reduced BC migration and invasion, possibly through the suppression of the

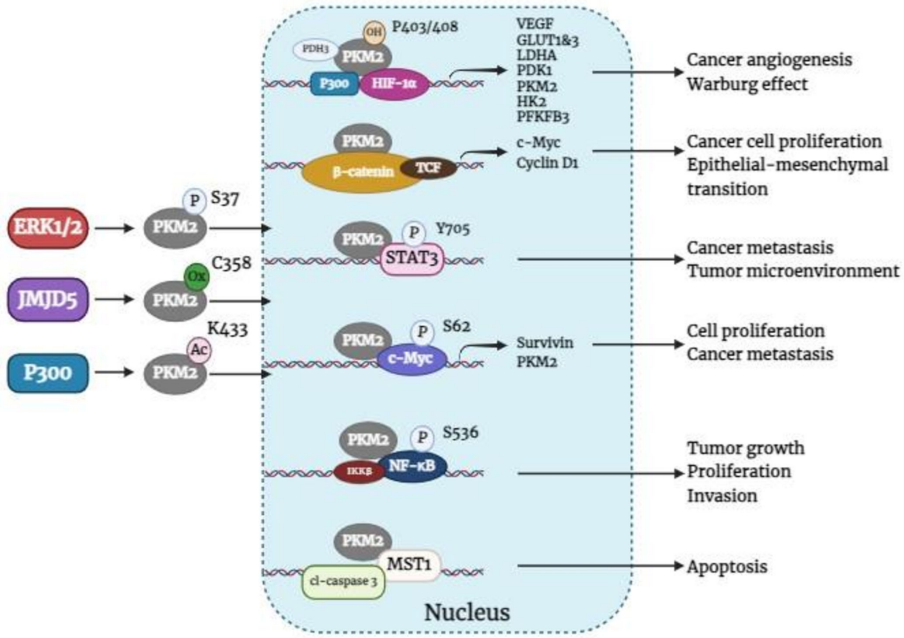

FIGURE 3
Nuclear PKM2 regulates the expression of related genes affecting BC tumorigenesis and development. ERK1/2, extracellular signal-regulated kinase 1/2; JMJD5, jumonji C domain-containing protein 5; PKM2, pyruvate kinase M2; HIF-1α, hypoxia-inducible factor-1α; PDH3, prolyl hydroxylase domain 3; TCF, T-cell factor; VEGF, vascular endothelial growth factor; GLUT1&3, glucose transporters 1 and 3; LDHA, lactate dehydrogenase A; PDK1, pyruvate dehydrogenase kinase 1, HK2, hexokinase 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3; c-Myc, cellular-myelocytomatosis oncogene; MST1, mammalian sterile 20-like kinase 1; cl-caspase 3, cleaved-Caspase-3.

PKM2/β-catenin pathway and the overexpression of PKM2, which decreased cryptotanshinone sensitivity in BC cells (54). Consequently, these findings suggest that targeting the PKM2/β-catenin signaling pathway may hold promise for treating BC.

PKM2/STAT3

STAT3 is activated in several types of tumor cells and can promote the malignant transformation of cells and inhibit apoptosis, suggesting that the STAT3 signaling pathway could be a new target for tumor gene therapy (55, 56). The Janus kinase (JAK)/STAT3 pathway is responsible for regulating the gene expression of several enzymes involved in glucose metabolism. Recently, interest in understanding the importance of the PKM2/STAT3 pathway in the advancement of cancer has increased (57). PKM2 overexpression facilitates the nuclear translocation of STAT3, a transcription factor crucial for PKM2-driven metastasis. The protein kinase activity of PKM2 mediates the nuclear translocation and up-regulation of STAT3, which regulates the aggressive progression of colorectal cancer (58). Nuclear PKM2 activates the transcription of MAP kinase kinase 5 (MEK5/ERK5) by phosphorylating STAT3 at Y705, resulting in cell proliferation (18, 59). Some investigations related to the STAT3 oncogene have laid the foundation for the interpretation and treatment of BC formation mechanisms (60). Guan et al. reported that knocking down PKM2 in BC cells resulted in a decrease in the expression of STAT3 and STAT3 (Tyr(P)-705), leading to the suppression of gene transcription and the inhibition of BC cell proliferation (61). *In vitro* and *in vivo* studies have confirmed that pY705 modification of STAT3 is necessary for tumor growth, autophagy, and metastasis and suggest that STAT3 is an effective approach for treating cancer (62). Furthermore, clinical studies using STAT3 inhibitors indicate encouraging outcomes in malignant conditions. For example, yuanhuacine (YHC), a daphnane-type diterpenoid as the main active ingredient, may inhibit BC cell proliferation and induce apoptosis *in vivo* and *in vitro* by regulating the STAT3 pathway and glycolysis through targeting PKM2 (63). The suppression of STAT3 leads to apoptosis, inhibition of growth, reduced tumor cell invasion, and increased sensitivity to treatment in BC cells (64). These results emphasize the potential significance of targeting PKM2/STAT3 as a therapeutic approach for BC.

PKM2/C-MYC

c-Myc is a frequently activated oncogene that is closely associated with the initiation and progression of cancer in humans (65). It functions as an oncoprotein involved in various cellular processes such as deoxyribonucleic acid (DNA) replication, transcription, and RNA splicing (66). One crucial transcriptional target of c-Myc is survivin, which is an inhibitor of the apoptosis protein family and plays a significant role in tumorigenesis (67–69). Survivin is highly expressed in BC and promotes the proliferation and migration of cancer cells (67, 70). Transcriptional factors play a role in the development and advancement of cancer, such as metastasis and cellular proliferation (71). PKM2's nuclear translocation enables it to serve as a transcriptional activator for

the c-Myc gene (70, 72, 73). Yu et al. (70) revealed that PKM2 interacts with c-Myc and regulates its phosphorylation at Ser-62. This interaction suggests that c-Myc may be a novel substrate of PKM2. Inhibiting PKM2 reduces c-Myc phosphorylation, leading to the downregulation of c-Myc protein expression through the promotion of its degradation in BC cells. The results of c-Myc knockdown indicated decreased survivin protein and mRNA levels, suggesting that PKM2 regulates survivin via c-Myc (70, 74). Thus, a novel approach to inhibit BC cell proliferation and migration could involve focusing on the PKM2-c-Myc-survivin pathway.

PKM2/NF-κB

The nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) family of transcription factors plays important roles in regulating inflammation, immunological response, cell differentiation, proliferation, and survival (75). NF-κB consists of five subunits: p65/RelA, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2, which form unique protein complexes that bind to consensus DNA sequences at gene promoter regions to control various biological activities (76, 77). PKM2 plays a role in controlling the NF-κB signaling pathway in cancer cells. For example, Azoitei et al. reported that PKM2 stimulated the release of VEGF-A by activating NF-κB and HIF-1α, which in turn affected tumor angiogenesis in pancreatic cancer (41). In addition, Zheng et al. found that PKM2 facilitates the movement of NF-κB/P65 to the nucleus, resulting in the promotion of ovarian cancer cell migration and invasion (78). In BC, there has also been evidence of positive crosstalk between NF-κB and PKM2 (79, 80). Knockdown of PKM2 in TNBC cells significantly reduces the activity of NF-κB by decreasing the phosphorylation of p65 at serine 536 and suppressing the expression of NF-κB target genes (80, 81). However, the exact mechanism by which PKM regulates the activity of NF-κB through p65 phosphorylation remains unclear. IKKβ is known to play a critical role in phosphorylating RelA/p65 at serine 536 and controlling the activation of NF-κB (81). Dimeric PKM2 acts as an active protein kinase that can phosphorylate certain nuclear proteins (18, 82). Therefore, future investigations will explore whether the localization of PKM2 differs between the cytoplasm and nucleus and whether dimeric PKM2 can phosphorylate the p65 protein at serine 536 or influence IKKβ signaling molecules. These studies may reveal additional mechanisms and metabolic changes in PKM2/NF-κB in TNBC cells.

PKM2/MST1

Mammalian sterile 20-like kinase 1 (MST1), also known as STK4 and Krs-2, is widely present in all human tissues and cell lines and serves as a crucial upstream kinase in the Hippo signaling pathway (83). MST1 plays a vital role in regulating mammalian cell shape, controlling organ size, managing apoptotic responses, and influencing cancer development (83, 84). PKM2 physically interacts with MST1 *in vivo*. The phosphorylation and proteolysis of MST1 regulated by PKM2 could affect downstream signaling pathways controlled by MST1, which are involved in regulating cell death (85, 86). Treatment with 4-hydroxytamoxifen reduces the levels of PKM2 and disrupts its interaction with MST1, leading to the activation of caspase-3. This further enhances the cleavage of MST1 mediated by caspase-3 in BC cells. The cleaved form of MST1 relocates to the nucleus, where it causes chromatin condensation and ultimately triggers cell apoptosis (85). Silencing PKM2 can activate Caspase-3, which then cleaves its substrate to enhance the apoptotic signaling cascade induced by tamoxifen treatment (85, 87). These findings offer new insight for the development of therapies against BC.

Other transcription factors or signaling pathways affected by nuclear PKM2

Besides, studies have revealed that the activation of PKM2 induces the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK), resulting in the phosphorylation and subsequent inhibition of acetyl-CoA carboxylase. This suggests that heightened PK activity results in an energy deficit (88). AMPK activation is known to hinder mammalian target of rapamycin (mTOR) signaling during low-energy conditions, exerting a cytostatic effect (89). The classic antidiabetic drug metformin, for example, has been extensively studied for its anticancer effects via AMPK signaling (90, 91). Existing studies have demonstrated that PKM2 interacts directly with the histone methyltransferase enhancer of zeste homolog 2 (EZH2) to orchestrate the epigenetic silencing of the carnitine transporter, SLC16A9. Inhibiting PKM2 disrupts the binding of EZH2 to SLC16A9, which de-represses the expression of SLC16A9 and increases intracellular carnitine influx, thus programming TNBC cells toward fatty acid oxidation-dependent and luminal-like cell states (92). According to previous reports, nuclear PKM2 promotes the proliferation of liver, breast, colon, and lung cancer cells by phosphorylating nuclear sterol regulatory element-binding protein (SREBP)-1a T59, which prevents SREBP-1a from being ubiquitinated and degraded and consequently enhances the expression of SREBP-1a's target genes related to lipid metabolism. This study demonstrated that PKM2 functions as a transcriptional co-activator by promoting SREBP-1a's transcriptional activation function and thus driving the proliferation of BC cells (93).

The effect of regulating nonmetabolic enzyme function of PKM2 on breast cancer tumorigenesis and development

The above studies revealed that the nuclear localization of dimeric PKM2 can affect a variety of biological processes in BC cells. As a result, controlling the nonmetabolic function of PKM2 is critical for intervening in BC carcinogenesis and progression. Currently, a growing body of research is focused on exploring the potential role of natural products and noncoding RNAs in regulating the nonmetabolic function of PKM2 to affect BC tumorigenesis and development.

Natural products regulate the nonmetabolic function of PKM2

Shikonin

Shikonin, an active compound extracted from *Lithospermum erythrorhizon*, has exhibited various pharmacological effects and has been found to possess antitumor capabilities in a variety of human cancer types (94, 95). Specifically, it can impede the glycolytic function of PKM2 in BC cells (96, 97), and suppress its nonmetabolic enzyme function to impact the biological processes of these cells (36, 97–99). Shikonin works by binding with the R399/400 residues of PKM2 to hinder its nuclear translocation, hence leading to the disruption of the PKM2-HIFs-PFKFB3 pathway (36). As PFKFB3 facilitates aerobic glycolysis, reducing its levels increases the reliance on OXPHOS to meet ATP needs (100). Cancer cell growth is known to be inhibited by a transition from a glycolysis-dependent state to an OXPHOS-dependent state (101). Pandkar et al. found that shikonin inhibits the hypoxic activation of PFKFB3 by engaging with a putative nuclear localization signal (NLS) on PKM2, thereby blocking its movement into the nucleus during hypoxia. Furthermore, studies in mice carrying WT BBS MCF7 tumors revealed that shikonin treatment led to significantly reduced tumor growth, emphasizing the crucial role of targeting PKM2 signaling to inhibit the progression of tumors (36). Moreover, LEE et al. showed that shikonin is a robust and rapid inducer of late apoptosis in MDA-MB-231 cells, implying that inhibitors of PKM2 may be validated treatment targets for TNBC. Targeting PKM2 in TNBC paves the way for the development of PKM2 inhibitors as potential anti-TNBC agents (98). Therefore, focusing on targeting PKM2 in BC sets the stage for developing promising anti-BC agents in the form of PKM2 inhibitors.

Cryptanshinone

Cryptotanshinone is a liposoluble monomer of tanshinones isolated from the dried roots and rhizomes of *Salvia miltiorrhiza* (102). Cryptotanshinone has been recognized as a medication with anti-inflammatory and anti-oxidative properties, and it has demonstrated its ability to combat different forms of cancer by impeding cell growth, movement, and infiltration (103). Notably, significant evidence suggests that cryptotanshinone has the capacity to inhibit the metabolism of glucose in ovarian cancer cells, suggesting that it could be an effective anti-cancer agent by controlling glycolysis (104). Zhou et al. showed that cryptotanshinone has an anti-cancer effect on BC cells by inhibiting cell proliferation, migration, and invasion *in vitro*. In addition, it significantly decreases the expression of glycolysis-related proteins and PKM2/β-catenin signaling in BC cells (54). PKM2 is a crucial regulator that controls the transactivation of β-catenin, which is responsible for numerous critical functions of cancer cells, including invasion, migration, and angiogenesis (105). Cryptotanshinone successfully hindered the migration and invasion of BC, which could be attributed to the suppression of the PKM2/β-catenin pathway (54). Therefore, cryptotanshinone may hold promise for the advancement of novel targeted medications for BC.

Cyclosporine A

Cyclosporin A is an immunosuppressant that is not toxic to cells and was first identified in the 1970s. Initially, it was utilized to suppress immune responses after organ and marrow transplantation (106). Afterwards, it has been utilized in various medical fields where autoimmune or inflammatory processes are involved in pathology (107, 108). Recent studies have highlighted the potential antitumor activity of this compound against different cancer cells, including BC. Jiang et al. found that cyclosporin A inhibited the cell proliferation, cell cycle progression, and G1/S phase transition of BC cell lines (109). Several studies have reported that cyclosporin A hinders the expression of some oncogenes. Cyclosporin A can inhibit the proliferation of cancer cells, possibly by controlling the expression levels of c-Myc, p21, and proliferating cell nuclear antigen (PCNA) through the suppression of calcineurin (CaN)/nuclear factor of activated T cells (NFAT) activity (110). It also suppresses cancer-specific PKM2, which supports the advancement of the tumor (109, 111). The function of the PKM2 enzyme was also suppressed in MCF-7 cells when they were exposed to cyclosporin A. Pyruvate kinase catalyzes the final step in the glycolytic pathway and is responsible for net ATP production; hence, the production of ATP was evaluated in MCF-7 cells treated with cyclosporin A and was found to be significantly reduced (109). This finding shows that cyclosporin A hampers the expression of PKM2, leading to a reduction in intracellular ATP within tumor cells, which ultimately slows down cell growth and may even trigger cell death (109, 111). Thus, cyclosporin A acts as a suppressor of BC growth by targeting tumor-related PKM2.

Lapatinib

Lapatinib is a small-molecule tyrosine kinase inhibitor that targets EGFR and HER2 (112, 113). Clinical studies have revealed that lapatinib is well-tolerated and can be used alone or in combination with other drugs for the treatment of BC (114, 115). The specific molecular mechanism of lapatinib involves regulating PKM2-mediated STAT3 tyrosine phosphorylation (18, 61). Guan et al. (61) demonstrated that lapatinib hinders the proliferation of BC cells by influencing the expression of PKM2, which decreases the levels of STAT3 and phosphorylated STAT3. Downregulation of PKM2 expression by lapatinib-mediated EGFR and HER2 suppression decreases STAT3 and phosphorylated STAT3 expression, resulting in decreased gene transcription and prevention of tumor cell proliferation.

Mangifera indica

M. indica, also known as mango, is a member of the *Anacardiaceae* flowering family (116). Various sections of the M. indica plant contain different types of phytochemicals, and they have traditionally been used to treat a wide range of health issues such as gastrointestinal, genitourinary, ophthalmic, and respiratory conditions (117, 118). Preclinical research on extracts derived from diverse plant sections has indicated their anti-cancer, anti-inflammatory, antimicrobial, antioxidant, and immunomodulatory properties (119, 120). Specifically, various investigations have reported the anti-cancer properties of the pulp extracts of M.

indica in BC. These findings suggest that consuming M. indica fruits could be beneficial for BC treatment (121). M. indica extracts (leaf, bark, and seed coats) were also found to have anticancer activity against TNBC (122, 123). These studies revealed that M. indica extracts could inhibit PKM2, but the exact mechanism underlying these effects is still not fully understood. Further research is also suggested to examine their potential in *in vivo* studies.

Carpesium abrotanoides (L.)

Carpesium abrotanoides Linn. (CA) is a popular medicinal plant recognized for its anti-inflammatory effects and wide range of therapeutic applications (46). Some studies have demonstrated that CA extracts exhibit promising antitumor effects on various cancer cells *in vitro*, including non–small cell lung, ovarian, skin, and colon cancer cells (124, 125). As a traditional herbal remedy, it is commonly used as an oral decoction for treating chronic inflammatory illnesses (126). Currently, various compounds have been isolated from the whole plant of CA that display significant cytotoxicity against MCF-7 and MDAMB-231 cells, indicating its potential anti-BC effects (46, 127). In addition, Chai et al. found that CA has dose-dependent antiproliferative effects on both metastatic (MDA-MB-231) and nonmetastatic (MCF-7) BC cells while inhibiting their migration ability. Furthermore, CA downregulates the expression of glycolysis-related proteins (PKM2, LDHA, HK2, and GLUT1) and suppresses the PKM2/HIF-1α/VEGF signaling pathway to exert its anticancer effects (46). In cancer cells, PKM2 activates the transcription of HIF-1α and its target gene VEGF in the nucleus; this stimulates the secretion of VEGF to promote angiogenesis and enhance tumor growth (41). Angiogenesis is a key event in tumor invasion and migration. Moreover, VEGFA has been linked to promoting cancer stem cell self-renewal and EMT while contributing to tumor metastasis in BC cells (43). Clinical studies have also revealed that patients with metastatic BC tend to have higher circulating levels of VEGFA (45). Consequently, targeting the PKM2/HIF-1α axis holds promise as a feasible approach for regulating VEGF in BC.

Yuanhuacine

Yuanhuacine, a daphnane-type diterpenoid as the main active ingredient, has been previously documented to exhibit cytotoxic effects *in vitro* against a wide range of human cancer cell lines (128–130). For instance, yuanhuacine has notable inhibitory effects on human lung cancer cells (131). Recent studies have revealed that yuanhuacine significantly inhibits effect BC cell growth (63, 132). Yuanhuacine reduced the growth of BC cells and caused apoptosis both *in vivo* and *in vitro*. The mechanism of action has been linked to yuanhuacine, which has been hypothesized to potentially disrupt the interactions of PKM2 and STAT3, hence inhibiting downstream proteins. Besides, yuanhuacine suppresses BC cells by targeting PKM2, which controls the STAT3 pathway and glycolysis. The combination of yuanhuacine and PKM2 siRNA treatment significantly hindered the phosphorylation of STAT3 (Y705) and its downstream effects, in contrast to yuanhuacine treatment alone (63). This finding could also indicate a synergistic effect of yuanhuacine when it is used with other cancer-specific treatments.

Cantharidin

Cantharidin, a sesquiterpenoid bioactive component, is among the active ingredients found in *mylabris*. Many *in vitro* studies have investigated cantharidin's antitumor activity, which includes hindering migration, inducing apoptosis, halting the G2/M transition, and suppressing invasion (133). Yang et al. showed that EGFR activation results in the translocation of PKM2 into the nucleus, leading to increased expression of GLUT-1 and LDHA, which work together to enhance aerobic glycolysis in a positive feedback loop (105). This mechanism relies on ERK signaling, which is facilitated by importin α5 (23). Pan et al. (134) investigated the mechanism by which cantharidin influences PKM2 translocation into the nucleus in BC cells. Cantharidin reduces GLUT1 transcription and glucose uptake by blocking the transformation of PKM2 dimers into tetramers and their entry into the nucleus. Most importantly, the inhibitory effect of cantharidin on migration and invasion was significantly reversed when FBP and L-cysteine were introduced (134). These findings suggest that cantharidin and its derivatives have strong potential as anti-metastatic agents, making them highly valuable in clinical applications.

Beta-elemene

Beta-elemene is a prominent non-cytotoxic anticancer ingredient extracted from *Curcuma zedoary* that triggers cancer cell death, halts cell cycle progression, and improves radiotherapy and chemotherapy sensitivity without causing myelosuppression or notable harm to the liver or kidney (135–137). In cancer cells, the balance between tetrameric and dimeric forms of PKM2 regulates the glucose metabolic pathway, influencing energy production and the synthesis of anabolic precursors. Additionally, this interconversion between dimeric and tetrameric PKM2 helps to maintain a dynamic equilibrium (37, 138). Pan et al. showed that β-elemene inhibited the spread of BC by impeding aerobic glycolysis. Specifically, β-elemene hindered the transformation of dimeric and tetrameric forms of PKM2, thus inhibiting its pyruvate kinase activity, resulting in reduced utilization of glucose and generation of pyruvate and lactate. Besides its metabolic function, PKM2 acts as a nonmetabolic protein kinase and transcriptional coactivator for HIF-1α and c-MYC, which are crucial for tumorigenesis induced by the activation of EGFR. Activated EGFR facilitates the translocation of PKM2 to the nucleus through importin α5 (23, 105). β-elemene inhibited the EGFR-importin α5-mediated movement of PKM2 into the nucleus and the expression of GLUT1, monocarboxylate transporter 1 (MCT1), MCT4, and LDHA (139). Collectively, these studies suggest that β-elemene can inhibit BC metastasis by disrupting PKM2-mediated metabolic signaling to exert an anti-BC effect.

Noncoding RNAs regulate nonmetabolic function of PKM2 in breast cancer

Current studies are focused on the impact of long noncoding RNA (lncRNA), circular RNA (circRNA), and microRNA (miRNA) on PKM2 in BC tumorigenesis and development (140–142). Noncoding RNA can influence BC cell metabolic reprogramming

by targeting PKM2's metabolic enzyme function as well as its nonmetabolic enzyme function (141, 143). In some cases, miRNAs have been found to regulate PKM2 expression in BC cells. For instance, miR-122 is abundantly released by tumor cells and promotes metastasis by adapting to the metabolic environment in the pre-metastatic niche, whereas down-regulation of PKM2 and GLUT1 limits glucose consumption in BC cells (144). miR-Let-7a inhibited MDA-MB-231 cell proliferation and particularly upregulated the levels of PMK2, oxidative phosphorylation, and reactive oxygen species (ROS) in TNBC and improved the sensitivity of BC cells to the tumor suppressor doxorubicin in BC metastasis (145). CircKIF4A, which is largely found in the cytoplasm, may interact with miR-335, while aldolase A (ALDOA) and octamer-binding transcription factor 4 (OCT4) serve as miR-335's downstream targets. ALDOA and OCT4 are metabolic proteins that control glycolytic proteins such as HK2 and PKM2 (146–148). Increased miR-335 expression resulted in decreased levels of ALDOA/HK2 and OCT4/PKM2 proteins. As a result, circKIF4A regulates glucose metabolism through the miR-335-ALDOA/OCT4-HK2/PKM2 pathway (149).

Zheng et al. demonstrated the importance of HIF-1α antisense lncRNA (HIFAL) in maintaining and increasing HIF-1α-driven transactivation and glycolysis. Functionally, HIFAL recruits PHD3 to PKM2, causing its prolyl hydroxylation and facilitating the entry of the PKM2/PHD3 complex into the nucleus by interacting with heterogeneous nuclear ribonucleoprotein (hnRNP) F. This promotes HIF-1α transactivation, glucose absorption, and lactate generation in BC cells (150). Besides, Yao et al. revealed that miR-Let-7a-5p suppresses aerobic glycolysis by modulating the STAT3/hnRNP-A1/PKM2 signaling pathway. They showed that hnRNP-A1 controls PKM2 gene selective cleavage, resulting in enhanced PKM2 synthesis. STAT3 may increase hnRNP-A1 transcription, whereas miR-Let-7a-5p suppresses STAT3 and hence reduces PKM2 (141, 151).

A study conducted by Wen et al. revealed that miR-152 suppresses growth and angiogenesis in BC by suppressing both β-catenin and PKM2. β-catenin, which is a downstream target of insulin-like growth factor 1 (IGF-1), plays a role in controlling cell proliferation (53). Recent evidence has shown that miR-148a/152 activation contributes to the suppression of PKM2 and NF-κB p56 expression in TNBC cells. NF-κB p56 interacts with PKM2 to regulate early growth response 1 (EGR1) expression. EGR1 can bind to multiple sites on miRNA gene promoters, thereby controlling miR-148a and miR-152 expression (152).

Conclusion and future perspectives

In this review, we reviewed how PKM2 has emerged as a crucial player in BC; through its role as a transcriptional co-activator and regulator of gene expression, PKM2 has been demonstrated to contribute to the epigenetic regulation of gene transcription. Understanding the diverse functions of PKM2 beyond its metabolic role opens new avenues for targeting this enzyme in BC treatment. The nonmetabolic role of PKM2 controls the expression of cancer-related genes implicated in various aspects of BC progression, such as cell cycle, proliferation, programmed cell death, angiogenesis, migration and invasion, tumor microenvironment, and others. Natural products such as shikonin, cryptanshinone, cyclosporine A, lapatinib, mangifera indica, beta-elemene, yuanhuacine, cantharidin, and others control PKM2's nonmetabolic function and affect BC progression. In addition, the interaction of PKM2 with noncoding RNAs has garnered increasing interest. Noncoding RNAs regulate PKM2's nonmetabolic enzyme function and influence BC growth. In the future, it will be imperative to further investigate the nonmetabolic function of PKM2 in BC in order to gain a comprehensive understanding of its involvement in the growth and advancement of tumors. In addition, exploring the interaction between metabolic reprogramming and PKM2-mediated nonmetabolic functions in BC is an intriguing area for further research. This knowledge could potentially lead to the use of PKM2 as a molecular marker for BC diagnosis and prognosis, which could be highly important for the therapeutic targeting of BC.

Author contributions

MJ: Conceptualization, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. MW: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. GA: Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. BT: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. TB: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. EM: Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EO: Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. NC: Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. AA: Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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A novel arginine methylation-associated lncRNA signature effectively predicts prognosis in breast cancer patients

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Breast cancer (BC) is a disease highly associated with epigenetic modification, and arginine methylation is particularly important in its genetic regulation. However, the role of arginine methylation related lncRNAs in breast cancer has not been studied. First, we identified the related lncRNAs (from TCGA database) according to the differentially expressed genes related to arginine methylation in breast cancer. Then the lncRNAs related to protein arginine methylation were obtained by regression analysis, and the risk score model was constructed. Finally, the cell experiment and subcutaneous tumor model verified that the arginine methylation related lncRNA z68871.1 in the model had a significant effect on the proliferation and invasion of breast cancer cells. In conclusion, we successfully constructed an arginine methylation related lncRNA model, which has strong predictive ability. At the same time, this study provides an experimental basis for exploring the mechanism of arginine methylation in BC and helps to find new biomarkers of BC.

KEYWORDS

breast cancer, arginine methylation, lncRNA, prognosis, cancer biomarkers

Introduction

Breast cancer (BC) is an incurable disease that causes great harm to women (1). Despite considerable progress in early diagnosis and individual therapy over the past decades, drug resistance, recurrence, metastasis, and cancer heterogeneity remain important causes of cancer-related deaths and are significant issues hindering the successful management of BC (2). Individualized specific targeted therapies are receiving increasing attention in clinical work. Therefore, it is necessary to explore new biomarkers with diagnostic and therapeutic significance.

Arginine methylation is a common post-translational modification of proteins (3), similar to phosphorylation and ubiquitination (4), it plays a vital role in cell biology, where it can affect protein function, stability, and localization, as well as participate in cancer-related epigenetics and signaling, RNA metabolism, and DNA repair (5–7). Recent research has demonstrated that arginine methylation plays a role in the progression of breast cancer (8, 9). Therefore, identifying key regulatory factors of arginine methylation is crucial for basic research and diagnosis and treatment of tumors.

Currently, long non-coding RNA (lncRNA) plays an important role in a variety of genetic material regulation processes (10), including proliferation, apoptosis, metastasis, metabolism and drug resistance (11, 12). In addition, the emergence of genomics and next-generation sequencing technologies has provided new evidence for the impact of lncRNAs on gene regulation (13). lncRNAs, as one of the critical regulators of genetic material, affect the stability and various modifications of genetic material by regulating nuclear structure and transcription (14). With further research, it was discovered that lncRNA boosts tumor progression by regulating arginine methylation and have the potential to predict the deterioration of various cancer prognoses (15–17). Therefore, in cancer research, it is important to identify key lncRNAs closely related to arginine methylation. It is important in the treatment and prognosis evaluation of cancer.

Our study conducted an in-depth survey of the dataset containing lncRNA expression profiles in BC from TCGA database. Our screening process focuses on identifying lncRNAs with prognostic significance associated with arginine methylation. Therefore, we identified 8 different lncRNA signals associated with arginine methylation prognosis, which may accurately predict the survival outcomes of cancer patients.

Materials and methods

Data source

All data (including age, gender, histologic grade, OS time, survival status and gene expression) were from the TCGA database. After data collation, 1096 breast cancer and 112 standard tissue samples were finally obtained (excluding data with survival time less than 30 days and unrecorded data collection).

Identification of arginine methylation-related lncRNAs

The arginine methylation-related genes included in the study were obtained from the GeneCards database. First, we investigated the differentially expressed genes (DEGs, (|log2 FC|> 1). Next, Cox regression analysis was performed to identify key LncRNAs as prognostic features of BC.

Predictive risk mode

We constructed a risk score (RS) based on 8 predictive features. $risk\ score = \sum_{i=1}^{n} coef\ AMT\ LncSigi\ X\ EXP\ AMT\ LncSigi$.

The "Coef AMT LncSigi" in the formula represents the value of the multifactorial regression coefficient, and the "EXP AMT LncSigi" indicates the expression of lncRNA.

Immune cell function and drug analysis

Immune cell activity and pathways were calculated for each sample using single-sample GSEA (ssGSEA), and then the proportion of infiltrating immune cells in the tumor samples was assessed and immune-related drugs were evaluated and predicted using the "pRRophetic" R package.

Cell transfection

We purchased two cell lines (MCF-7 and MDA-MB-231) from the Cell Bank of the Chinese Academy of Sciences. Cells with good growth conditions were taken and digested with trypsin. Then, the cells were inoculated in 6-well plates(1×10^5) and placed in the 5% CO2 cell culture incubator for lentiviral transfection experiments until the cell confluence was 20~30%. Lentiviral expression vectors with Z68871.1 sequence were purchased from Shanghai Jikai Gene Medical Technology Co. According to the instructions of the reagent supplier, cells were infected with packaged lentivirus.

Clone formation experiment and radiosensitivity analysis

MCF-7, MDA-MB-231 experimental group and control group were digested with trypsin and resuspended as a single cell suspension, respectively; 1000 cells/well were put into the corresponding cells in 6-well Petri dishes and placed in the cell incubator for two weeks. The number of colonies was counted for analysis. Colony formation was used to assess the proliferative capacity of the cells. At the same time, in order to further understand the effect of z68871.1 on the radiosensitivity of breast cancer cells, MCF-7 cells were irradiated with doses of 0Gy, 3Gy and 6Gy, and the cell survival fraction was observed by colony formation assay.

Transwell invasion experiment

Transwell chambers (Corning Costar, 8 μm) were primed with Matrigel matrix gel. The transfected control and experimental cells were resuspended with an FBS-free DMEM medium. After 24 hours of cultivation, we fixed the cells with 4% PFA, stained and counted them.

CCK-8 cell proliferation experiment

Cells in the logarithmic growth phase were taken to prepare a single-cell suspension. The cells were counted with a glass plate, and the cell concentration was 2 × 104/ml. Take the cell culture plate, and each group of cells is set at 2000/100 μ l/well, with 3 parallel samples in each group, and add the whole culture medium. Continue to cultivate in the incubator. CCK8 reagent (10 μL/well) was added to 96 well plates after 0, 24, 48, 72 and 96 hours, respectively. Cell-free medium wells can be used as blank control. Continue to incubate in the cell incubator for 1-4 hours. The microplate reader measured the absorbance value of 450nm. Draw the growth curve of each group of cells.

Subcutaneous tumor formation experiments in mice

Five-week-old female nude mice (Guangdong Pharmachem Biotechnology Co., Ltd., Guangzhou, China) were divided into two groups. MCF-7 cells were divided into the NC and Z68871.1-ox groups, respectively. The number of cells injected subcutaneously into each mouse was 1×106, and weekly live imaging was performed to record the growth of tumors. This study was approved by the ethics committee of the Cancer Hospital Affiliated with Shandong First Medical University.

Statistical analysis

R software (https://www.r-project.org/) was used for all analyses. The results of clone formation and transwell invasion experiments were analyzed using ImageJ software, and GraphPad Prism 8.0 was used to analyze the statistical significance of the difference between the two groups. Correlation coefficients were examined using the Pearson correlation test. $P < 0.05$ was considered significant.

Results

Screening of arginine methylation-associated lncRNAs

Based on the GeneCards database, 111 arginine methylation-associated genes were obtained, and the screening criterion was a correlation score of >10 for protein-coding genes (Supplementary Data Sheet 1). Subsequently, 21 arginine methylation-associated DEGs were obtained by differential analysis of BC and normal breast tissues (Figures 1A, B). Enrichment analysis demonstrated that the above genes were enriched in cell growth and division, hormone and immune, cytokine receptor and tumor-related biological function signaling pathways (Figures 1C, D). Then, 1199 arginine methylation-associated lncRNAs (Supplementary Data Sheet 2) were screened by arginine methylation-associated DEGs. Univariate Cox regression screened 48 prognostic-related lncRNAs (Supplementary Material 3). Multivariate Cox regression identified 8 lncRNAs with the best predictive correlation: AL122010.1, OTUD6B-AS1, EGOT, AP005131.2, Z68871.1, AC024361.1, LINC00987, ST7-AS1 (Table 1), and we further visualized the correlation between lncRNAs and mRNAs using Cytoscape (Figures 1E, F), showing that Z68871.1, AP005131.2 and AC024361.1 were most closely associated with mRNAs.

Construction of predictive risk model and internal validation

We used the RS = (0.542214503 × Z68871.1 expression) + (0.571403384 × OTUD6B-AS1 expression) - (0.8962291962× AC024361.1 expression) - (0.576665716 × LINC0098 expression) - (0.76381661 × AL122010.1 expression) - (0.717527719 × ST7-AS1 expression) - (0.497395506 × AP005131.2 expression) - (0.411082836 × EGO expression) to calculate the score of each patient. Patients in the model were categorized into two groups (high- and low-risk groups), and patient scores were positively related to prognosis (Figures 2A, B). lncRNA expression levels also showed significant differences (Figure 3C). The ROC curves for the model training set data were 0.75 (1 year), 0.762 (3 years), and 0.777 (5 years), and the K-M curves also demonstrated a poor prognosis for patients with high RS (Figures 2D, E).

Using the same threshold, the validation set was divided into two groups. Patient distribution was similar to the training set (Figure 2F), and high scores were positively associated with worse prognosis (Figure 2G). The validation set AUC value and lncRNA expression levels were similar to the training set (Figures 2H, I). K-M analysis proved that the model predicted well ($p<0.05$, Figure 2, J).

Construction of nomogram diagrams and validation of accuracy

The results of unifactorial and multifactorial analyses of clinical characteristics demonstrated that the model could predict patient prognosis independently of other clinicopathologic data (Figures 3A, B). Next, we constructed a nomogram diagram based on clinicopathologic data T, N, M grading, and risk score, which was used to calculate survival probabilities for clinical workup (Figure 3C). Upon further analysis, the risk model was significantly correlated with patient staging and prognosis (Figures 3D, E). The ROC curves suggest that predictive risk models perform best when compared to other clinical data (Figure 3F). Calibration curves show good agreement between model predictions and actual conditions (Figures 3G–I).

To further validate the accuracy of the risk model, we analyzed the survival curves of individual genes in the model (Figures 4A–H). We found that similar to the multifactorial results, high expression levels of AC024361.1, LINC00987, AL122010.1, EGOT, ST7-AS1, and AP005131.2 were correlated with a better prognosis; on the

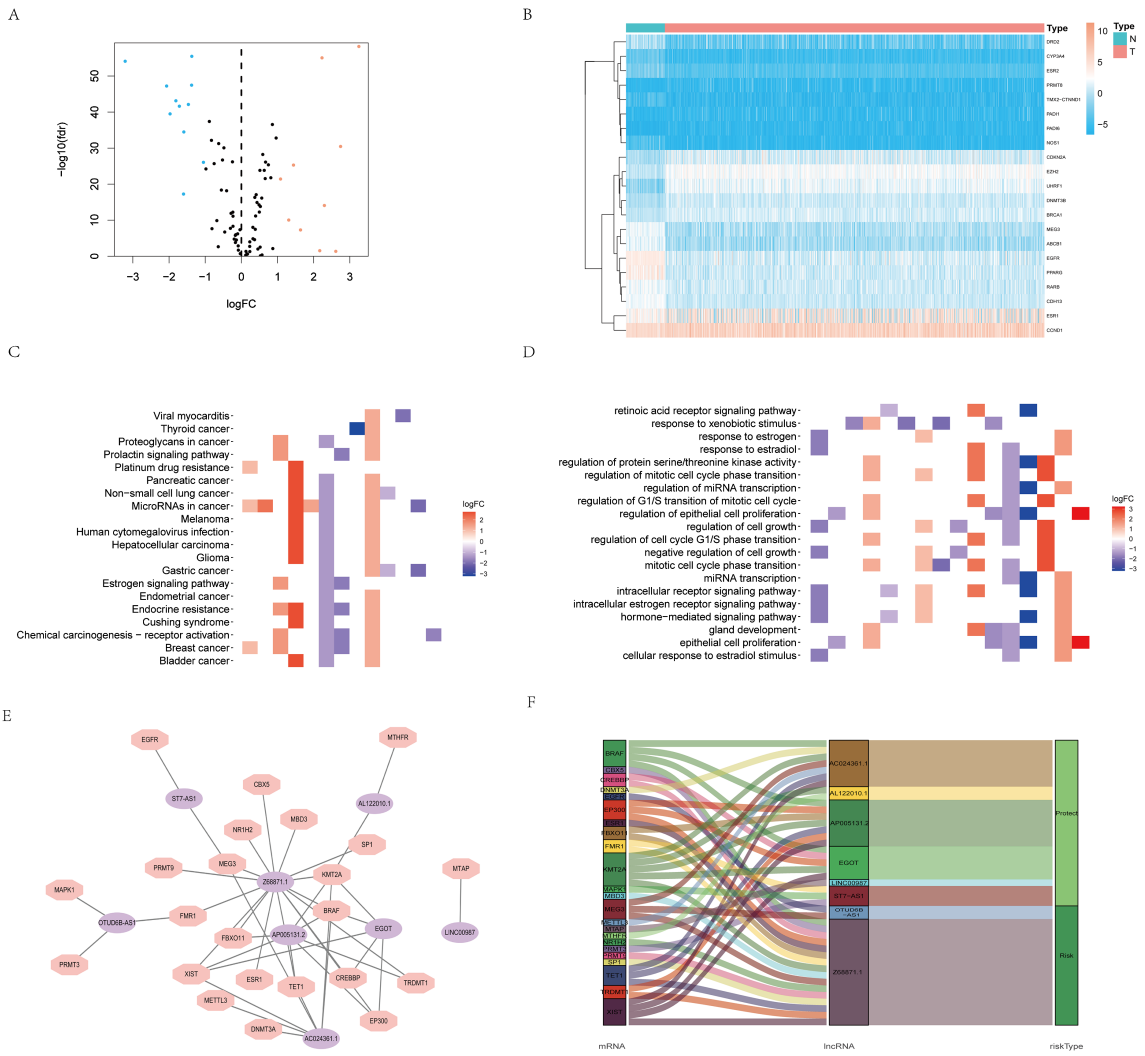

FIGURE 1
Screening of arginine methylation-related lncRNAs. **(A)** Volcano plot of 10 up-regulated and 11 down-regulated DEGs in BC. **(B)** Heatmap of 21 protein arginine methylation-associated DEGs. **(C)** KEGG analysis of protein arginine methylation-associated DEGs. **(D)** GO analysis of protein arginine methylation-associated DEGs. **(E)** The co-expression network of prognostic protein arginine methylation-associated lncRNAs. **(F)** Sankey diagram of prognostic protein arginine methylation-associated lncRNAs. N, normal; T, tumor.

TABLE 1 Prognostic arginine methylation-associated lncRNAs.

id	coef	HR	HR.95L	HR.95H	pvalue
AC024361.1	-0.896229	0.4081056	0.1720781	0.9678756	0.0419446
LINC00987	-0.576666	0.5617683	0.3409932	0.9254839	0.0235749
Z68871.1	0.5422145	1.7198112	0.8856762	3.3395393	0.1092875
AL122010.1	-0.763817	0.4658849	0.2552217	0.8504324	0.0128608
OTUD6B-AS1	0.5714034	1.7707504	1.0852829	2.8891607	0.02216
ST7-AS1	-0.717528	0.4879571	0.2257422	1.0547524	0.0680878
AP005131.2	-0.497396	0.6081124	0.3017806	1.2253959	0.1641129
EGOT	-0.411083	0.662932	0.4442858	0.9891805	0.0440894

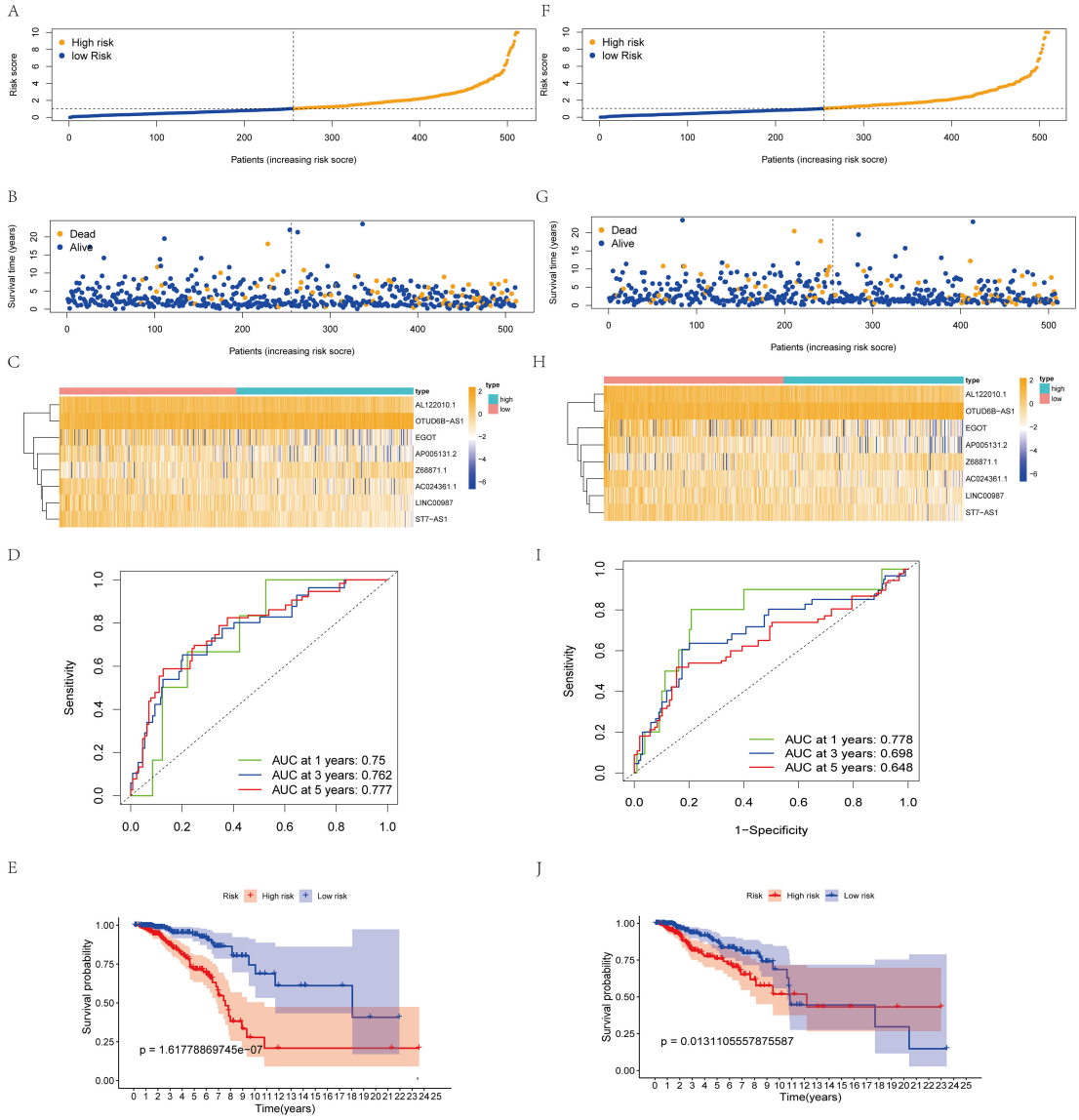

FIGURE 2
Construction and validation of the prognostic protein arginine methylation-associated lncRNAs signature for survival prediction. **(A-E)** Distribution of RS; survival time and status of patients; heatmap of protein arginine methylation-associated lncRNAs of RS; ROC curve; K-M curve for training Data. **(F-J)** Distribution of RS; survival time and status of patients; heatmap of protein arginine methylation-associated lncRNAs of RS; ROC curve; KM curve for validation Data.

contrary, high expression levels of OTUD6B-AS1 and Z68871.1 were associated with a poor prognosis of patients.

Immune cell infiltration and functional analysis

We further investigated the biological functions of prognostic factors using GSEA and found that many immune-related pathways and cell metabolism-related pathways were associated with high-risk populations (Figures 5A, B). we further performed ssGSEA enrichment analysis and found that in the high-risk populations, most cells (DC cells, Th1 cells, NK cells, Tregs, etc.) were significantly elevated (Figure 5C). APC co-stimulation, CCR, T cell co-inhibition, and checkpoint were significantly observed in the high-risk populations (Figure 5D). Meanwhile, in order to better understand their correlation, we specifically quantified the relationship coefficients between immune-related pathways and the proportion of immune cells (Figures 5E–G). Given the importance of immune checkpoints for BC, we also analyzed 21 common immune checkpoints and found that PDCD1LG2, CD86, IDO1, CD276, TNFSF9, and NRP1 were higher in the high-risk populations (Figure 5H). Finally, relevant drug prediction analysis showed that the IC50 values of Etoposide, Rapamycin, Lenalidomide, and Cisplatin were higher in the high-risk populations (Figure 5I).

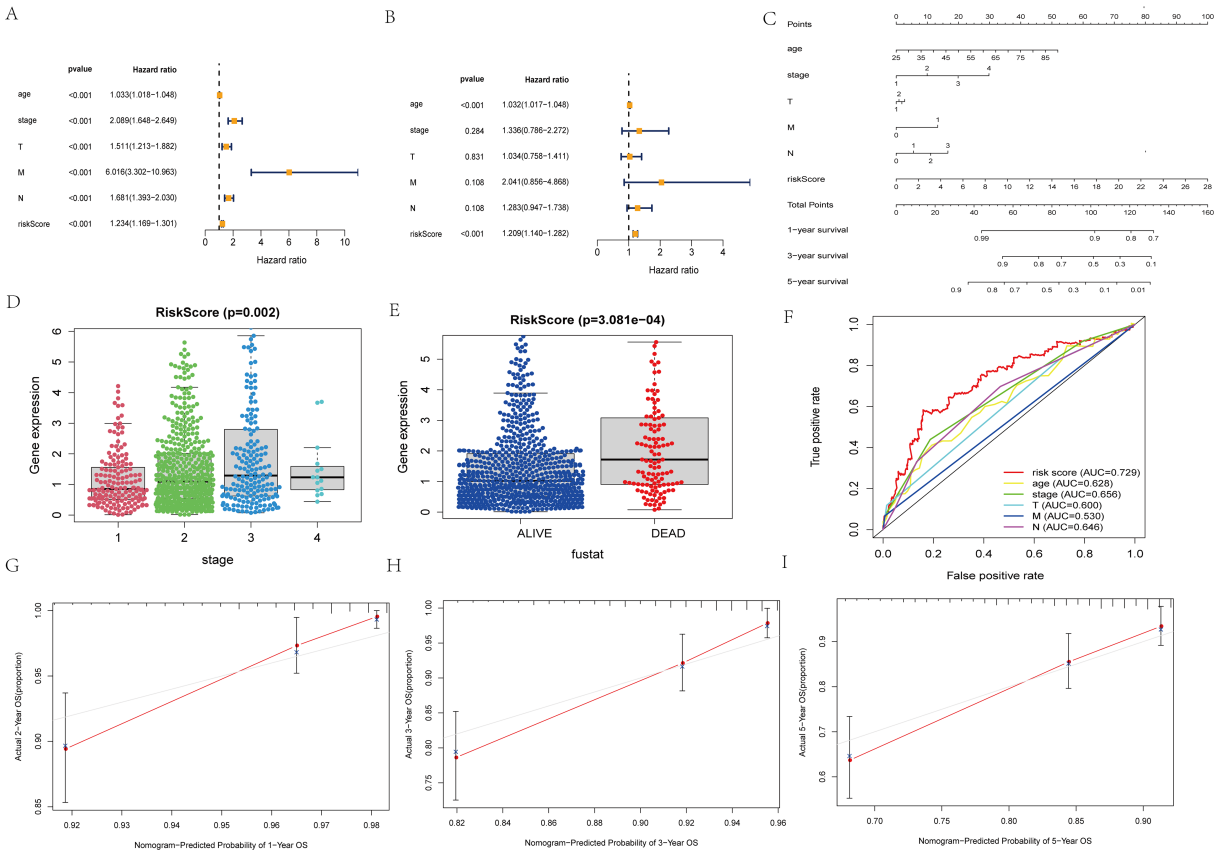

FIGURE 3
Independent prognosis analysis of risk score. **(A)** Univariate COX Forest plot of risk score. **(B)** Multivariate COX Forest plot of risk score. **(C)** A nomogram based on prognostic features. **(D, E)** Relationship between stage staging, survival status and risk score of patients. **(F)** ROC curve of risk score and clinical characteristics. **(G–I)** Calibration plots of the nomogram for predicting the probability of OS at 1, 3, and 5 years.

Overexpression of Z68871.1 enhances proliferation and invasion of BC

Among these eight screened genes, most of them, except Z68871.1 and AC024361.1, have sufficient basic experimental studies in cancer. According to our preliminary analysis, the closest association between Z68871.1 and mRNA was found, so it is reasonable to suspect that it plays a crucial role in BC. Therefore, in the following experiments, we investigated whether Z68871.1 plays a role in BC development. First, we overexpressed Z68871.1 in two BC cell lines, and in a cellular functionalization assay, a cell cloning assay showed that overexpression of Z68871.1 enhanced cell viability in BC cells (Figures 6A, B). Next, we also examined the invasive ability of cells and found that overexpression of Z68871.1 significantly enhanced their invasive ability (Figures 6C, D). Next, we used the CCK8 assay to detect the effect of z68871.1 on cell proliferation. The results showed that in the cell line, there was no significant change in each group at 24 hours. After 72 hours, the proliferation ability of overexpressing z68871.1 cells was stronger (Figures 6E, F). In order to further understand the effect of z68871.1 on the radiosensitivity of breast cancer cells and provide useful information for clinical treatment and prognosis, MCF-7 cells were irradiated with doses of 0Gy, 3Gy and 6Gy, respectively. The radiosensitivity of MCF-7 cells was evaluated by plate clone formation assay (Figures 6G, H). The survival rate of the nc group at 3Gy and 6Gy doses was lower than that of the ox group (P <0.05). The overexpression of z68871.1 attenuated the sensitivity of MCF-7 cells to radiation. Finally, to further test the functional effects of Z68871.1 on BC, we established an animal tumor model to explore the biological effects of Z68871.1. After the mouse tumor model was established, each mouse was subjected to *in vivo* imaging at intervals of 1 weekday to monitor the tumor growth process and plot the growth curve of intracranial meningiomas. The fluorescence values of the intracranial tumors in the two groups of nude mice did not show any significant difference in the first 2 weeks after cell injection, and the fluorescence values of the tumors in the two groups began to change on day 21 (P <0.05). Twenty-eight days later, the fluorescence values of tumors overexpressing Z68871.1 were remarkably higher than those of the control group (Figures 6I, J P < 0.001). These suggest that overexpression of Z68871.1 enhances the proliferation and invasion of BC in a nude mouse tumor model.

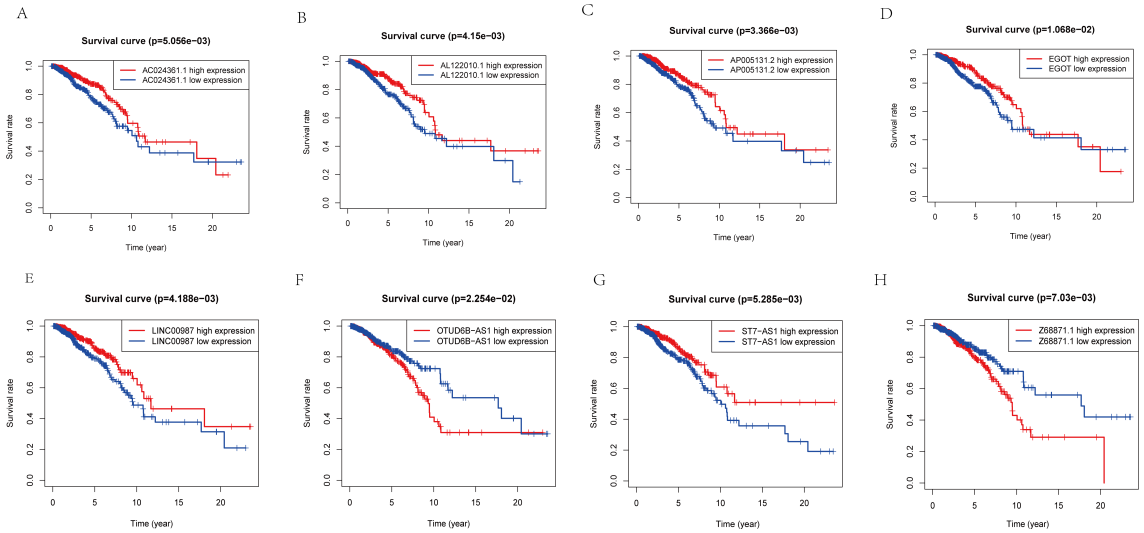

FIGURE 4
K-M analysis of lncRNA expression level in predictive model and prognosis of patients. **(A)** The relationship between the expression level of AC024361.1 and prognosis. **(B)** The relationship between the expression level ofAL122010.1 and prognosis. **(C)** The relationship between the expression level of AP005131.2 and prognosis. **(D)** The relationship between the expression level of EGOT and prognosis. **(E)** The relationship between the expression level of LINC00987 and prognosis. **(F)** The relationship between the expression level of OTUD6B-AS1 and prognosis. **(G)** The relationship between the expression level of ST7-AS1 and prognosis. **(H)** The relationship between the expression level of Z68871.1 and prognosis.

Discussion

Currently, despite the significant advances in the treatment of BC patients, BC accounts for 23% of all cancer-related deaths and poses a major threat to women's survival (18–20). Therefore, the need to explore new molecular targets and individualized treatments for BC is urgent (21).

Numerous studies have fully demonstrated that arginine methylation is crucial in transcriptional regulation, cell division, cell cycle regulation, DNA repair and substance metabolism in multiple genetic and developmental aspects and critical processes (22). Not only that, arginine methylation has been well studied in cancers (breast, lung, and colon cancers as well as leukemia), and most of the protein arginine methyltransferases have been associated with cancer-related epigenetic and chromatin regulation, transcription, signaling, and metabolic regulation (23). In recent years, research has proved that lncRNAs carry out different functions in the physiological processes of tumors (24–26). Nevertheless, the functions of arginine methylation-associated lncRNAs in BC patients have never been investigated. Therefore, the present study aimed to identify arginine methylation-related lncRNAs, construct a risk model with predictive properties, and characterize the genetic and immune functions of lncRNAs.

Firstly, we screened 21 genes relevant to arginine methylation and then used reliable biological analysis to determine the predictive characteristics of 8 prognostic lncRNAs related to arginine methylation. After analysis, AC024361.1, LINC00987, AL122010.1, EGOT, ST7-AS1, and AP005131.2 have been identified as protective factors; OTUD6B-AS1 and Z68871.1 have been identified as risk factors. Then, GSEA analysis revealed that cell cycle and TGF-β signaling pathways were more marked in the high-risk population. Afterwards, the results regarding TIICs demonstrated that the infiltration abundance of DCs, Th1, NK and Tregs cells was higher in the high-risk population. Finally, basic experiments on lncRNA in the model were conducted again through BC cells and mice experiments, and the results showed that Z68871.1 affected the viability, proliferation and invasion ability of BC cells, and had an impact on the sensitivity of cells to radiation. Therefore, the arginine methylation-associated lncRNAs successfully screened in our work could predict the outcome of BC patients. More importantly, it provides ideas for further exploring the personalized therapeutic value of arginine methylation-related lncRNAs.

In previous studies, lncRNAs were studied, and their biological functions were confirmed. Wang et al. discovered that lncRNA ST7-AS1 was critical in promoting LUAD cell viability, affecting invasive ability and leading to EMT. The mechanism of its carcinogenic activity is mainly achieved by sponge-like miR-181b-5p, thereby relieving the inhibition of KPNA4 (27). In addition, other studies have found that the low expression of ST7-AS1 may change the regulation of the cell cycle, affect the repair ability after DNA damage, and affect the distribution and activity of immune cells in the microenvironment of breast cancer, thus leading to the development of tumors and therapeutic effects (28). Li et al.'s experimentation has shown that overexpression of OTUD6B-AS1 can inhibit the malignant biological characteristics of thyroid cancer cells and is achieved by targeting miR-183-5p (29). However, there are also studies that overexpression of OTUD6B-AS1 can promote tumor progression, such as lncRNA OTUD6B-AS1 promoting malignant transformation of liver cancer cells through Wnt/β-catenin signaling (30). The biological function of LINC00987 has also been studied in lung cancer. Specifically, the progression of

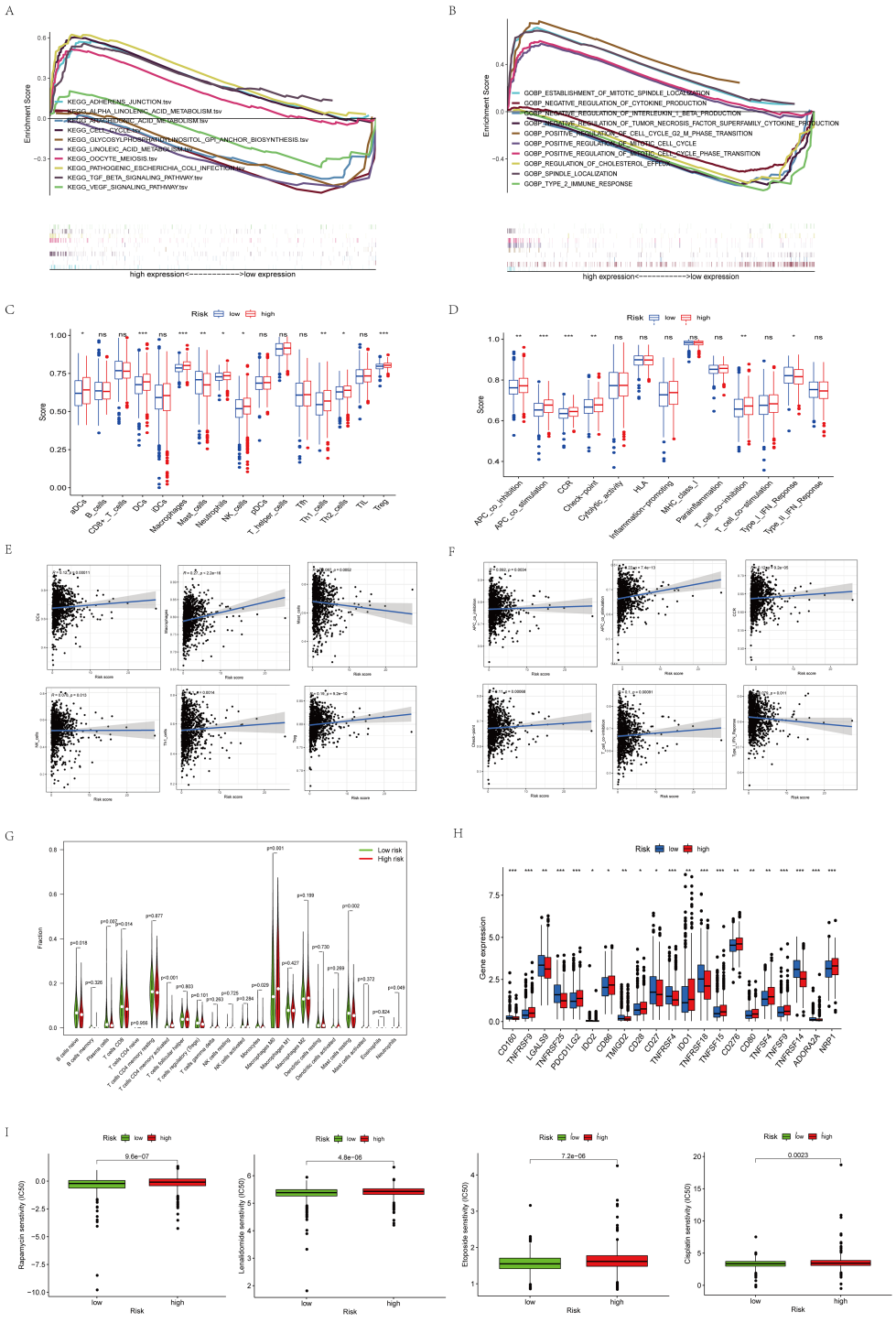

FIGURE 5
Functional enrichment analysis of 8 prognostic arginine methylation-associated lncRNAs. **(A)** KEGG analysis of 8 prognostic arginine methylation-associated lncRNAs. **(B)** GO analysis of 8 prognostic arginine methylation-associated lncRNAs. **(C, E)** Infiltration level and correlation of 16 kinds of immune cells. **(D, F)** The correlation between the predictive signature and 13 immune-related functions. **(G)** Analysis of immune cell infiltration between high-risk group and low-risk group. **(H)** Analysis of immune checkpoints between high-risk group and low-risk group. **(I)** IC50 of Rapamycin, Lenalidomide, Etoposide, and Cisplatin in high and low-risk groups. (ns $P > 0.05$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.).

lung adenocarcinoma is induced by the downregulation of low methylation related LINC00987, which inhibits phosphorylation-mediated SND1 degradation (31). AL122010.1 and AP005131.2 have previously been reported as immune and metabolic prognostic features of breast cancer (32, 33). AC024361.1 can serves as a risk model of autophagy-associated lncRNA composition (34), but this study did not demonstrate its function experimentally. Studies on EGOT have found that the expression of EGOT is negatively

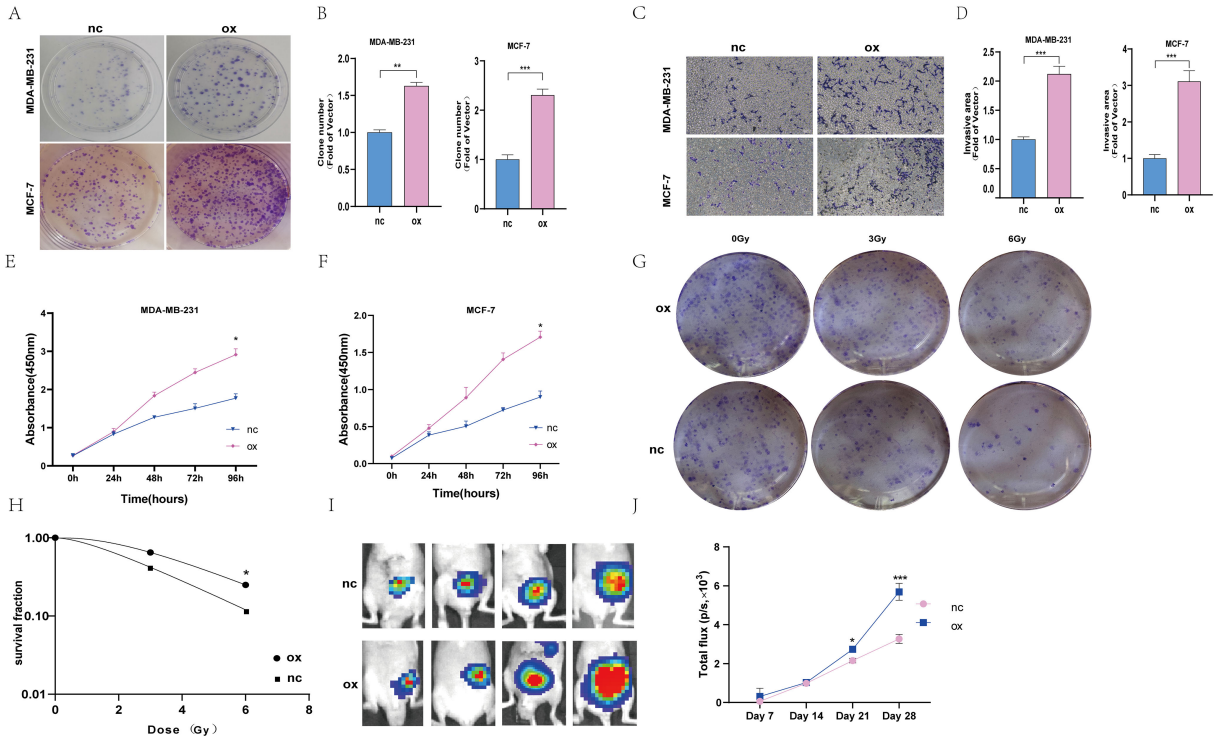

FIGURE 6
Overexpression of the z68871.1 gene can enhance the proliferation and invasion of MCF-7 and MDA-MB-231 cells. **(A, B)** Cell clones were used to detect the proliferation of MCF-7 and MDA-MB-231 cells. **(C, D)** The transwell method was used to detect the migration and invasion of MCF-7 and MDA-MB-231 cells. **(E, F)** Use CCK-8 method to detect the proliferation ability of MCF-7 and MDA-MB-231 cells. **(G, H)** Cell cloning experiment to detect the effect of Z68871.1 on the radiation sensitivity of MCF-7 cells. **(I, J)** *In vivo* imaging detection of subcutaneous tumour growth in mice. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.).

correlated with the survival of breast cancer patients, which is mainly caused by the inactivation of lncRNA EGOT through the Hedgehog pathway (35). Similarly, EGOT research in gastric cancer also found that the loss of EGOT led to the down-regulation of Hedgehog signaling pathway, and could inhibit the proliferation function by preventing the G1 phase cycle process of GC cells (36). Although Z68871.1 has recently been reported as a methyladenosine-modified lncRNA that can predict outcomes for BC patients (37), Zhao et al. did not conduct basic experiments to verify this finding in their study. Therefore, our functional research on Z68871.1 in cells and animals provides relevant evidence for their conclusion to some extent.

Next, we conducted GSEA analysis further to explore other functions of lncRNA models in BC. KEGG analysis shows that high-risk populations are mainly enriched in pathways closely concerned with cell cycle. Functional analysis showed that DEGs were mainly enriched in immune pathways such as anti-tumor factor action, and negative interleukin blockade. Based on the above analysis, we found a correlation between the predictive factors in the model and tumor immunity. Therefore, further study of immune cells and pathways showed that DCs, Th1 cells, NK cells, Tregs, etc. have a higher proportion in high-risk populations, and CCR, T cell co inhibition, and checkpoints are more significant. Previous experimentations have shown that the proportion of different immune cells in BC can affect the prognosis of patients (38). Moreover, it was found that M2 macrophages release cytokines that promote tumor growth. These factors not only stimulate tumor cell proliferation and angiogenesis but also promote tumor metastasis and regulate the extracellular matrix, leading to poor prognosis in BC patients (39). Our research findings are consistent with the appeal's conclusions, further confirming and strengthening the reliability of our risk prediction model in terms of predictive ability. Finally, our research on immune control points and immune-related drugs aims to explore new directions for immunotherapy of BC. Fortunately, many of the latest experimental results have fully confirmed that the medicines we predicted have achieved remarkable results in improving the poor outcomes of BC patients and animal models of breast cancer (40, 41). This not only validates our research but also brings new hope and strategies for breast cancer patients to provide more accurate and personalized treatment.

This study successfully constructed a prediction model based on high-throughput sequencing data and verified the effectiveness of the model through basic experiments. However, there are still some limitations, such as the need for more sequencing data and prospective studies to verify the accuracy of the model. In addition, the link between z68871.1 and arginine methylation has not been established in this study, and the specific mechanism and function of lncRNA and arginine methylation still need to be further explored, and more basic experiments are needed to help study how z68871.1 promotes the proliferation and invasion of breast cancer cells through arginine methylation. In the future, we

will conduct in-depth studies on arginine methylation-related lncRNAs and verify the prediction accuracy of this model through more samples and experiments so as to promote its application in clinical work.

In summary, we have successfully identified lncRNAs with significant predictive value in the process of arginine methylation and established a highly accurate prediction model based on them. And this model not only provides a new perspective for the development of cancer treatment strategies, but also opens up new avenues for future basic research on lncRNAs related to arginine methylation.

Data availability statement

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

Ethics statement

The animal study was approved by the ethics committee of the Cancer Hospital Affiliated with Shandong First Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CW: Conceptualization, Software, Writing – original draft, Writing – review & editing. SW: Conceptualization, Software, Writing – original draft, Writing – review & editing. YH: Data curation, Investigation, Writing – original draft. JW: Data curation, Investigation, Writing – original draft. KR: Data curation, Investigation, Writing – original draft. MZ: Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1472434/full#supplementary-material

SUPPLEMENTARY MATERIAL 3
lncRNAs were associated with patient prognosis.

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Mechanisms of tamoxifen resistance: insight from long non-coding RNAs

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Breast cancer(BC) is the second most prevalent tumor in the world and one of the most lethal tumors in women. Patients with estrogen receptor-positive breast cancer can obtain significant advantages from endocrine therapies including tamoxifen, aromatase inhibitors, and others. However, the development of primary or acquired drug resistance ultimately leads to discontinuation of treatment with adverse consequences for breast cancer patients, and the underlying mechanisms have not been fully elucidated. Long non-coding RNAs (lncRNAs) play pivotal roles in orchestrating fundamental biochemical and cellular processes. They exert regulatory control over various processes including epigenetics, gene transcription, post-transcriptional modifications, and translation. Additionally, they influence key biological events like cell cycle progression, cell differentiation, and development. For the past few years, the relationship between lncRNAs and endocrine resistance has gained increasing attention, leading to a surge in related studies. LncRNAs mediate tamoxifen resistance in cancer by utilizing a variety of molecular mechanisms, including enhanced estrogen receptor (ER) signaling, inhibition of apoptosis, autophagy, exosome-mediated transfer, epigenetic alterations, epithelial-to-mesenchymal transition, and acting as competitive endogenous RNAs(ceRNAs). In this comprehensive review, we systematically summarize the critical role and intricate molecular mechanisms by which lncRNAs influence the development of tamoxifen resistance in breast cancer. Furthermore, we propose the potential clinical significance of lncRNAs as innovative therapeutic targets and prognostic biomarkers for breast cancer.

KEYWORDS

breast cancer, lncRNA, tamoxifen, drug resistance, mechanism

1 Introduction

Breast cancer (BC) holds the highest incidence rate among diagnosed malignancies in women and presents a great threat to female health worldwide (1). Approximately 70% of breast tumors exhibit estrogen receptor positive (ER+) (2) Consequently, specifically targeting the ER signaling pathway offers an efficacious approach for treating (ER+) breast cancer.

Tamoxifen, known as selective estrogen receptor modulators (SERMs), is widely used in endocrine therapy. However, the development of tamoxifen resistance remains a daunting challenge during the treatment, which may lead to a poor prognosis. Tamoxifen resistance encompasses intricate mechanisms, involving somatic alterations, epigenetic modifications, and shifts in the tumor microenvironment (3).

Noncoding RNAs (ncRNAs) represent a burgeoning category of transcripts coded by the genome, yet predominantly lacking protein-coding functionality. ncRNAs are categorized as small non-coding RNAs (sncRNAs, 18~200 nucleotides) and long non-coding RNAs (lncRNAs, >200 nucleotides) according to their sizes (4). lncRNAs are involved in diverse functions, such as regulation of transcription, epigenetic modifications, protein/RNA stability, translation, and posttranslational modifications by interacting with DNA (5). In recent years, numerous investigations have demonstrated the dysregulation of lncRNAs in various cancers, influencing tamoxifen resistance through interactions with other RNAs and proteins associated with chemoresistance. These lncRNAs actively contribute to the intricate regulatory network in breast cancer by participating in various pathways and factors, ultimately influencing tamoxifen resistance (6). At present, an assortment of abnormally expressed lncRNAs are identified in breast cancer including DILA1, UCAT1, H19,

LINP1, SNHG6, CYTOR, HOTAIR (7), and other lncRNAs are summarized below.

In the current review, We conducted a comprehensive literature review to elucidate the intricate mechanisms by which lncRNAs either enhance or diminish tamoxifen resistance in breast cancer, thus suggesting lncRNAs promising biomarkers and therapeutic targets of breast cancer.

2 Classification of lncRNA in human breast cancers

LncRNAs are categorized into numerous distinct groups to explore their underlying mechanisms of action (8) (Figure 1).

1. Intergenic lncRNAs (lincRNAs) are transcribed intergenically and play a crucial role in regulating gene expression through diverse mechanisms (9). While they can influence the expression of nearby genes, they can also regulate other genes by mechanisms such as miRNA sponging, modulation of transcriptional activity, and interaction with chromatin modifiers (10). lincRNAs undergo transcriptional activation most similar to mRNAs, demonstrating higher conservation and stability compared to intron transcripts.
2. Intronic lncRNAs, on the contrary, originate from intronic regions of protein-coding genes.
3. Sense lncRNAs are generated from the sense strand of protein-coding genes, incorporating exonic regions derived from protein-coding genes. These lncRNAs can partially overlap with protein-coding genes or encompass the entire sequence of a protein-coding gene.
4. Antisense lncRNAs, contrary to sense lncRNAs, originate from the antisense strand of protein-coding genes. Antisense transcripts may be in common use both in human and animal.

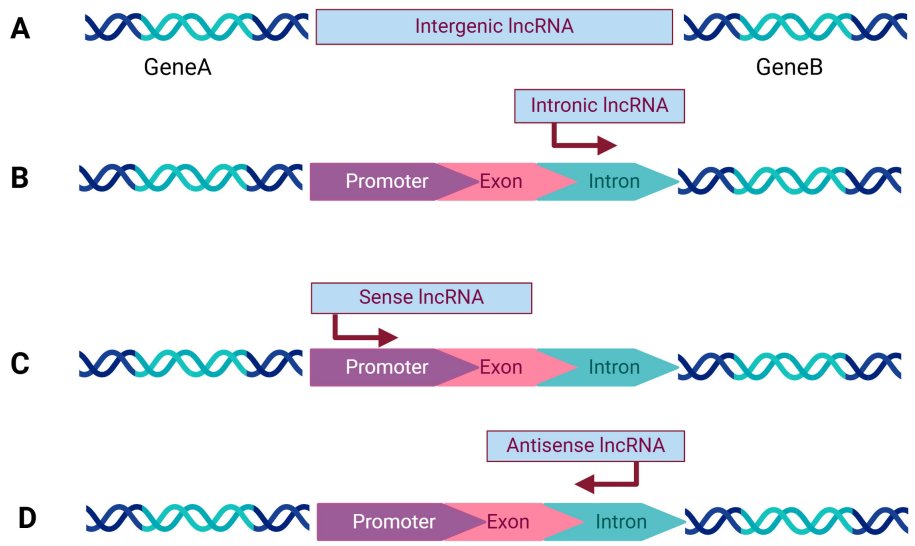

FIGURE 1
Categorization of lncRNAs: **(A)** Intergenic lncRNAs originate from the space between two protein-coding genes and are transcribed independently. **(B)** Intronic lncRNAs are composed of long introns transcribed from within the introns of other protein-coding genes. **(C)** Sense lncRNAs are are located within genes and share the same transcription orientation as the adjacent protein-coding gene. **(D)** Antisense lncRNAs are transcribed in the opposite direction to the corresponding protein-coding gene. Created with bioRender.com.

3 Functions of lncRNA

LncRNAs have been proposed to present a variety of functions, including cis- or trans-transcriptional regulation, nuclear domain organization, and protein or RNA molecule regulation (11). (Figure 2) lncRNAs interacting with the mentioned molecules contribute to the coordination of various physiological processes, and their aberrant function are involved in a range of human diseases. Currently, they are recognized for their significant involvement in the regulation of cancer initiation, development and progression (12).

The functions of lncRNA are based on their unique subcellular localization. A large portion of lncRNAs are located in the nucleus (13) and participate in gene regulation at both the epigenetic and transcriptional levels (14), including histone modifications, alterations to chromatin structure, along with engagement with nuclear chromatin modification complexes, transcription factors and proteins. A handful of lncRNAs located in the cytoplasm engage in gene regulation at post-transcriptional and translational tiers. This involves interactions with cytoplasmic proteins and the modulation of mRNA metabolism, acting as ceRNAs that interact with microRNAs. To sum up, lncRNA plays a significant role in influencing cancer cell proliferation, migration, invasion, and resistance to therapeutic drugs.

For example, the lung cancer-related transcript 1 (LUCAT1) was first discovered in non-small cell lung cancer but highly expressed in various cancer (15). LUCAT1 is upregulated in breast cancer, including triple-negative breast cancer, where it promotes tumor progression and is linked to poor prognosis (16).Numerous studies have demonstrated that suppression of LUCAT1 expression can inhibit the advancement of breast cancer,indicating it as a novel therapy target. Mou et al. found that in TNBC tissues and cell lines, LUCAT1 expression is markedly elevated and strongly associated with unfavorable outcomes. Reducing LUCAT1 levels can suppress TNBC cell growth, movement, invasiveness, and epithelial-mesenchymal transition (EMT), while enhancing cell apoptosis. LUCAT1 operates through the competing endogenous RNA (ceRNA) mechanism, binding competitively to miR-5702 and consequently diminishing its expression.

4 LncRNA-mediated mechanisms of tamoxifen resistance

A great number of lncRNAs have been found to be abnormally expressed with the vast majority upregulated in breast cancer and involved in tamoxifen resistance through regulating different target genes. (Table 1) Most lncRNAs mediating tamoxifen resistance in BC play a facilitating role like LncRNA DILA1、H19、UCAT1 (17). This review uncovers several mechanisms through which lncRNAs influence tamoxifen resistance in breast cancer

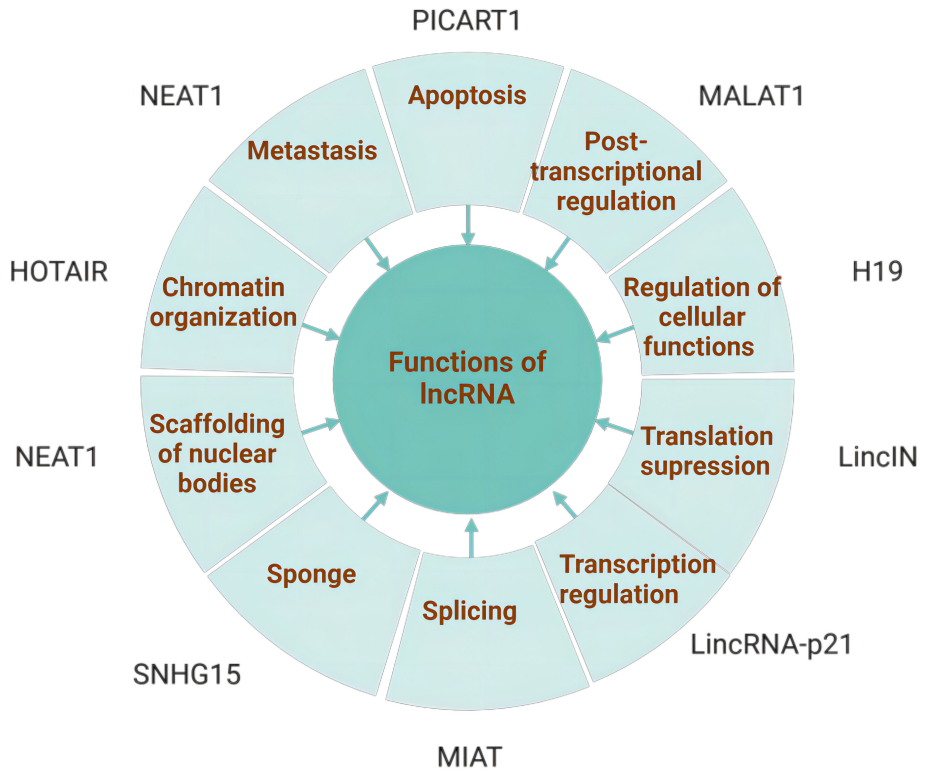

FIGURE 2
Functions of lncRNAs: The major functions of lncRNAs include chromatin organization, transcriptional regulation, sponging, scaffolding, apoptosis, metastasis, splicing modulation, post-transcriptional regulation, and regulation of cellular functions. A variety of lncRNAs involved in these functions are also associated with the tumorigenesis and progression. Created with bioRender.com.

TABLE 1 LncRNAs contribute to tamoxifen resistance in breast cancer.

LncRNAs	Expression	Targeting Genes or Pathways	Effect on Resistance	Reference
DILA1	↑	GSK3β	Promoting	(17)
Lnc-DC	↑	STAT3	Promoting	(18)
UCAT1	↑	miR-7-5p,SOX2,miR-5702	Promoting	(16)
H19	↑	Beclin1	Promoting	(19)
LINP1	↑	ER signaling	Promoting	(20)
DSCAM-AS1	↑	miR-137	Promoting	(21)
MIR497HG	↓	miR-195、miR-497 PI3K/AKT	Reversing	(22)
ADAMTS9-AS2	↓	miR-130a-5p	Promoting	(23)
SNHG6	↑	miR-101	Promoting	(24)
ATXN8OS	↑	miR-16-5p	Promoting	(25)
CYTOR	↑	miR–125a–5p	Promoting	(26)
LncRNA-42060	↑	miR-204-5p/SOX4	Promoting	(27)
SBF2-AS1	↑	PI3K/AKT/MTOR	Promoting	(28)
HOTAIR	↑	ER signaling	Promoting	(29)
TUG-1	↑	miR-186	Promoting	(30)
MAFG-AS1	↑	miR-339-5p	Promoting	(31)
ELOVL2-AS1	↓	miR-1233-3p	Promoting	(32)
AGPG	↑	E2F1 signaling	Promoting	(33)
BNAT1	↑	ER signaling	Promoting	(34)
BDNF-AS	↑	mTOR signaling	Promoting	(35)
HNF1A-AS1	↑	miR-363/SERTAD3	Promoting	(36)
CCAT2	↑	ERK/MAPK signaling	Promoting	(37)
LINC00894-002	↑	TGF-β Signaling	Promoting	(38)
ROB	↑	Beclin-1 and light chain 3 (LC3)	Promoting	(39)

lncRNAs up-regulated (↑) in tamoxifen-resistant breast cancer cells.
lncRNAs down-regulated (↓) in tamoxifen-resistant breast cancer cells.
This table shows 24 lncRNAs whose expression levels and underlying pathways in tamoxifen resistance to breast cancer.

including enhanced ER signaling, suppression of apoptosis, autophagy, exosome-mediated transfer, epigenetic alterations, epithelial-to-mesenchymal transition and acting as ceRNA.

4.1 LncRNA and enhanced ER signaling

Estrogen receptor(ER) plays a critical role in breast cancer, acting as a master transcriptional regulator that shapes the phenotype of breast cancer and serves as a central target for molecular therapy (40). Targeting of proteins and genes within ER nuclear and nonnuclear pathways has generated a range of endocrine therapies. SERMs operate by "occupying" estrogen receptors in breast cells so that estrogen cannot bind to the receptors in breast cells and the cells do not receive estrogen signals to grow and reproduce. Tamoxifen, a type of SERM, is the cornerstone of endocrine therapy for ER(+) breast cancer but the development of drug resistance remains a challenging obstacle.

Recent studies have revealed that lncRNA HOTAIR is upregulated in tamoxifen-resistant breast cancer cells compared to primary breast cancer cells, which contributes to enhanced ER signaling, even in the absence of estrogen (29). HOTAIR enhances tamoxifen resistance through regulating ER at the level of transcription. Furthermore, the research indicates that reducing HOTAIR levels in tamoxifen-resistant MCF7 breast cancer cells markedly inhibits their growth. This finding implies that reversing tamoxifen resistance might be achievable by targeting and diminishing HOTAIR.

4.2 LncRNA and suppressed apoptosis

Apoptosis, often referred to as programmed cell death, is a highly regulated and controlled process that occurs naturally in the body. It plays a crucial role in maintaining the balance and health of tissues by eliminating senescent, damaged, or unnecessary cells.

Cancer is one of the outcomes where too little apoptosis occurs, resulting in abnormal proliferation of cancer cells (41).Thus, de-regulated apoptotic signaling, including the intrinsic mitochondrial pathway and the extrinsic death receptor pathway, leads to drug resistance and recurrence of cancer (42). Long non-coding RNAs safeguard cancer cells by suppressing apoptosis triggered by oxidative stress or DNA damage caused by tamoxifen. For example, LncRNA PRNCR1 promotes breast cancer proliferation and inhibits apoptosis by modulating microRNA-377/CCND2/MEK/MAPK Axis (43). PRNCR1 inhibits miR-377, leading to the upregulation of CyclinD2 (CCND2), which is a distinct gene among the three D-type cyclins. This upregulation subsequently activates the MEK/MAPK pathway, resulting in the suppression of apoptosis and the regulation of proliferation in breast cancer cells.

4.3 LncRNA and autophagy

Autophagy is a cellular process that involves the degradation and recycling of cellular components, such as damaged organelles and misfolded proteins, to maintain cellular homeostasis and survival. Mutations in autophagy-related processes are associated with various physiological and pathological conditions, including stress response, aging, infection, and cancer (44). In multiple settings, autophagy is regarded as a double-edged sword in tumor therapy (45). In healthy cells, autophagy typically prevents the transformation into cancer cells. However, in cancer cells, efficient autophagic responses can aid in tumor progression and resistance to treatments. LncRNAs may interact with key targets to influence the resistance of breast cancer cells to tamoxifen during the autophagy process.

Li Y et al. explored the mechanism underlying the lncRNA regulator of reprogramming (ROR) modulating autophagy on tamoxifen resistance in breast cancer. They demonstrated that lncRNA ROR expression in the BC tissues was five times higher than in the paraneoplastic tissues.

In vitro studies have demonstrated that silencing ROR through small interfering RNA can enhance autophagy and elevate tamoxifen sensitivity by elevating the levels of two key autophagic markers—Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3) (39).On the contrary, Wang J et al. found that lncRNA H19 promotes tamoxifen resistance in breast cancer via autophagy as well. *In vitro* cell culture revealed that the expression of H19 in tamoxifen-resistant MCF-7 BCE cells and tumor tissues was upregulated. Downregulation of H19 could inhibit autophagy and reverse resistance to tamoxifen (19).

In the case of tamoxifen, the response differs significantly, primarily due to the diverse types of BC cells. While the intricate molecular interplay between lncRNAs and autophagy is not fully understood, it is clear that various lncRNAs influence autophagy by regulating crucial autophagy-related genes, resulting in distinct outcomes in tamoxifen-resistant BC. A deeper comprehension of the multifaceted role of autophagy in tamoxifen resistance could pave the way for innovative strategies to overcome this resistance and identify new therapeutic targets for effective management.

4.4 Exosome-mediated transfer of lncRNA

Exosomes, which are a subset of extracellular vesicles (EVs), contain nucleic acids, amino acids, proteins, and lipids, with lncRNAs being one of the major components of their cargo (46). Cancer cells typically release more exosomes compared to healthy cells, and these cancer-derived exosomes possess a potent ability to alter microenvironments both locally and distantly (47). Furthermore, EVs play significant roles in mediating drug resistance through various mechanisms. In recent studies, lncRNAs associated with exosomes have been identified as key factors in promoting drug resistance in BC.C-G Xu et al. (48) compared the loading of Urothelial carcinoma-associated 1(UCA1) in exosomes released from tamoxifen-sensitive MCF-7 cells and tamoxifen-resistant LCC2 cells and found that UCA1 is significantly increased in exosomes from tamoxifen-resistant cells than that from tamoxifen sensitive cells. Furthermore, mTOR signaling is hypothesized to be an important downstream signaling pathway of UCA1 in tamoxifen resistance which requires further studies.

4.5 Epigenetic modifications of LncRNAs

Building upon the foundation of genomic and epigenomic mechanisms, lncRNAs introduce an extra layer of regulation. They facilitate both transcriptional and post-transcriptional control by engaging with proteins and nucleic acids that modulate gene expression within the nucleus and cytoplasm. Numerous lncRNAs, including HOTAIR, ANRIL, ROR, and H19, play a role in suppressing transcription through the recruitment of proteins involved in chromatin remodeling or histone modification.

Cyclin D1 is a key oncoprotein that promotes the proliferation of cancer cells and is linked to tamoxifen resistance in breast cancer. The lncRNA DILA1, which interacts with Cyclin D1, is found to be upregulated in tamoxifen-resistant breast cancer cells (17). Mechanistically, DILA1 prevents the phosphorylation of Cyclin D1 at the Thr286 site by directly binding to it, thereby inhibiting its degradation. This leads to an increased level of Cyclin D1 protein in breast cancer cells. Yu et al. (33) investigated that in endocrine-resistant breast cancer cells, the expression of the lncRNA actin gamma 1 pseudogene 25 (AGPG) was found to be elevated, a phenomenon attributed to the epigenomic activation of an enhancer. AGPG engaged in a physical interaction with PURα, which in turn freed E2F1 from PURα's grasp, thereby triggering the activation of E2F1 signaling pathways in ERα-positive breast cancer cells.

4.6 LncRNA induced EMT

The epithelial-to-mesenchymal transition (EMT) is a cellular program crucial for diverse pathological events, such as wound repair, tissue scarring, and the advancement of cancer (49). An increasing number of research has revealed the activation of EMT throughout the Development of malignant tumors (50). Khan and Ahmad (24) found that the expression of SNHG6 is elevated in tamoxifen-resistant cells and positively regulates acquired resistance against tamoxifen. It sponges miR-101, leading to the activation of EMT and increased EMT cell markers including E-cadherin, vimentin, ZEB1 and ZEB2, contributing to tamoxifen resistance. Silencing SNHG6 sensitizes resistant cells to tamoxifen, reverses EMT, and reduces invasion and clonogenicity, implicating the SNHG6-miR-101 axis as a potential target for overcoming tamoxifen resistance in breast cancer.

4.7 lncRNAs act as competitive endogenous RNA

MicroRNAs (miRNAs) are sncRNAs that play important roles in posttranscriptional gene regulation. They act by inhibiting translation and decreasing the stability of target RNAs (30). Salmena et al. hypothesized that lncRNAs can function as competitive endogenous RNA(ceRNA) to bind specific miRNAs, which in turn could alleviate the repression of their target mRNAs. Additionally, microRNAs can also be "sponged" or titrated away by lncRNAs, thereby exerting their effects independently of protein coding.

In addition to the inducing of EMT mentioned above, SNHG6 was also found to sponge and inhibit miR-101, with the endogenous expression levels of SNHG6 and miR-101 being inversely correlated. Moreover, silencing SNHG6 in tamoxifen-resistant cells resulted in miR-101 inhibition and reversed EMT. SNHG6 directly correlated with increased stem cell markers Sox2, Oct4, and EZH2. Manipulation of miR-101 levels affected tamoxifen sensitivity, with pre-miR-101 sensitizing resistant cells to tamoxifen and anti-miR-101 inducing resistance in parental cells. MiR-101 was found to attenuate SNHG6-mediated effects on tamoxifen resistance, EMT, and stem cell markers, presenting a crucial role for the SNHG6-miR-101 axis in tamoxifen resistance of ER-positive breast cancer cells.

Chen X et al. (51) found that LINC02568 regulates estrogen receptor-induced transcriptional activation of target genes in the cytoplasm by competitively binding miR-1233-5p to the ESR1 mRNA of estrogen receptor, thereby trans-regulating ESR1 mRNA stability. Meanwhile, LINC02568 plays a specific role in maintaining the pH homeostasis of tumors by controlling the activity of the carbonic anhydrase enzyme CA12 in the nucleus through a cis-regulatory mechanism. Targeting the antisense nucleotide (ASO) of LINC02568 significantly inhibited the growth as well as tumor formation of estrogen receptor-positive breast cancer cells and restored the sensitivity of tamoxifen-resistant breast cancer cells to tamoxifen.

5 Cancer therapy for targeting lncRNAs

Tamoxifen, prescribed as a gold standard treatment for ER-positive BC, has been shown to substantially reduce the recurrence rate by 40% and the mortality rate by 30%. However, even with 5 years of tamoxifen treatment, one-third of these patients experience a relapse within 15 years (52). Endocrine-resistant patients, who may constitute up to 25% of all breast cancer cases, continue to pose a significant clinical challenge. Extensive efforts have been pursued to treat tamoxifen-resistant patients. At ESMO 2022, third-generation oral SERM lasofoxifene shined. Phase II ELAINE 1 study results show lasofoxifene delivers durable complete remission (CR) in ER-positive, HER-negative, metastatic breast cancer patients harboring ESR1 mutation (53).

In this context, lncRNAs emerge as promising therapeutic targets due to their involvement in regulating oncogenic molecular networks and their cell type-specific expression. They can act as oncogenes or tumor suppressors, influencing various cancer hallmarks such as cell proliferation, invasion, and metastasis. Despite their potential, targeting lncRNAs faces several hurdles including their structural complexity, diversity in function, and poor annotation.

Currently various pharmaceutical strategies targeting lncRNAs aim to achieve loss-of-function (LOF) in cancer therapy (54). LOF involves the use of oligonucleotides such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) to downregulate harmful lncRNAs. Gapmer ASOs bind to target lncRNAs based on sequence complementarity. Once bound, they induce degradation of the lncRNA by recruiting RNase H1 in the nucleus. This approach is effective regardless of the lncRNA's subcellular localization (55). SiRNAs are double-stranded RNA molecules that also target lncRNAs through sequence complementarity. The active strand of the siRNA is incorporated into the RNA-induced silencing complex (RISC), which then binds to and cleaves the complementary lncRNA, leading to its degradation. SiRNAs mainly function in the cytoplasm, though ongoing debate exists about their activity in the nucleus. Several oncogenic lncRNAs have been successfully targeted using siRNAs, including SNHG6 (24), ELOVL2-AS1 (32), BNAT1 (34).

Zhimin Shao et al. conducted a clinical trial (NCT02641847) aimed at evaluating the efficacy and safety of two chemotherapy regimens in high-risk triple-negative breast cancer (TNBC) patients, as identified by an integrated mRNA-lncRNA signature. The trial aims to validate this signature's ability to predict recurrence risk in TNBC patients and explore optimal chemotherapy strategies. Patients classified as high-risk are randomized to receive either a regimen of docetaxel, doxorubicin (or epirubicin), and cyclophosphamide followed by gemcitabine and cisplatin, or doxorubicin (or epirubicin) and cyclophosphamide followed by docetaxel. The primary goal is to assess recurrence-free survival, with secondary endpoints including safety and overall survival.

An ongoing clinical trial (NCT06307249) has been launched to explore the therapeutic potential of combining Palbociclib, a CDK4/6 inhibitor, with Bevacizumab, a VEGF inhibitor, in treating solid tumors, with an initial focus on colorectal cancer and plans to extend to other cancers, including breast cancer. The study is particularly innovative in its incorporation of LncRNAs as biomarkers, which have shown promise in predicting treatment response and prognosis across various cancers. By analyzing the molecular profiles through LncRNA markers, the trial aims to enhance the efficacy of this combination therapy, offering a targeted and individualized treatment option for patients with solid tumors, ultimately striving for better outcomes and improved survival rates.

Advanced genomic technologies and molecular tools are used to identify and validate lncRNA targets. These include high-throughput RNA sequencing, CRISPR-Cas technologies for genomic editing, and various *in vivo* and *in vitro* models for functional validation (56).

6 Conclusion

As master gene regulators, lncRNAs have been implicated in the regulation of diverse cellular functions and disease processes. Substantial knowledge on how lncRNAs contribute to cancer has accumulated in the last 10 years and extensive efforts have been fostered to discover mechanisms of drug resistance and potential targets for the treatment of breast cancer patients. ER is not only a good diagnostic marker for breast cancer but also serves as a therapeutic target. Tamoxifen is one of the important SERMs for ER(+)breast cancers and it competitively inhibits the recruitment of transcription coactivators by ER, hence shutting down the transcription of ER-responsive genes. However, a critical challenge is the development of tamoxifen resistance.

Mechanisms of tamoxifen resistance are complex and may involve multiple factors such as AKT, HER2, and R-Ras. These studies have been focusing on protein-coding genes. On the other hand, much less is known about the role of lncRNAs in tamoxifen resistance although the number of lncRNAs is much larger than that of protein-coding genes. Thus, our study provides new insight into the lncRNA-mediated mechanisms of tamoxifen resistance in BC including enhanced ER signaling, suppression of apoptosis, autophagy, exosome-mediated transfer, epigenetic alterations, epithelial-to-mesenchymal transition and acting as ceRNA. A comprehensive mechanism related to recently identified various novel lncRNA implicated in BC tamoxifen resistance has been summarized. (Figure 3) Combination of lncRNAs-based therapeutic interventions with traditional chemotherapy or targeted therapy may be a promising option to conquer tamoxifen resistance in ER(+)breast cancer patients. However, it is still a challenge how to choose crucial target lncRNAs from a large amount of candidate lncRNAs. Future translational studies or clinical trials are warranted to develop lncRNAs-based

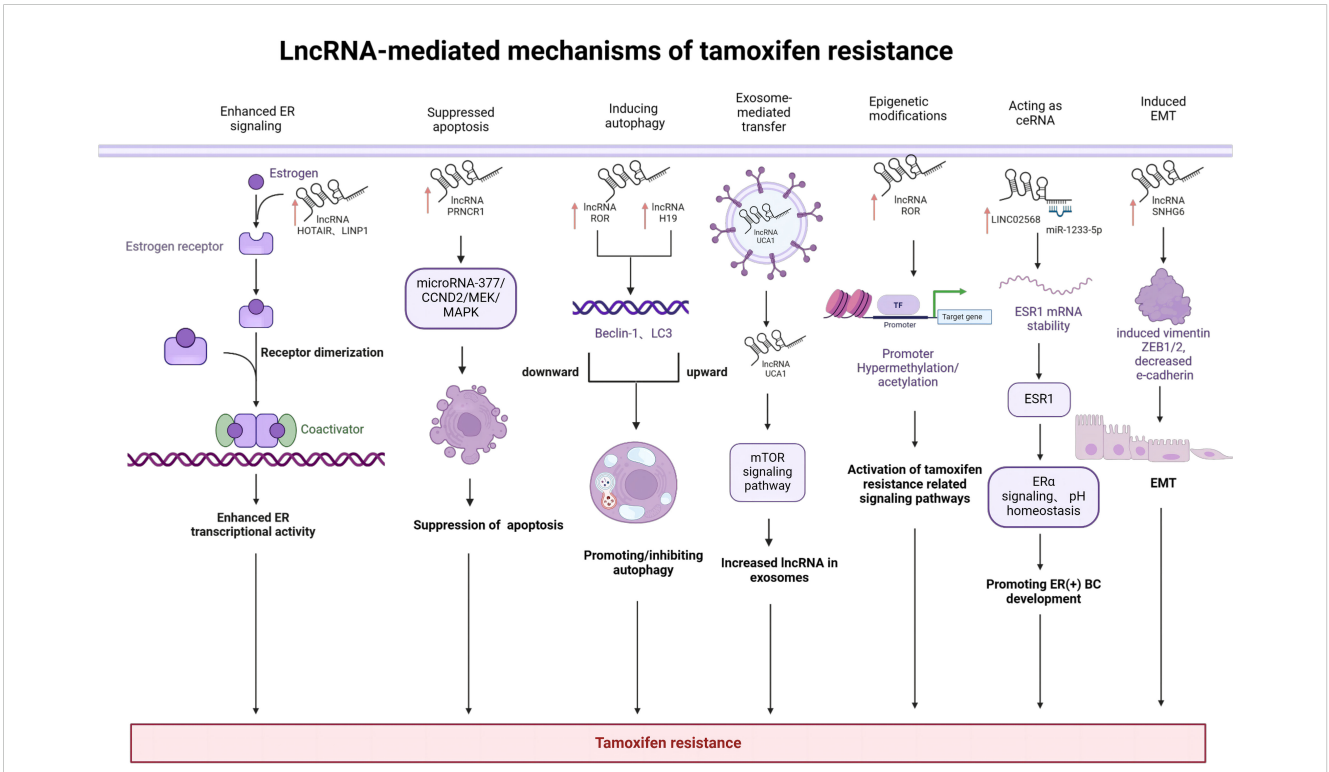

FIGURE 3
LncRNA-mediated mechanisms of tamoxifen resistance in breast cancer: LncRNAs mediate drug resistance by various molecular mechanisms including enhanced ER signaling, suppression of apoptosis, autophagy, exosome-mediated transfer, epigenetic alterations, epithelial-to-mesenchymal transition and acting as ceRNA. Created with bioRender.com.

therapeutics, which may eventually improve the prognosis of tamoxifen-resistant breast cancer patients.

Author contributions

YY: Writing – original draft, Writing – review & editing. JZ: Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Roles of lncRNAs related to the p53 network in breast cancer progression

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The p53 is a crucial tumor suppressor and transcription factor that participates in apoptosis and senescence. It can be activated upon DNA damage to regulate the expression of a series of genes. Previous studies have demonstrated that some specific lncRNAs are part of the TP53 regulatory network. To enhance our understanding of the relationship between lncRNAs and P53 in cancers, we review the localization, structure, and function of some lncRNAs that are related to the mechanisms of the p53 pathway or serve as p53 transcriptional targets.

KEYWORDS

p53, lncRNAs, breast cancer, gene expression, prognosis

1 Introduction

Breast cancer is one of the most common malignancies affecting women worldwide (1), characterized by various genetic and epigenetic alterations (2). Among the critical genes involved in breast cancer, TP53, which encodes the tumor suppressor protein p53 (3), is the most commonly altered gene in cancer (4) and is involved in the development of both sporadic and some hereditary breast tumors (3). Non-coding RNAs (ncRNAs) have emerged as critical regulators in the development and progression of breast cancer, among which lncRNAs account for less than 1% (5). However, lncRNAs play critical roles in transcription, post-transcriptional processing, and translation in breast cancer (6). Recent research has uncovered specific role of lncRNAs in gene regulation, interaction with p53, and their potential as therapeutic targets. The tumor suppressor gene p53 and various lncRNAs are significant in regulating breast cancer progression, treatment response, and patient prognosis (Figure 1). This study highlights the latest findings in clinical and basic research on p53-associated lncRNAs in regulating breast cancer.

2 Long non-coding RNAs and breast cancer

Long non-coding RNAs (lncRNAs) are a diverse group of non-coding RNAs (ncRNAs) longer than 200 nucleotides (7). Instead of evident protein coding capacity, lncRNAs

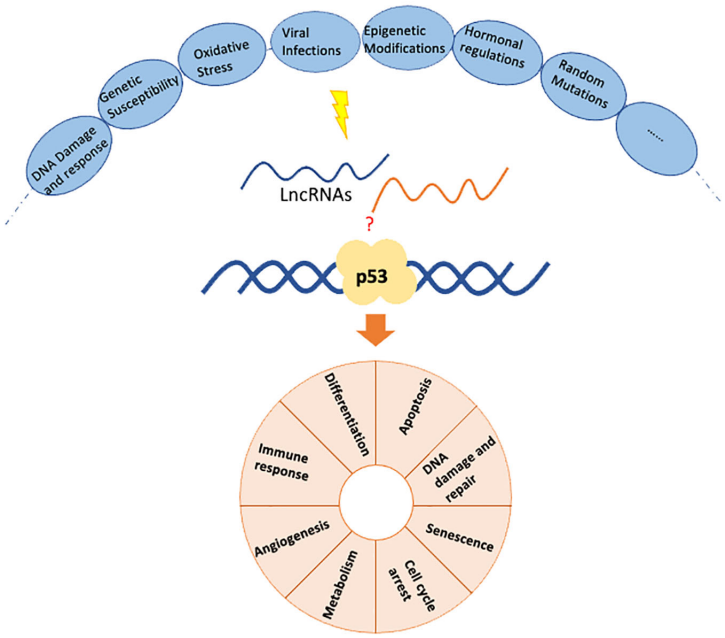

FIGURE 1
The role of p53 and lncRNAs in regulating breast cancer progression.

modulate gene expression at various levels, including cellular proliferation, differentiation and development, dosage compensation, chromosomal imprinting, and genomic stability (8), leading to tumor-suppressing or oncogenic functions (9). Statistics from the Human GENCODE exhibit that there are more than 16,000 lncRNAs in the human genome, while others suggest there are exceeding 100,000 human lncRNAs (10, 11). Compared with mRNAs, a higher proportion of lncRNAs are expressed almost only in the nucleus, showing functions related to nuclear processes to control gene expression. lncRNAs can be divided into two categories, cis- or trans-acting, depending on whether the lncRNA impacts nearby genes or performs on long-distance regions (12). Plenty of lncRNAs function in the cytoplasm after being transported from the nucleus, acting as miRNA sponges, regulating mRNA degradation or mRNA translation (13). As significant regulators emerging in cancer biology, lncRNAs also add to the complexity of breast cancer progression (13).

Some lncRNAs can affect the proliferation of breast cancer cells by interacting with key cell cycle regulatory factors. For example, HOTAIR is highly expressed in breast cancer and may promote cell cycle progression by binding to cell cycle-related proteins, leading to excessive proliferation of cancer cells (14, 15). Specific lncRNAs play an important role in the metastasis and invasion of breast cancer cells. They can regulate the epithelial-mesenchymal transition (EMT) process, enabling cancer cells to acquire the ability to migrate and invade. In addition, some lncRNAs can also create favorable conditions for the metastasis of cancer cells by affecting processes such as extracellular matrix remodeling and angiogenesis. Some lncRNAs are closely related to chemotherapy resistance and DNA damage repair in breast cancer. For example, NEAT1 may promote homologous recombination repair or non-homologous end joining repair pathways and affect the response of breast cancer cells to DNA damage caused by radiotherapy and chemotherapy (16).

3 Role of p53 in breast cancer

p53 is a key tumor suppressor protein involved in maintaining genomic stability (17). As the major regulatory transcription factor, p53 is reported being activated during the stress response liking replicative stress, oxidative stress, hypoxia, DNA damage (18), nutrient deprivation, and telomere shortening (8). Mutations in the p53 gene are frequent in breast cancer and are associated with a loss of tumor suppressive function or gain of oncogenic properties (19). These mutations contribute to tumor progression, metastasis, and resistance to therapies.

While in breast cancer, the mutation of the p53 gene can be triggered by a variety of factors including: 1) DNA Damage and Repair Deficiencies. Exposure to ultraviolet (UV) radiation, tobacco smoke, chemical carcinogens, and ionizing radiation can cause DNA damage, leading to mutations in the p53 gene (20). Mutations in genes responsible for DNA repair, such as BRCA1 and BRCA2, increase the likelihood of p53 mutations (21). 2) Genetic Susceptibility. Familial breast cancer often involves inherited mutations in BRCA1, BRCA2, and other genes. Li-Fraumeni syndrome, associated with inherited p53 mutations, significantly increases the risk of breast cancer and other cancers

(22). 3) Oxidative Stress: Oxidative stress generated by reactive oxygen species (ROS) can cause oxidative DNA damage, leading to mutations (23). 4) Viral Infections: Infections with certain viruses, such as human papillomavirus (HPV), can lead to degradation or inactivation of p53 protein (24). 5) Epigenetic Modifications: Abnormal methylation patterns can lead to genomic instability and increased mutation rates in the p53 gene (25). 6) Hormonal Factors: Estrogen, for instance, promotes the proliferation of breast epithelial cells, potentially increasing the risk of mutations during cell division (26). 7) Random Mutations: Spontaneous DNA Replication Errors accumulate over time, particularly in rapidly dividing cells, contributing to mutations in genes like p53 (27). In breast cancer, mutations in p53 are common and can lead to the loss of its tumor suppressor functions, thereby contributing to cancer progression, resistance to therapy, and poor prognosis. Understanding these processes helps in developing targeted therapies that can restore p53 function or mimic its activity.

Furthermore, p53 is involved in the following biological processes related to breast cancer. 1) induce cell cycle arrest in response to DNA damage (28). 2) promotes apoptosis in cells with irreparable DNA damage (29). 3) enhances the DNA repair process by upregulating genes involved in nucleotide excision repair and base excision repair pathways (30). 4) induce cellular senescence in response to oncogenic stress or extensive DNA damage (31). 5) influences cellular metabolism by regulating genes involved in glycolysis, oxidative phosphorylation, and antioxidant defense (32). 6) suppress angiogenesis, which is essential for tumor growth and metastasis (33). 7) has a role in modulating the immune response against tumor cells (34).

4 The direct or indirect regulation between p53 and lncRNAs in breast cancer

p53 can directly or indirectly regulate the expression of some lncRNAs. Some lncRNAs can interact with p53 to regulate the transcriptional activity or protein stability of p53, thereby affecting the regulatory role of p53 on breast cancer cells. p53 and lncRNA may have a synergistic effect in the occurrence and development of breast cancer. For example, mutation or loss of function of p53 may lead to abnormal expression of some lncRNAs, and these lncRNAs further promote the progression of breast cancer. At the same time, abnormal expression of some lncRNAs may also affect the function of p53, thereby aggravating the malignancy of breast cancer. Therefore, both p53 and lncRNA play important roles in breast cancer, and there is a complex association between them. In-depth study of the relationship between lincRNA and p53 in breast cancer can help us uncover new molecular mechanisms, identify potential biomarkers, develop targeted therapies, and improve treatment efficacy. The expression levels or activities of specific lincRNAs and p53 may serve as biomarkers for breast cancer diagnosis, prognosis, or response to treatment. According to the publications reviewed, the relationship between p53 and lncRNAs could be stated from the following aspects (Figure 2; Table 1). We have searched the recently published works about p53-related lincRNAs from the Pubmed database, mainly using the keywords as breast cancer, p53, and lincRNAs. The following roles are what we were able to access until the paper was submitted.

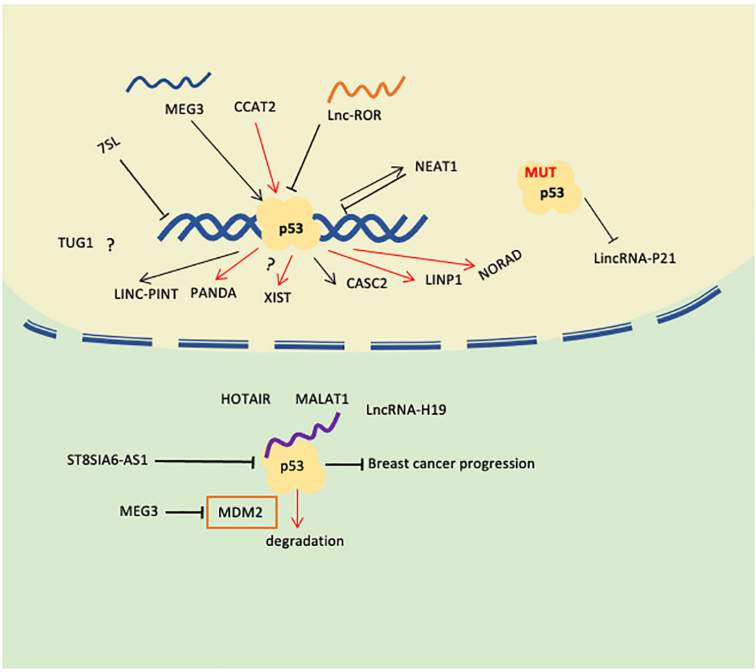

FIGURE 2
Role of lncRNAs correlated with p53 in Breast Cancer(rows in red mean promoting breast cancer progression or chemotherapy).

TABLE 1 lncRNAs related with P53 network in breast cancer.

lncRNA	Functions	subtype	interact with p53	reference
MALAT1	promote migration and invasion	Breast cancer	Depletion of MALAT1 resulting in activation of p53 and its target genes	(35–43)
MEG3	tumor suppressor	TNBC	induces accumulation of p53 by reducing the levels of MDM2 expression, which mediates p53 degration	(44–47)
7SL	upregulated in cancer cells	Breast cancer	The interaction of 7SL with TP53 mRNA reduced p53 translation	(48–50)
HOTAIR	elevated expression correlated with metastasis and death		HOTAIR may affect proliferation, apoptosis, migration and invasion of MCF-7 cells through regulating the P53/Akt/JNK signaling pathway	(14, 15, 51–55)
NEAT1	promoting the progression	Breast cancer	Neat1 ablation slightly decreased DNA damage in PyVT tumors, as determined by phosphorylation of ATM/ATR including p53,	(56–62)
lincRNA-RoR	negative regulator of p53	in er positive breast cancer cells	form an autoregulatory feedback loop : p53 transcriptionally induces RoR expression, and in turn, RoR inhibits p53 translation through hnRNP I.	(63)
ST8SIA6-AS1	promotes cell proliferation and metastasis	TNBC	target miR-145-5p/CDCA3 to inactivate the p53/p21 signaling pathway	(64–66)
CASC2 (Cancer Susceptibility Candidate 2)	tumor suppressor	her2 1+ higher than her2 negative	p53 binds to specific response elements in the CASC2 promoter region, activating its transcription.	(67–69)
XIST	high XIST expression had poor outcomes	BRCA1-deficient patients	p53 is required for normal Xist expression and X chromosome inactivation	(70, 71)
PANDA	high levels of PANDA expression had poor prognosis and resistant to chemotherapy		unknow in breast cancer	(69, 72)
LINK-PINT	tumor suppressor	TNBC	p53-induced LINK-PINT transcription	(73, 74)
LINP1	a potential oncogene	TNBC	p53 was identified as a regulator of LINP1	(75, 76)
LincRNA-p21	positively associated with the response to neoadjuvant chemotherapy	ER-positive breast tumor	ERα inhibited lincRNA-p21 expression by redirecting mutp53 to increase tDDB2 transcription, leading to enhanced DNA repair and chemoresistance	(77–82)
LincRNA-p21	lincRNA-p21 knockdown macrophages could mitigate breast cancer progression	TAM	Knocking down lincRNA-p21 led to the polarization of macrophages towards the pro-inflammatory M1 phenotype, which was attributed to MDM2 promoting the proteasome-dependent degradation of p53, consequently activating the NF-κB and STAT3 pathways	(83–85)
lncRNA H19	promote cell proliferation, migration, and invasion, induce cell cycle arrest, and lead to abnormal expression of EMT markers	TNBC	counteract the tumor suppressor p53 and in turn promotes the EMT process	(86)
NORAD	high levels of NORAD expression had poor prognosis	TNBC	is activated in response to DNA damage in a manner dependent on the p53 protein	(87, 88)
(CCAT2)	High CCAT2 expression had lymph node metastasis	ER-positive breast tumor	CCAT2 inhibits p53-induced activation of miR-145, and its overexpression enhances drug resistance	(89, 90)
(TUG1)	promote cell proliferation, cell migration, and invasion	TNBC	low TUG1 levels notably associated with mutant p53 expression and lymph node metastasis	(91–95)

4.1 MALAT1

MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) is a broadly studied lncRNA with a length of ~8000 nt, which was firstly founded as a prognostic marker in non-small cell lung cancer (35). As a conserved lncRNA, MALAT1 is specially localized in nuclear speckles, a sub-nuclear domain suggested to coordinate RNA polymerase II transcription, pre-mRNA splicing and mRNA export, in a transcription-dependent manner (36–38). Cancer associated MALAT1 regulates cellular proliferation by modulating the expression and/or pre-mRNA processing of cell cycle-regulated transcription factors (39). Associated with

hyperproliferation and metastasis, MALAT1 is not only highly expressed in lung cancer (40), but also regulate migration and invasion of breast cancer cells (41). Depletion of MALAT1 leads to cell cycle arrest with prominently reduced cellular proliferation, at the same time, resulting in activation of p53 and its target genes (39). Magdalena Pruszko et al. demonstrated that MALAT1 could be bridged to mutant p53 and ID4 proteins in breast cancer cells (42). The mutant p53 and ID4 delocalize MALAT1 from nuclear speckles and favor its association with chromatin, which enables aberrant recruitment of MALAT1 on VEGFA pre-mRNA. The VEGFA isoforms expression was associated with ID4 expression specifically in basal-like breast cancers carrying p53 mutations (42). Abdel-Latif M et al. showed a repression of MALAT1 in MQG (Methoxylated Quercitin Glycoside) treated MDA-MB-231 (43).

4.2 MEG3

MEG3 (Maternally Expressed Gene 3), as an imprinted lncRNA on human chromosome 14q (44), induces accumulation of p53 by reducing the levels of MDM2 expression, which mediates p53 degration (45). But tumor suppression by MEG3 was found to be both p53-dependent and p53-independent (45). MEG3 contains a 356-nucleotide nuclear retention element related to U1 snRNP, which in turn retains MEG3 in the nucleus (46). The overexpression of MEG3 resulted in increased breast cancer cell apoptosis, not only via upregulating the ER-stress and proapoptotic proteins, but by activating the NF-κB and p53 signaling pathways. Pharmacological inhibition of NF-κB completely abolished MEG3 induced activation of p53 (47), making it a potential biomarker and therapeutic target in breast cancer.

4.3 7SL

The 300-nt long lncRNAs 7SL (NR 002715; gene name RN7SL1) forms a ribonucleoprotein complex (RNP)with six signal recognition proteins(SRPs) (48) and. was exhibited to be broadly upregulated in several cancer cell types (liver, lung, breast, stomach) (49). 7SL forms a partial hybrid with the 3'-untranslated region (UTR) of p53 mRNA, which encodes the tumor suppressor p53, leading to the reduction of p53 translation (50). On the contrary, silencing 7SL results in enhanced binding of HuR to p53 mRNA, leading to the promotion of p53 translation (50). The competition between 7SL and HuR for binding to p53 3'UTR determined the magnitude of p53 translation, in turn affecting p53 levels and the growth suppressive function of p53 (50). Therefore, targeting 7SL may be effective in the treatment of cancers with reduced p53 levels.

4.4 HOTAIR

HOTAIR (HOX Transcript Antisense RNA) is a conserved 2.1kb transcript produced from the HOXC locus on chromosome twelve and is composed of six exons, acting not only as a molecular guide but a scaffold for several chromatin-modifying complexes (51). As one of the most remarkably tumor associated lncRNAs (52), HOT genes are tightly regulated during their development and dysregulated (mostly over-expressed) in different cancer types (15), especially highly induced in breast cancer samples. The elevated HOTAIR expression was correlated with metastasis and death (53). Y. Yu et al. exhibited that knockdown of HOTAIR in MCF-7 cells induced significant increase in the expression of P53, and obvious decrease in the expression of MDM2(Mouse double minute 2 homolog), AKT, JNK on both mRNA and protein level. MDM2 is a negative regulator of p53 (54). Akt and JNK are crucial in the carcinogenesis of breast cancer (55). Therefore, HOTAIR may affect proliferation, apoptosis, migration and invasion of MCF-7 cells through regulating the P53/Akt/JNK signaling pathway (14). That means HOTAIR may have the chance to become a therapeutic target for the treatment of breast cancer.

4.5 NEAT1

NEAT1 (Nuclear Enriched Abundant Transcript 1) is a ~3.7 knt single-exon lncRNA (56). It localizes to paraspeckle sub-organelles and underlies the complex organization and functions of paraspeckles (57), acting as a transcriptional hub for numerous oncogenes (58). NEAT1 could promote the growth and survival cancer cells (59, 60) and chemoresistance in breast cancer cells (61). NEAT1 is a confirmed target gene of p53 (62). p53 regulates NEAT1 expression to promote paraspeckle formation, and in turn, NEAT1 paraspeckles reduce replication-associated DNA damage and p53 activation. This reveals an autoregulatory negative feedback loop that mitigates p53 activity in DNA-damaged cells (16). In addition, NEAT1 is up-regulated by 5-fluorouracil in wild-type p53 breast cancer cells but not in mutant-p53 cells (61). the histone acetylation at the NEAT1 promoter was induced by knocking down of MED12 (Mediator Complex Subunit 12), resulting in elevated NEAT1 mRNA levels and a chemoresistant phenotype. This chemoresistant phenotype can be partially reversed by NEAT1 knockdown in p53 wild-type cells (61). This novel mechanism of chemoresistance dependent on NEAT1 in p53 wild-type breast cancer cells provide a promising target for breast cancer therapies (61).

4.6 LincRNA-RoR

The human lincRNA-RoR (Regulator of Reprogramming) has been identified as a powerful negative regulator of p53, through direct interaction with heterogeneous nuclear ribonucleoprotein I (hnRNP I), leading to the inhibition of p53 translation after DNA damage (63). A critical 28-base sequence within RoR, which contains hnRNP I binding motifs, is essential and sufficient for this repression of p53. RoR disrupts p53-mediated cell cycle arrest and apoptosis via an autoregulatory feedback loop where p53 transcriptionally induces RoR expression, and in turn, RoR inhibits p53 translation through

hnRNP I. This RoR-hnRNP I-p53 axis may constitute an additional surveillance mechanism for cells in breast cancer (63).

4.7 ST8SIA6-AS1

ST8SIA6-AS1, also known as APAL, is an lncRNA that is overexpressed in several cancers and associated with a poor prognosis. Its suppression triggers mitotic catastrophe and substantial apoptosis in human cancer cells (64). It has been found that ST8SIA6-AS1 facilitates breast tumorigenesis by linking up PLK1 and Aurora A to increase PLK1 phosphorylation (64). In triple-negative breast cancer (TNBC) cells, ST8SIA6-AS1 is notably overexpressed. Knockdown of this lncRNA results in decreased cell proliferation, cell cycle arrest, reduced migration and invasion *in vitro*, and inhibited tumor growth *in vivo*. Mechanistically, ST8SIA6-AS1 boosts the expression of CDCA3 (cell division cycle associated protein 3) and inhibits the p53/p21 pathway by targeting miR-145-5p. Through its interaction with miR-145-5p, ST8SIA6-AS1 enhances CDCA3 expression and inactivates p53/p21 signaling, contributing to its oncogenic role in TNBC. Therefore, ST8SIA6-AS1 inactivates p53/p21 signaling to promote breast cancer progression. These insights indicate that ST8SIA6-AS1 could be a promising molecular target for the treatment of TNBC (65, 66).

4.8 CASC2

CASC2 (Cancer Susceptibility Candidate 2) has been explored in various cancers, including breast cancer, for its potential tumor-suppressing roles. The expression of CASC2 is triggered by p53 in response to cellular stress or DNA damage. p53 binds to specific response elements in the CASC2 promoter region, activating its transcription. Additionally, CASC2 exerts its tumor-suppressive effects through various mechanisms, interacting with miRNAs, proteins, and other molecular targets to modulate their activity. For instance, CASC2 acts as a competitive endogenous RNA (ceRNA), sponging miRNAs that target tumor suppressor genes, thereby protecting these genes from miRNA-mediated repression. CASC2 also interacts with the protein PTEN, enhancing its tumor-suppressive functions (67). Reduced CASC2 expression in breast cancer is linked to poor prognosis, higher tumor grade, and increased metastatic potential. CASC2 could be a valuable biomarker for breast cancer diagnosis and prognosis. CASC2 interacts with key signaling pathways involved in cancer progression, such as the PI3K/AKT and Wnt/β - catenin pathways (68). Research suggests that CASC2 can sensitize cancer cells to chemotherapeutic agents, making it a promising target for enhancing treatment efficacy The expression levels of p53, MEG3, CASC2, and PANDA were significantly lower in tumor samples compared to non - tumor samples. CASC2 expression was higher in Her2 1+ cases compared to Her2 negative cases (Beta = 1.85, P value = 0.037). Among these genes, CASC2 exhibited the best performance as a biomarker, with an area under the curve value of 0.78, and sensitivity and specificity values of 56.33% and 88.73%, respectively (P value < 0.0001) (69).

4.9 XIST

Previous studies have associated high XIST expression and low 53BP1 (p53 binding protein) expression with poor outcomes in systemic therapy and therapy resistance in BRCA1 - deficient mouse tumor models. However, these associations have not yet been evaluated in BRCA1-deficient patients. The expression levels of XIST and 53BP1 were examined to predict the outcome of HD chemotherapy in 28 BRCA1 - like patients. It was reported that high RNA expression of XIST and low protein expression of 53BP1 did not overlap. Patients with either high XIST or low 53BP1 expression had poor outcomes after HD chemotherapy. In contrast, patients with low XIST and high 53BP1 expression showed significant benefits from this regimen in terms of recurrence - free survival, disease-free survival, and overall survival. Therefore, XIST and 53BP1 may serve as predictive biomarkers in BRCA1 - like breast cancer (70). Delbridge et al. provide evidence that p53 is essential for normal Xist expression and X chromosome inactivation. They demonstrate in two models that partial failure of X chromosome inactivation is linked to female - biased neural tube defects (71). However, the relationship between Xist and p53 needs to be further studied.

4.10 PANDA

PANDA (P21 associated ncRNA DNA damage activated) is transcribed from a genomic region adjacent to the CDKN1A (p21) gene, a renowned p53 target involved in cell cycle regulation. Upon DNA damage, p53 binds to the promoter region of PANDA, resulting in its transcriptional activation. PANDA has been demonstrated to inhibit apoptosis in cancer cells (osteosarcoma) by sequestering the transcription factor NF-YA, which is implicated in the expression of pro-apoptotic genes (72). In breast cancer, high levels of PANDA expression have been correlated with poor prognosis and increased resistance to chemotherapy PANDA interacts with various proteins and other non-coding RNAs to exert its effects (69). Targeting PANDA or its interacting partners could offer new therapeutic strategies for treating breast cancer treatment, particularly in cases where p53 is functional but the apoptotic pathways are disrupted. The interplay between p53 and PANDA underscores the complexity of p53 signaling in cancer and the significance of non-coding RNAs in modulating these pathways. Understanding the mechanisms by which p53 controls PANDA expression and function can provide valuable insights into breast cancer pathogenesis and open up new avenues for treatment.

4.11 LINC-PINT

LINC-PINT (Long intragenic non-coding RNA p53-induced transcript) functions as a tumor suppressor. LINC-PINT is a direct transcriptional target of p53. In response to cellular stress or DNA

damage, p53 binds to its putative response element in the promoter region of LINC-PINT, activating its transcription. Reduced expression of LINC-PINT is often associated with poor prognosis and more advanced clinical stages of tumors. Moreover, the specific regulatory mechanisms of LINC-PINT may provide novel targets for therapeutic interventions, potentially leading to more effective cancer treatments (73). In paclitaxel-resistant cells, a significant reduction in the expression of LINC-PINT was observed. When LINC-PINT is ectopically expressed, both paclitaxel-resistant and wild-type triple-negative breast cancer (TNBC) cells showed increased sensitivity to paclitaxel. Mechanistically, LINC-PINT was found to bind to the RNA-binding protein NONO. Overexpressing LINC-PINT leads to the degradation of NONO in a proteasome-dependent manner. Additionally, analysis of patient samples via an online database indicates that LINC-PINT and NONO have antagonistic functions in various types of breast cancer (74). This suggests that the balance between LINC-PINT and NONO may be crucial in determining the outcome of breast cancer.

4.12 LINP1

LINP1 is involved in breast cancer cell proliferation, metastasis, and chemoresistance. Through a genetic screening method guided by clinical data, a lncRNA called LINP1 was found to be significantly upregulated in human triple-negative breast cancer. It was revealed that LINP1 plays a role in enhancing the repair of DNA double-strand breaks by acting as a scaffold that connects Ku80 and DNA-PKCs, thereby coordinating the nonhomologous end joining (NHEJ) pathway. LINP1 is controlled by both p53 and epidermal growth factor receptor (EGFR) signaling, and LINP1 overexpression counteracts the metastatic effects of p53. Notably, inhibition of LINP1 enhances the sensitivity of breast cancer cells to radiotherapy (75). Knockdown of LINP1 leads to reduced breast cancer cell growth by inducing G1-phase cell cycle arrest and promoting apoptosis. Additionally, LINP1 facilitates breast cancer cell metastasis and modulates the expression of markers related to epithelial-mesenchymal transition (EMT). LINP1 levels are found to be elevated in cells resistant to doxorubicin and 5-fluorouracil, indicating its role in inducing chemoresistance. Clinical data show that elevated LINP1 levels in tumors are associated with poorer overall survival and disease-free survival in breast cancer patients (76). Therefore, targeting LINP1 could be a promising therapeutic strategy to reduce chemoresistance and improve patient outcomes.

4.13 LincRNA-p21

Mutant p53 (mutp53) often loses its ability to bind to p53 response elements (p53REs) and cannot fully trigger apoptosis (77–79). The detailed mechanism has been reported to be correlated with the expression of long intergenic noncoding RNA-p21 (lincRNA-p21), which enhances chemotherapy resistance by targeting its G-quadruplex structure instead of the p53RE on its promoter (80, 81). Surprisingly, estrogen receptor alpha (ERα) inhibits mutp53-mediated lincRNA-p21 expression by redirecting mutp53 to increase the transcription of damaged DNA binding protein 2 (DDB2), leading to enhanced DNA repair and chemoresistance. The levels of lincRNA-p21 are positively associated with the response of breast cancer patients to neoadjuvant chemotherapy and show an inverse relationship with ER status and DDB2 levels. This reveals that the ER status determines how mutp53 functions in promoting chemoresistance as it switches its target gene preference from lincRNA-p21 to DDB2 (82). These results suggest that inducing lincRNA-p21 expression and targeting DDB2 could be effective strategies to enhance the chemosensitivity of breast cancer patients with mutp53.

Besides, a significant upregulation of lincRNA-p21 was revealed in 4T1-educated macrophages. Knocking down lincRNA-p21 leads to the polarization of macrophages towards the pro-inflammatory M1 phenotype in the tumor microenvironment. This effect may be attributed to MDM2 promoting the proteasome-dependent degradation of p53, consequently activating the NF-κB and STAT3 pathways (83, 84). Tumor-associated macrophages (TAMs) with lincRNA-p21 knockdown induce apoptosis in cancer cells and inhibit their migration and invasion. Furthermore, *in vivo* studies showed that adoptive transfer of lincRNA-p21 knockdown macrophages could mitigate breast cancer progression (85). These results highlight lincRNA-p21 as a critical regulator of TAMs in the tumor microenvironment and suggest potential therapeutic targets for tumors characterized by monocyte/macrophage infiltration.

4.14 LncRNA H19

LncRNA H19 (Long non-coding RNA H19) is known to play a role in breast cancer tumorigenesis and metastasis by influencing the epithelial-mesenchymal transition (EMT). Elevated expression levels of both lncRNA H19 and TNFAIP8 are observed in breast cancer tissues and cell lines, particularly in TNBC. Knockdown of either lncRNA H19 or TNFAIP8 has been shown to suppress cell proliferation, migration, and invasion, induce cell cycle arrest, and lead to abnormal expression of EMT markers. Mechanistically, lncRNA H19 appears to counteract the tumor suppressor p53, thereby increasing the expression of TNFAIP8, which in turn promotes the EMT process. Additionally, silencing either lncRNA H19 or TNFAIP8 has been found to inhibit tumorigenesis and lymph node metastasis in xenograft mouse models using MDA-MB-231 cells (86). These findings shed light on a new mechanism involving lncRNA H19 in breast cancer tumorigenesis and metastasis, highlighting the H19/p53/TNFAIP8 axis as a potential therapeutic target, especially for TNBC.

4.15 NORAD

The human long non-coding RNA NORAD is a newly discovered molecule that is activated in response to DNA damage in a manner dependent on the p53 protein. NORAD expression has been observed in various cancers, including breast cancer (87).

Elevated levels of NORAD expression are detected in a series of human epithelial breast cancer cell lines (MDA-MB-231, MDA-MB-436, and MDA-MB-468), which are classified as the most aggressive subtypes known as triple-negative breast cancer. These findings are consistent with previous research indicating that high levels of NORAD expression in basal-like tumors are linked to a poor prognosis. NORAD plays a crucial role in safeguarding genomic stability by interacting with Pumilio proteins, thereby preventing the repression of their target mRNAs (88). Disruption of NORAD function leads to chromosomal instability and aneuploidy (87). Consequently, reducing NORAD levels was found to sensitize triple-negative breast cancer cells to chemotherapy, potentially due to an increased accumulation of genomic abnormalities and a reduced ability to detect DNA damage.

4.16 CCAT2

The long noncoding RNA (lncRNA) CCAT2 (colon cancer-associated transcript 2) is found to affect the cell growth, migration, invasion, and drug sensitivity of breast cancer (BC) cells to 5-fluorouracil (5-Fu), involving miR-145 and p53. High CCAT2 expression is associated with lymph node metastasis, positive progesterone receptor, estrogen receptor, and Ki-67 in BC cells. Silencing CCAT2 upregulates miR-145, which is lowly expressed in drug-resistant BC cells (89). p53 is found to bind to the miR-145 promoter region and increase miR-145 expression. The upregulation of miR-145 induced by CCAT2 silencing is reversed by p53-siRNA (90). In conclusion, CCAT2 inhibits p53-induced activation of miR-145, and its overexpression enhances drug resistance in BC cells to 5-Fu.

4.17 TUG1

TUG1 (Taurine-upregulated gene 1) is a long non-coding RNA recently linked to the development of various human cancers. TUG1 is demonstrated to be upregulated in breast cancer (91). Knockdown of TUG1 significantly slows down cell proliferation, cell migration, and invasion in breast cancer cell lines MDA-MB-231 and MDA-MB-436. In MDA-MB-231 and BT549, cisplatin-induced cell growth arrest is remarkably augmented by overexpression of TUG1 and was significantly reduced by TUG1 silencing (92). However, in another study, TUG1 expression is significantly decreased in TNBC cell lines compared with normal breast epithelial cell lines and cell lines of other subtypes of breast cancer. Zhang et al. demonstrated that TUG1 is a direct transcriptional target of p53. In lung cancer, p53 binds to its putative response element in the promoter region of TUG1 (93). In breast cancer, TUG1 expression is significantly reduced in breast cancer tissues and cell lines compared to normal controls, and low TUG1 levels are notably associated with mutant p53 expression and lymph node metastasis (94). *In vitro*, overexpressing TUG1 markedly inhibits cell proliferation by inducing cell cycle arrest and apoptosis in breast cancer cells, while silencing TUG1 leads to increased cell growth by promoting cell cycle progression and altering the expression of cyclinD1 and CDK4. Further functional tests demonstrated that TUG1 overexpression significantly enhances cell migration and invasion, whereas TUG1 knockdown has the opposite effects (95). The mechanism should be further investigated in breast cancer.

5 Suggestions for clinical research translation

The study of p53-related lncRNAs regulation is of great significance for clinical breast cancer and can be translated into clinical applications as follows:

5.1 Diagnostic significance

P53-related RNAs, such as specific lncRNAs, can serve as potential biomarkers for breast cancer. The abnormal expression levels of these RNAs in breast cancer tissues compared to normal tissues can provide important clues for early diagnosis. For example, detecting the expression of lncRNAs like MEG3, CASC2, and others can help clinicians identify breast cancer at an early stage and improve diagnostic accuracy. Non-invasive or minimally invasive diagnostic methods can be developed by detecting these RNAs in blood or other body fluids, increasing patient acceptance.

5.2 Prognostic value

The expression patterns of p53-related RNAs can predict the prognosis of breast cancer patients. For instance, certain lncRNAs may be associated with poor prognosis, higher tumor grade, and increased metastatic potential. This information can help clinicians make more informed decisions about treatment options and patient management.

5.3 Therapeutic implications

Targeting p53-related RNAs can provide new therapeutic strategies. For example, inhibiting oncogenic lncRNAs or restoring the function of tumor suppressor lncRNAs can inhibit the growth, metastasis, and drug resistance of breast cancer cells. Understanding the role of p53-related RNAs in chemotherapy resistance can help develop strategies to overcome resistance and improve treatment efficacy. For instance, by targeting specific lncRNAs that are abnormally expressed in drug-resistant cells, the sensitivity of breast cancer cells to chemotherapy drugs can be enhanced.

The knowledge gained from studying p53-related RNA regulation can lead to the development of novel targeted therapies. By understanding the specific functions and interactions of these RNAs, researchers can design drugs or

therapeutic strategies that specifically target these molecules to inhibit breast cancer progression. Combining traditional treatment methods with RNA-targeted therapies based on p53 regulation can potentially improve treatment outcomes and reduce the risk of recurrence and metastasis.

6 Limitations and future directions

Despite significant progress, several challenges remain in the study of p53-related lncRNAs in breast cancer. These include the functional characterization of lncRNAs, elucidation of their precise mechanisms of action, and development of effective therapeutic strategies targeting these molecules. Developing efficient delivery systems for lncRNA-based therapies to ensure specificity and minimize off-target effects is a major hurdle. Additionally, large-scale clinical trials are needed to validate the prognostic and therapeutic potential of lncRNAs. Future research should focus on addressing these challenges to harness the full potential of p53-related lncRNAs in breast cancer management. Exploring the interaction networks of lncRNAs, miRNAs, and mRNAs could provide a comprehensive understanding of their roles in cancer biology. Continued research in this field is likely to lead to breakthroughs in personalized medicine, offering new hope for patients with advanced and resistant forms of breast cancer.

7 Conclusion

Breast cancer is a major global health challenge. Understanding its molecular mechanisms, classification, and treatment options is crucial. P53-related long non-coding RNAs are a hot research field in breast cancer biology. By elucidating the interaction between p53 and lncRNAs, it is expected to reveal new diagnostic markers and therapeutic targets and improve patient prognosis. Resolving existing controversies, optimizing screening and treatment strategies, and developing new targeted inhibitors are essential for reducing the burden of breast cancer worldwide.

Author contributions

JS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. QC: Writing – original draft, Writing – review & editing. JG: Writing – review & editing.

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Conflict of interest

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

Publisher's note

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EIF4A3-induced circ_0022382 promotes breast cancer cell progression through the let-7a-5p/PI3K/AKT/mTOR signaling pathway and SLC7A11 axis

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Introduction: Breast cancer is one of the most common cancers in women and poses a serious threat to women's health. Circular RNAs (circRNAs) have been found to be specifically expressed in cancers and regulate the growth and death of tumor cells. The role of circRNAs in breast cancer remain unknown. In this study, we explored the impacts of circRNAs on the progression of breast cancer cells.

Methods: Using bioinformatics analysis, we screened out one up-regulated circRNA in breast cancer, and its function and regulatory mechanisms were confirmed by quantitative real-time PCR, cell counting kit-8 experiment, migration assay, dual luciferase reporter assay, Kyoto Encyclopedia of Genes and Genomes enrichment analysis, cell immunofluorescence, clone formation assay, scratch wound healing experiment, RNA immunoprecipitation and subcutaneous tumor-bearing experiments.

Results: Circ_0022382 was highly expressed in breast cancer cell lines MDA-MB-231, MCF-7 as well as breast cancer tissues, and promoted the proliferative and migratory capacity of breast cancer cells. In terms of regulatory mechanisms, circ_0022382 activated PI3K/AKT/mTOR signaling pathway and SLC7A11 by sponging let-7a-5p, while knockdown of circ_0022382 contributed to the occurrence of disulfidptosis. In addition, EIF4A3 promoted the expression of circ_0022382 in MDA-MB-231 and MCF-7. Consistently, knockdown of circ_0022382 inhibited the growth of breast cancer cells *in vivo*.

Discussion: Circ_0022382 and its related molecules may be effective targets for diagnosis or targeted therapy of breast cancer.

KEYWORDS

breast cancer, circ_0022382, let-7a-5p, PI3K/Akt/mTOR signaling pathway, SLC7A11, disulfidptosis, EIF4A3

1 Introduction

Breast cancer has a high incidence and mortality among women (1). Though advances in treatment approaches have benefited many breast cancer patients, drug resistance remains due to the genomic instability inherent of the tumor itself (2, 3). Circular RNAs (circRNAs), as non-coding RNA, were first thought to be meaningless and byproducts of the gene-splicing process (4). However, with the widespread development of next-generation sequencing in clinical diagnosis and treatment, circRNAs have been found to be specifically expressed in many cancers and affect the proliferation, metastasis, drug resistance and immune response of tumor cells (5, 6). In addition, circRNAs with closed loop structure are more stable than linear RNAs. Therefore, circRNAs could act as stable and specific molecular markers of cancers. Mechanically, most of the circRNAs such as circ_104348 and circ_001783 are mainly located in the cytoplasm and exert regulatory functions by sponging microRNAs (7, 8), then regulate signaling pathways such as PI3K/AKT/mTOR signaling pathway (9). Recent studies reported correlations between circRNAs and various types of cell death such as apoptosis and ferroptosis in breast cancer (10, 11). Disulfidptosis is a type of cell death arising from accumulated cystine within cells with high expression of SLC7A11 (12, 13). However, the relationship between circRNAs and disulfidptosis in breast cancer remains unknown. In the present study, bioinformation analysis was carried out in the GEO database, which found that circ_0022382 was highly expressed in breast cancer. For mechanism of circ_0022382, we revealed that knockdown of circ_0022382 inhibited the proliferation and migration of breast cancer cells via sponging let-7a-5p, down-regulated p-AKT and SLC7A11, promoted the occurrence of disulfidptosis. RNA-binding proteins such as AUF1 and EIF4A3 were reported to induce the production of circRNAs (14, 15), in this study, we also demonstrated EIF4A3 could promote the production of circ_0022382 in breast cancer.

2 Materials and methods

2.1 Clinical samples and cells

Three paired human breast cancer tissues and adjacent tissues were obtained from the Department of Oncology Surgery (Thyroid & Breast), Affiliated Hospital of Jiangsu University. The study was approved by the Ethics Committee of Jiangsu University. Breast cancer cells including MDA-MB-231, MCF-7, normal breast epithelial cell MCF-10A and 293T were purchased from American Type Culture Collection (Manassas, VA, USA).

2.2 Bioinformatics analysis

Searching for the keywords “breast cancer”, “circRNA” and “miRNA” in the GEO database (https://www.ncbi.nlm.nih.gov/geo/), selected the corresponding datasets for “circRNA” and “miRNA”, the threshold of $|log_2\ (fold\ change,\ FC)|>2$ and *adj.* $p<0.05$ was set and perform differential analysis of GSE165884, similarly, the threshold of $|log_2\ (FC)|>1.4$ and *adj.* $p<0.05$ was set and perform differential analysis of GSE45498, GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was a part of GEO database to analyze and visualize the differential genes of samples, the potential miRNAs combined with circRNA were predicted by ENCORI (https://starbase.sysu.edu.cn/), and these miRNAs and down-regulated miRNAs were intersected by Venn diagram(https://bioinformatics.psb.ugent.be/webtools/Venn/), and the circRNA combined with the intersecting miRNA was taken as the research object. DAVID v6.8 (https://david-d.ncifcrf.gov/), Wei Sheng Xin platform (https://www.bioinformatics.com.cn/login/) was used to perform and visualize the results of enrichment analysis of KEGG signaling pathways on the basis of mRNAs bound by miRNA, CircInteractome (https://circinteractome.nia.nih.gov/) was used to predict the RNA-binding proteins combined with circRNA.

2.3 RNA extraction and quantitative real-time PCR

Total RNA was extracted from cells according to the instructions for Trizol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) and we measured the concentration using a Nanodrop 1000 ultramicro spectrophotometer (NanoDrop, Wilmington, DE, USA). For quantitative analysis of mRNA, we first reverse transcribed total RNA into cDNA according to the mRNA reverse transcription reagent instructions (Vazyme Biotech, Nanjing, China; R323-01), and then amplified the cDNA according to the qPCR instructions (Vazyme; Q711), using *GAPDH* as the internal reference, the primer sequences can be seen in the Table 1. For quantitative analysis of miRNA, the miRNA was first specifically reverse transcribed into cDNA using the stem loop method according to the miRNA reverse transcription reagent instructions (Vazyme; MR101-02). Then, the cDNA was amplified according to the qPCR instructions (Vazyme; MQ101-02), using U6 as the internal reference, the primer sequences can be seen in the Table 1. The expression of circ_0022382, let-7a-5p and SLC7A11 was measured after siRNA transfection for 36 h respectively.

2.4 Cell transfection

On the day before transfection, MDA-MB-231 and MCF-7 in the logarithmic growth phase were inoculated into a 6-well plate at a density of 1×10^5 cell/mL. The next day, when the cell density had reached approximately 70%, cells were transfected with siRNA or miRNA mimic respectively, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor. specific procedures can be seen in the instructions for Lipofectamine$^{TM\ 2000}$ (Thermo Fisher Scientific), the sequences of transfection primers can be seen in the Table 1.

2.5 Cell counting kit-8 experiment

MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor for 24 h, 48 h and 72 h were digested with trypsin respectively and inoculated into 96-well plates at a cell density of 5×10^4 cells/mL. After 24 h, 10 μL of Cell Counting Kit-8 reagent (Biosharp, Beijing, China) was added to each well and optical density was measured by an enzyme-linked immunosorbent assay reader at a wavelength of 450 nm.

2.6 Clone formation experiment

After transfecting si-circ_0022382 for 24 h, MDA-MB-231 and MCF-7 were digested with trypsin respectively and inoculated at a density of 500 cells per well into a 6-well plate and cultured in a CO_2 incubator. The medium was changed every 3 days. When the number of cells in the clone group under the microscope exceeded 50, cell culture was stopped. The original culture medium was discarded and cells were washed three times with PBS, followed by addition of 1 mL 4% paraformaldehyde fixative for 20 min. The fixative was discarded, the cells were washed three times with PBS, and 1 mL of 0.1% crystal violet staining solution was added to each well for 20 min. The staining solution was discarded and the cells were washed three times with PBS before taking photos.

2.7 Scratch wound healing experiment

After transfecting si-circ_0022382 for 24 h, MDA-MB-231 and MCF-7 were digested with trypsin respectively and inoculated into 6-well plates, two days later, the cells were overgrown. A scratch was made across the cell layer using a 200 μL pipette. The culture medium was discarded and the cells were washed three times with PBS. DMEM nutrient solution with 2% FBS was then added and the cells were photographed and recorded as 0 h. After 24 h, the medium was removed and the cells were photographed again and recorded as 24 h.

2.8 Migration assay

MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor for 24 h were digested with trypsin respectively and resuspended in serum-free high-glucose DMEM cell culture medium. The cells were inoculated into Transwell chambers at a density of 1×10^5 cells/well, and the cells were placed in 600 μL of high-sugar DMEM cell culture medium containing 10% fetal bovine serum. After 12 h, the Transwell chambers were removed and washed 3 times with PBS. We used a cotton swab to wipe-off any cells that had not penetrated through the Transwell chamber, then fixed the inserts in 4% paraformaldehyde solution for 30 min. The fixative was removed and the cells were washed 3 times with PBS. Finally, crystal violet solution was used to stain the cells at room temperature for 30 min before washing 3 times with PBS, cleaning the remaining substances and wiping the upper chamber cells clean with a cotton swab. After drying at room temperature, we imaged and counted the migrating cells on the basolateral side of the chamber by Image J.

2.9 Dual luciferase reporter assay

Co-transfect circ_0022382 dual luciferase reporter vector (wild type plasmid or mutant type plasmid) and let-7a-5p (negative control sequence or overexpression sequence), judge whether let-7a-5p could bind to circ_0022382 or not from luciferase density, if

TABLE 1 qRT-PCR primer sequences and transfection primer sequences.

	Forward (5′-3′)	Reverse (5′-3′)
Circ_0022382	GATTCCTACCCTCATCACGGC	GAAGGCGCGGAAGGCAT
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
SLC7A11	TGTGTGGGGTCCTGTCACTA	CAGTAGCTGCAGGGCGTATT
Si-circ_0022382-1	CACUUAAAGGAUGCCUUCCTT	GGAAGGCAUCCUUUAAGUGTT
Si-circ_0022382-2	UUAAAGGAUGCCUUCCGCGTT	CGCGGAAGGCAUCCUUUAATT
Let-7a-5p	GCGCGTGAGGTAGTAGGTTGT	AGTGCAGGGTCCGAGGTATT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Si-EIF4A3	CGAGCAAUCAAGCAGAUCATT	UGAUCUGCUUGAUUGCUCGTT
Let-7a-5p mimic	UGAGGUAGUAGGUUGUAUAGUU	CUAUACAACCUACUACCUCAUU
Let-7a-5p inhibitor	AACUAUACAACCUACUACCUCA	
LV-shcirc_0022382	GCCACTTAAAGGATGCCTTCC	

luciferase density decline, let-7a-5p could interfere the expression of circ_0022382 plasmid, indicating that let-7a-5p could bind to circ_0022382. Among of them, mutant type plasmid was used for positive control, negative control sequence was used for negative control. Specific procedures can be seen in the instructions for the Dual Luciferase Reporter Assay Kit (Vazyme; DL 101-01).

2.10 RNA immunoprecipitation

EIF4A3 protein and the genes that interact with EIF4A3 could be pulled down together by magnetic beads containing the EIF4A3 antibody_(AiFang; AF301768), and the final concentration of EIF4A3 antibody stock in solution is 1 : 40, the specific procedure of RNA immunoprecipitation follows the instruction of the RIP reagent kit (Merck, DARMSTADT; 17-701).

2.11 Western blot

After lysing with the cell lysis buffer (LEAGENE, Beijing, China; PS0013), the cell lysate was obtained by scraping and centrifuged at 12000 g rotational speed. The supernatant was separated and 5 × loading buffer was added at a ratio of 4 : 1, and 2 μL of PMSF was added at the same time to prevent protein degradation. The total protein extract was divided into protein bands of different molecular weights by electrophoresis and membrane transfer, and then the membranes containing the different bands were blocked with 5% skim milk. The membranes were immunoblotted with primary antibodies, including anti-p-AKT_(Cell Signaling Technology; 4060T), anti-SLC7A11_(Abclonal, Wuhan; A2413), anti-EIF4A3_(AiFang; AF301768) and HRP-conjugated anti-β-actin_(Santa Cruz; sc-47778). All primary antibody stocks were diluted with TBST at a dilution ratio of 1 : 1000 and incubated with the membranes at 4°C

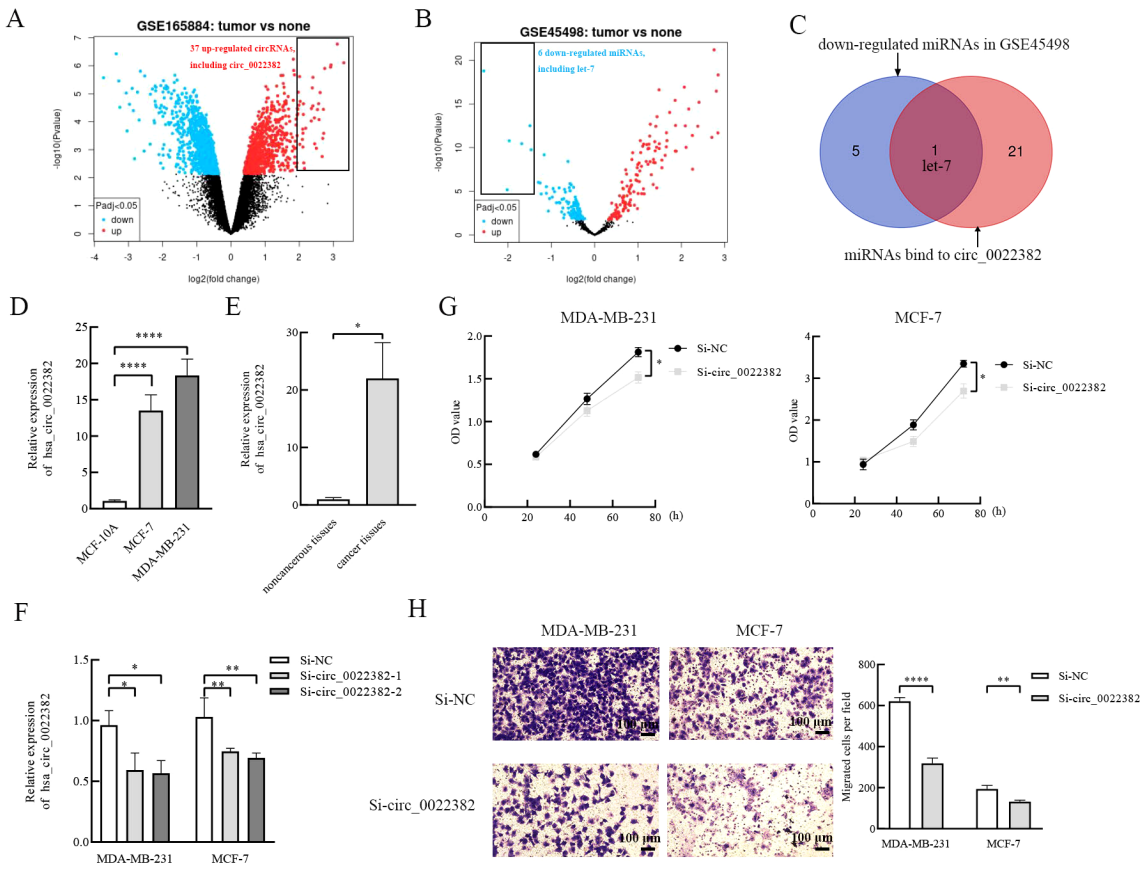

FIGURE 1
The highly expressed circ_0022382 screened from the GEO database promotes the proliferation and migration of breast cancer cells. **(A)** The volcano plot of GSE165884 was generated by GEO2R, red scatter points showed circRNAs that are up-regulated in breast cancer, including circ_0022382. **(B)** The volcano plot of GSE45498 was generated by GEO2R, blue scatter points showed miRNAs that are down-regulated in breast cancer, including let-7. **(C)** The intersection of miRNAs bind to circ_0022382 and down-regulated miRNAs was generated by Venn diagram. **(D)** QRT-PCR was used to measure the expression of circ_0022382 in MDA-MB-231, MCF-7 and MCF-10A. ****p<0.0001 vs. MCF-10A group. Data are the means ± SEM of three independent experiments. **(E)** QRT-PCR was used to measure the expression of circ_0022382 in three pairs of breast cancer and adjacent noncancerous tissues. *p<0.05 vs. adjacent noncancerous tissue group. Data are the means ± SEM of three independent experiments. **(F)** Knockdown efficiency of si-circ_0022382-1 and si-circ_0022382-2 in MDA-MB-231 and MCF-7 were measured by qRT-PCR. *p<0.05, **p<0.01 vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(G)** Cell Counting Kit-8 (CCK8) was used to detect speed of proliferation in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382-1 (si-circ_0022382) for 24 h, 48 h and 72 h. *p<0.05 vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(H)** Migration assay was used to detect numbers of migrated MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382 for 24 h. **p<0.01, ****p<0.0001 vs. Si-NC group; scale bar = 100 μm. Data are the means ± SEM of three independent experiments.

overnight. The membranes were then washed with TBST three times and incubated for 2 h at room temperature in secondary antibody stock solution diluted in TBST at a dilution ratio of 1 : 2000. The membranes were washed in TBST three times and placed on the ECL (Vazyme; E412-02-AA) in developer (GE, Boston, MA, USA; LAS4000Mini) for imaging.

2.12 Lentiviral transfection and subcutaneous tumor injection

MDA-MB-231 cells were inoculated into 6-well plates, and when the cell density reached 30%, the culture medium was discarded and washed with PBS, and a lentiviral transfection mixture consisting of 2 mL of DMEM nutrient solution and 20 μL of lentiviral solution (LV-NC or LV-shcirc_0022382) was added to each well, gently mixed and placed in the incubator to continue culture for 24 h, then the culture medium was replaced after 24 h, one part of cells were used to detect the knockdown efficiency in MDA-MB-231 cells transfected with LV-shcirc_0022382, another part of cells were digested and resuspended with PBS for subcutaneous tumor-bearing experiments. Ten 4-week-old female BALB/C nude mice weighing between 15 g and 20 g were used for subcutaneous tumor-bearing experiments. 1×10^6 cells were injected into the back of each mouse and the tumor size was measured every 3 days. Two weeks later, the mice were euthanatized by cervical dislocation, the tumors were photographed and weighed. Tumor volume = 0.52 × width^2 (mm^2) × length (mm). The sequence of LV-shcirc_0022382 can be seen in the Table 1.

2.13 Immunohistochemistry

Prepared the tumor tissues into paraffin slides, which were deparaffinized and hydrated with different concentrations of xylene and ethanol solutions, washed the slides with water and placed them in boiling citrate retrieval solution for antigen retrieval, soaked the slides in H_2O_2 after washing with PBS, washed the slides with PBS and added goat serum to the slides, incubated for 1 h at room temperature, added the primary antibody dilution to the slides, i.e, anti-Ki-67 (AiFang; AF300540), anti-SLC7A11 (Proteintech, Wuhan, China; 26864-1-AP), or anti-p-AKT (Cell Signaling Technology), incubated overnight at 4°C, washed the slides with PBS, added the secondary antibody dilution to the slides, i.e, Cy3-IgG (Abclonal, Wuhan, China; AS007), incubated for 1 h at room temperature, washed the slides with PBS and add HRP-Streptavidin, incubated for 30 min at room temperature, washed the slides with PBS and add DAB chromogenic solution, incubated for 2 min at room temperature, washed the slides with water and add hematoxylin, incubated for 1 min at room temperature, washed the slides with water, added different concentrations of ethanol and xylene solutions.

2.14 Cellular immunofluorescence

MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor for 24 h were digested with trypsin and inoculated into crawling slides in 24-well plates for 24 h, then the culture medium was discarded and the cells were washed three times with PBS. The cells were fixed in 4% paraformaldehyde for 15 min and then washed three times in PBS. Then, the cells were permeabilized with 0.5% Triton X-100 for 20 min. The cells were washed three times in PBS and blocked with goat serum at room temperature for 30 min. After discarding the blocking solution, primary antibody, i.e., anti-p-AKT (Cell Signaling Technology), or anti-SLC7A11 (Proteintech) was added.

TABLE 2 miRNAs bind to circ_0022382 and down-regulated miRNAs in GSE45498.

down-regulated miRNAs at the threshold of $\|\log_2(FC)\|>1.4$ and adj. $p<0.05$ in GSE45498	the miRNAs that bind to circ_0022382
hsa-miR-548	hsa-miR-214-3p
hsa-miR-125	hsa-miR-761
hsa-miR-145	hsa-miR-3619-5p
hsa-miR-204	hsa-miR-4640-5p
hsa-miR-199	hsa-miR-4726-5p
hsa-let-7	hsa-miR-4761-5p
	hsa-miR-3127-5p
	hsa-miR-3918
	hsa-miR-670-3p
	hsa-miR-766-5p
	hsa-miR-665
	hsa-miR-4640-5p
	hsa-miR-6720-5p
	hsa-miR-6512-3p
	hsa-miR-5586-5p
	hsa-let-7b-5p
	hsa-let-7c-5p
	hsa-miR-98-5p
	hsa-let-7i-5p
	hsa-let-7a-5p
	hsa-let-7e-5p
	hsa-let-7f-5p
	hsa-let-7g-5p
	hsa-let-7d-5p
	hsa-miR-4500
	hsa-miR-3614-5p
	hsa-miR-3187-3p
	hsa-miR-552-3p
	hsa-miR-4458
	hsa-miR-624-3p

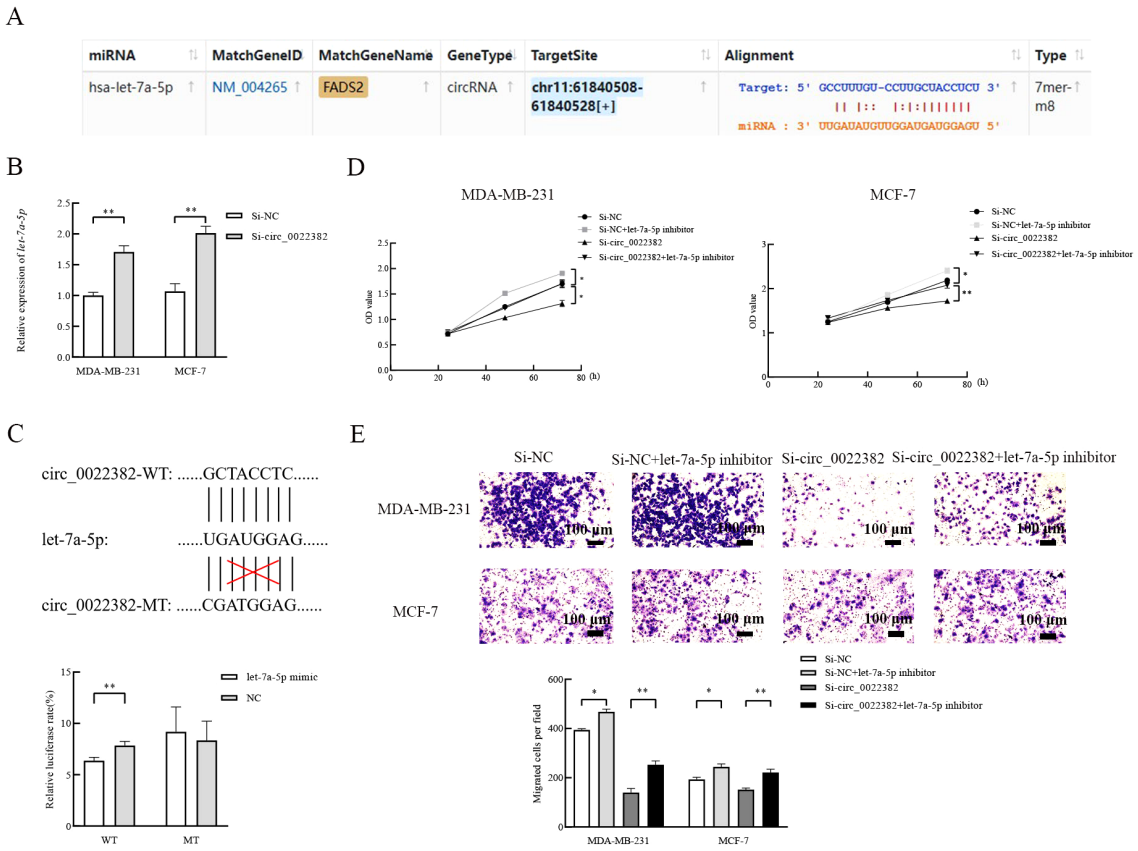

FIGURE 2
Let-7a-5p is a direct target to affect the proliferation and migration of breast cancer cells. **(A)** Let-7a-5p was predicted as a target of circ_0022382 by ENCORI. **(B)** QRT-PCR was used to measure the expression of let-7a-5p in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382. $^{**}p<0.01$ vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(C)** Dual-luciferase reporter assay was used to measure the luciferase of circ_0022382 wild type plasmid (WT) and circ_0022382 mutant type plasmid (MT) in 293T cells transfected with negative control (NC) or let-7a-5p mimic. $^{**}p<0.01$ vs. NC group. Data are the means ± SEM of three independent experiments. **(D)** CCK8 was used to detect speed of proliferation in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor for 24 h, 48 h and 72 h. $^{*}p<0.05$, $^{**}p<0.01$ vs. Si-NC group or Si-circ_0022382 group. Data are the means ± SEM of three independent experiments. **(E)** Migration assay was used to detect numbers of migrated MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382, co-transfected with si-NC and let-7a-5p inhibitor or si-circ_0022382 and let-7a-5p inhibitor for 12 h. $^{*}p<0.05$, $^{**}p<0.01$ vs. Si-NC group or Si-circ_0022382 group; scale bar = 100 μm. Data are the means ± SEM of three independent experiments.

The cells were incubated overnight at 4°C, the primary antibody was removed and the cells were washed three times with PBS, then incubated with Cy3-conjugated secondary antibody (Abclonal; AS007) at room temperature for 1 h. The cell slides were inverted onto anti-fluorescence quencher containing DAPI, and images were captured under a confocal microscope.

2.15 Glucose concentration detection

After transfecting si-circ_0022382 for 24 h, the culture medium supernatant of MDA-MB-231 and MCF-7 were collected respectively. The glucose concentration in the cell supernatant was detected according to the glucose kit (Nanjing Jiancheng Bioengineering Institute, Nanjing; A154-2-1).

2.16 Detection of the concentrations of NADPH and $NADP^+$

After transfecting si-circ_0022382 for 24 h, MDA-MB-231 and MCF-7 were digested with trypsin and collected respectively. The ratio of the concentrations of NADPH and $NADP^+$ were detected according to the kit (Nanjing Jiancheng Bioengineering Institute, Nanjing; A115-1-1).

2.17 Statistical analysis

Except for clone formation experiment, scratch wound healing experiment and cellular immunofluorescence, other experiments were performed three times, whose data were parametric and

GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used to analyze the data, which were expressed as mean ± Standard error of the mean (SEM). The significance of differences between the two groups was determined using the Student's t-test, and one-way analysis of variance was used to compare multiple groups.

3 Results

3.1 Circ_0022382 is highly expressed in breast cancer and promotes proliferation and migration of breast cancer cells

Firstly, we roughly screened up-regulated circRNAs in breast cancer from GEO database, GSE165884 is a GEO dataset related to breast cancer and circRNA, by performing differential analysis of circRNAs, the volcano plot was drawn by GEO2R, which showed up-regulated and down-regulated circRNAs in breast cancer tissues respectively, and there were 37 up-regulated circRNAs at the threshold of $|log_2\ (FC)|>2$ and *adj.* $p<0.05$, including circ_0022382 (Figure 1A). Secondly, since most of circRNAs act as miRNA sponges, we further narrowed the scope of circRNAs screened in the first step, consistently, we screened down-regulated miRNAs in breast cancer from GEO database, the volcano plot was drawn by GEO2R in GSE45498 to reveal differential expressed miRNAs in breast cancer tissues, and there were 6 down-regulated miRNAs at the threshold of $|log_2\ (FC)|>1.4$ and *adj.* $p<0.05$, including let-7 (Figure 1B). By predicting miRNAs bound by circRNAs from the first step and intersecting with miRNAs from the second step (Table 2), we revealed an intersection between miRNAs bound by circ_0022382 and 6 down-regulated miRNAs, i.e, let-7, so we took circ_0022382 as the research object (Figure 1C). Then, expression of circ_0022382 was evaluated by qRT-PCR in both breast

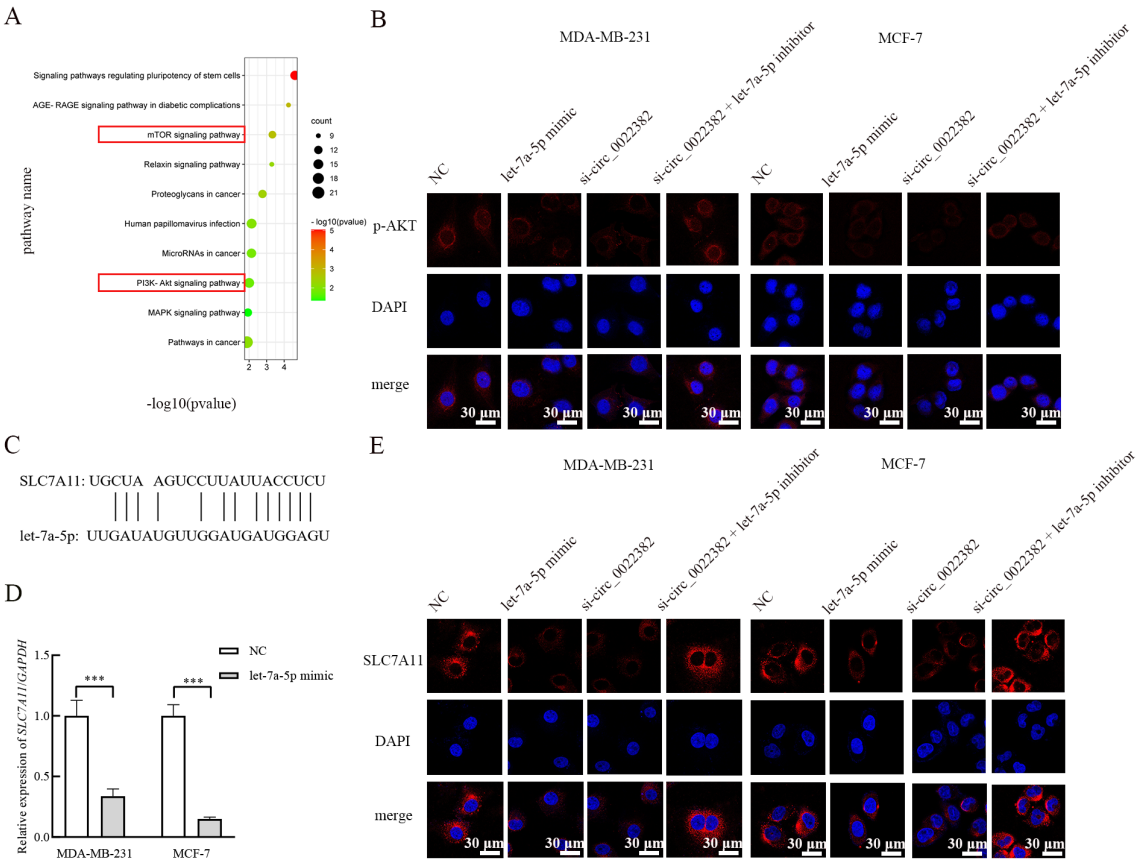

FIGURE 3
The enrichment of PI3K/AKT/mTOR signaling pathway and SLC7A11 is regulated by circ_0022382/let-7a-5p axis. **(A)** The top 10 enriched KEGG signaling pathways of downstream target genes of let-7a-5p were visualized through bubble plots. **(B)** The luciferase density of Cy3-P-AKT in MDA-MB-231 and MCF-7 cells transfected with negative control (NC), let-7a-5p mimic, si-circ_0022382 respectively, co-transfected with si-circ_0022382 and let-7a-5p inhibitor was verified by cellular immunofluorescence; scale bar = 30 μm. **(C)** SLC7A11 was predicted as a target of let-7a-5p by ENCORI. **(D)** QRT-PCR was used to measure the expression of SLC7A11 mRNA in MDA-MB-231 and MCF-7 cells transfected with NC or let-7a-5p mimic. ***$p<0.001$ vs. NC group. Data are the means ± SEM of three independent experiments. **(E)** The luciferase density of Cy3-SLC7A11 in MDA-MB-231 and MCF-7 cells transfected with NC, let-7a-5p mimic, si-circ_0022382 respectively, co-transfected with si-circ_0022382 and let-7a-5p inhibitor was verified by cellular immunofluorescence; scale bar = 30 μm.

associated cells and tissues, the expression of circ_0022382 in breast cancer cells (MDA-MB-231 and MCF-7) were significantly higher than in MCF-10A (p<0.0001; Figure 1D), breast cancer tissues had higher expression of circ_0022382 than in adjacent noncancerous tissues (p<0.05; Figure 1E). In addition, we explored effects of circ_0022382 on biological functions of breast cancer cells by interfering with circ_0022382 expression, knockdown efficiency of two siRNAs (si-circ_0022382-1 and si-circ_0022382-2) was evaluated by qRT-PCR, which found two siRNAs could both effectively knock down circ_0022382 expression in MDA-MB-231 and MCF-7 (p<0.05 or p<0.01; Figure 1F). Then, si-circ_0022382-1 was chosen for subsequent experiments due to si-circ_0022382-1 has less aggregated G or C bases at the 3' end (Table 1). CCK8 was performed to evaluate cell viability, the results showed the speed of cell proliferation was significantly slower after transfection with si-circ_0022382 (p<0.05; Figure 1G). Meanwhile, migration experiment was performed to evaluate cell migration, the results showed numbers of migrated cells were also significantly reduced after transfection with si-circ_0022382 (p<0.01 or p<0.0001; Figure 1H).

3.2 Circ_0022382 promotes the proliferation and migration of breast cancer cells by sponging let-7a-5p

In terms of mechanism, miRNA sponges have been studied the most (16), the above results showed that let-7 were potential targets of circ_0022382, so we screened the miRNA candidate from let-7 homologs by the lowercase letters of its suffix, surprisingly, ENCORI showed that let-7a-5p could bind to circ_0022382 and the binding capacity could reach 7 mer to 8 mer (Figure 2A). Let-7a-5p expression significantly increased after transfection with si-circ_0022382 in MDA MB-231 and MCF-7 (p<0.01; Figure 2B). Moreover, we performed dual luciferase reporter assay to further confirm this result, by co-transfecting let-7a-5p mimic and circ_0022382 wild-type plasmid with luciferase reporter, the fluorescence intensity in 293T cells significantly reduced, which indicated let-7a-5p had binding sites on circ_0022382 wild-type plasmid, on the contrary, the inhibitory effect of let-7a-5p mimic on fluorescence intensity could not be reflected when circ_0022382 plasmid was mutated (p<0.01; Figure 2C). Then, we tested whether

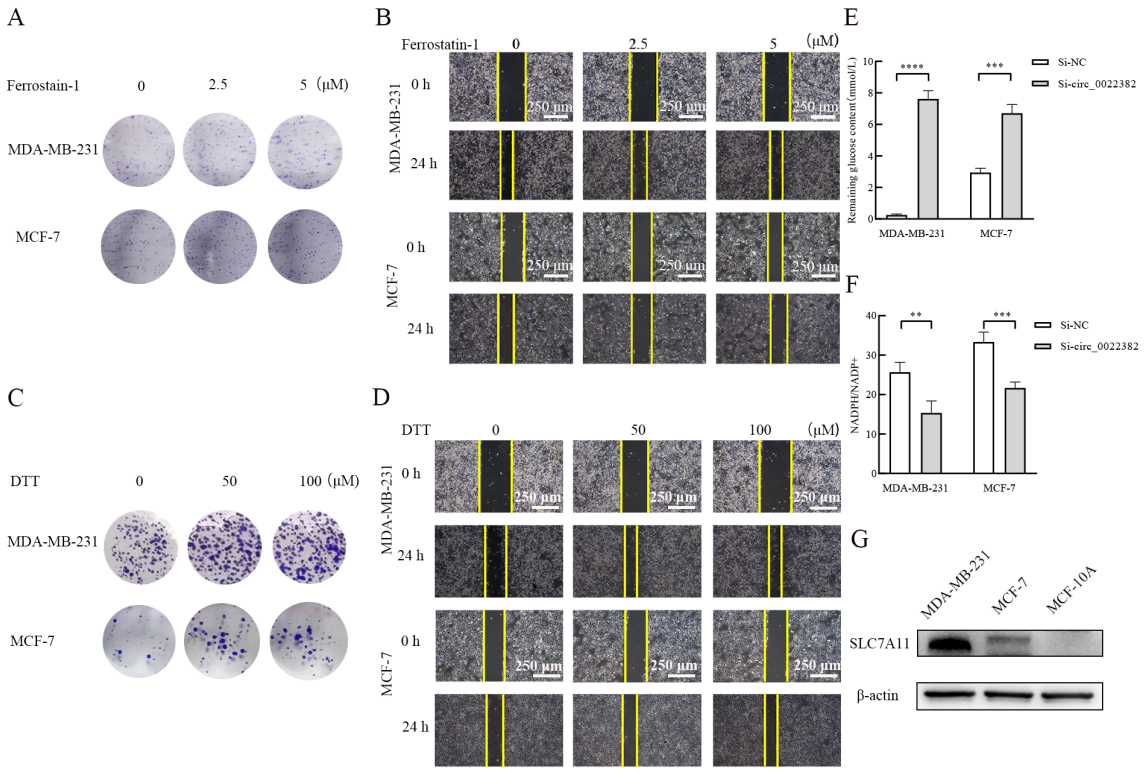

FIGURE 4
Knockdown of circ_0022382 could contribute to the occurrence of disulfidptosis rather than ferroptosis. **(A, B)** Clone formation and scratch wound healing experiment were used to detect effects of different concentrations of Ferrostatin-1 (ferroptosis inhibitor) on the proliferative and migratory capacity of MDA-MB-231 and MCF-7 cells transfected with si-circ_0022382, scale bar = 250 μm. **(C-D)** Clone formation and scratch wound healing experiment were used to detect effects of different concentrations of dithiothreitol (DTT, cysteine inhibitor) on the proliferative and migratory capacity of MDA-MB-231 and MCF-7 cells transfected with si-circ_0022382, scale bar = 250 μm. **(E)** The concentration of residual glucose in the culture medium supernatant of MDA-MB-231 and MCF-7 cells transfected with si-circ_0022382. ***p<0.001, ****p<0.0001 vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(F)** The ratio of the concentrations of NADPH and $NADP^+$ in MDA-MB-231 and MCF-7 cells transfected with si-circ_0022382. **p<0.01, ***p<0.001 vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(G)** SLC7A11 protein expression in MDA-MB-231, MCF-7 and MCF-10A was detected by Western blot.

circ_0022382 promoted the proliferation and migration of breast cancer cells by sponging let-7a-5p. The results of CCK8 showed the speed of cell proliferation was significantly faster after co-transfection with si-NC and let-7a-5p inhibitor, co-transfection with si-circ_0022382 and let-7a-5p inhibitor could rescue the effect of si-circ_0022382 on the proliferation ($p<0.05$ or $p<0.01$; Figure 2D), we observed similar effect of let-7a-5p inhibitor from migration experiment ($p<0.05$ or $p<0.01$; Figure 2E). Therefore, the above results suggested that circ_0022382 and let-7a-5p had opposite effect, circ_0022382 could interact with let-7a-5p to affect the proliferation and migration of breast cancer cells.

3.3 Circ_0022382 regulates PI3K/AKT/mTOR signaling pathway and SLC7A11 by sponging let-7a-5p

As shown in the bubble plots, the downstream mRNAs of let-7a-5p were enriched in signaling pathways involved in cancer progression, i.e, mTOR signaling pathway, PI3K-AKT signaling pathway (Figure 3A). To further confirm this enrichment, expression of signaling pathway-related proteins was detected by cellular immunofluorescence, the results showed p-AKT expression was reduced after overexpressing let-7a-5p, while co-transfection with si-circ_0022382 and let-7a-5p inhibitor could rescue effects of si-circ_0022382 on the p-AKT expression (Figure 3B). Moreover, SLC7A11 was reported to be related to PI3K/AKT/mTOR signaling pathway in cancers, such as gastric cancer (17), glioma (18), pancreatic carcinoma (19), neuroendocrine tumors (20), but the interaction has not been clarified in breast cancer. To confirm the relationship, firstly we tested whether let-7a-5p regulated SLC7A11, as shown in ENCORI, let-7a-5p could bind to SLC7A11 (Figure 3C), the expression of SLC7A11 mRNA in MDA-MB-231 and MCF-7 transfected with let-7a-5p mimic was significantly decreased ($p<0.001$; Figure 3D). Moreover, the results of cellular immunofluorescence showed SLC7A11 protein expression was also reduced after overexpressing let-7a-5p, while co-transfection of si-circ_0022382 and let-7a-5p inhibitor could rescue effects of si-circ_0022382 on the SLC7A11 expression (Figure 3E).

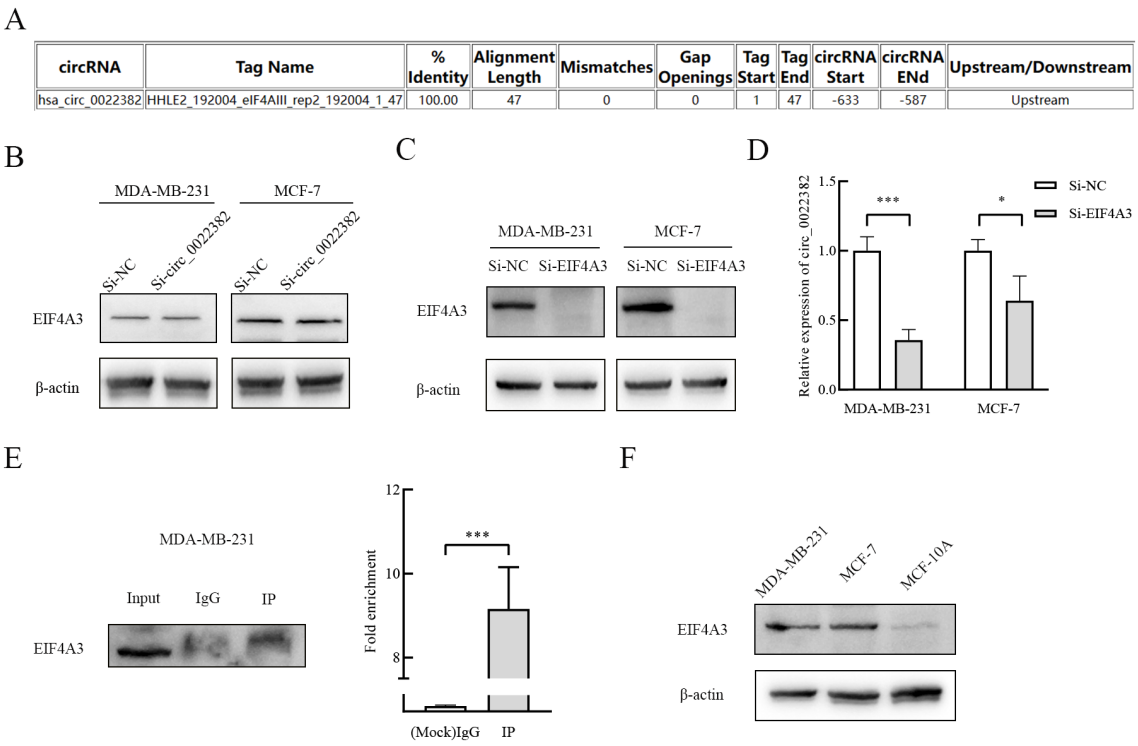

FIGURE 5
EIF4A3 could promote the production of circ_0022382 in breast cancer cells. **(A)** CircInteractome was used to predict the binding sites of circ_0022382 and EIF4A3. **(B)** EIF4A3 protein expression in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-circ_0022382 was detected by Western blot. **(C)** EIF4A3 protein expression in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-EIF4A3 was detected by Western blot. **(D)** Circ_0022382 expression in MDA-MB-231 and MCF-7 cells transfected with si-NC or si-EIF4A3 was detected by qRT-PCR. *$p<0.05$, ***$p<0.001$ vs. Si-NC group. Data are the means ± SEM of three independent experiments. **(E)** The interaction between EIF4A3 and circ_0022382 in MDA-MB-231 was tested by RNA immunoprecipitation (RIP), left was the enrichment of EIF4A3 protein in the whole cell lysate (Input), eluent for magnetic beads incubated with IgG (IgG), eluent for magnetic beads incubated with anti-EIF4A3 (IP), with IgG as a negative control, Input as a positive control, right was the enrichment of circ_0022382 in the IgG group and IP group. ***$p<0.001$ vs. IgG group. Data are the means ± SEM of three independent experiments. **(F)** The expression of EIF4A3 protein in MDA-MB-231, MCF-7 and MCF-10A was detected by Western blot.

3.4 Knockdown of circ_0022382 contributes to SLC7A11-mediated disulfidptosis

Then, previous study had reported that SLC7A11 was associated with ferroptosis and disulfidptosis (11, 12), especially disulfidptosis, consistent with PI3K/AKT/mTOR signaling pathway, was related with metabolism of glucose (12, 21), which provided evidence SLC7A11 may indirectly interact with PI3K/AKT/mTOR signaling pathway. To further explore the relationship, we added Ferrostatin-1 (ferroptosis inhibitor) and dithiothreitol (DTT, cysteine inhibitor) respectively in MDA-MB-231 and MCF-7 cells transfected with si-circ_0022382, the results of clone formation and scratch wound healing experiment showed dithiothreitol instead of Ferrostatin-1 could rescue effects of si-circ_0022382 on the proliferation and migration of cells (Figures 4A–D), which indicated cysteine accumulated in cells transfected with si-circ_0022382, moreover, the remaining glucose concentration in culture medium of cells transfected with si-circ_0022382 significantly increased ($p<0.001$ or $p<0.0001$; Figure 4E) and the ratio of the concentrations of NADPH and $NADP^+$ in cells transfected with si-circ_0022382 significantly decreased ($p<0.01$ or $p<0.001$; Figure 4F). The above results revealed that knockdown of circ_0022382 in MDA-MB-231 and MCF-7 increased the accumulation of cysteine, meanwhile reduced the consumption of glucose and the production of NADPH within cells, which was consistent with the characteristics of disulfidptosis. In addition, SLC7A11 expression in MDA-MB-231 and MCF-7 was higher than in MCF-10A (Figure 4G), indicating that SLC7A11 could also be a potential target.

3.5 EIF4A3 promotes the production of circ_0022382 in breast cancer cells

Through CircInteractome database, we found that EIF4A3 had binding sites on the upstream sequences of circ_0022382 (Figure 5A), which indicated circ_0022382 could also bind to RNA-binding proteins. To further clarify the interaction between EIF4A3 and circ_0022382, expression of EIF4A3 and circ_0022382 were detected in MDA-MB-231 and MCF-7 cells transfected with

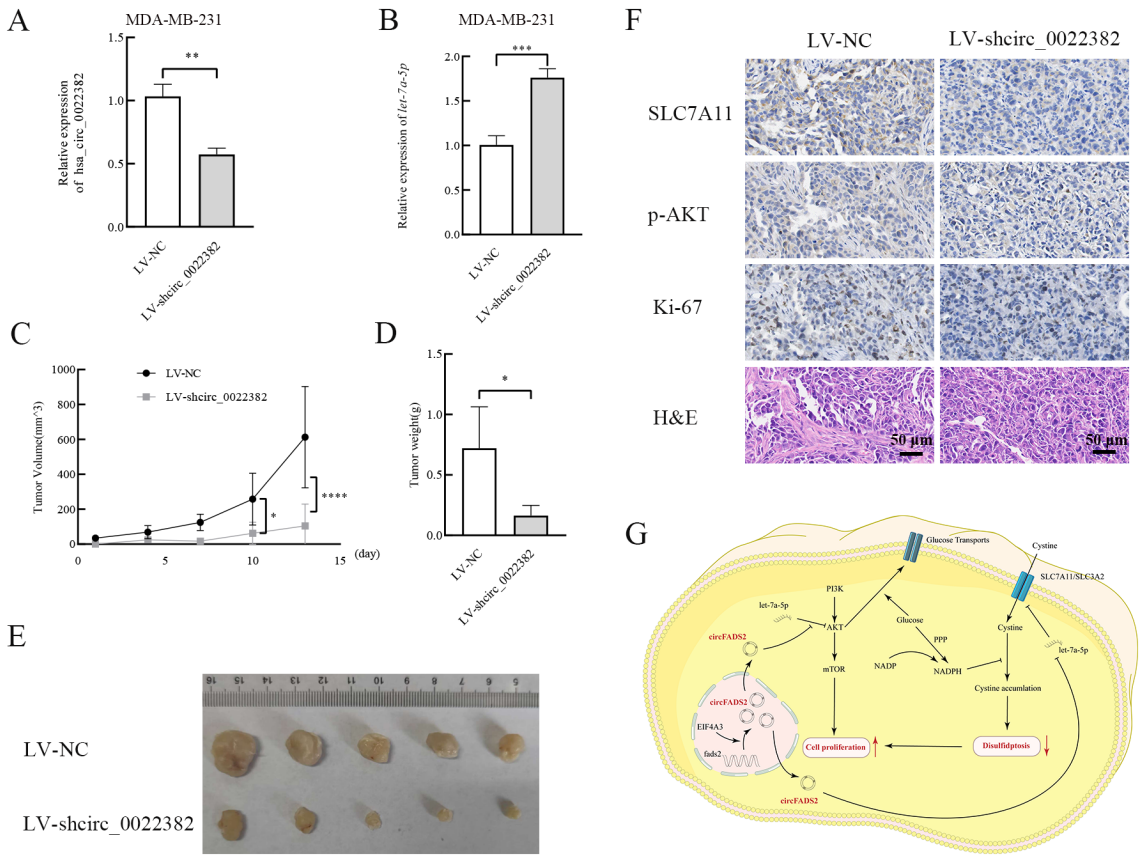

FIGURE 6
Circ_0022382 promotes the growth and progression of breast cancer *in vivo*. **(A, B)** The expression of circ_0022382 and let-7a-5p in MDA-MB-231 transfected with LV-NC or LV-shcirc_0022382 was detected by qRT-PCR. $^{**}p<0.01$, $^{***}p<0.001$ vs. LV-NC group. Data are the means ± SEM of five independent experiments. **(C)** The volume of tumors derived from LV-NC group mice (n = 5) and LV-shcirc_0022382 group mice (n = 5) was measured every 3 days. $^{*}p<0.05$, $^{****}p<0.0001$ vs. LV-NC group. Data are the means ± SEM of five independent experiments. **(D)** The weight of tumors from the above two groups was measured after two weeks. $^{*}p<0.05$ vs. LV-NC group. Data are the means ± SEM of five independent experiments. **(E)** The size of tumors from the above two groups. **(F)** Tumor tissues from the above two groups were immunohistochemistry stained with anti-SLC7A11, anti-p-AKT, anti-Ki-67 and H&E; scale bar = 50 μm. **(G)** The mechanism of circ_0022382/let-7a-5p/PI3K/AKT/mTOR signaling pathway and SLC7A11 axis was illustrated by a diagram.

si-circ_0022382 and si-EIF4A3 respectively. It is worth noting that EIF4A3 protein expression remained unchanged in cells transfected with si-circ_0022382 (Figure 5B), indicating the regulatory effect of circ_0022382 did not depend on EIF4A3 expression, in contrast, circ_0022382 expression dramatically decreased in cells transfected with si-EIF4A3 ($p<0.05$ or $p<0.001$; Figures 5C, D), in addition, the results of RNA immunoprecipitation (RIP) showed a prominent enrichment of circ_0022382 in anti-EIF4A3 (IP) group than in IgG group ($p<0.001$; Figure 5E), which further demonstrated the role of EIF4A3 in the process of circ_0022382 production in breast cancer cells. We also tested the EIF4A3 expression among breast cancer cells and normal cells, the results showed that MDA-MB-231 and MCF-7 had higher EIF4A3 expression than MCF-10A (Figure 5F), which was consistent with high expression of circ_0022382 in breast cancer cells.

3.6 Knockdown of circ_0022382 inhibits the growth of breast cancer cells *in vivo*

To further confirm the role of circ_0022382 played in the proliferation of breast cancer *in vivo*, we prepared MDA-MB-231 cells transfected with LV-NC or LV-shcirc_0022382, the results of qRT-PCR showed circ_0022382 expression in cells transfected with LV-shcirc_0022382 markedly decreased ($p<0.01$; Figure 6A), while let-7a-5p expression significantly increased ($p<0.001$; Figure 6B), then the cells were collected and injected into five BALB/C nude mice respectively by subcutaneous tumor-bearing experiment, the results showed the growing rate of tumors in LV-shcirc_0022382 group was significantly slower than in LV-NC group ($p<0.05$ or $p<0.0001$; Figure 6C), two weeks later, we observed lighter weights of tumors in LV-shcirc_0022382 group than in LV-NC group mice ($p<0.05$; Figure 6D), as shown in Figure 6E, effects of circ_0022382 on cell proliferation *in vivo* were consistent with the results of CCK8. Moreover, the tumors were prepared into paraffin slides and the expression of p-AKT, SLC7A11, Ki-67 was detected by immunohistochemistry staining respectively, the results showed p-AKT, SLC7A11 as well as Ki-67 expression decreased in tumors derived from LV- shcirc_0022382 group mice (Figure 6F), which were also consistent with the findings *in vitro*. Lastly, the mechanism of circ_0022382/let-7a-5p/PI3K/AKT/mTOR signaling pathway and SLC7A11 axis was depicted in the diagram (Figure 6G).

4 Discussion

The expression of circ_0022382 in breast cancer tissues and cells were higher than in normal breast tissues and cells, and knockdown of circ_0022382 could inhibit the proliferation and migration of breast cancer cells. Therefore, circ_0022382 may serve as an oncogene of breast cancer. In terms of mechanism, circ_0022382 could bind to let-7a-5p, it was reported that the down-regulated let-7a-5p in breast cancer could regulate the growth and progression of breast cancer cells, for example, the let-7a-5p/DUSP7 axis was associated with paclitaxel resistance (22), the let-7a-5p/GLUT12 axis (23) and the let-7a-5p/STAT3/hnRNP-A1/PKM2 axis (24) were associated with glycolysis in breast cancer. The occurrence of disulfidptosis often happens in cells with high SLC7A11 expression, allowing the accumulation of cystine within cells and then exposes a targetable metabolic vulnerability in cancer (12, 13). However, in this paper, we found expression of SLC7A11 in breast cancer cells transfected with si-circ_0022382 was decreased, which seems not to meet the common condition for disulfidptosis to occur. Of note, in the recent study, Yan et al. drew the conclusion that cells with moderate levels rather than high or low levels of SLC7A11 could undergo disulfidptosis rather than oxidative stress (25), which broke the boundaries of disulfidptosis cognition. As for why dithiothreitol rather than Ferrostatin-1 could rescue effects of si-circ_0022382 on the proliferation and migration of breast cancer cells, this is because both the consumption of glucose and the production of NADPH within cells transfected with si-circ_0022382 markedly decreased, thereby preventing the conversion of cystine to cysteine, which may also cause the accumulation of cystine within cells. Therefore, we have reason to suspect that MDA-MB-231 and MCF-7 have high levels of SLC7A11, and knocking down circ_0022382 will put SLC7A11 at a medium level rather than a low level, but when the circ_0022382 is knocked out, the expression level of the SLC7A11 may be further reduced, reaching a so-called low level, cells are more likely to undergo ferroptosis rather than disulfidptosis at that time, although these speculations need to be further verified. EIF4A3, as an RNA helicase, plays an important role in transcription and post transcriptional regulation (26), and the interaction between EIF4A3 and RNA in cancer has been widely studied (27, 28). In addition, EIF4A3 was found highly expressed in many cancers, such as prostate cancer (29), bladder cancer (30), endometrial cancer (31), so EIF4A3 may be an oncogene in cancer. In conclusion, circ_0022382 may serve as a potential marker for diagnosis and treatment of breast cancer, and its regulatory mechanism needs further exploration.

Data availability statement

Original datasets are available in a publicly accessible repository: The original contributions presented in the study are publicly available. These data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165884 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45498.

Ethics statement

The studies involving humans were approved by The Ethics Committee of Jiangsu University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by The Ethics Committee of Jiangsu University. The study was conducted in accordance with the local legislation and institutional

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

Author contributions

WL: Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. JZ: Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. JWZ: Data curation, Writing – review & editing. YY: Data curation, Writing – review & editing. JQZ: Methodology, Writing – review & editing. QY: Data curation, Writing – review & editing. TL: Data curation, Writing – review & editing. XS: Supervision, Writing – review & editing. HC: Supervision, Writing – review & editing.

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Conflict of interest

Authors WL, TL, and HC were employed by the company Health BioMed Co., Ltd.

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

Publisher's note

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