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The Role of miRNA-9 as a Predisposing Factor for Metastasis in Breast Cancer among Iraqi Patients from Localized to Locally Advanced and Metastatic Stages

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Abstract: Breast cancer (BC) is the highly prevalent malignancy afflicting women worldwide, with an alarming 2 million new cases diagnosed in 2020. The escalating incidence and mortality rates of this disease have demonstrated a consistent upward trend over the past three decades. These trends are primarily attributed to modifications in risk factor profiles, advancements in cancer registration methodologies, and enhanced capabilities in cancer detection techniques. This study was aimed at the Oncology Teaching Hospital/Baghdad Medical City and the Oncology Unit at Al-Yarmouk Teaching Hospital in Baghdad, encompassing a cohort of 150 samples divided into two groups: a blood group comprising 90 samples (control, localized, locally advanced, and metastatic BC patients) and a tissue group comprising 60 samples (benign and malignant BC). The study spanned from March 2022 to January 2023, involving patients aged 24 to 75 years. The primary objective of this investigation was to assess miRNA-9 gene expression across all sample types, with gene expression levels normalized to the housekeeping gene U6 and quantified using the ΔCt value and the fold change (2-ΔΔCt) method. The results concluded an upregulated fold expression of *miRNA-9*, with the highest expression observed in locally advanced and metastatic BC (fold expression 2.404±0.1364 compared to other groups. In localized breast cancer, the fold expression was 1.795±0.092, and in malignant tissue, it was 1.972±0.119, both compared to the apparently healthy control group.

Keywords: *miRNA-9*; breast cancer; localized tumor; locally advanced tumor; metastasis.

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Introduction

Breast cancer (BC) is characterized by the abnormal growth and proliferation of cells within the breast, resulting in the formation of a lump or tumor (American Cancer Society, 2019)(1). The pathogenesis of breast cancer is multifaceted, influenced by both genetic. Predispositions and environmental factors (2). Notably, in its early stages, BC often presents without discernible symptoms. The most prevalent indication is the presence of a painless lump. As the disease progresses, it may extend to the lymph nodes, giving rise to swelling or lumps in proximity to the original breast tumor. Additionally, less frequent signs and symptoms encompass sensations of breast heaviness or discomfort, swelling, skin thickening or reddening, and alterations in nipple appearance, including nipple discharge (3). Subsequently, upon exiting the ducts or lobules, cancer cells acquire the ability to disseminate via the bloodstream or lymphatic system, frequently targeting distant organs like the bones, lungs, liver, or brain (4). The process of cancer metastasis involves a series of intricate

steps, including local invasion of the primary tumor into adjacent tissues, the intravasation of tumor cells into blood vessels, their circulation in the bloodstream, their extravasation from blood vessels at distant sites, and the establishment of colonies in these remote locations. This ultimately culminates in the formation of both microscopic and macroscopic metastases. The specific organ colonization relies on the interplay between cancer cells and their local microenvironment (5). MicroRNAs (miRNAs), a category of non-coding RNAs, have emerged as pivotal players in various critical facets of breast cancer development, encompassing apoptosis, heightened cell proliferation, and augmented metastatic potential (6). Among these, miRNA-9, a brief non-coding RNA gene implicated in gene regulation, is situated on human chromosome 1q22 and demonstrates aberrant expression in numerous cancer types, functioning as an oncogene in breast cancer (7). The generation of mature miRNAs from precursor hairpin sequences is facilitated by the enzyme Dicer. In the case of miR-9, the predominant mature miRNA arises from both the 5' arm of the mir-9 precursor and the 3' arm of the mir-79 precursor. These mature miRNAs are believed to exert regulatory control by binding to complementary mRNA sequences. MiR-9 exhibits high expression in the brain and plays a role in the regulation of neuronal differentiation. Several potential targets of miR-9 have been identified, including the transcription factor REST and its co-repressor CoREST (8). Recent investigations by Liu DZ *et al*. (9) have substantiated that miR-9 can heighten the migratory and invasive capabilities of breast cancer cells by attacking the forkhead box O1 (FOXO1) gene. Ma et al. (2010) (10) have demonstrated that the miR-9 expression is stimulated by the oncogenes MYC and MYCN, leading to enhanced cellular invasiveness and motility by directly interacting with the pivotal metastasis-suppressing protein E-cadherin. Initially associated with neurogenesis (11), miR-9 exhibits varying expression patterns in different tumor types, functioning as a pro-metastatic miRNA in breast cancer (12, 13) while acting as a tumor suppressor in melanoma (14). Furthermore, miR-9 appears to exert dual effects, combining roles in neurogenesis and angiogenesis (15), and influencing lymphatic inflammatory and lymphangiogenic pathways (16), suggesting its potential involvement in neovascularization processes.

Materials and methods

This case-control study enrolled women spanning a wide age range, from 20 to 75 years old, who were recruited from two medical institutions: the Oncology Teaching Hospital in Medical City and the Oncology Unit at Al-Yarmouk Teaching Hospital in Baghdad. The study was conducted from March 2022 to the end of January 2023, involving a total of 150 subjects, categorized into two groups:

1. Blood Group (N=60):

This group consisted of 60 samples collected from women diagnosed with breast cancer, subdivided as follows:

- 30 samples from patients with localized breast cancer.
- 30 samples from patients with locally advanced and metastatic breast cancer.

Additionally, 30 samples were obtained from healthy women serving as a control group.

2. Tissue Group (N=60):

This group encompassed 60 samples collected from cases involving mastectomy for breast cancer, involving the removal of cancerous masses and biopsies using Tru-cut needles.

Furthermore, 30 samples were derived from control cases, which included instances of fibroadenoma, accessory breast tissue, mastitis, lipoma, ductectasia, benign breast masses, as well as cases of mastectomy and quadrectomy due to haemorrhagic cysts and haemorrhagic capsule. Additionally, normal breast tissues from mammoplasty cases were included in this group. All samples, both from patients and controls, underwent an investigation of miRNA-9 expression through quantitative real-time polymerase chain reaction (qRT-PCR), utilizing the relative quantitation method. Gene expression was normalized to the expression level of the housekeeping gene U6, and quantification was achieved using the fold change ($2^{-\Delta\Delta CT}$) method.

Samples collection and preparation

Blood samples

From each participant, we collected five milliliters (mL) of venous blood samples (inclusive of all patients and healthy control women) using disposable syringes. These 5 mL of collected samples were carefully placed into gel tubes known as serum separation tubes (SST). Subsequently, they were allowed to coagulate at room temperature for approximately 30 minutes. Following coagulation, the samples underwent centrifugation at a rate of 5000 rounds per minute (rpm) for 10 minutes to separate the serum from the blood components. From the separated serum, 0.4 mL (400 µl) was extracted and combined with 0.6 mL (600 µl) of TRIzol™ Reagent. This resulting lysate was thoroughly homogenized by repeated pipetting and then stored at -20°C, awaiting further analysis of microRNA-9 gene expression.

Fresh tissue samples

Tissue samples were obtained and promptly added to 0.6 mL (600 µl) of TRIzol™ Reagent. The lysate was homogenized by gently pipetting up and down multiple times to ensure proper mixing. Subsequently, these prepared samples were stored at -20°C, ready for the analysis of microRNA-9 gene expression.

Total RNA extraction with TRIzol

Total RNA, including microRNAs from the samples, was meticulously extracted using TRIzol™ reagent, adhering to the manufacturer's protocol (Thermo Fisher, USA).

RNA quantitation by Qubit 4.0

The quantification of total RNA was performed using a non-traditional yet precise method employing the Qubit® RNA HS Assay Kits. This approach yielded a range of RNA concentrations, spanning from low concentrations (4.7-46.1 ng/µl) to high concentrations. Notably, there were no statistically noticeable variations observed in the concentrations of total RNA between the tumor and control samples. Furthermore, the purity of the RNA exhibited no significant variations within the same sample groups.

Synthesis of complementary DNA (cDNA) for miRNA-9

To synthesize complementary DNA (cDNA) for miRNA-9, we employed the TransScript® miRNA First-strand cDNA preparation SuperMix kit. This synthesis procedure was conducted in a reaction volume of 20µl, meticulously following the manufacturer's instructions. A total RNA volume of 20µl was utilized for the reverse transcription process, as detailed in (Table 1). The concentration of cDNA was thoroughly assessed for efficiency, and this efficiency was subsequently validated through qRT-PCR analysis. Remarkably, all steps in the cDNA synthesis yielded exceptional results, signifying a remarkably efficient reverse transcription process.

Table (1): Reaction Volume and Contents of Reverse Transcription Reaction for cDNA Preparation from Total RNA

Contents	Volume(µl)Reaction
TransScript® miRNA RT Enzyme Mix	1µl
Total RNA	6 µl
2×TS miRNA Reaction Mix	10 µl
RNase-free water	To 20 µl

The primers

The primers utilized in this investigation were fabricated by Macrogen (South Korea) and were stored in a lyophilized state until their intended use. Prior to their use, the specificity of the miRNA-10b primers was meticulously verified using the BLAST tool (http://blast.ncbi.nlm.nih.gov/BLAST.cg) to ensure their suitability for the intended purpose.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The qRT-PCR system relies on the measurement of fluorescent light to quantify the amount of complementary DNA (cDNA) specific to a given gene. Here is a summary of the qRT-PCR methodology used in this study:

RNA isolation and reverse transcription

Total RNA was initially extracted from the samples and subjected to reverse transcription using the High Capacity cDNA Kit, following the kit's prescribed protocol. To ensure accurate normalization, the levels of miRNA were normalized using the endogenous control gene U6 small nuclear RNA.

qRT-PCR procedure

The qRT-PCR was conducted utilizing the smart cycler Real-time PCR System from Bioer (Japan). Fold changes and gene expression levels were determined by estimating the cycle threshold (Ct) values, utilizing components from the Wizbio pure™ (SYBR) qPCR Kits. Each reaction was performed in duplicate and contained negative controls, which encompassed a non-template control (NTC), a non-primer control (NPC), and a non-amplification control (NAC).

Reverse transcription and SYBR-green reagents

Total RNA was reverse transcribed employing the TransScript® miRNA First Strand cDNA Synthesis Kit. For the qRT-PCR analysis of miRNA levels, SYBR-Green Reagents were employed.

Step 1: qPCR reaction setup:

Prior to synthesizing the qPCR reactions, the Wizbio pure (SYBR®) qPCR Master Mix, template DNA, and primers were meticulously mixed. The appropriate volume of each element was determined based on the specifications provided in (Table 2).

Table (2): Components of qRT-PCR for miRNA-9 and U6 Expression Experiment

Components	Volume (µl)	Consternation
Master mix syper green	10	
Forward primer	0.5	10 pmol
Reverse primer	0.5	10 pmol
CDNA	6	
Nuclease free water (N.F.W)	3	
Total	20	

Step 2: The qPCR Reaction run

The cycling protocol for the qPCR reaction was programmed based on the thermal profile presented in (Table 3). Below is a summary of the thermal profile used for the qPCR reaction.

Table (3): Thermal Profile for miRNA-10b Gene Expression

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	8 seconds	1
Denaturation **Extension**	95 °C 60 °C	15 seconds 30 seconds (+plate read)	50
Melt Curve	60-95 °C	40 minutes	1

Gene expression calculation

The fold changes in the quantified expression of mature RNAs were determined utilizing the relative CT method, represented as ($2^{-\Delta\Delta Ct}$). This method was originally introduced by Livak and Schmittgen in 2001 (17). Livak and Schmittgen's method allows for a robust and widely used approach to assess the relative changes in gene expression, providing valuable insights into the regulatory mechanisms underlying miRNA expression alterations.

Statistical analysis

The data obtained from the study was summarized, analyzed, and presented using GraphPad Prism version 9. GraphPad Prism is a widely used software for statistical analysis and graphical representation of experimental data. It provides various tools for data visualization, hypothesis testing, and statistical modeling, making it valuable for drawing meaningful conclusions from research findings.

Result and discussion

Expression of *miRNA-9*

The analysis of miRNA-9 expression was carried out following normalization with the U6 gene. Amplification plots for both the target miRNA-9 and U6 were generated to determine the Ct value for each. (Figures 1,2) show the corresponding data and plot.

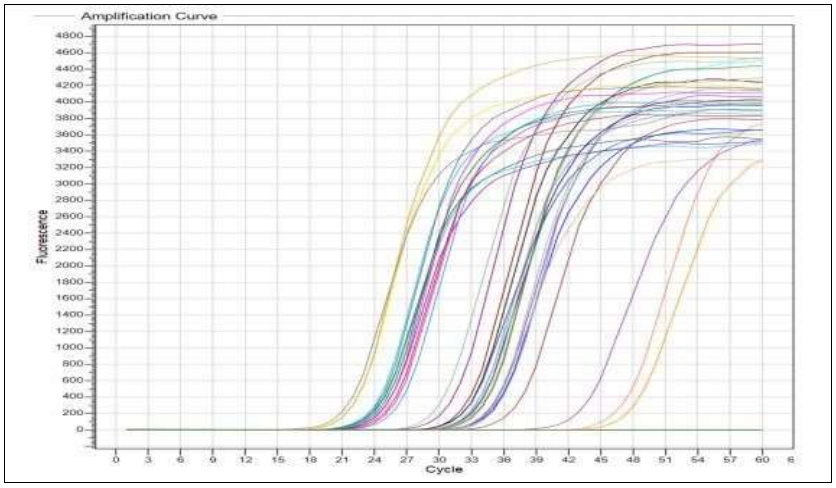

Figure (1): Amplification plots for *miR-9* Expression obtained by Real-Time PCR.

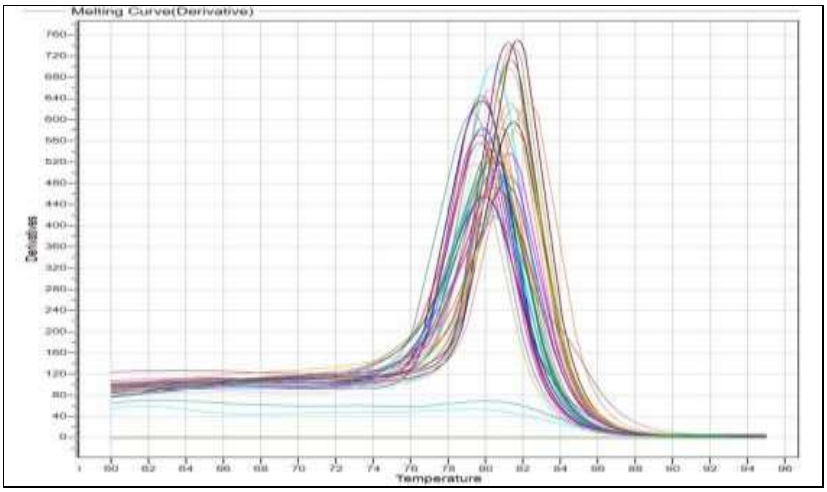

Figure (2): The *miR-9* expression Melting Curve

An upsurge in *miRNA-9* levels was observed in patients with locally advanced and metastatic breast cancer (BC) when compared to both localized BC patients and the malignant BC tissue group. Specifically, the fold mean of *miRNA-9* demonstrated a significant increase in locally advanced and metastatic BC patients, with a value of (2.404±0.1364), in contrast to the control group. In localized BC patients, the relative expression of miRNA-9 was also notably increased, showing a fold change of (1.795±0.092). Similarly, in the malignant BC tissue group, a substantial increase was detected compared to the control group, with a fold expression of (1.972±0.119). Detailed results and graphical representation are available in (Figure 3). These findings underscore the potential significance of *miRNA-9* as a biomarker linked with the progression of breast cancer, particularly in cases of locally advanced and metastatic disease. Further analysis and investigations may shed light on the precise role of *miRNA-9* in breast cancer development and metastasis.

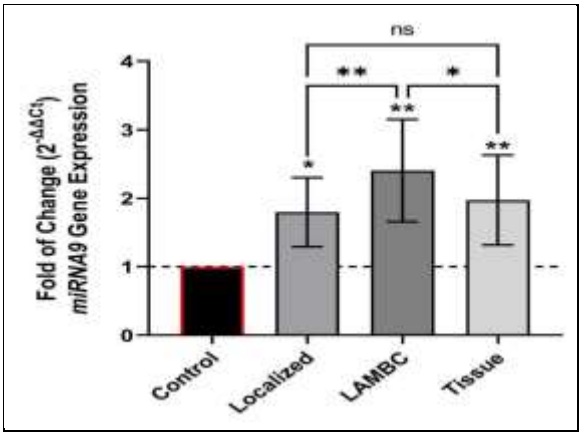

Figure (3): Fold of change of *miRNA-9* gene expression
***LAMBC= locally advanced and metastatic breast cancer**

Cancer cells exhibit distinct metabolic reprogramming and undergo significant epigenetic modifications, both of which are recognized as characteristic features. Throughout tumorigenesis and cancer progression, the activity of metabolic pathways undergoes dynamic changes, indicating

a carefully regulated metabolic adaptability. These metabolic shifts are often closely intertwined with epigenetic alterations, including changes in the expression or activity of enzymes involved in epigenetic modifications. These changes can exert either direct or indirect influences on cellular metabolism.

Significantly, elevated expression of *miR-9* seems to play a pivotal role in the development of breast cancer, particularly by enhancing its metastatic potential. Recent research has predominantly supported the notion that *miR-9* acts as a promoter of breast cancer development, with its primary effect being the enhancement of breast cancer metastasis. This highlights the intricate interplay between genetic and epigenetic factors in cancer progression, emphasizing the potential significance of *miR-9* as a key player in the metastatic cascade of breast cancer. Further elucidation of the molecular mechanisms underlying *miR-9'*s involvement may provide valuable insights for the development of targeted therapies and interventions in breast cancer management.

MiR-9 has emerged as a separated prognostic factor included with low disease-free survival, underscoring its significance as a critical marker in the context of breast cancer. Bertoli *et al.* in 2015(12) conducted a comprehensive review of the role of miRNAs in breast cancer initiation and progression. They highlighted *miR-9* as an emerging biomarker for prognosis and diagnosis, suggesting that *miR-9*-based therapies hold promise for breast cancer treatment. Furthermore, in 2016 and 2018, Bertoli *et al.*(18) summarized various miRNAs, including *miR-9*, as potential diagnostic markers for breast cancer.

The research findings from Sporn JC *et al*. in 2019 (19), which utilized data from The Cancer Genome Atlas (TCGA), provided insights into the association between miR-9 levels and breast cancer patient prognosis. They observed that low miR-9 expression is linked to a protective effect, resulting in improved overall survival (OS), smaller tumor sizes, and a prevalence of estrogen receptor (ER)-positive (ER+) cancers. In the context of triple-negative breast cancer (TNBC), Jang MH *et al*. in 2017(20) confirmed that high *miR-9* levels are significantly correlated with poor disease-free survival and distant metastasis-free survival, indicating its potential as a prognostic marker for TNBC patients. Furthermore, research by Zhou X *et al*. in 2012 (21) demonstrated that miR-9 levels can predict the likelihood of local recurrence (LR) in breast cancer. Lower miR-9 levels were associated with better 10-year LR-free survival, particularly in cases with estrogen receptor-positive (ER+) breast cancer.

It's worth noting that *miR-9* displays varying expression patterns in different cancer types, functioning either as a proangiogenic oncomiR or a tumor suppressor, as exemplified in melanoma. Recent studies have elucidated miR-9's role as an angiogenic mediator, focusing on its ability to selectively target mRNA from genes involved in stimulating the expression of vascular endothelial growth factor (VEGF). Specifically, *miR-9* was found to inhibit VEGFA production by binding to the products of the ITGA6 gene. This integrin subunit, encoded by ITGA6, has been previously associated with increased VEGF expression through the activation of the mTOR pathway, shedding light on the intricate regulatory mechanisms involving *miR-9* in breast cancer angiogenesis (22, 23).

Moreover, *miR-9* enhances VEGFA expression in breast cancer by targeting E-cadherin, thereby stimulating beta-catenin signaling in animal cell lines and models. *MiR-9* targets CDH1 expression, resulting in increased nuclear localization and activity of β-catenin, both of which play pivotal roles in tumorigenesis(24). Finally, elevated levels of miR-9 in breast cancers were observed to promote angiogenesis, indicating its proangiogenic role in cancer development. Importantly, exosomal miRNAs, including miR-9, have been identified as signaling molecules that regulate various aspects of tumor biology, encompassing growth, angiogenesis, metastasis, chemotherapy response, and immune evasion (25).

Expression of (*miR-9*) according to stage of BC disease

In cases of localized breast cancer, our findings have uncovered a notable up-regulation in the expression of miR-9. Specifically, the fold changes observed were as follows: Stage 0 (1.84), Stage I (1.72), and Stage IIA (1.82), as detailed in Table 4. This suggests that miR-9 is actively involved in the regulation of gene expression in localized breast cancer, potentially contributing to disease progression.

It is essential to acknowledge that miRNAs exhibit distinct expression profiles within cancer cells and tissues. Moreover, miRNAs possess the ability to enter the body's circulatory system and exhibit considerable chemical stability. These distinctive qualities make miRNAs highly promising candidates for serving as non-invasive markers for various types of tumors, as emphasized by the findings of Naorem *et al*. (26). The differential expression of miR-9 in breast cancer stages and its potential as a non-invasive biomarker underscore its significance in the field of breast cancer research and diagnosis. Further investigations may elucidate the precise mechanisms through which miR-9 contributes to breast cancer progression and metastasis.

Table (4): Folding change of *miR-9* expression in localized breast cancer patients group according to stages of disease

Genes	Folding		
	Stage 0	Stage I	Stage IIA
MiR-9	1.84^{a}	1.72^{a}	1.82^{a}
	LSD at 0.05 probability		

In patients with locally advanced and metastatic breast cancer, our results have unveiled a notable up-regulation in the expression of miR-9. The fold changes observed were as follows: Stage IIB (2.53), Stage III (2.24), Stage IV recurrence (2.8), and Stage IV de novo (1.99), as outlined in (Table 5). This upregulation of *miR-9* is a consistent finding and indicates its active involvement in the molecular processes associated with advanced stages of breast cancer.

These findings align with the research conducted by Naorem *et al*. in 2019 (26), which also reported high expression levels of *miR-9*. This observation holds significant promise for enhancing the diagnosis and treatment strategies, particularly in the context of triple-negative breast cancer (TNBC). The consistent up-regulation of *miR-9* in advanced stages of breast cancer underscores its potential as a biomarker for disease progression and metastasis. Further exploration of the mechanisms underlying miR-9's role in breast cancer may open avenues for more effective diagnostic and therapeutic approaches, especially for patients with aggressive forms of the disease such as TNBC.

Table (5): Folding change of *miR-9* expression in locally advanced and metastatic breast cancer patients group according to stages of disease

Genes	Folding			
	Stage III	Stage IIB	Stage IV Recurrence	Stage IV DeNovo
MiR-9	2.24^{a}	2.53^{a}	2.8^{a}	1.99^{a}
	LSD at 0.05 probability			

In malignant breast cancer tissue, our results have uncovered a notable up-regulation in the expression of miR-9. The fold changes observed were as follows: Grade I (2.04), Grade II (2.16), and Grade III (1.76), as presented in (Table 6). This consistent upregulation of miR-9 in malignant breast cancer tissue suggests its potential role in the context of breast cancer malignancy, with higher levels observed in more advanced grades. This observation underscores the possibility that *miR-9* may contribute to the molecular processes associated with tumor aggressiveness and progression. Further research may provide insights into the specific mechanisms through which *miR-9* impacts the development and behavior of malignant breast cancer, potentially opening new avenues for targeted therapies and diagnostic strategies.

Table (6): Folding change of *miR-9* expression in malignant tissue of breast cancer women according to grade of disease

Genes	Folding		
	Grade I	Grade II	Grade III
miR9	2.04^{a}	2.16^{a}	1.76^{a}
	LSD at 0.05 probability		

In 2019, Shen *et al.*(27) conducted a study that unveiled a significant phenomenon related to breast cancer and chemotherapy. They discovered that chemotherapy can stimulate breast cancer cells to release a group of extracellular vesicle (EV) miRNAs, which includes miR-9, miR-195, and miR-203a-3p. These miRNAs collaboratively target the transcription factor known as One Cut Homeobox 2 (ONECUT2). This targeting leads to the activation of traits associated with cancer stem cells (CSCs) and confers resistance to chemotherapy. Crucially, among these miRNAs, *miR-9* emerged as particularly influential. It was found to significantly enhance breast cancer metastasis and promote CSC-like characteristics, especially in highly aggressive breast cancer subtypes such as triple-negative breast cancer (TNBC). These findings shed light on the complex interplay between miRNAs, chemotherapy, and the aggressive behavior of breast cancer cells, offering potential insights into novel therapeutic approaches.

Furthermore, *miR-9* was identified as closely associated with breast cancer recurrence and a poor prognosis. Therefore, it holds promise as a valuable biomarker for both the diagnosis and prognosis of breast cancer, as indicated by Xiaoman *et al*. in 2020 (28). These findings highlight the multifaceted role of *miR-9* in breast cancer, encompassing its involvement in chemotherapy resistance, cancer stem cell characteristics, metastasis promotion, and its potential as a clinically relevant biomarker for disease management. Further research may offer a deeper understanding of the intricate mechanisms regulated by

miR-9 and its implications for breast cancer diagnosis and treatment.

Conclusion

Breast cancer is the second most prevalent malignancy worldwide, following lung cancer, and remains a major contributor to cancer-related fatalities among women. In the context of breast cancer, various biological characteristics are routinely utilized for early detection, prognosis assessment, and the selection of appropriate therapeutic strategies. These characteristics encompass factors such as histologic subtype and grade. Among the microRNAs (miRNAs), miR-9 has emerged as a potential diagnostic marker in breast cancer. Importantly, there exists a robust connection between the level of miR-9 and the prognosis of breast cancer patients. MiR-9 has been identified as an independent prognostic factor associated with poor disease-free survival, and it exhibits a close association with breast cancer recurrence and prognosis. This underscores the potential utility of miR-9 as a valuable tool in the management and understanding of breast cancer. The unique characteristics of miR-9 make it a promising candidate for further research and clinical application, with the potential to enhance early diagnosis, prognostic assessment, and treatment selection in breast cancer patients.

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