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Upregulation of miR-99b-5p Modulates ESR1 Expression as an Adaptive Mechanism to Circumvent Drug Response via Facilitating ER/HER2 Crosstalk

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Background: Endocrine resistance remains a significant therapeutic challenge in estrogen receptor-positive (ER+) breast cancer, the most common subtype, contributing to increased morbidity and mortality. The interaction between ER and HER family receptors, particularly HER2 and epidermal growth factor receptor (EGFR), drives resistance to standard therapies such as tamoxifen and trastuzumab by activating key signaling pathways, including PI3K/AKT and RAS/MAPK. Dysregulated miRNAs, which are non-coding gene expression regulators, have been linked to therapy response.

Aims: To investigate the role of miR-99b-5p in ER-HER2/EGFR crosstalk in BT-474 cells.

Study Design: Experimental study.

Methods: The expression profile and prognostic significance of miR-99b-5p in breast cancer were analyzed using The Cancer Genome Atlas (TCGA) database. BT-474 cells were transfected with miR-99b-5p mimics and inhibitors, followed by treatment with tamoxifen and trastuzumab to assess their impact on cell proliferation and ER-HER2/EGFR crosstalk. Western blotting was performed to quantify EGFR, HER2, and ESR1 protein levels. Real-time proliferation analysis evaluated changes in cell growth following miRNA transfection and drug treatment.

Results: The study revealed that miR-99b-5p is significantly overexpressed in tumors compared to normal tissues and is associated with poor patient survival and enhanced ER signaling. Transfection with miR-99b-5p mimics increased ESR1 expression and cell proliferation, even in the presence of tamoxifen or trastuzumab, indicating that miR-99b-5p contributes to therapy resistance through receptor crosstalk. Conversely, miR-99b-5p inhibition significantly restored drug sensitivity, reducing proliferation and enhancing the effectiveness of tamoxifen and trastuzumab.

Conclusion: These findings establish miR-99b-5p as a key regulator of endocrine and HER2-targeted therapy resistance. Targeting miR-99b-5p could represent a potential therapeutic strategy to improve treatment outcomes in ER+/HER2+ breast cancer. Further research is needed to clarify the underlying molecular mechanisms and validate the therapeutic potential of miR-99b-5p inhibition in clinical applications.

INTRODUCTION

Annually, around 200,000 new cases of invasive breast cancer are diagnosed, making it the second leading cause of mortality among women worldwide.[1] Approximately 70% of breast cancer cases express the estrogen receptor (ER), which plays a crucial role in breast cancer pathogenesis by upregulating key proteins such as MYC, CCND1, BCL2, and VEGF. These proteins are essential for cell survival, cell cycle regulation, and angiogenesis.[2] As a result, endocrine therapy is the primary treatment for most patients with ER+ breast cancer. Since its introduction in the 1970s, tamoxifen has been the standard adjuvant therapy, significantly reducing the risk of breast cancer recurrence, particularly in premenopausal women.[3] However, tamoxifen resistance remains a major challenge in breast cancer treatment. Understanding the mechanisms driving this resistance is critical for developing next-generation targeted therapies. Research has identified ER and epidermal growth factor receptor (EGFR or HER) interactions as a key factor contributing to tamoxifen resistance.

In breast cancer, HER2 overexpression acts as an oncogene by persistently activating growth factor signaling pathways. Both pharmacological and genetic studies have confirmed that HER2 is crucial for tumor development in breast cancer models. Trastuzumab (herceptin), a humanized recombinant monoclonal antibody

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***Only the relation of miR-99b-5p with HER2/EGFR part of this manuscript was intersected with a small part of poster we presented at the EACR 2021 Congress, Innovative Cancer Science: Better Outcomes through Research held virtually from 09-12 June 2021.**

targeting the extracellular domain of HER2, has demonstrated antitumor efficacy in both HER2-positive breast cancer patients and preclinical models.[4,5]

Crosstalk and trans-phosphorylation between receptors promote breast tumor formation by activating multiple signaling pathways through the HER2 receptor, playing a key role in drug resistance. The reduced effectiveness of trastuzumab may be due to interactions and activation of other members of the ErbB family. Therefore, understanding how HER2 crosstalk with other growth factor receptors (GFR) affects anti-neoplastic therapies is essential for developing and evaluating new targeted treatments for HER2-overexpressing breast cancer.

The recent advancements in miRNA research, combined with the development of anticancer drugs, have created opportunities to identify new drug targets. These progressions build upon fundamental studies on the role of miRNAs in cellular regulation under disease conditions and their potential application in pharmacological interventions. miRNAs offer significant advantages as a new class of drug targets. Given the interactions between the ER-HER2/EGFR pathways, miRNAs-being receptor-independent intracellular molecules-could regulate these interactions, highlighting their potential as therapeutic agents.

A notable finding in the literature is the significant downregulation of miR-99b-5p across all studied cancer types compared to normal tissues. In non-small-cell lung cancer[6,7] and osteosarcoma,[8] miR-99b-5p expression was reduced, and its upregulation through mimic transfection demonstrated antitumor effects. In cervical cancer, miR-99b-5p exhibited anticancer properties by modulating the PI3K/AKT/mTOR signaling pathway.[9] In colorectal cancer, it was detected in exosomal circulation, with lower expression levels observed in cancer cases, suggesting its potential as a diagnostic biomarker.[10] Additionally, it was reported to inhibit liver metastasis in colorectal cancer by suppressing mTOR.[11] In gastric cancer, miR-99b-5p was shown to target IGF-1R, acting as a tumor suppressor when its expression was increased.[12] There is limited research on the role of miR-99b-5p in breast cancer, and no comprehensive study has yet examined its mechanism of action. In this study, we investigated the effect of miR-99b-5p on the ER-HER2/EGFR crosstalk mechanism in BT-474 cells, building on our previous findings that identified it as a modulator of tamoxifen and trastuzumab responses.[13]

MATERIALS AND METHODS

Cell culture and drug treatment

The BT-474 cell line, which overexpresses HER2 and is ER+, was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (HyClone, GE Healthcare, USA) supplemented with 1% penicillin/streptomycin (HyClone, GE Healthcare, USA) and 10% fetal bovine serum (HyClone, GE Healthcare, USA). Mycoplasma contamination was tested using polymerase chain reaction (PCR), and only cells used in the logarithmic growth phase were used for experiments.

Tamoxifen was sourced from TOCRIS (Minneapolis, MN, USA). Additional details on tamoxifen and trastuzumab are available in our previous study.[13]

miRNA transfection

miR-99b-5p mimics (MSY0000689, Qiagen, Germany), scrambled control (scr) (SI03650318, Qiagen, Germany), miR-99b-5p hairpin inhibitor (IH-300658-05-0005, GE Healthcare, USA), and scrambled hairpin control (IN-001005-01-05, GE, USA) were obtained from Qiagen and Dharmacon, respectively. Cells were transfected using DharmaFect 1 reagent (Dharmacon, GE, USA) following the manufacturer's protocol. After 72 h of transfection, cell lysates were prepared for subsequent experiments.

Western blot

BT-474 cells were seeded at a density of 0.3×10^6 cells per well in 6-well plates. The following day, cells were transfected with either an miRNA mimic or an inhibitor. After 24 h, the transfected cells were treated with 10 µM tamoxifen and/or 6 µg/ml trastuzumab. Three days post-transfection and treatment, cells were trypsinized and collected. Protein isolation was performed using the Complete Lysis-M kit (Roche, Switzerland), followed by western blot analysis. Additionally, protein isolation from cytoplasmic and nuclear fractions was conducted for scr, mimic, and inhibitor-transfected samples using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific).

A total of 10 µg of protein was loaded onto an SDS gel, and proteins were detected via antibody staining using the Odyssey imaging system (LI-COR) with the WesternBright Sirius kit (Advansta Inc., USA). Beta-actin was used as a loading control. The primary antibodies used in this study were ER-alpha (D8H8, Cell Signaling, 1:1000), HER2 (ab8054, Abcam, 1:1000), and EGFR (sc373746, Santa Cruz, 1:1000). The secondary antibodies included HRP-conjugated anti-rabbit (7074S, Cell Signaling, 1:10000) and HRP-conjugated anti-mouse (405306, BioLegend, 1:5000).

Real-time cell proliferation analysis

BT-474 cells were plated in 96-well plates at a density of 6×10^3 cells per well in 100 µl of complete RPMI 1640 medium (HyClone, GE Healthcare, USA) and cultured overnight. The next day, cells were transfected with the miR-99b-5p inhibitor, followed by the addition of tamoxifen (10 µM) or trastuzumab (6 µg/ml). Cell proliferation was monitored for 160 h using the incucyte real-time imaging system (Sartorius, Germany).

Bioinformatics analysis

miR-99b-5p expression levels in breast cancer were analyzed using the The Cancer Genome Atlas (TCGA) dataset via the expression analysis module of the UALCAN database.[14,15] Survival analysis was performed using Kaplan-Meier analysis, a key feature of UALCAN.[15]

Statistical analysis

Group differences were evaluated using the Student's t-test, with a p value of 0.05 considered statistically significant.

RESULTS

miR-99b-5p responsiveness in ER/HER2-positive breast cancer cells

In our previous study, we identified tamoxifen- and trastuzumab-responsive miRNAs using a quantitative real-time-polymerase chain reaction (qRT-PCR) array in BT-474, SK-BR-3, and MCF-7 cells.[13] miR-99b-5p was among the miRNAs downregulated following both drug treatments in all cell lines (Figure 1a). To assess the clinical relevance of miR-99b-5p in breast tumors, TCGA analysis was performed, revealing that miR-99b-5p expression was significantly higher in tumors than in normal tissues (Figure 1b). To investigate its role in cell proliferation, BT-474 cells were transfected with a miR-99b-5p mimic and monitored for 160 h. As shown in Figure 1c, miR-99b-5p overexpression significantly increased proliferation compared with scr-transfected cells, supporting its oncogenetic potential. Survival analysis (Figure 1d) demonstrated an inverse relationship between miR-99b-5p expression and overall survival. Patients with lower miR-99b-5p expression had longer survival times, whereas those with higher expression exhibited shorter survival durations (Figure 1d).

Downregulation of miR-99b-5p enhances drug efficacy in BT-474 cells

Given the oncogenic role of miR-99b-5p, we examined the impact of its suppression on cell proliferation, both independently and in combination with drug treatment. Transfection of BT-474 cells with a miR-99b-5p inhibitor led to a greater reduction in proliferation compared with trastuzumab treatment alone. Furthermore, the combination of the miR-99b-5p inhibitor with trastuzumab further enhanced the drug's effectiveness (Figure 2a). A similar effect was observed with tamoxifen (Figure 2b). These findings suggest that miR-99b-5p inhibition not only reduces cell proliferation on its own but also improves the efficacy of tamoxifen and trastuzumab when used in combination.

Impact of miR-99b-5p on ER-HER2 crosstalk in tamoxifen- or trastuzumab-treated ER+ and HER2-positive cells

To investigate the potential role of miR-99b-5p inhibition in drug sensitivity, we examined its effect on crosstalk signaling in BT-474 cells. ER-HER2 crosstalk plays a key role in promoting tumor cell proliferation and survival during endocrine therapy.[16,17] Based on this, we analyzed the impact of miR-99b-5p on key mediators of this

a

miRNAID	Fold Difference			
	BT-474		MCF-7	SK-BR-3
	Tamoxifen	Trastuzumab	Tamoxifen	Trastuzumab
miR-99b-5p	-1.72	-1.61	-1.72	-1.58

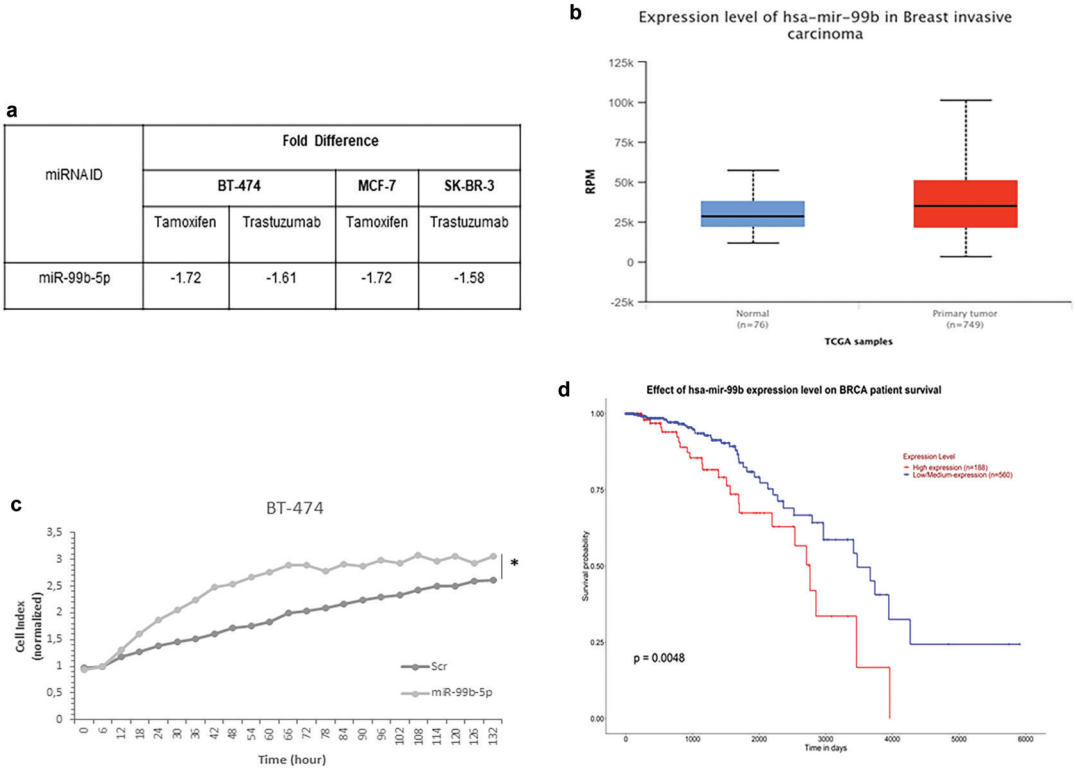

FIG. 1. The expression level of miR-99b-5p in breast cancer. (a) The expression of miR-99b-5p was analyzed by qPCR array system ($p < 0.01$ vs. vehicle control). (b) Graph showing the expression level of miR-99b-5p in normal breast tissues and primary breast tumors. Blue boxplot depicts expression level in normal samples, while red boxplot represents expression in primary tumors (UALCAN web-portal; $^{*}p = 1.34829925002578E-11$). (c) Cells were transfected with 25 nM miR-99b-5p mimic or scrambled control (scr) and monitored for 140 h. Up-regulation of miR-99b-5p promoted cell proliferation in BT-474 cells ($n = 3$, $p < 0.05$). (d) Red and blue lines in the Kaplan-Meier survival plots show the analysis of patients with high and low miRNA expression levels, respectively.

qPCR, quantitative polymerase chain reaction.

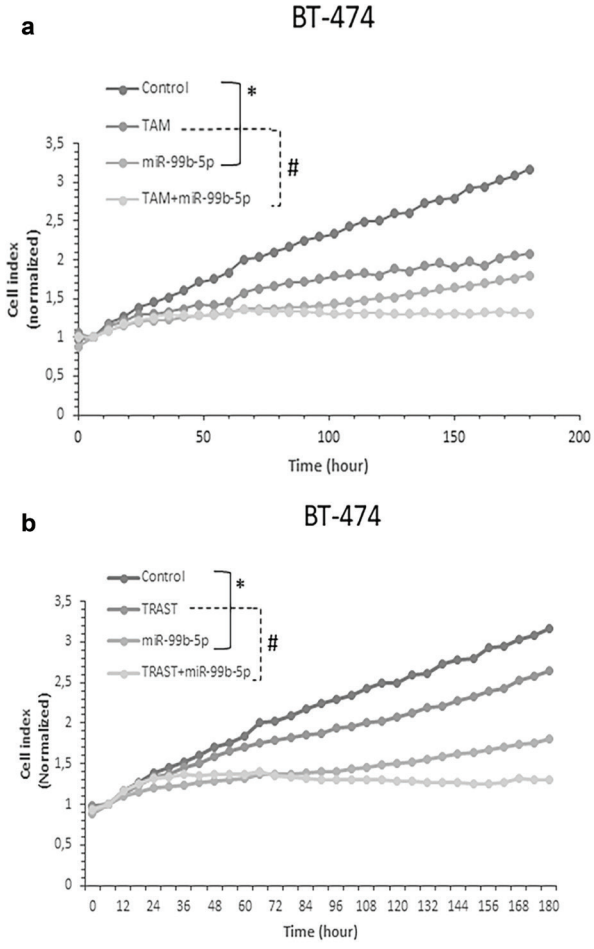

FIG. 2. Inhibiting miR-99b-5p contributes to tamoxifen or trastuzumab sensitivity in BT-474 cells. Suppression of cell proliferation by miR-99b-5p inhibitor/drug combinations compared to untreated control cells (n = 3, $^{*}p < 0.05$, $^{\#}p < 0.01$). Cells were transfected with 100 nM inhibitor for 48 hours then were treated with 10 μM tamoxifen (a) or 6 μg/ml trastuzumab (b). Cells were monitorized for 180 h. TAM and TAM+ miR-99b-5p represented only tamoxifen and tamoxifen with with miR-99b-5p inhibitor, respectively (a). TRAST and TRAST+ miR-99b-5p represented only trastuzumab and trastuzumab with with miR-99b-5p inhibitor, respectively (b).

interaction-ER, HER2, and EGFR-at the protein level. Notably, cells transfected with the miR-99b-5p mimic exhibited increased ESR1 protein levels compared with scr control cells following tamoxifen or trastuzumab treatment (Figure 3a, b).

Additionally, treatment with the miR-99b-5p mimic in combination with tamoxifen or trastuzumab significantly reduced HER2 and EGFR protein levels in BT-474 cells (Figures 3a, b). ESR1 expression was also assessed at the RNA level, revealing a significant increase in ESR1 mRNA levels in cells transfected with the miR-99b-5p mimic compared with the scrambled control consistent with the observed rise in protein levels (Figure 3c). To further explore how ESR1 expression changes under miR-99b-5p modulation, we evaluated its levels following miR-99b-5p inhibition. ESR1 expression was significantly lower in cells treated with the miR-99b-5p inhibitor compared with the scr (Figure 3d). As ESR1 (ER-alpha) is a nuclear protein, we also assessed its expression in both cytoplasmic and nuclear fractions. While cytoplasmic ESR1 expression increased in the presence of the miR-99b-5p mimic, no notable changes were detected at the nuclear level (Figure 3e).

DISCUSSION

Breast cancer remains the most commonly diagnosed cancer in women. According to GLOBOCAN data, approximately 2.3 million cases and 684,996 deaths were reported globally in 2020, with projections estimating an increase to 3.2 million cases by 2050.[18,19] Around 70% of breast cancer cases express ER.[20] One of the main intracellular signaling pathways driving tumor growth and disease progression in ER+ breast tumors is ER signaling. Endocrine therapies such as tamoxifen and fulvestrant are commonly used to target ER by binding to its ligand-binding domain.

miRNAs are endogenous, single-stranded, non-coding RNA molecules measuring 17-22 nucleotides in length. They regulate gene expression by either promoting or degrading mRNA targets.[21] Dysregulated miRNA expression is a characteristic molecular feature of breast cancer. Similar to oncogenes or tumor suppressor genes, miRNAs are considered potential therapeutic targets for breast cancer. miRNA-based therapies focus on either inhibiting oncogenic miRNAs or restoring tumor suppressor miRNAs.[22] Additionally, miRNAs are expected to play a significant role in improving therapeutic responses to trastuzumab in ER- patients and tamoxifen in advanced ER+ cases.[23,24]

The current scientific literature includes only one bioinformatics-based study investigating the role of miR-99b-5p in breast cancer.[25] Additionally, in January 2024, a study analyzed a panel of microRNAs in tumor samples from patients with hormone receptor-positive/HER2-negative metastatic breast cancer receiving endocrine therapy in combination with the CDK4/6 inhibitor (CDK4/6i) palbociclib. This analysis identified seven microRNAs, including miR-99b-5p.[26] However, there is still no comprehensive research detailing the mechanistic pathways through which miR-99b-5p exerts its effects. This study underscores the crucial role of miR-99b-5p in regulating drug responses in ER/HER2-positive breast cancer cells, particularly in the context of tamoxifen and trastuzumab treatment. Our findings reveal that miR-99b-5p is significantly overexpressed in tumors compared to normal tissues, as shown by TCGA analysis. This overexpression is associated with poorer patient survival outcomes, indicating that miR-99b-5p may serve as a negative prognostic factor in breast cancer.

Mutations or amplifications in the HER family contribute to cancer progression. The crosstalk between ER and HER2 regulates the RAS/RAF/MAPK pathway, promoting tumor cell proliferation and survival in endocrine resistance,[16] alongside the PI3K/mTOR signaling pathway, which frequently remains active in breast cancer.[27,28] Most preclinical studies suggest that inhibiting specific GFR is an effective strategy for overcoming endocrine resistance. For instance, blocking

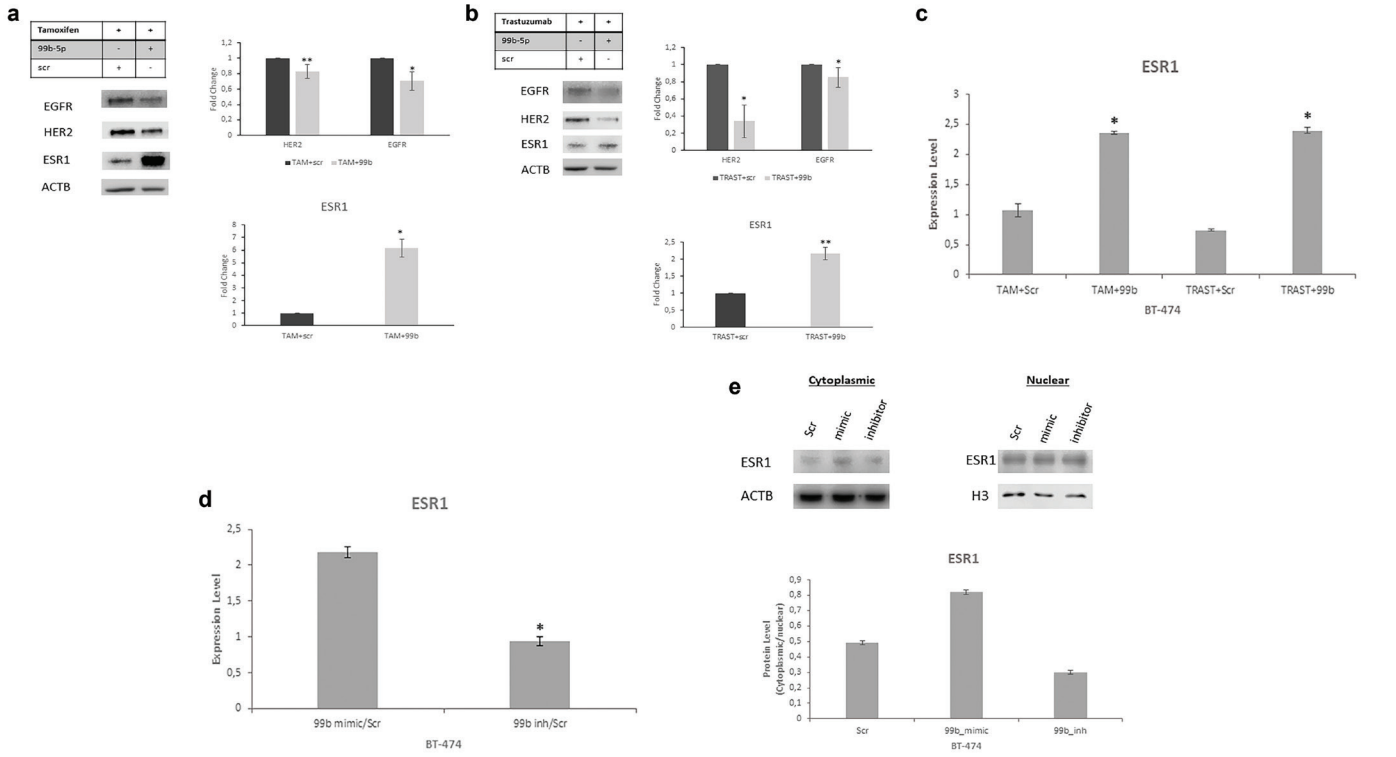

FIG. 3. Effect of miR-99b-5p on ER-HER crosstalk signaling in BT474 cells. We investigated the effect of miR-99b-5p, tamoxifen, trastuzumab or the combination on ER-HER2 crosstalk signaling in BT-474 cells. Western blotting was performed to analyze ESR1, HER2 and EGFR. Bar graphs indicated relative levels of proteins vs. scrambled control normalized to β-actin (n = 2, $^{*}p < 0.05$, $^{**}p < 0.01$). Drug treatments were used at IC50 concentrations. 25 nM miR-99b-5p mimic or scrambled control (scr) concentrations were applied. TAM+ scr and TAM+ 99b represented tamoxifen with scrambled control (scr) and tamoxifen with miR-99b-5p mimic, respectively (a). TRAST+ scr and TRAST+ 99b represented trastuzumab with scrambled control (scr) and trastuzumab with miR-99b-5p mimic, respectively (b). (c) ESR1 expression in miR-99b-5p mimic-transfected samples was assessed at the RNA level under the treatment conditions of tamoxifen or trastuzumab ($^{*}p < 0.01$). (d) ESR1 expression was evaluated at the RNA level in the presence of both miR-99b-5p mimic and inhibitor, with a decrease in ESR1 expression observed in samples transfected with the miR-99b-5p inhibitor ($^{*}p < 0.01$). (e) Western blot analysis of ESR1 was conducted using cytoplasmic and nuclear extracts from BT-474 cells transfected with either a scrambled control, miR-99b-5p mimic, or inhibitor.

ER, estrogen receptor; EGFR, epidermal growth factor receptor.

EGFR has been shown to restore the sensitivity of endocrine-resistant cells to tamoxifen's inhibitory effects.[29] Combination therapy, which involves endocrine agents and inhibitors targeting specific upregulated molecules, is considered a potential approach to preventing or managing endocrine resistance. Our findings indicate that the miR-99b-5p mimic also modulates HER2 and EGFR signaling, suggesting that miR-99b-5p may facilitate crosstalk between these pathways and contribute to a prosurvival environment in breast cancer cells.

In tumor biology, it is well established that gene amplification within a specific chromosomal region can lead to the overexpression of the corresponding protein. Overexpression of HER2 or FGFR1 has been implicated in the development of endocrine resistance in ER+ breast tumors.[30,31] Additionally, *ESR1* gene amplification occurs in approximately 30% of ER+ breast cancer cases, with studies linking it to tamoxifen resistance and poor prognosis in this subgroup.[32,33] Our protein-level analysis revealed a significant increase in ESR1 expression in cells treated with miR-99b-5p mimics alongside tamoxifen. This suggests that miR-99b-5p may enhance ER signaling, promoting cell survival and proliferation despite treatment. Furthermore, it indicates that the ESR1 pathway may remain active, potentially reducing the therapeutic efficacy of tamoxifen and contributing to resistance development.

Tamoxifen acts as either an antagonist or a partial agonist of the ER.[34] When ER activity is involved in ER/HER2 crosstalk, receptor tyrosine kinase (RTK) expression decreases, indicating a negative correlation between them.[35] In our study, increased miR-99b-5p expression in the presence of tamoxifen led to higher ESR1 expression while simultaneously reducing HER2 and EGFR expression. Trastuzumab, an ErbB2 antagonist, naturally downregulated this RTK. Similarly, we observed that miR-99b-5p upregulation in the presence of trastuzumab also lowered HER2 and EGFR expression while increasing ESR1 levels. These findings suggest that miR-99b-5p upregulation disrupts ER/HER2 crosstalk by enhancing ESR1 expression, which in turn suppresses HER2 and EGFR expression.

EGFR and HER2 (ErbB2) are key RTKs that frequently exhibit aberrantly elevated activation. These receptors phosphorylate downstream signaling molecules, thereby enhancing ER-mediated transcriptional activity.[36] ER+ tumors with ErbB2 amplification have been associated with reduced ER expression, diminished responsiveness to ER-targeted therapies, and poor outcomes with tamoxifen treatment.[37] However, the combined targeting of ER and HER2 has emerged as a clinically effective strategy in breast cancer. Experimental models have extended this concept to other RTKs expressed in ER+ breast cancer. Notably, studies have identified both transcription-dependent and non-genomic functions of ER in conjunction with EGFR.[38] Additionally, clinical trials investigating the use of EGFR inhibitors in endocrine therapy-resistant breast cancer underscore the need for further exploration of the mechanisms underlying RTKs-ER interactions. In this study, inhibition of miR-99b-5p significantly increased drug sensitivity. BT-474 cells transfected with a miR-99b-5p inhibitor exhibited a greater reduction in proliferation than with tamoxifen or trastuzumab treatment alone. Moreover, when combined with these drugs, the miR-99b-5p inhibitor further enhanced therapeutic efficacy. These findings suggest that reducing miR-99b-5p levels can restore drug sensitivity and potentially improve clinical outcomes.

A persistent challenge in breast cancer treatment is the occurrence of cases that either fail to respond or develop resistance to existing therapies. Developing combination treatments that effectively target key signaling pathways in breast cancer is essential for improving therapeutic outcomes. Our findings suggest that miR-99b-5p plays a crucial role in ER/HER2 signaling and significantly influences drug sensitivity in breast cancer. Targeting miR-99b-5p could offer a novel therapeutic approach to enhance the efficacy of established treatments, such as tamoxifen and trastuzumab, particularly in patients with elevated levels of this miRNA. Further research is necessary to elucidate the underlying mechanisms and assess the clinical potential of miR-99b-5p inhibition as a therapeutic strategy.

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- S.N., B.G.D.; Design- S.N.; Supervision- B.G.D.; Fundings- B.G.D.; Materials- B.G.D.; Data Collection or Processing- S.N.; Analysis and/or Interpretation- S.N.; Literature Review- S.N.; Writing- S.N., B.G.D.; Critical Review- B.G.D.

Conflict of Interest: The authors declare that they have no conflict of interest.

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