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The dysregulation of a subset of microRNAs in triple-negative breast cancer is associated with poor survival following neoadjuvant chemotherapy

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ABSTRACT

Introduction: Triple-negative breast cancer (TNBC) is a highly aggressive breast cancer subtype characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. Despite significant research efforts, current therapies are often ineffective against TNBC, resulting in poor survival rates. Therefore, identifying novel molecular markers to predict the response to neoadjuvant chemotherapy (NAC) is essential. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and play crucial roles in various biological processes, including cancer progression. This study aimed to investigate the expression of five miRNAs (miR-26a, miR-125b, miR-181c, miR-181a, and miR-340-5p) in formalin-fixed, paraffin-embedded (FFPE) samples from 20 patients with TNBC and to evaluate their association with clinicopathological features. **Methods:** miRNAs were isolated from FFPE samples of TNBC patients, and their expression levels were assessed using qRT-PCR. An interactive network-based pathway analysis was performed using IPA software to identify key targets regulated by these miRNAs. **Results:** Our findings revealed the downregulation of miR-181a, miR-181c, miR-26a, and miR-340-5p in patients who subsequently received NAC using the AC-T regimen. Notably, higher expression levels of these miRNAs were associated with improved overall survival, as demonstrated by Kaplan–Meier survival analysis. Additionally, the pathway analysis identified 17 potential targets regulated by miR-340-5p. **Conclusion:** Taken together, these findings suggest that the downregulation of specific miRNAs in samples from patients with TNBC may serve as a potential biomarker for predicting the response to NAC.

KEYWORDS: triple-negative breast neoplasms; neoadjuvant therapy; microRNAs.

INTRODUCTION

Breast cancer constitutes a group of neoplasms that accounts for 25% of cancer incidence worldwide. Approximately 15%–25% of breast cancer cases are classified as triple-negative breast cancer (TNBC)[1–3]. TNBC is one of the most aggressive breast cancer subtypes, characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[4]. Additionally, these tumors exhibit high vascularity and frequently metastasize to various organs, including the lungs and brain[5]. As a result, current therapies are often ineffective against TNBC, leading to a poor prognosis and a low 5-year survival rate[6].

Neoadjuvant chemotherapy (NAC) is a standard treatment for patients with TNBC, utilizing a combination of cytotoxic drugs to reduce tumor size before surgery. The AC-T regimen, consisting of doxorubicin and cyclophosphamide followed by paclitaxel, is widely used in the treatment of TNBC[7]. Achieving a pathologic complete response (pCR) after NAC means having no invasive residual disease in the breast or lymph nodes; therefore, it is recognized as a marker of sensitivity to systemic therapy[7,8].

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However, the development of chemoresistance remains one of the major challenges in patients with TNBC[8].

MicroRNAs (miRNAs) are small non-coding RNAs approximate 18–25 nucleotides in length that have been implicated as essential components of complex regulatory networks that modulate physiological cellular functions, including post-transcriptional regulation through either translation inhibition or mRNA degradation[3,9–11]. Currently, emerging evidence has revealed the roles of miRNAs in predicting and monitoring responses to NAC. Preclinical and clinical observational reports have shown that the expression levels of several miRNAs (including miR-181a[12,13], miR-181c[12], miR-26a[14], miR-125b[8,15,16], and miR-340-5p[17,18]) were associated with neoadjuvant chemotherapeutic response or survival outcomes in patients with TNBC.

In this paper, the expression of five miRNAs (miR-26a, miR-125b, miR-181c, miR-181a, and miR-340-5p) was investigated in formalin-fixed, paraffin-embedded (FFPE) samples from patients with TNBC and their association with clinicopathological features such as clinical stage and NAC.

METHODS

Study design

The study protocol was approved by the Institutional Ethics Committee of Instituto Mário Penna (CAAE No. 39741820.4.0000.9507). Retrospective research was conducted on 20 female patients with TNBC analyzed at the Instituto Mário Penna Surgical Pathology Lab between 2011 and 2021. Based on the medical records system, data on age, histological differentiation grade, Ki-67 labeling index, breast cancer clinical staging, overall survival, lymphovascular invasion, perineural invasion, presence of inflammatory infiltrate, pCR, and NAC status were collected.

Formalin-Fixed, Paraffin-Embedded RNA isolation and microRNA evaluation

Three 20-µm sections of each FFPE samples were taken, and total RNA was isolated using an AllPrep DNA/RNA FFPE kit (Qiagen, USA). Transcript analysis of miR-181a, miR-181c, miR-340-5p, miR-125b, and miR-26a (Table 1) was performed by quantitative real-time polymerase chain reaction (qRT-PCR). The RNU gene was included as the housekeeping gene. A simple, two-step protocol (Applied Biosystems™) was performed for reverse transcription using a miRNA-specific primer (TaqMan® MicroRNA Reverse Transcription), followed by real-time PCR with TaqMan probes using TaqMan™ Fast Advanced and TaqMan MicroRNA Assays (Applied Biosystems™). Cycling and data collection were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems™). Gene expression was quantified by relative quantification (RQ) using the $2^{-\Delta\Delta Ct}$ method[19].

Enrichment and visual network pathway analysis

An enrichment analysis of transcription factor regulation by miRNAs was performed using the freely available TransmiR database, version 3.0 (http://www.cuilab.cn/transmir). The STRING software, version 12 (https://string-db.org/) search tool was used for protein–protein interaction analysis. Ingenuity Pathway Analysis (IPA; Qiagen) was used to predict the potential targets of the studied miRNAs and to infer the biological impact of these relationships based on the identified expression profile.

Data analysis

Statistical analyses were conducted using SPSS 20 software (SPSS Inc., Chicago, Illinois, USA) or GraphPad Prism (v.8.0.2). Survival analysis was performed using the Kaplan–Meier method. A Cox regression model was used for multivariate analysis of miRNA expression-associated survival. Data were expressed as the mean ± standard deviation (SD). In all cases, p-values <0.05 were considered statistically significant.

RESULTS

Characterization of the patient cohort

In this study, a total of 20 FFPE tissue samples were analyzed. Clinical features of the study participants are summarized in Table 2. The ages of the patients with TNBC enrolled in our study ranged from 34.0 to 88.0 years, with a median age of 58 years. Lymphovascular invasion was observed in 15 cases (75%), and perineural invasion was present in eight patients (40.0%). Inflammatory infiltrate was observed in 14 patients (70%). There was a significant difference in the mean age according to the presence of any of these three parameters, showing that

Table 1. Detail of the TaqMan® Gene Expression Assays used for qPCR.

miRBase ID	Mature miRNA sequence	Assay ID (Applied Biosystems™)
hsa-miR-26a-1-3p	CCUAUUCUUGGUUACUUGCACG	02443
hsa-miR-125b-5	UCCCUGAGACCCUAACUUGUGA	000449
hsa-miR-181a-2-3p	ACCACUGACCGUUGACUGUACC	002317
hsa-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU	000482
hsa-miR-340-5p	UUAUAAAGCAAUGAGACUGAUU	002258

inflammatory infiltrate, lymphovascular invasion, and perineural invasion were mainly present in patients under 60 years of age (Figure 1A–C). A significant difference was observed when survival was compared among age groups, showing that patients under 50 years of age had lower survival compared with women over 70 years of age (Figure 1D).

A Ki-67 labeling index ≥45% was detected by immunohistochemistry in 12 patients (60%), whereas 4 patients showed an index under 45% (20%). Most patients (30%) were TNM stage IIIA, followed by 4 patients in stage IIA (20%), 4 patients in stage IIB (20%), 3 patients in stage IIIB (15%), 3 patients in stage IV (10%), and only one patient in stage IIIC (5%). The mean age of TNBC patients with Ki-67 expression below 45% was higher than that of patients with a Ki-67 labeling index above 45% (Figure 1E). The survival rate was lower in patients with a Ki-67 labeling index below 45% compared with those with a Ki-67 labeling above 45% (Figure 1F). The Ki-67 labeling index was more pronounced in patients with TNM stage II compared with those in the stage III/IV group (Figure 1G), and the survival rate was lower in patients with TNM stage III/IV compared with those with stage II disease (Figure 1H).

NAC was administered in 45% of cases (9 patients), whereas 55% (11 patients) did not receive NAC. None of the 20 patients achieved pCR. The next step was to investigate whether NAC affected the survival rate. The data showed that patients treated with NAC had a lower survival rate compared with those who did not receive NAC (Figure 1I).

Table 2. Clinicopathological characteristics of the study cohort.

Characteristics	All Patients (n=20)
Age, n (%)	
<50	6 (30)
≥50 <70	10 (50)
≥70	4 (20)
Survival (months), n (%)	
≤24	10 (50)
>24	10 (50)
Grade, n (%)	
2	10 (50)
3	10 (50)
TNM staging, n (%)	
IIA	4 (20)
IIB	4 (20)
IIIA	6 (30)
IIIB	3 (15)
IIIC	1 (5)
IV	2 (10)
Ki-67 index, n (%)	
≤45%	4 (20)
>45%	12 (60)
Not informed	4 (20)
Lymphovascular invasion, n (%)	
Present	15 (75)
Absent	5 (25)
Perineural Invasion, n (%)	
Present	8 (40)
Absent	12 (60)
Inflammatory infiltrate, n (%)	
Present	14 (70)
Absent	6 (30)
NAC, n (%)	
Yes	9 (45)
No	11 (55)
pCR, n (%)	
Yes	0
No	7 (35)
Surgery not performed	2 (10)
Not applicable	11 (55)

NAC: Neoadjuvant chemotherapy; pCR: Pathological complete response

miR-340-5P as a promising molecular signature in triple-negative breast cancer patients treated with neoadjuvant chemotherapy

The heatmap analysis (Figure 2A) highlighted that the targets evaluated in TNBC samples were clustered into two groups. The first group encompassed the targets miR-181a, miR-181c, and miR-340-5p, whereas the second group included miR26a and miR-125b. Our results showed that only three patients exhibited upregulated miR-125b expression, and none of them subsequently received NAC.

At first glance, no differences in miRNA expression profiles were observed among TNBC clinical stages (Figure 2B). To further explore the role of miRNAs in the survival of patients with TNBC, an analysis was conducted based on the global expression levels of each miRNA (upregulated or downregulated). The results showed that patients with upregulation expression of the miRNAs evaluated in this study had a positive effect on cumulative survival, whereas downregulation had the opposite effect (Figure 2C).

The predictive value of the expression of each miRNA for NAC response was evaluated in 20 FFPE samples from stage II to IV breast cancer patients. It was observed that patients who subsequently received NAC were those who exhibited lower expression of four miRNAs (miR-181a, miR-181c, miR-340-5p, and miR-125b) compared with those who did not receive NAC (Figure 2D–H). Kaplan-Meier analysis demonstrated that patients with high miR-340-5p expression had a positive effect on cumulative

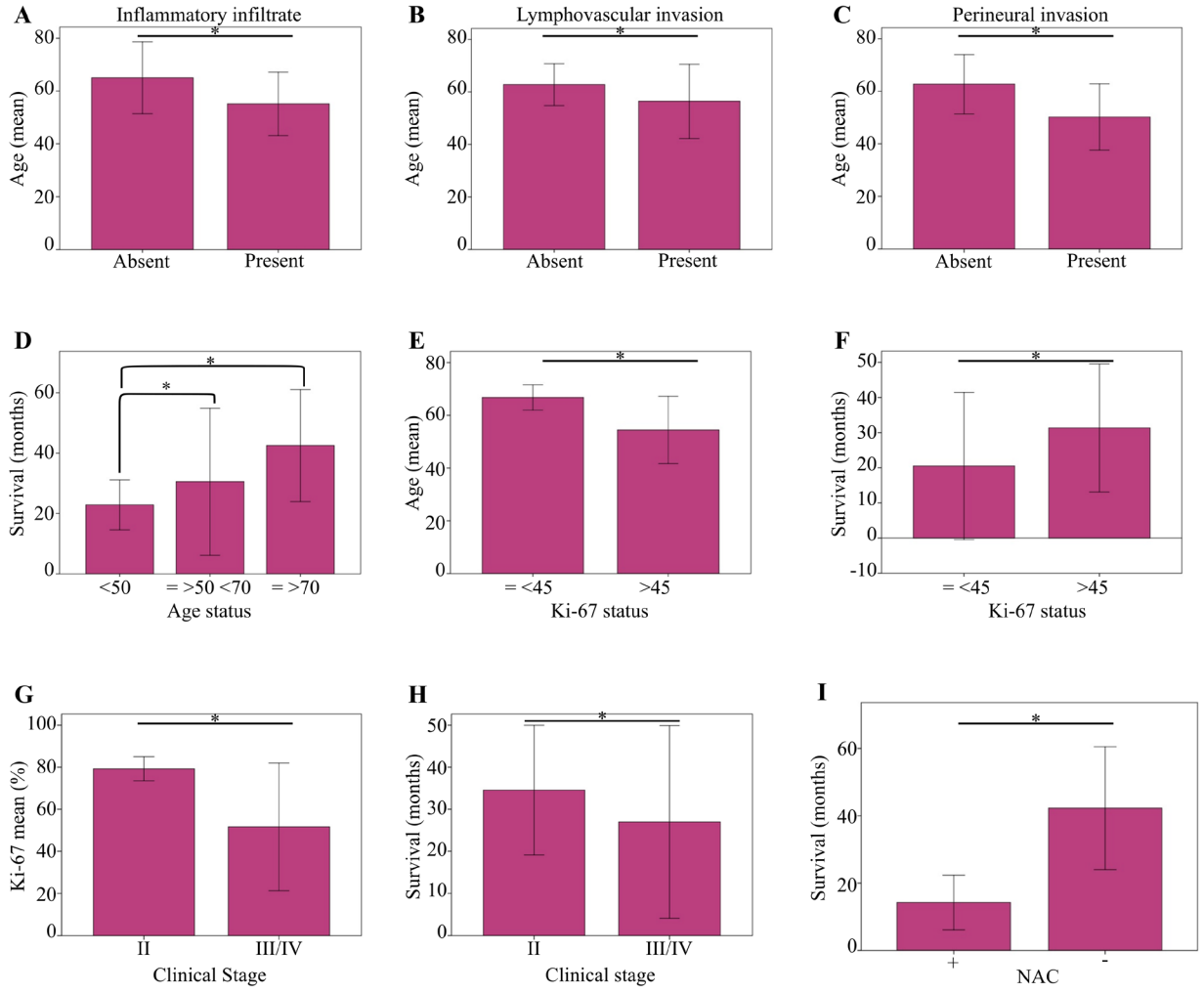

Figure 1. Characterization of the patient cohort. (A-C) There was a significant difference in the mean age according to the presence of any of these three parameters, showing that inflammatory infiltrate, lymphovascular invasion, and perineural invasion were mainly present in patients under 60 years of age. (D) A significant difference was observed when survival was compared among age groups, showing that patients under 50 years of age had lower survival compared with women over 70 years of age. (E) The mean age of TNBC patients with Ki-67 expression below 45% was higher than that of patients with a Ki-67 labeling index above 45%. (F) The survival rate was lower in patients with a Ki-67 labeling index below 45% compared with those with a Ki-67 labeling index above 45%. (G) The Ki-67 labeling index was more pronounced in patients with TNM stage II compared with the stage III/IV group. (H) The survival rate was lower in patients with TNM stage III/IV compared with those with stage II disease. Mann-Whitney test and Kruskal-Wallis Post Hoc Dunn's test. Data are presented as the mean ± standard deviation. n=20. Statistical significance was set at $p<0.05$.

survival compared with those with low expression (median survival: 44vs.17 months; log-rank p=0.045). High expression was associated with high cumulative survival (HR=0.38, 95% confidence interval (95%CI) 0.15–0.98) (Figure 2I).

Interactive pathway analysis

The next step was to investigate potential transcription factors (TFs) regulated by the miRNA targets. To this end, the freely available TransmiR database was utilized. The analysis revealed that miR-340-5p was the primary miRNA involved in the regulation of a wide array of TFs compared with the other miRNAs (Table 3). A protein-protein interaction analysis using STRING demonstrated interactions among the following targets: BCL11A, SALL4, EZH2, TP53, and NFYA (Figure 3A). The subsequent step involved evaluating the putative regulation of target genes by the miRNAs. Target selection was refined by including only those with statistical significance ($p<0.05$). This analysis identified 17 targets potentially downregulated by miR-340-5p when this microRNA is upregulated (Figure 3B). It was observed that miR-340-5p can regulate targets with different final locations in the cell. Additionally, this microRNA was found to negatively regulate the expression of three other non-coding RNAs: the long non-coding RNAs (lncRNAs) LINC03047, LINC00857, and B3DALT5-AS1. Furthermore, among other targets, miR-340-5p can negatively regulate the expression of TGFA and MED28, both of which are implicated in breast carcinogenesis.

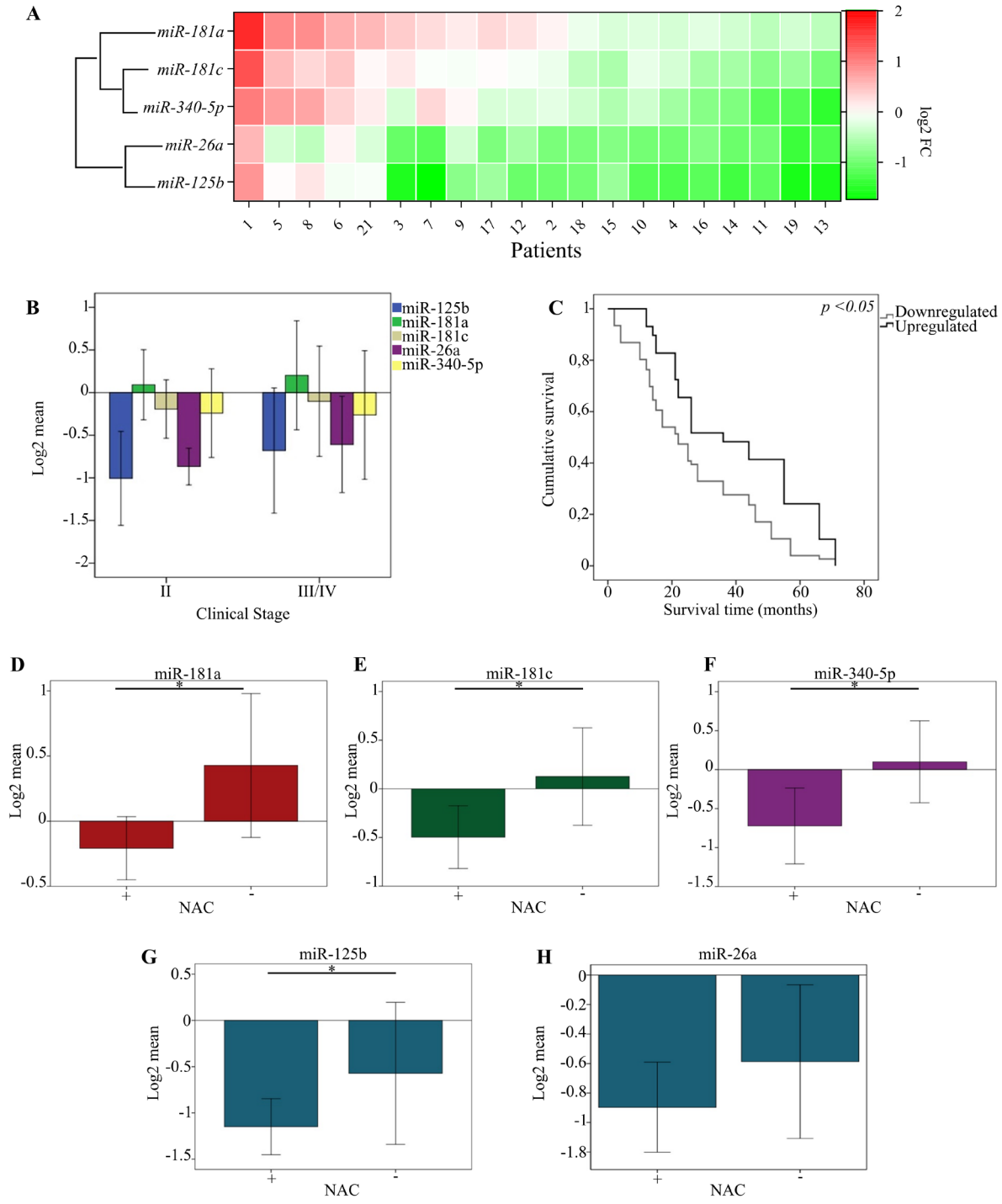

Figure 2. MicroRNA expression profile in treatment-naïve triple-negative breast cancer patients and its association with neoadjuvant chemotherapy response. (A) Heatmap showing qRT-PCR data of microRNA expression in triple-negative breast cancer samples collected prior to treatment. The analysis revealed two main clusters: one comprising miR-181a and miR-181c, and another including miR-340-5p, miR-26a, and miR-125b. (B) No significant differences in microRNA expression profiles were observed across triple-negative breast cancer clinical stages. (C) Upregulation of the evaluated microRNAs was associated with improved cumulative survival, whereas downregulation was associated with poorer outcomes. (D–H) Patients who subsequently received neoadjuvant chemotherapy exhibited lower expression levels of four microRNAs (miR-181a, miR-181c, miR-340-5p, and miR-125b) compared with those who did not receive neoadjuvant chemotherapy. (I) Patients with high miR-340-5p expression had improved cumulative survival compared with those with low expression (log-rank p=0.045). Survival analysis was performed using the Kaplan–Meier method to evaluate the prognostic value of these microRNAs in patients with triple-negative breast cancer. Data are presented as the mean ± standard deviation (n=20). Statistical significance was set at p<0.05. Relative microRNA expression ($2^{-\Delta\Delta Ct}$ method) was calculated using normal breast tissue samples as calibrators.

Table 3. TransmiR database analysis.

Transcription factor	Overlapped miRNAs	p-value
AKNA	miR-340-5p	0.0221
BCL11A	miR-340-5p, miR-181c	0.049
CREB3	miR-340-5p	0.0462
EZH2	miR-26a, miR-125b, miR-181c, miR-181a, and miR-340-5p	0.0155
MORC2	miR-340-5p	0.0366
NF-Y	miR-181a	0.0172
SALL4	miR-340-5p	0.039
TEF	miR-340-5p	0.039
THAP8	miR-340-5p	0.0366
TP53	miR-26a, miR-125b, miR-181c, miR-181a, and miR-340-5p	0.0161
TSHZ2	miR-340-5p	0.027
ZFP37	miR-340-5p	0.0486
ZNF197	miR-340-5p	0.0414
ZNF33B	miR-340-5p	0.039
ZNF511	miR-340-5p	0.0366
ZNF584	miR-340-5p	0.039

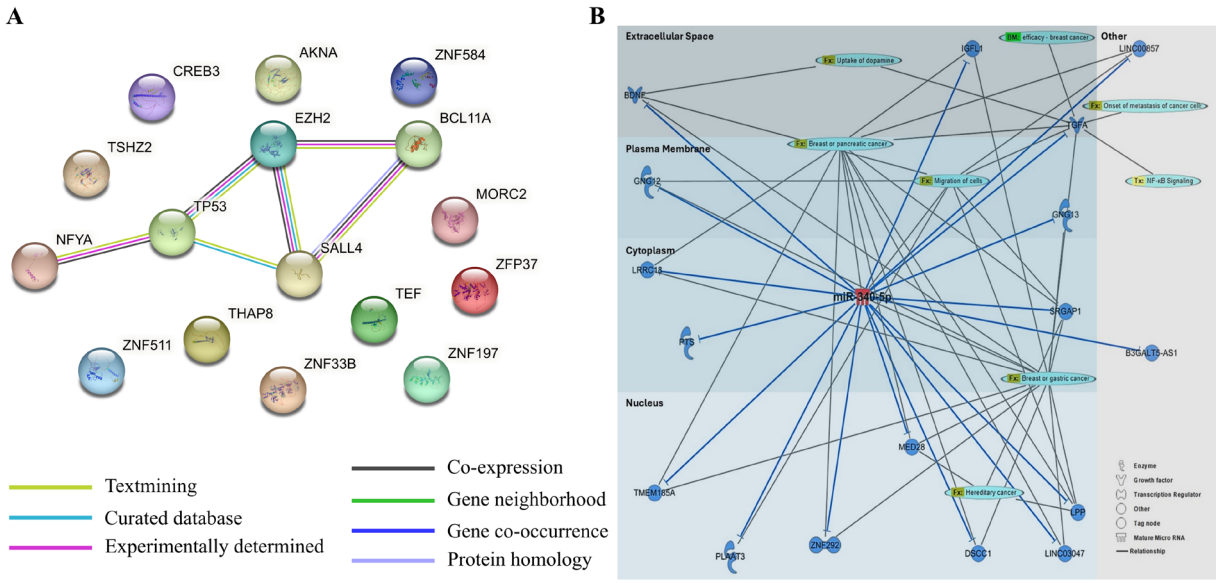

Figure 3. miR-340-5p is the primary microRNA involved in the regulation of a wide array of genes compared to the other microRNAs. (A) STRING analysis of 16 transcription factors regulated by miR-340-5p. Edges represent different types of protein-protein interactions. Protein-protein interaction enrichment p-value: 0.036. (B) Interactive pathway analysis. This analysis identified 17 targets potentially downregulated by miR-340-5p when this microRNA is upregulated. miR-340-5p was found to regulate targets with different final cellular locations. Additionally, this microRNA negatively regulates the expression of three other non-coding RNAs: the long non-coding RNAs (lncRNAs) LINC03047, LINC00857, and B3DALT5-AS1. Furthermore, among other targets, miR-340-5p can negatively regulate the expression of TGFA and MED28, both implicated in breast carcinogenesis.

DISCUSSION

Gene and miRNA expression profiles have been utilized to classify various tumor types[1,20–23]. Previous studies have highlighted the critical role of miR-340-5p in cancer development and progression[17,18,24–26]. Dysregulated miR-340-5p expression has been reported in numerous types of cancer, including human TNBC. miR-340-5p expression levels are inversely correlated with the development of late-stage disease, poor prognosis, and TNBC cell aggressiveness[17].

Wang et al.[27] documented that the viability, migration, and survival of miR-181a-transfected MDA-MB-231 cells were all

significantly reduced. In addition, miR-181a inhibited glycolysis by reducing glucose uptake and lactate production rates in a TNBC cell line. Zhang et al.[14] showed that miR-26a upregulation promotes cisplatin sensitivity and DNA damage in TNBC cell lines. The present study analyzed the expression of miR-26a, miR-181a, miR-181c, miR-340-5p, and miR-125b. Furthermore, no differences were observed among clinical stages; upregulation of these miRNAs positively affects the cumulative survival of patients with TNBC and could be associated with a favorable prognostic marker.

Liu et al.[15] investigated the predictive value of serum miRNAs for the response to NAC and the prognosis of 118 patients with breast cancer (stage II/III) and 30 healthy adult women. The authors showed that lower miR-125b expression during NAC was associated with a favorable chemotherapy response and disease-free survival (DFS). The present study detected differences in miRNA expression profiles between patients who underwent NAC and those who did not. Although none of the 20 patients achieved pCR, those who received NAC exhibited longer overall survival and downregulation of the studied miRNAs, including miR-125b.

In the interactive pathway analysis, miR-340-5p was found to negatively regulate the expression of three lncRNAs — LINC03047, LINC00857, and B3DALT5-AS1 — as well as the proteins TGFA and MED28. A large number of lncRNAs have been described as markers of epithelial-mesenchymal transition (EMT) modulation. EMT is a highly orchestrated event involving a series of changes that lead to the loss of epithelial cell properties and the acquisition of a mesenchymal phenotype and invasive migratory capacities. This event is closely related to TNBC carcinogenesis, metastasis, and chemoresistance[28–32].

Zhang et al.[33] documented that LINC03047 promoted EMT in a lung cancer cell line. Xia et al.[34] demonstrated that LINC00857 is capable of inducing EMT in hepatocellular carcinoma and that its inhibition can trigger apoptotic cell death. Additionally, LINC00857 promotes cell migration and proliferation in colorectal cancer cell lines[35]. Upregulation of TGFA has been reported in a subset of TNBC[36], and enhanced TGFA expression is associated with breast cancer development and progression[37]. Huang et al.[38] demonstrated that MED28 regulates EMT through the NFKB pathway in the human TNBC cell line MDA-MB-231.

In this study, downregulation of miR-181a, miR-181c, miR-340-5p, and miR-125b was observed in patients who subsequently received NAC compared with those who did not receive NAC. NAC is increasingly used to treat early and locally advanced breast cancer, enabling an objective evaluation of treatment efficacy. Proposed as a surrogate endpoint for survival, pCR is usually employed as the primary endpoint of NAC. However, owing to breast cancer heterogeneity, substantial differences in pCR rates exist among subtypes. Therefore, reliable methods for predicting the response to NAC, aimed at preventing side effects and poor outcomes resulting from ineffective treatment, are clinically needed. Obtaining tumor tissue or blood samples before, during, and after NAC is feasible, facilitating the discovery of novel predictive biomarkers such as miRNAs[8].

The molecular mechanisms of non-coding RNAs, particularly miRNAs, in TNBC warrant further characterization, especially with respect to the crosstalk among diverse RNA classes and the deregulation of intricate signaling pathways[28,29]. Furthermore, detecting these molecules in samples from patients with TNBC may facilitate the identification of clinically relevant molecular signatures[39]. The present study revealed intricate relationships between miRNA expression and NAC in TNBC, highlighting the potential utility of these biomolecules as valuable predictive biomarkers (Figure 4).

CONCLUSION

Dysregulation of miRNAs was strongly hypothesized as a possible mechanism of NAC resistance. In this cohort, nine patients were indicated for treatment with NAC. Among these patients,

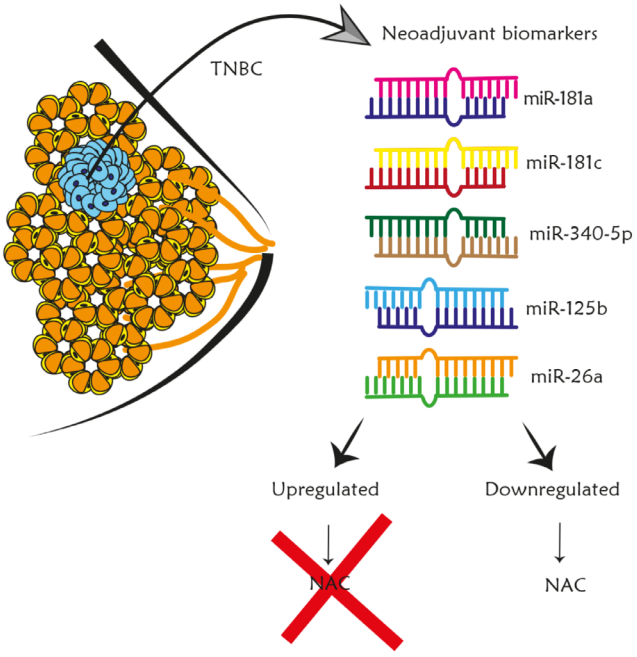

Figure 4. Candidate microRNAs associated with neoadjuvant chemotherapy response in triple-negative breast cancer. Schematic representation of the microRNAs identified as potential predictive biomarkers of neoadjuvant chemotherapy response in triple-negative breast cancer. Differential expression analysis revealed that miR-181a, miR-181c, miR-340-5p, miR-125b, and miR-26a were differentially expressed in patients with triple-negative breast cancer, highlighting their potential utility as predictive biomarkers and their possible involvement in mechanisms associated with chemotherapy sensitivity and resistance.

seven were diagnosed with TNM stage III/IV disease. Together, these results demonstrate a promising molecular signature for patients with TNBC who subsequently received NAC.

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AUTHORS' CONTRIBUTIONS

BOPG: Data curation, Formal Analysis, Writing – original draft, Writing – review & editing. BDSS: Methodology, Data curation, Formal Analysis. FRQ: Methodology, Data curation, Formal Analysis. CEML: Methodology, Data curation, Formal Analysis. ALFMG: Methodology, Data curation, Formal Analysis. PGOS: Methodology, Data curation, Formal Analysis. LMS: Conceptualization, Formal Analysis, Writing – review & editing. LCB: Conceptualization, Funding acquisition, Formal Analysis, Supervision, Writing – review & editing.

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Conflict of interest: nothing to declare.

Artificial Inteligence usage: none.

Data Availability statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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