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Correlative expression of exosomal miRNAs in chemotherapy resistance of triple-negative breast cancer

An observational study

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Abstract

Drug resistance in tumors is the primary contributor to clinical treatment failures, and aberrant expression of small RNA molecules, specifically microRNAs (miRNAs), in tumor tissues is intricately associated with drug resistance. The aim of this study is to investigate the targets and mechanisms through which exosomal miRNAs from triple-negative breast cancer (TNBC) regulate chemotherapy resistance in tumor cells. Utilizing high-throughput sequencing technology, we conducted exosomal miRNA sequencing on serum samples obtained from TNBC patients who were either sensitive or resistant to AC-sequential T chemotherapy. Subsequently, we identified and screened differentially expressed miRNAs. The observed differences in miRNA expression were further validated through quantitative reverse transcription-polymerase chain reaction. In comparison to TNBC patients who exhibited sensitivity to the AC-sequential T regimen chemotherapy, we identified significant differences in the expression of 85 miRNAs within serum exosomes of patients displaying chemotherapy resistance. Furthermore, we observed a substantial difference in the expression of hsa-miR-6831-5p between TNBC patients who were responsive to chemotherapy and those who were drug-resistant and underwent treatment with the AC-sequential T regimen. hsa-miR-6831-5p holds the potential to serve as a diagnostic marker for assessing the chemosensitivity of the AC-sequential T regimen in TNBC.

Abbreviations: ER = estrogen receptor, HER-2 = human epidermal growth factor receptor 2, MDR = multidrug-resistant, miRNAs = microRNAs, OS = overall survival, PR = progesterone receptor, qRT-PCR = quantitative reverse transcription-polymerase chain reaction, TNBC = triple-negative breast cancer.

Keywords: AC-sequential T scheme, drug resistance, microRNAs, therapeutic target, triple-negative breast cancer

1. Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression. TNBC, a subtype of breast cancer, falls under the category of advanced multidrug-resistant (MDR) types and stands as one of the most prevalent causes of mortality among women worldwide. TNBC exhibits a notably high recurrence rate and shorter overall survival (OS) following initial diagnosis.[1–3] The factors contributing to these variations in survival outcomes are multifaceted, encompassing genetic predisposition,[4] lifestyle choices,[5] and environmental factors.[6]

Currently, in terms of diagnosis, TNBC is primarily reliant on imaging and tissue biopsy, which may not effectively lead to early detection. In terms of treatment, the main therapeutic approaches for TNBC include surgery, neoadjuvant therapy, chemotherapy, targeted therapy for signaling pathways, and radiotherapy. AC sequential T chemotherapy is commonly used for TNBC. However, there are no established effective chemotherapy targets for TNBC, leading to generally poor prognosis. Therefore, TNBC lacks specific diagnostic or chemotherapy markers to address the high heterogeneity of tumors.[7,8] New potential diagnostic and treatment targets need to be explored.

MicroRNAs (miRNAs) are endogenous RNA molecules approximately 15 to 23 nucleotides in length. They exert their effects by negatively regulating the expression of target genes through base-pairing interactions.[9,10] Previous research has indicated that miRNAs play pivotal roles in promoting various aspects of tumor development, including growth, migration, invasion, angiogenesis, cell survival, and immune evasion.[11,12]

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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MiRNAs have demonstrated potential as biomarkers for various diseases, including cancer, and have been shown to be sensitive to various antitumor drugs.[13,14]

Exosomes are small vesicles with diameters ranging from 40 to 100 nanometers, released by various cell types. They contain specific proteins, lipids, cytokines, and genetic material. Exosomes derived from different tissues carry distinct protein signatures and contain molecules critical to their functions.[15–17] In recent years, research on exosomes has expanded into diverse fields, including tumor therapy, foundational medical research, and immunology. Clinical investigations have explored their roles in cardiovascular, endocrine, metabolic systems, and more.

In summary, exosomal miRNA may offer new avenues of research and diagnostic and therapeutic targets for triple-negative breast cancer (TNBC). Furthermore, there have been no comprehensive analyses of serum exosomal miRNA in patients with TNBC to date. This project aims to employ high-throughput sequencing technology for miRNA sequencing on serum exosome samples from TNBC patients who are either sensitive or resistant to AC-sequential T chemotherapy. The goal is to identify differentially expressed miRNAs and investigate their roles in breast cancer chemosensitivity. The study seeks to elucidate the clinical relevance of these miRNAs and provide novel insights into breast cancer prognosis.

2. Materials and methods

2.1. Collection of clinical specimens

This study was approved by the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (2022 No. 57). Serum samples were collected from TNBC patients who received sequential AC (Adriamycin and Cyclophosphamide) followed by T (Taxane) chemotherapy at the Affiliated Tumor Hospital of Xinjiang Medical University from 2017 to 2021.

Inclusion criteria: histologically confirmed as TNBC; agreement to neoadjuvant chemotherapy; tolerable physical condition for chemotherapy.

Exclusion criteria: concurrent other malignancies; non-consent for follow-up; lack of pathological results in the hospital; concurrent other diseases that prevent standard chemotherapy; patients with incomplete clinical data; patients with psychiatric disorders.

Our drug resistance diagnosis is based on the guidelines for breast cancer diagnosis and treatment from the Chinese Society of Clinical Oncology (CSCO) as well as the RECIST criteria. *Drug resistance criteria:* Assessed by ultrasound and magnetic resonance imaging, tumor regression or progression was evaluated post-chemotherapy. Patients with no significant regression or progression were considered drug-resistant.

A total of 36 patient samples were collected, including 6 samples for miRNA sequencing (3 chemotherapy-sensitive patients in the "F" group and 3 drug-resistant patients in the "NF" group). The remaining 30 samples were used for an expanded sample cohort in RT-qPCR validation experiments, consisting of 15 drug-sensitive and 15 drug-resistant cases. Informed consent was obtained from all patients for sample collection.

2.2. High-throughput RNA sequencing

Patient serum was initially subjected to exosome rapid extraction reagent kit (YEASEN) for exosome isolation. Subsequently, total RNA was extracted using TRIzol reagent following the standard protocol. In brief, an appropriate volume of TRIzol was mixed with the isolated exosomes based on the sample quantity. Then, an equal volume of chloroform was added, thoroughly mixed, and centrifuged at 12,000 × g for 15 minutes. The upper aqueous phase was carefully collected, and 1.5 times the volume of isopropanol was added. After centrifugation under the same conditions, RNA was washed with 75% ethanol and then centrifuged to air-dry the ethanol. RNA was resuspended in RNase-free water. The concentration and purity of RNA were determined using a Nanodrop 2000 spectrophotometer. RNA integrity was assessed by agarose gel electrophoresis and RNA gel electrophoresis.

For RNA sequencing, TruSeqTM stranded mRNA libraries were constructed, and RNA sequencing was performed on the Hiseq4000 platform (Illumina, USA).

2.3. Bioinformatics analysis

Cutadapt software (National) was utilized for filtering sequencing adapters, while BLASTN software was employed to eliminate rRNA, tRNA, miscRNA, snRNA, and snoRNA. The miRBase 21.0 database was systematically queried for filtered sequences (http://mirbase.org/) to discover novel miRNAs. miRNA expression levels were calculated using DEGseq. The threshold for identifying differentially expressed miRNAs as statistically significant is set at a corrected P value < .05 and a fold change (FC) > 2.

For predicting the target genes of differentially expressed miRNAs, miRNA–target prediction software, miRDB (http://www.mirdb.org/), and miRWALK (http://mirwalk.umm.uni-heidelberg.de/), were employed. The target genes regulated by differentially expressed miRNAs were functionally categorized using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) through the Blast2GO software. Furthermore, a miRNA–target gene coexpression network was constructed using Cytoscape 3.6.1 (https://cytoscape.org/).

2.4. qRT-PCR experiment

Total RNA was extracted from cells using the Trizol reagent (ET111, Transgene) following the recommended protocol. Subsequently, the RNA was reverse transcribed into cDNA using a reverse transcription kit.[18] The primers used in this study were synthesized by Xinjiang Ouyi Biotechnology Co., Ltd. The PCR was carried out with the following conditions: initial denaturation at 95 °C for 5 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 2 minutes, and a total of 40 cycles. The relative miRNA levels were calculated using the 2-ΔΔCt method. The qRT-PCR primer sequences are provided in Table 1.

2.5. Statistical analysis

Statistical analysis was performed using R software version 4.0.1, and a significance level of 0.05 was set for all analyses. All tests were 2-sided. Descriptive statistics were presented as the mean ± standard deviation for measurement data that followed a normal distribution. Student t test was applied to compare data that met the normal distribution assumption. The characteristics of the 2 data groups were described and compared using either chi-square or t tests, as appropriate.

Table 1

qRT-PCR primers.

Primer name	Sequence (5′ to 3′)
hsa-miR-3646-F	GCGCAGAAAATGAAATGAGC
hsa-miR-4741-F	CTGTCCGGAGGGGTC
hsa-miR-937-5p-F	GCAGGTGAGTCAGGGT
hsa-miR-6730-3p-F	CTGACACCCCATCTGC
hsa-miR-6831-5p-F	CAGTAGGTAGAGTGTGAGGAG
hsa-miR-8485-F	AGCACACACACACACAC

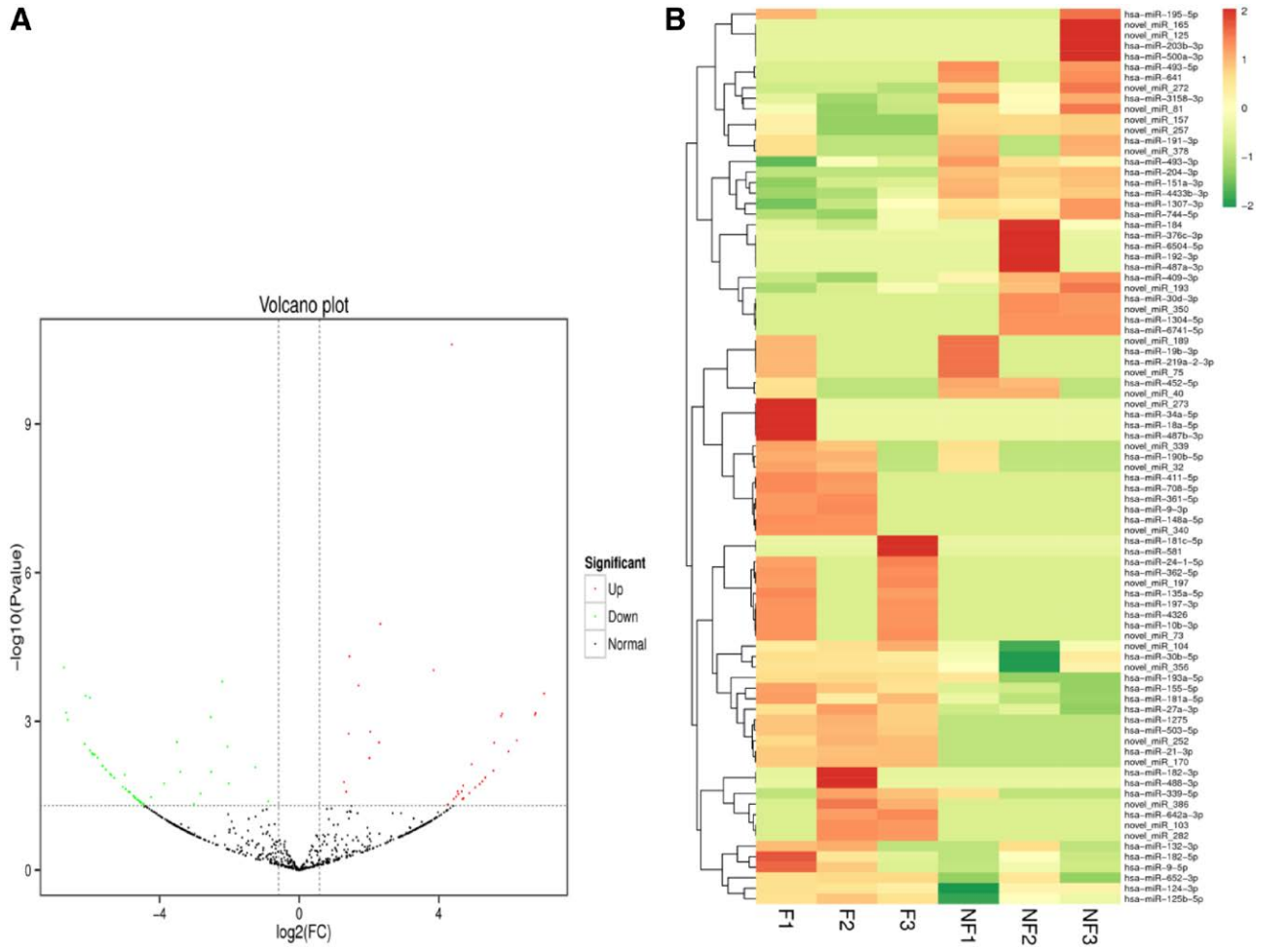

Figure 1. Differential miRNA analysis. (A, Left) The differential miRNA volcano plot depicts each data point as a representation of a miRNA. Upregulated miRNAs are depicted in red, downregulated ones in blue, and those with no significant difference in expression are shown in gray. It is important to note that the *P* value mentioned here refers to the corrected *P* value. (B, Right) The differential miRNA clustering heatmap displays the sample names on the *x* axis and miRNA names on the *y* axis. Upregulated expressions are indicated in red, while downregulated expressions are shown in green.

Table 2

Statistics of miRNA–target gene prediction results in different samples.

Sample ID	All miRNA	miRNA with target	Target gene
F1	464	413	18,330
F2	428	365	17,951
F3	468	417	18,284
NF1	387	341	17,833
NF2	364	320	17,714
NF3	348	302	17,843

3. Experimental results

3.1. miRNA sequencing analysis

Selection of differentially expressed miRNAs through miRNA sequencing. As shown in Figure 1, the F group represents the chemotherapy-sensitive group, while the NF group represents the chemotherapy-resistant group. Compared to breast cancer patients in the chemotherapy-sensitive group, there were 85 differentially expressed genes in the serum of chemotherapy-resistant breast cancer patients, including 37 upregulated miRNAs and 48 downregulated miRNAs (Fig. 1).

3.2. Target prediction of miRNA–target genes miRNA

Exosomal miRNAs have the ability to cross cellular boundaries and regulate intercellular processes. Understanding the targets of exosomal miRNAs can further elucidate the downstream targets and major signaling pathways in drug-resistant TNBC. To further investigate the regulatory network of miRNAs, we analyzed the target genes of differentially expressed miRNAs using miRWALK and MiRDB. The F group represents the chemotherapy-sensitive group, while the NF group represents the chemotherapy-resistant group. A total of 85 differentially expressed miRNAs predicted 19,197 target genes (Table 2).

3.3. miRNA–target gene enrichment analysis

To further investigate the functional role of miRNAs, we conducted an analysis of target gene enrichment using GO and KEGG pathways. The results revealed significant differences in a total of 11,092 GO lists and 10,491 KEGG pathways.

GO enrichment analysis indicated that target genes were primarily associated with biological regulation, immune response, and cellular metabolism processes (Fig. 2A).

Furthermore, KEGG analysis demonstrated that the target genes were predominantly involved in axon guidance, MAPK signaling pathways, and the Hippo signaling pathway (Fig. 2B).

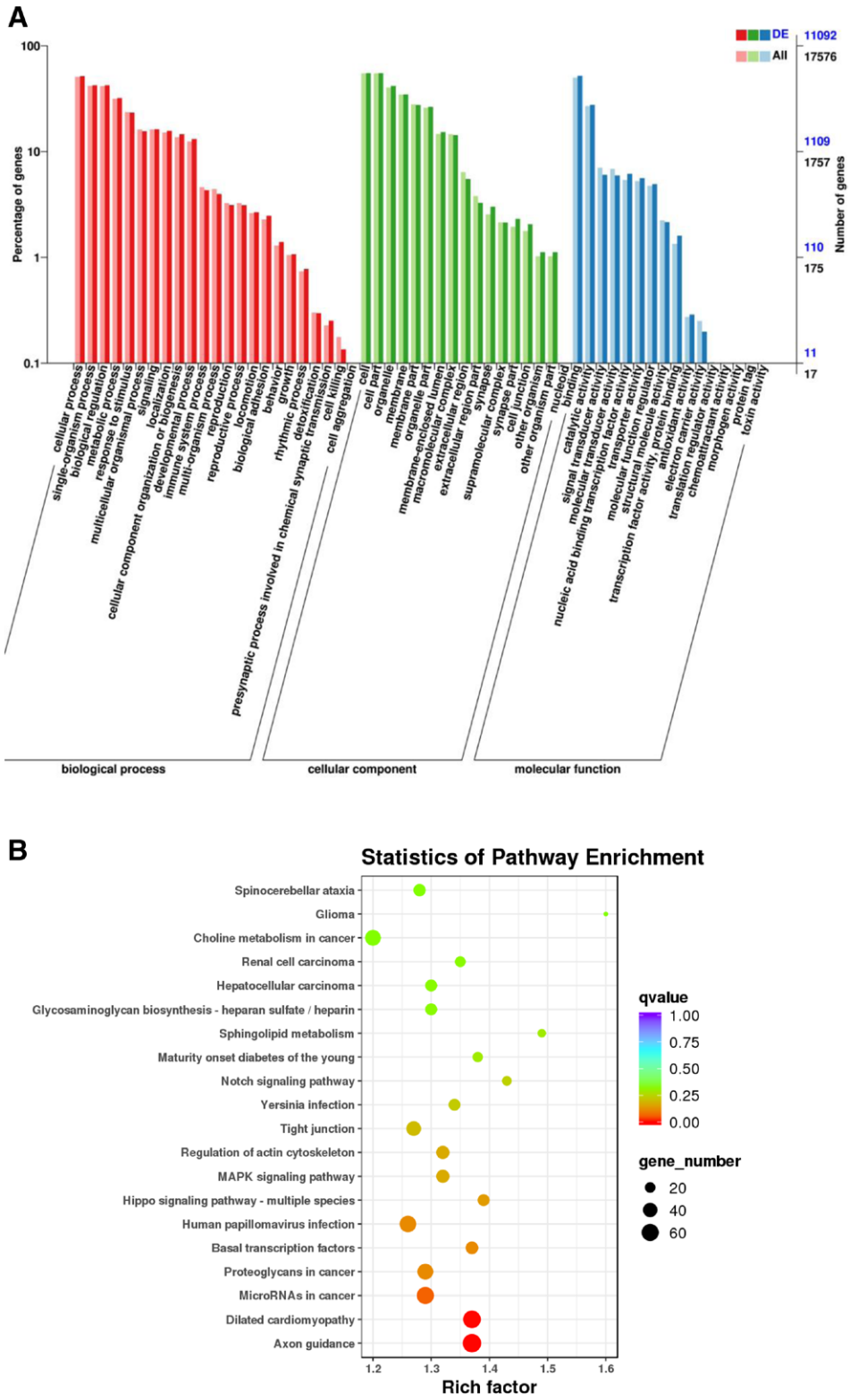

Figure 2. Target gene functional enrichment analysis. (A) Target gene GO enrichment analysis; (B) KEGG enrichment analysis of target gene. GO = Gene Ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

3.4. miRNA expression verification

In order to screen for miRNAs associated with the chemotherapy sensitivity of TNBC as potential diagnostic markers, we selected 6 differentially expressed miRNAs that are documented in the literature to potentially affect breast cancer chemotherapy sensitivity, including hsa-miR-182, hsa-miR-1246, hsa-miR-378-e, hsa-miR-6730-3p, hsa-miR-6831-5p, and hsa-miR-373.[19–22] We collected 30 serum samples, including 15 cases in the sensitive group and 15 cases in the Drug resistance group, and conducted clinical qRT-PCR experiments in an expanded cohort to obtain the relative expression values of miRNAs. hsa-miR-6831-5p showed differential expression between chemotherapy-sensitive and resistant breast cancer patients ($P < .05$, Table 3).

Table 3

qRT-PCR analysis of miRNA levels in human serum ($\bar{x} \pm s$).

Group	hsa-miR-182	has-miR-1246	hsa-miR-378-e	has-miR-6730-3p	hsa-miR-6831-5P	has-miR-373
Sensitive group (n = 15)	3.68 ± 0.46	2.99 ± 0.60	0.65 ± 0.12	1.72 ± 1.69	2.72 ± 2.94	6.16 ± 1.56
Drug resistance group (n = 15)	3.62 ± 0.55	3.08 ± 0.64	0.70 ± 0.11	1.48 ± 1.74	0.64 ± 1.32	6.21 ± 1.45
F value	0.324	0.397	1.190	0.152	6.229	0.091
P value	.748	.694	.244	.70	.019*	.928

*Statistical significance.

4. Discussion

Breast cancer stands as one of the most prevalent malignancies among women worldwide.[23,24] At present, checkpoint inhibitors have been preliminarily applied to TNBC, and some studies have explored the feasibility of using PD-1 and PD-L1 inhibitors to treat TNBC. While significant progress has been made in the treatment of breast cancer, drug resistance remains a major obstacle in clinical chemotherapy, often resulting in poor prognosis. TNBC, a subtype known for its worse clinical prognosis and susceptibility to multidrug resistance, poses particular challenges.[25]

Exosomes, emerging as key intercellular communication mediators, have garnered increasing attention due to their crucial role in regulating tumor pathophysiology. They are widely implicated in tumor neovascularization, malignant invasion, distant metastasis, and immune evasion. Exosomes contain proteins, messenger RNA, and microRNAs (miRNAs), making them effective carriers for delivering these molecules to recipient cells and inducing significant changes. Notably, miRNAs have been found to maintain high stability in the body fluids of cancer patients, partly attributed to their encapsulation and transport by exosomes, positioning them as novel clinical diagnostic and prognostic indicators.[26,27] Prior research has also suggested that drug-resistant tumor cells can secrete a large quantity of exosomes, facilitating horizontal transfer of genetic material between cells.

In this study, high-throughput sequencing was employed to identify differentially expressed miRNAs by sequencing exosomal miRNAs in serum samples from patients treated with the AC-sensitive and drug-resistant breast cancer regimens. The findings revealed significant differences in 85 miRNAs within the serum exosomes of chemotherapy-resistant patients when compared to AC-sequential T regimen-sensitive TNBC patients. Furthermore, an exploration of the regulatory network of miRNAs predicted 19,197 target genes for these 85 differentially expressed miRNAs, utilizing miRWALK and MiRDB. Subsequent target gene analysis unveiled substantial differences in 11,092 GO categories and 10,491 KEGG pathways. GO enrichment analysis highlighted the involvement of target genes in biological regulation, immune response, and cellular metabolism processes. Target genes were predominantly associated with axon guidance, MAPK signaling, and the Hippo signaling pathway.[28,29]

Moreover, qRT-PCR validation confirmed significant differences in hsa-miR-6831-5p expression between TNBC patients sensitive and resistant to AC-sequential T chemotherapy. This suggests that hsa-miR-6831-5p holds promise as a diagnostic marker for chemotherapy sensitivity in AC-sequential T regimen-treated TNBC.

However, it is important to acknowledge certain limitations of this study, including its relatively small sample size and single-hospital source, which may introduce bias into the results. Future research endeavors should prioritize larger sample sizes and multicenter studies to address these limitations.

5. Conclusion

hsa-miR-6831-5p is expected to be a diagnostic marker for the chemosensitivity of AC sequential T regimen in TNBC.

Author contributions

Conceptualization: Le Yang, Jingjing Fan, Binlin Ma.
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Writing – review & editing: Le Yang.
Methodology: Chao Dong.
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Supervision: Binlin Ma.

References

[1] Qattan A. Novel miRNA targets and therapies in the triple-negative breast cancer microenvironment: an emerging hope for a challenging disease. Int J Mol Sci . 2020;21:8905.
[2] Ding L, Gu H, Xiong X, et al. MicroRNAs involved in carcinogenesis, prognosis, therapeutic resistance and applications in human triple-negative breast cancer. Cells. 2019;8:1492.
[3] Deepak KGK, Vempati R, Nagaraju GP, et al. Tumor microenvironment: challenges and opportunities in targeting metastasis of triple negative breast cancer. Pharmacol Res. 2020;153:104683.
[4] Desmedt C, Yates L, Kulka J. Catalog of genetic progression of human cancers: breast cancer. Cancer Metastasis Rev. 2016;35:49–62.
[5] Newman LA, Kaljee LM. Health disparities and triple-negative breast cancer in African American women: a review. JAMA Surg. 2017;152:485–93.
[6] Costa A, Kieffer Y, Scholer-Dahirel A, et al. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. Cancer Cell. 2018;33:463–79.e10.
[7] Kanugo A, Gautam RK, Kamal MA. Recent advances of nanotechnology in the diagnosis and therapy of triple-negative breast cancer (TNBC). Curr Pharm Biotechnol. 2021 [Epub ahead of print].
[8] Thakur V, Kutty RV. Recent advances in nanotheranostics for triple negative breast cancer treatment. J Exp Clin Cancer Res. 2019;38:430.
[9] Khazaei-Poul Y, Shojaei S, Koochaki A, et al. Evaluating the influence of human umbilical cord mesenchymal stem cells-derived exosomes loaded with miR-3182 on metastatic performance of triple negative breast cancer cells. Life Sci. 2021;286:120015.
[10] Salvador-Coloma C, Santaballa A, Sanmartín E, et al. Immunosuppressive profiles in liquid biopsy at diagnosis predict response to neoadjuvant chemotherapy in triple-negative breast cancer. Eur J Cancer. 2020;139:119–34.
[11] Gong C, Tian J, Wang Z, et al. Functional exosome-mediated co-delivery of doxorubicin and hydrophobically modified microRNA 159 for triple-negative breast cancer therapy. J Nanobiotechnology. 2019;17:93.
[12] Yu M, Gai C, Li Z, et al. Targeted exosome-encapsulated erastin induced ferroptosis in triple negative breast cancer cells. Cancer Sci. 2019;110:3173–82.
[13] Li Y, Liang Y, Sang Y, et al. MiR-770 suppresses the chemoresistance and metastasis of triple negative breast cancer via direct targeting of STMN1. Cell Death Dis. 2018;9:14.
[14] Todorova VK, Byrum SD, Gies AJ, et al. Circulating exosomal microRNAs as predictive biomarkers of neoadjuvant chemotherapy response in breast cancer. Curr Oncol. 2022;29:613–30.
[15] Fridrichova I, Zmetakova I. MicroRNAs contribute to breast cancer invasiveness. Cells. 2019;8:1361.

[16] El-Ashmawy NE, Khedr EG, Abo-Saif MA, et al. Long noncoding RNAs as regulators of epithelial mesenchymal transition in breast cancer: a recent review. Life Sci. 2024;336:122339.
[17] Lu G, Li Y, Ma Y, et al. Long noncoding RNA LINC00511 contributes to breast cancer tumourigenesis and stemness by inducing the miR-185-3p/E2F1/Nanog axis. J Exp Clin Cancer Res. 2018;37:289.
[18] Yao X, Wang Y, Wang L, et al. Expression patterns of serum MicroRNAs related to endothelial dysfunction in patients with subclinical hypothyroidism. Front Endocrinol (Lausanne). 2022;13:981622.
[19] Lakshmi S, Hughes TA, Priya S. Exosomes and exosomal RNAs in breast cancer: a status update. Eur J Cancer. 2021;144:252–68.
[20] Song W, Wu S, Wu Q, et al. The microRNA-141-3p/ CDK8 pathway regulates the chemosensitivity of breast cancer cells to trastuzumab. J Cell Biochem. 2019;120:14095–106.
[21] Zheng Z, Chen M, Xing P, et al. Increased expression of exosomal AGAP2-AS1 (AGAP2 Antisense RNA 1) in breast cancer cells inhibits trastuzumab-induced cell cytotoxicity. Med Sci Monit. 2019;25:2211–20.
[22] Li B, Cao Y, Sun M, et al. Expression, regulation, and function of exosome-derived miRNAs in cancer progression and therapy. FASEB J. 2021;35:e21916.
[23] Banerji S, Cibulskis K, Rangel-Escareno C, et al. Sequence analysis of mutations and translocations across breast cancer subtypes. Nature. 2012;486:405–9.
[24] Dandawate PR, Subramaniam D, Jensen RA, et al. Targeting cancer stem cells and signaling pathways by phytochemicals: novel approach for breast cancer therapy. Semin Cancer Biol. 2016;40-41:192–208.
[25] Denkert C, Liedtke C, Tutt A, et al. Molecular alterations in triple-negative breast cancer – the road to new treatment strategies. Lancet. 2017;389:2430–42.
[26] Weidle UH, Dickopf S, Hintermair C, et al. The role of micro RNAs in breast cancer metastasis: preclinical validation and potential therapeutic targets. Cancer Genomics Proteomics. 2018;15:17–39.
[27] Wood SL, Westbrook JA, Brown JE. Omic-profiling in breast cancer metastasis to bone: implications for mechanisms, biomarkers and treatment. Cancer Treat Rev. 2014;40:139–52.
[28] Srivastava AK, Wang Y, Huang R, et al.; GUSTO study group. Human genome meeting 2016: Houston, TX, USA. 28 February–2 March 2016. Hum Genomics. 2016;10(Suppl 1):12.
[29] Liu J, Yu H, Cui H, et al. lncRNA LINC000466 predicts the prognosis and promotes the progression of triple-negative breast cancer via modulating miR-539-5p. Clin Breast Cancer. 2022;22:374–80.