



MiR-103a-3p Promotes Tumorigenesis of Breast Cancer by Targeting ETNK1

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Abstract

Background: We aimed to elucidate the molecular mechanism of miR-103a-3p regulating breast cancer progression.

Methods: Firstly, clinical tissues was obtained from 2019-2023 at Yancheng Third People's Hospital, Yancheng, China. miR-103a-3p or *ETNK1* expression in clinical tissues or breast cancer cell lines was analyzed with qRT-PCR. MDA-MB-231 cells were performed with miR-103a-3p inhibitor or mimic, and OE-*ETNK1*. The proliferation and apoptosis ability were detected by CCK-8 and TUNEL assay. The xenograft models were established by inoculating transfected MDA-MB-231 cells to BALB/c mice.

Results: miR-103a-3p showed an overexpression and was related to poor prognosis in breast cancer. miR-103a-3p-deprived MDA-MB-231 cells displayed weaker levels of cell proliferation and higher rates of apoptosis. In contrast, *ETNK1* was downregulated in breast cancer and proved to be a downstream target of miR-103a-3p. Xenograft models subjected to either miR-103a-3p antagomir treatment or *ETNK1*-knockdown resulted in tumor growth suppression.

Conclusion: miR-103a-3p might promote breast cancer progression by inhibiting *ETNK1*.

Keywords: Breast cancer; miR-103a-3p; Proliferation; Apoptosis

Introduction

Breast cancer is the leading cause of cancer related death among women worldwide. The occurrence, growth, metastasis, invasion, angiogenesis and significant possibility of recurrence of breast cancer have made it become a complex neoplastic disease (1-3). To develop further the early diagnosis and detection methods of breast cancer to reduce effectively the mortality of patients, there is an urgent need for further progress in

prevention, detection and treatment (4). Therefore, analyzing the molecular mechanism of breast cancer occurrence and development and looking for breast cancer related markers have powerful significance for the clinical prediction and therapeutic feedback.

microRNAs (miRNAs) are small noncoding RNAs, of which more than 50% were found to be located in cancer-related genomic region in



cancer development (5), dooming that miRNA are highly correlated with human tumors (6-9). The role of miRNA in cancer has duplicity. It can be oncomiR or tumor suppressive miRNA (TS-miR). OncomiR can promote the growth, metastasis and angiogenesis of cancer cells by regulating downstream target genes and signal pathways. There are many representative oncomiRs acting as clinical biomarkers and therapeutic targets in tumorigenesis. miR-21 is considered to be the most stable and highly expressed miRNA in all human cancer types (10), and researchers believe that as a therapeutic agent, anti-miR-21 has great prospects in combined cancer treatment (11). In contrast, as a typical member of TS-miR, miR-34 is the first miRNA confirmed to be directly regulated by tumor suppressor gene *p53* (12). The first tumor targeting miRNA drug MRX34 based on miR-34a mimic has been completed in the phase I clinical trial (NCT01829971) (13). Interestingly, there are still some miRNAs proved "double-edged sword", which exist in different cancer types as cancer promoting and tumor inhibiting RNA (14). For example, miR-125b functioned as its oncogenic and tumor-suppressive role through targeting apoptotic proteins (15). As a oncomiR, miR-103a-3p has been previously studied and reported in multiple cancers, including lung cancer (16), cervical cancer (17), colorectal cancer (18), glioblastoma (19), liver cancer (20), thyroid cancer (21), etc. Nevertheless, whether miR-103a-3p participates in the regulation of breast cancer and its molecular mechanisms remain to be further studied. The imbalance between tumor cell apoptosis and proliferation is considered one of the basic characteristics of tumorigenesis (22, 23). In this study, the influence of miR-103a-3p on the proliferation and apoptosis of breast cancer *in vivo* and *in vitro* was explored, as well as the underlying mechanisms.

Methods

Cell culture and treatments

MCF-10A (normal breast cells) and the corresponding cancer cells SKBR3, MDA-MB-231, and MCF7 are acquired from ATCC. And the culture conditions were referred to the previous studies (24).

After growing to confluence, MDA-MB-231 cells were performed different transfected treatments using Lipofectamine 2000 (Life Technologies, USA). In addition, the sequences used are as follows: miR-103a-3p mimic (5'-AGCAGCAUUGUACAGGGCUAUGA-3'), mimic NC (5'-ACGGUAGACCGAUUCCGAAUCCGCG-3'), miR-103a-3p inhibitor (5'-TCATAGCCCTGTACAATGCTGCT-3'), inhibitor NC (5'-CAGTACTTTTGTGTAGTACAA-3'), sh-ETNK1 (5'-CAGCTCTTCACAGATGGAATCACAA-3'), and sh-NC (5'-TGCGCTAGGCCTCGGTTGC-3').

Clinical samples

Sixteen pairs of breast cancer tissues and paracancerous tissues were collected from clinic patients (aged 26 to 83 years) in The Sixth Affiliated Hospital of Nantong University, Yancheng Third People's Hospital, Yancheng. All patients underwent surgical resection followed up to May 2021. The obtained each tissue sample has been diagnosed as breast cancer by two different professional pathologists.

All selected patients have signed written informed consent.

qRT-PCR analysis

Total RNA was isolated from tissue samples or cells by using a simple total RNA kit (BioTeke, China). cDNA was obtained using the M-MLV Reverse Transcriptase (BioTeke, China). *ETNK1* and miR-103a-3p were quantified by using SYBR Green Master Mix (TaKaRa, China). The primer sequences used are list as Table 1.

Table 1: Primer sequences used for RT-qPCR

<i>Genes</i>	<i>Primer sequence</i>
miR-103a-3p	F: 5'-ATCCAGTGCGTGTCGTG-3' R: 5'-TGCTAGCAGCATTTGTACAGG-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
ETNK1	F: 5'-AAGGATCCACCAGTCTGTCCG-3' R: 5'-ATGCTCCTCCTGATCCTGAA-3'
β -actin	F: 5'-GGGAAATCGTGCGTGACAT-3' R: 5'-CTGGAAGG TGGACAGCGAG-3'

Protein extraction and western blot

Total proteins were extracted from MDA-MB-231 cells or tissue samples with lysis buffer, and then quantified by BCA. We separated proteins via SDS-PAGE and transferred them on PVDF membranes (Invitrogen, USA) by electroblotting. Subsequently the membranes were blocked and then incubated with corresponding primary antibodies: ETNK1 (1:1000, ab236615, Abcam), GAPDH (1:1000, ab8245, Abcam) and secondary antibody IgG (1:2000, ab109489, Abcam). The enhanced chemiluminescence kit (BeyoECL Moon, P0018FS, Beyotime) was in usage to measure the protein bands.

Dual luciferase reporter assay

Briefly, the wild- or mutant-type target gene *ETNK1* (ETNK1-WT/MUT) luciferase reporter vectors were constructed. Cells seeded on a 96-well plate were co-transfected with ETNK1-WT/MUT luciferase plasmids and miR-103a-3p mimic, NC mimic, miR-103a-3p inhibitor or NC inhibitor. Finally, Dual Luciferase Reporter System (Promega, USA) was applied to measure the luciferase activity.

Colony formation experiment

MDA-MB-231 cells, after stably transfection, were plated into culture dishes at 37°C and 5% CO₂. After incubating for about 2 weeks, the cells were collected and dyed by crystal violet solution (0.1%), then observed and counted with Gel Imaging System (Bio-Rad, USA).

CCK-8 assay

MDA-MB-231 cells, after stably transfection, were seeded into 96-well plates. Thereafter, 10 μ L CCK-8 solution (Beyotime, China) was added to each well at each preset time, and then the plates were continuously incubated at 37 °C for 2 h. After that, the absorbance value was monitored using a microplate reader at 450 nm.

TUNEL assay

The cell apoptosis was measured using TUNEL assay kit (Abcam, China). In short, the cells were incubated in TUNEL detection reagent for 1 h, then stained with DAPI solution (Abcam, China) for 3 min to stain the nucleus and mounted in an anti-fade reagent (Beijing Solarbio Science & Technology, China).

For the detection of apoptosis in tumor tissues, the tissue sections were waxed and hydrated. Next, sections were reacted with protease K solution for 20 minutes, and then treated with DNaseI reaction solution. Then after exposing to TDT enzyme reaction solution for 1 hour, each sample was cultured with streptavidin HRP for 30 minutes. Subsequently, the sections were stained with DAB and counterstained with hematoxylin, and finally sealed with cover glass.

The cells or tissue sections were observed with a confocal microscope (Leica, Germany) to quantify apoptosis level, and the images were processed with ImageJ.

Animal experiment

Fifteen male BALB/c nude mice (Age: 5~6 weeks, Weight: 17~19 g) were obtained from the Model Animal Research Center of Nanjing University (Jiangsu, China). 1×10^6 MDA-MB-231 cells were subcutaneously inoculated to set up the transplanted tumor model of nude mice. At the 10th day, each mice was treated with 100 nmol antagomir NC (n = 5) or miR-103a-3p antagomir (n = 10) through tail vein injection twice a week. The mice were divided into 3 groups: antagomir NC + sh-NC, miR-103a-3p antagomir + sh-NC and miR-103a-3p antagomir + sh-ETNK1. The tumor volume was recorded every week. After 4 weeks, tumor samples were collected for further study.

All animal experiments were performed under the approval of the animal ethics committee of Nanjing Agricultural University.

Immunohistochemistry (IHC)

Paraffin-embedded subcutaneous tumor tissues were consecutively cut into 4 μ m slices and then mounted on slides. The sections were dewaxed and then washed with Tris-buffered saline (TBS). Antigen retrieval was performed using EDTA antigen repair solution. After soaking and blocking, the sections were incubated with the specific antibody, anti-ETNK1 (1:100, ab236615, abcam) overnight at 4°C and anti-rabbit secondary antibody for 40 min. After that, the sections were stained with hematoxylin and then sealed. Finally, the sections were observed under a microscope (Olympus, Japan).

Statistical analysis

Graphpad Prism 8.0 software was used to analyze statistically all graphs in this study. The differences between two groups were compared with Student's t-test. Data of this study were shown as mean \pm SD. $P < 0.05$ was considered statistically significant.

Results

The up-regulated miR-103a-3p leads to adverse prognosis of breast cancer.

Detection of their expression in clinical tissues revealed that hsa-miR-103a-3p was the most up-regulated in breast cancer tissues in comparison with others (Fig. 1A). In addition, TCGA database analysis indicated the overexpressed miR-103a-3p in breast cancer (Fig. 1B). Subsequently, we extracted RNA from SKBR3, MDA-MB-231, MCF7, and MCF10A cells, and performed qRT-PCR quantification. miR-103a-3p exhibited markedly higher levels in SKBR3, MDA-MB-231 and MCF7 cells than that in MCF10A cells. Among them, the highest expression of miR-103a-3p was found in MDA-MB-231, which was chosen for the subsequent cell experiments (Fig. 1C). Finally, TCGA database demonstrated that the patients who had overexpression of miR-103a-3p were accompanied by the low survival rate (Fig. 1D).

miR-103a-3p regulates proliferation and apoptosis of breast cancer cells.

To further determine the effect of differential expression of miR-103a-3p on the function of breast cancer in vitro, we transfected miR-103a-3p silencing vectors into MDA-MB-231, and detected its transfection efficiency (Fig. 2A). Then function tests were carried out. In CCK-8 and colony formation assays, the proliferation of MDA-MB-231 cells in miR-103a-3p inhibitor group was much lower than inhibitor NC group (Fig. 2B-C). In addition, we observed that the positive MDA-MB-231 cells in miR-103a-3p inhibitor group increased significantly by TUNEL staining (Fig. 2D), which confirmed that miR-103a-3p down-regulation apparently restrained MDA-MB-231 apoptosis.

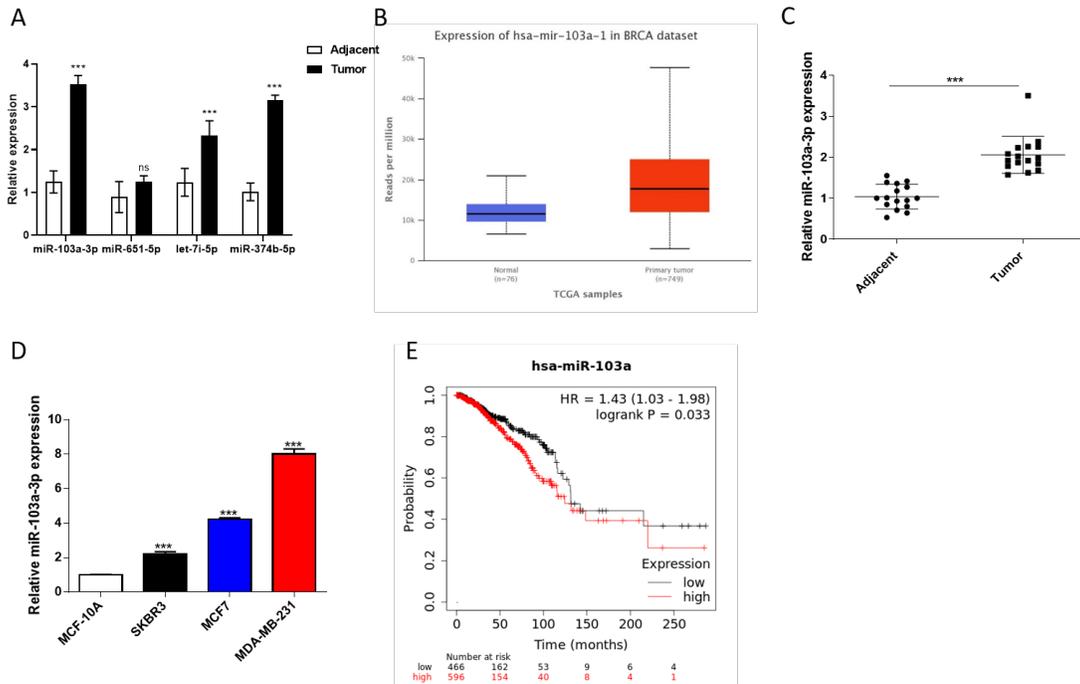


Fig. 1: miR-103a-3p is highly overexpressed in breast cancer and leads to poor prognosis

(A) The expression of miR-374b-5p, let-7i-5p, miR-651-5p, and miR-103a-3p in 5 pairs of breast cancer clinical specimens was quantified by qRT-PCR. (B) TCGA database was used to analyze miR-103a-3p level in breast cancer. (C) miR-103a-3p expression in 16 pairs of clinical tissues was detected by qRT-PCR. (D) TCGA database showed relationship between miR-103a-3p and survival rate of breast cancer patients. ns, not significant; ***, $P < 0.001$

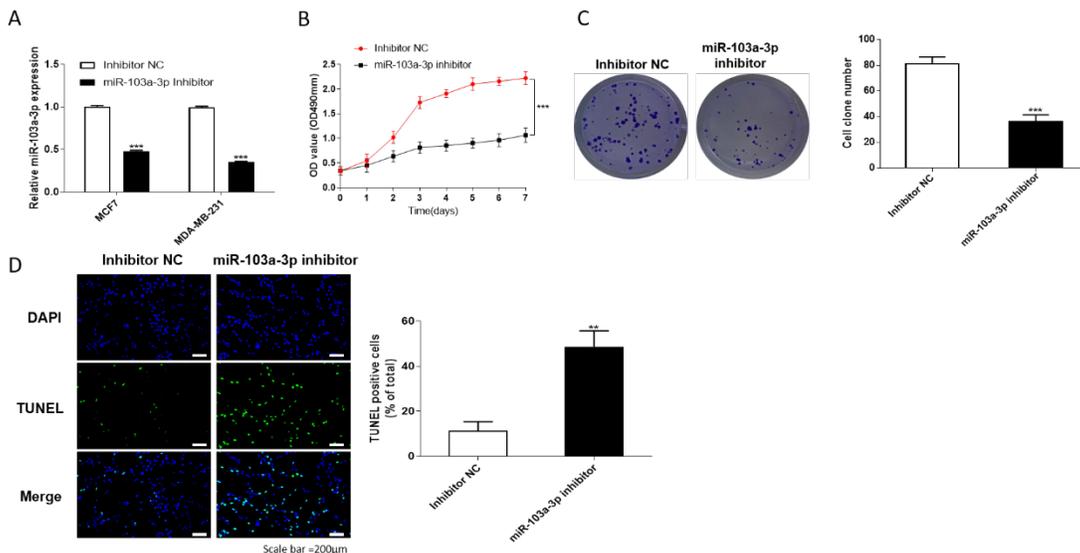


Fig. 2: miR-103a-3p regulates proliferation and apoptosis of MDA-MB-231 cells.

(A) Transfection efficiency of miR-103a-3p was detected by qRT-PCR. (B) CCK-8 and (C) Colony formation assay were carried out for the detection of cell proliferation ability. (D) TUNEL staining was carried out to detect the apoptosis level in each group. **, $P < 0.01$; ***, $P < 0.001$

miR-103a-3p targets binding ETNK1

ETNK1 exhibited a notably lower expression in breast cancer cell lines (Fig. 3A). As expected, the low expression of *ETNK1* corresponded to the low survival rate of breast cancer patients by using BioProfiling website analysis (Fig. 3B). Subsequently, the binding sites of miR-103a-3p and *ETNK1* were predicted using Targetscan website

(Fig. 3C). Then, the luciferase activity in *ETNK1* WT group was greatly decreased in miR-103a-3p mimic group and increased by miR-103a-3p inhibitor, but no significant changes was observed in *ETNK1* MUT group (Fig. 3D). In addition, inhibition of miR-103a-3p apparently enhanced the mRNA and protein expression of *ETNK1* in MDA-MB-231 cells (Fig. 3E-F).

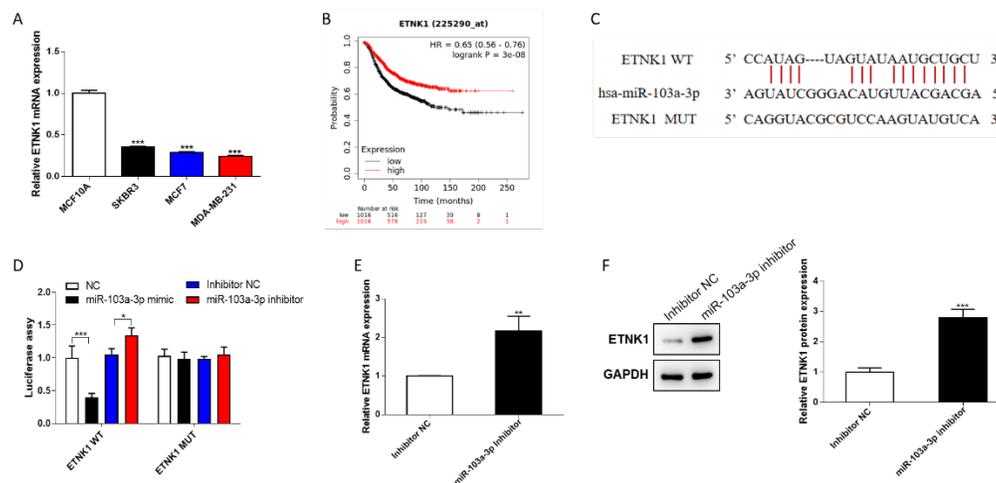


Fig. 3: miR-103a-3p targets binding ETNK1.

(A) The expression of *ETNK1* in different cell lines was quantified by qRT-PCR. (B) TCGA database was used for the analyzing of the relationship between *ETNK1* and survival rate of breast cancer patients. (C) The binding sites of miR-103a-3p and *ETNK1* were determined by using Targetscan website. (D) Dual-luciferase reporter assay verified the binding relationship between *ETNK1* and miR-103a-3p. (E) QRT-PCR and (F) western blot were performed for the detection of the mRNA and protein expression of *ETNK1* in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

miR-103a-3p regulates proliferation and apoptosis of breast cancer cells through targeting ETNK1.

To investigate whether miR-103a-3p affects the function of breast cancer cells through regulating of *ETNK1*, we carried out a rescue experiment. We overexpressed miR-103a-3p or/and *ETNK1* in MDA-MB-231 cells and assayed transfection efficiency by qRT-PCR (Fig. 4A). After that, CCK-8 and colony formation assay suggested that the proliferation of MDA-MB-231 cells markedly elevated with up-regulating miR-103a-3p, while *ETNK1* overexpression dramatically reversed the changes of cell proliferation caused by overexpression of miR-103a-3p (Fig. 4B-C). TUNEL staining showed that the apoptosis level

of MDA-MB-231 cells was obviously inhibited after miR-103a-3p mimic treatment, which was creased after overexpression of *ETNK1* (Fig. 4D).

miR-103a-3p/ETNK1 axis affects breast cancer development in vitro.

We inoculated MDA-MB-231 into the mouse subcutaneous tissue and established a subcutaneous transplantation model for breast cancer. The tumor tissue was collected and photographed after 4 weeks (Fig. 5A). The tumor volume and weight in miR-103a-3p antagomir group were apparently lower than those in the control group, but increased significantly after further inhibition of *ETNK1* (Fig. 5B-C). QRT-PCR results sug-

gested the decreased miR-103a-3p and increased *ETNK1* in miR-103a-3p antagonist group (Fig. 5D). Moreover, miR-103a-3p antagonist significantly up-regulated the protein expression of *ETNK1* observed by IHC results (Fig. 5E). The

results of TUNEL staining were consistent with that of cell experiments. The apoptosis level in miR-103a-3p antagonist group was markedly increased, while the apoptosis level decreased notably after *ETNK1* knockdown (Fig. 5F).

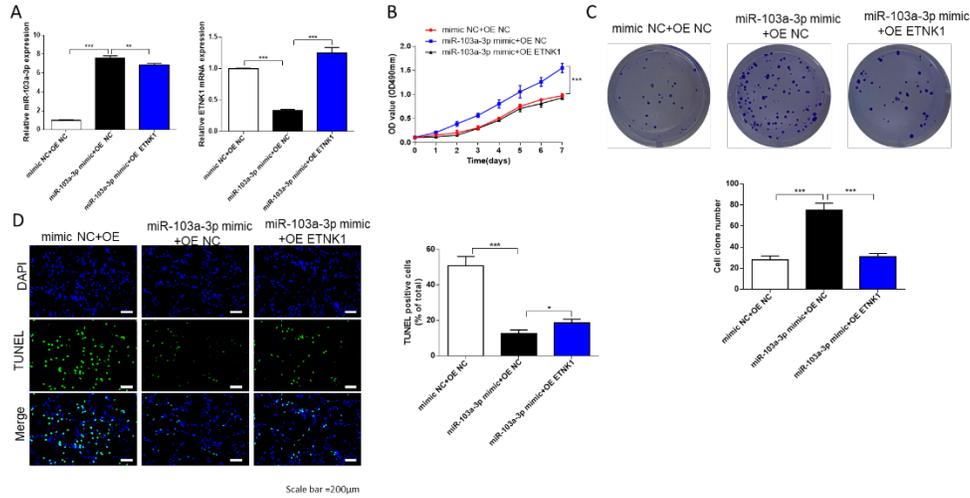


Fig. 4: miR-103a-3p regulates proliferation and apoptosis of breast cancer cells through ETNK1.

MDA-MB-231 cells were transfected with mimic NC+OE NC, miR-103a-3p mimic+OE NC, miR-103a-3p mimic+OE-ETNK1. (A) The expression of miR-103a-3p and *ETNK1* was quantified by qRT-PCR. (B) CCK-8 and (C) colony formation assay were performed for the detection of the cell proliferation. (D) TUNEL staining was used to detect the apoptosis level. **, $P < 0.01$; ***, $P < 0.001$

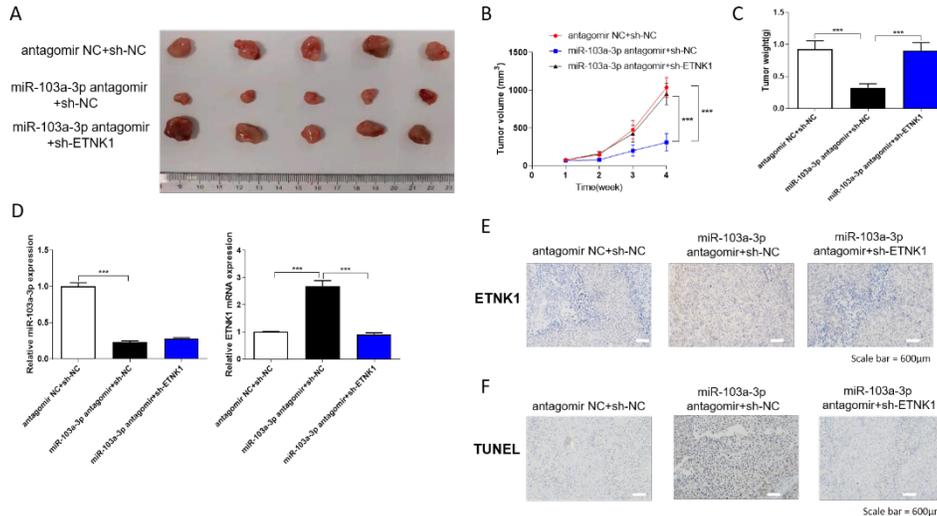


Fig. 5: miR-103a-3p regulates proliferation and apoptosis of mouse breast cancer through ETNK1.

We used stable transfected MDA-MB-231 cells to construct breast cancer xenografts in nude mice, the group name of which was: antagonist NC + sh-NC, miR-103a-3p antagonist + sh-NC, miR-103a-3p antagonist+sh-ETNK1. (A) The mouse tumor was dissected and photographed. (B) Tumor volume and (C) weight were measured. (D) QRT-PCR was performed to qualify the related factors in tumor tissues. (E) *ETNK1* expression was analyzed by IHC. (F) The apoptosis level was detected by TUNEL staining. ***, $P < 0.001$

Discussion

Benefit from the continuous progress of science and technology and the deepening of research, the treatment methods of breast cancer are also developing gradually. The level and effect of surgery, chemotherapy, radiotherapy and endocrine therapy for breast cancer have been prominently improved, as well as the survival rate of patients is also improving year by year (25). Nevertheless, human health is still threatened and troubled by the new occurrence, recurrence and metastasis of breast tumors (26, 27). Therefore, exploring the mechanism of occurrence, development and prognosis of breast cancer is still a hot topic for researchers.

It is well known that miRNAs can regulate downstream signal pathways, activating or blocking downstream effector molecular signal transduction, through affecting the key genes expression in cancer (28, 29). With the deepening of research in recent years, miRNA has the ability to participate in cell proliferation, apoptosis, migration, invasion and other physiological processes, which have been confirmed (30, 31). There are 12 miRNAs were characterized, of which 4 miRNAs (miR-139-5p, miR-425-5p, miR-145, and miR-130a) had high diagnostic accuracy (95%) (32). Furthermore, miR-216b can enhance the apoptosis and autophagy level of breast cancer through mediating mTOR pathway (33). CircRNA FOXC2 elevates the occurrence of breast cancer through IGF2BP3/miR-370 axis (34). miR-103a-3p as a sponge of lncRNA MEG3 participated in endoplasmic reticulum stress and affected the progression of colorectal carcinoma (35). Su et al. found that lncRNA FGD5-AS1 can promote the progression of glioblastoma through miR-103a-3p and promote the proliferation and metastasis of tumor cells (19). Similar studies also include prostate carcinoma, as well as oral squamous cell carcinoma (36, 37). For all that, the regulated mechanism of miR-103a-3p in breast cancer is rarely reported.

ETNK1 is mainly involved in the first step of phosphorylating ethanolamine to phosphoethanolamine (38). Previous studies on *ETNK1* were mostly related to leukemia, *ETNK1* had recurrent missense somatic mutations in chronic myelomonocytic leukemia (39, 40). However, some studies have shown that *ETNK1* is associated with several malignancies (41, 42). miRNAs could confer a sensitivity of laryngeal and lingual squamous cell carcinoma cells to cisplatin exposure by modulating *ETNK1* (43). Similarly, Chang et al. and Li et al. proved *ETNK1* was related to the progression and prognosis of gastric cancer, as well as the sensitivity of chemotherapy (44, 45). However, the biological function of *ETNK1* has not been reported in breast cancer. Our study for the first time first reported that *ETNK1* was regulated by miR-103a-3p. Besides, it exploited a vital role in breast cancer.

Conclusion

Herein, with TCGA database analysis, we found that miR-103a-3p overexpression would lead to poor prognosis of breast cancer and reduce the survival rate of patients. Additionally, miR-103a-3p was markedly expressed in breast cancer tissues and cell lines. Subsequent data revealed that differentially expressed miR-103a-3p could affect the viability and apoptosis of MDA-MB-231, showing a cancer promoting effect. Through further molecular analysis and combined with cell salvage experiments, we confirmed that miR-103a-3p might exacerbate breast cancer by down-regulating *ETNK1* in vitro. Finally, by constructing a subcutaneous tumor model of nude mice with breast cancer, we verified that miR-103a-3p affecting breast cancer progression through *ETNK1* in vivo. This study is actively exploring potential biomarkers associated with breast cancer and their underlying mechanisms, which will help to prevent and diagnose tumors more comprehensively and provide new targets and theoretical basis for finding more effective treatment options.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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No funding was received in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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