TMEM189 as a target gene of MiR-499a-5p regulates breast cancer progression through the ferroptosis pathway

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MicroRNA (miR)-499a-5p has been reported to regulate the progression of various tumours. However, the role of miR-499a-5p in breast cancer is unclear. The purpose of this study was to investigate the role and mechanism of miR-499a-5p in breast cancer. The growth effect of miR-499a-5p on breast cancer cells was investigated by the CCK-8 assay, wound healing assay and Transwell invasion assay. The luciferase activity assay was used to verify the downstream targets of miR-499a-5p. The levels of GSH, MDA, and ROS were detected by kits. Quantitative real-time PCR and Western blot were used to determine the expression levels of TMEM189, COX-2, GPX4, and other related genes in cells. miR-499a-5p was down-regulated in MDA-MB-231 cells and was shown to reduced the viability, migration and invasion of MDA-MB-231 cells. Further studies revealed that TMEM189 is a target of miR-499a-5p. miR-499a-5p inhibited breast cancer cell growth by downregulating TMEM189. Furthermore, the down-regulation of TMEM189 promotes ferroptosis in breast cancer cells. The low expression of TMEM189 inhibited the development of breast cancer through the ferroptosis pathway. We have demonstrated for the first time that miR-499a-5p inhibits breast cancer progression by targeting the TMEM189-mediated ferroptosis pathway.

Key Words: miR-499a-5p, TMEM189, ferroptosis, breast cancer

B reast cancer is the most common malignancy and the leading cause of cancer death in women worldwide.[1] It is a metastatic cancer that can often metastasise to distant organs such as the brain, bone, liver, lung, and distant lymph nodes.[2] Although the existing treatment methods have achieved good curative effects, it is still extremely difficult to cure advanced metastatic breast cancer.[3] The overall 5-year relative survival rate for patients with metastatic breast cancer is only 27%.[4] The development of genomics has made molecular therapy an emerging modality for early cancer screening and treatment.[5] Early detection of the disease can effectively prevent breast cancer and lead to good prognosis and high survival rates.[6] Therefore, there is an urgent need to determine the underlying mechanisms of breast cancer development and to develop more therapeutic targets for it.

miR-499a-5p is a class of microRNAs (miRNAs) with a length of 18~25 nt and plays an important role in various diseases, especially malignant tumors.[7] It has been reported that miR-499a-5p is significantly downregulated in endometrial cancer tissues and targets VAV3 to inhibit endometrial cancer growth and metastasis.[8] miR-499a-5p inhibits proliferation, invasion, migration and epithelial-mesenchymal transition by targeting eIF4E, and enhances the radiosensitivity of cervical cancer cells.[9] In addition, miR-499a-5p also inhibits tumour growth in non-small cell lung cancer.[7] In breast cancer, there is a significant correlation between miR-499a-5p and breast cancer risk.[10] However, the mechanism by which miR-499a-5p regulates breast cancer progression has not been investigated.

TMEM189 is a transmembrane protein containing a conserved motif and eight conserved histidine residues. This gene encodes plasma ethanolamine desaturase, which introduces vinyl ether double bonds into plasmalogens.[11] TMEM189 is involved in lipid biosynthesis, especially ether lipids.[12] The dynamic regulation of ether lipid biosynthesis alters the sensitivity of cancer cells, neurons, and cardiomyocytes to ferroptosis.[13] Ferroptosis is a novel iron-dependent form of cell death triggered by accumulation of lipid peroxides and reactive oxygen species (ROS).[14,15] Ferroptosis is often inhibited in various tumours, resulting in the uncontrolled proliferation of tumor cells.[16] Studies have shown that TMEM189 inhibits ferroptosis, and the deletion of TMEM189 sensitises cells to RSL3-induced ferroptosis.[17] In addition, the results from the genomics database showed that TMEM189 was highly expressed in various human cancers, and high levels of TMEM189 correlated with the high expression of GPX4.[17] Therefore, TMEM189 may regulate tumour progression through the ferroptosis pathway.

This study aimed to investigate the role and mechanism of miR-499a-5p and TMEM189 in the occurrence and development of breast cancer. We found that miR-499a-5p was significantly down-regulated in breast cancer cells while TMEM189 was remarkably up-regulated in breast cancer cells. miR-499a-5p directly targeted and negatively regulated TMEM189. miR-499a-5p promoted ferroptosis in breast cancer cells by downregulating the level of TMEM189, thereby inhibiting the excessive growth of breast cancer cells. miR-499a-5p and TMEM189 provide a preliminary theoretical basis for the clinical treatment of breast cancer.

Materials and Methods

Cell culture and transfection. Human breast cells (MCF-10) and human breast cancer cells (MDA-MB-231, MCF-7, and SUM159) were purchased from the Chinese Academy of Sciences typical culture preservation Center (Shanghai, China). The breast cells MCF-10 was cultured in RPMI 1640 medium (Hyclone, Logan, UT) and breast cancer cells (MDA-MB-231, MCF-7, and SUM159) were cultured in DMEM medium (Hyclone). The above media were supple-

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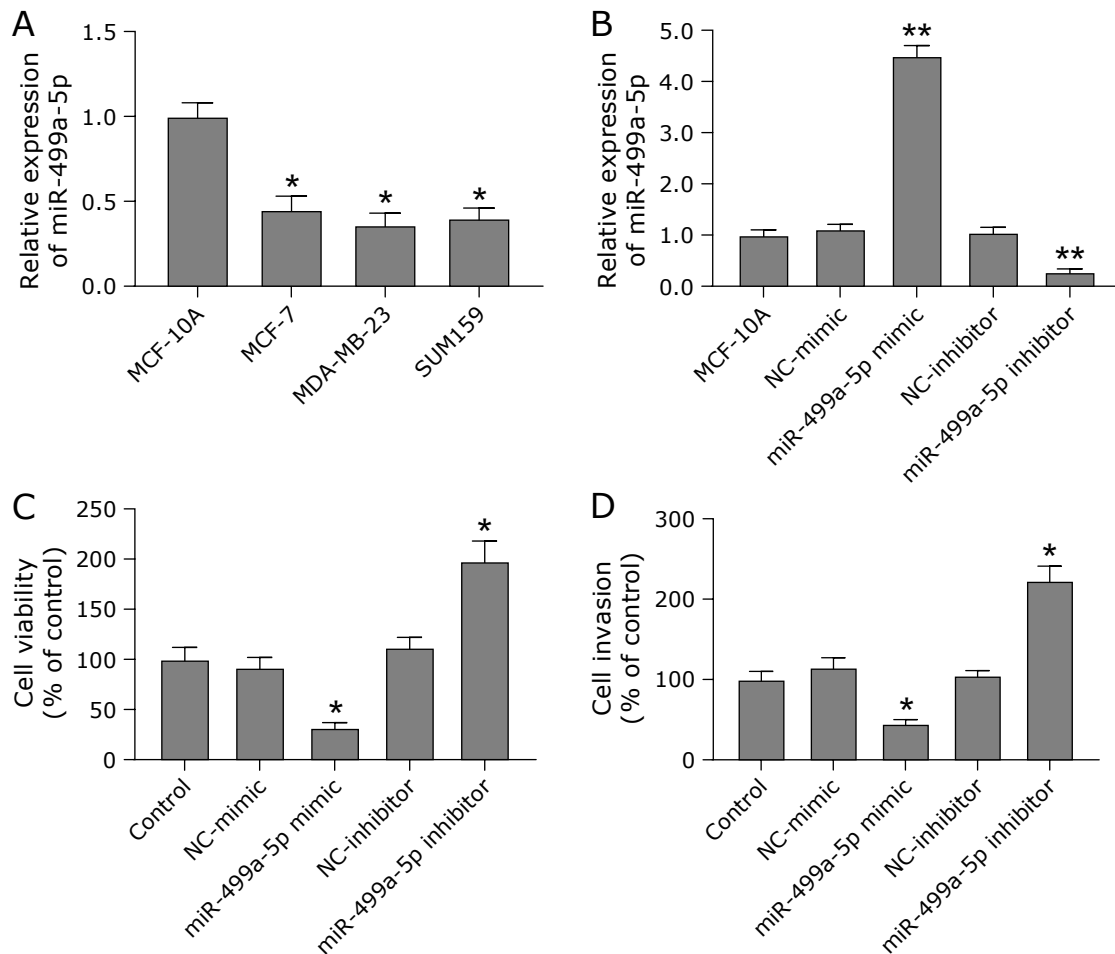J. Clin. Biochem. Nutr. | **September 2023** | vol. 73 | no. 2 | 154–160

Fig. 1. miR-499a-5p inhibited breast cancer cell growth. (A) RT-qPCR was used to detect the mRNA levels of miR-499a-5p in breast cells (MCF-10A) and breast cancer cells (MCF-7, MDA-MB-231, and SUM159). (B) Efficiency of overexpression and underexpression of miR-499a-5p. (C) Cell viability was measured by CCK-8 assay. (D) The invasive ability of cells in each group was determined by transwell assay. *$p<0.05$, **$p<0.01$.

mented with 10% foetal bovine serum (FBS) (Gibco, Grand Island, NY) and 1% penicillin-streptomycin (Procell Life, Wuhan, China). Cells were incubated at 37°C and 5% CO_2. All cells were regularly tested for mycoplasma with good results. miR-499a-5p mimic, miR-499a-5p inhibitor, PcDNA3.1-TMEM189, si-TMEM189 and negative control (GenePharma, Shanghai, China) were synthesised. miR-499a-5p mimic (forward 5'-GACACGGCTCCGTCAC-3'; reverse 5'-ATCCCT GATCAACTGTCCGCC-3') or its negative control (NC mimic) (forward 5'-AACCAGGCCTCCACCG-3'; reverse 5'-GCCACG CATGTCTTATACTGC-3'). miR-499a-5p inhibitors (5'-AAA CAUCACUGCAAGUCUUAA-3'); Negative control inhibitors (5'-CAGUACUUUUGUGUAGUACAAA-3'). When the cells reached 70% confluence, MDA-MB-231 cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The specific steps are carried out according to the manufacturer's instructions.

CCK-8 assay. Cells were seeded into 96-well plates at a density of 1×10^5 cells per well, and the cell viability of MDA-MB-231 was determined by CCK-8 method. Briefly, 10 μl of CCK-8 reagent (Beyotime, Beijing, China) was added to each well and incubated for 4 h. Absorbance was measured at a wavelength of 450 nm.

Wound healing assay. Cells grown to 90% confluency in six-well plates were used for wound healing assays. The cells were scraped with a 200 μl micropipette to create an even

scratch. Damaged cells were washed away with PBS and incubated in the incubator. Images were taken at 0 h and 24 h of the experiment to observe cell migration.

Transwell invasion assay. Invasion assays were performed using Matrigel (Sigma, St. Louis, MO)-coated Transwell wells (Corning, Batford County, Corning, NY). Then, 100 μl of cell suspension in serum-free medium was added to the upper chamber and 600 μl of medium containing 10% FBS was added to the lower chamber. Cells incubated for 24 h were fixed and then stained with crystal violet (Beyotime) for 15 min. Five different fields of view were selected for each well for photographing and counting.

Luciferase reporter gene assay. The binding sites of miR-499a-5p and TMEM189 were predicted by TargetScan software. TMEM189 WT, TMEM189 MUT, miR-499a-5p mimic, miR-499a-5p inhibitor and control vectors (GenePharma) were designed and constructed. Next, pmirGLO-TMEM189-WT and pmirGLO-TMEM189-Mut plasmids were co-transfected into MDA-MB-231 with miR-499a-5p mimic and mimic-NC. Cell lysates were collected 48 h after transfection. Luciferase activity was assessed using a dual-luciferase reporter gene assay system (Promega, Madison, WI). In this experiment, renilla luciferase activity was selected as the internal reference.

Determination of GSH, MDA and ROS levels. The level of GSH was measured using a total glutathione detection kit (Beyotime). The Cell Malondialdehyde (MDA) assay kit
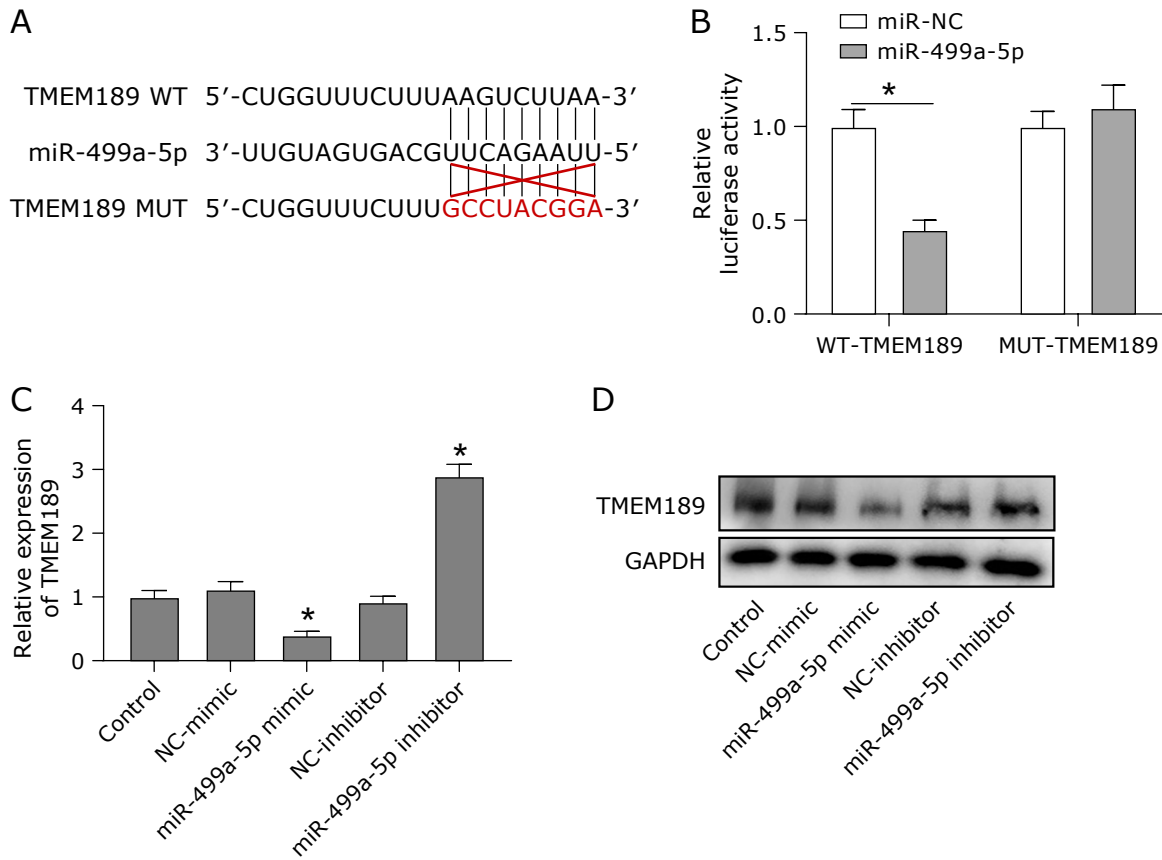
Fig. 2. miR-499a-5p directly targeted and negatively regulated TMEM189. (A) TMEM189 was predicted to have a binding site for miR-499a-5p by bioinformatics software. (B) Relative luciferase activity levels of TMEM189. (C) The protein expression level of TMEM189 under the condition of overexpression or underexpression of miR-499a-5p. (D) Quantification of protein results. *p<0.05.

(Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to detect the level of MDA. The level of ROS was discovered by reactive oxygen species detection kit (Nanjing Jiancheng Bioengineering Institute). The specific operation of the experiment was carried out according to the manufacturer's instructions.

RT-qPCR. Total RNA was extracted using TRIzol reagent (Invitrogen). RNA was reverse transcribed to cDNA using Qiagen One-Step RT-PCR kit (Qiagen Gmbh, Dusseldorf, Germany). cDNA, primers and SYBR™ Green PCR Master Mix (Thermo Fisher, Waltham, MA) were mixed and qRT-PCR was performed. GAPDH was used as an internal reference. The reaction conditions in the experiments were performed according to the manufacturer's instructions. The expression levels of target genes were determined using the $2^{-\Delta\Delta CT}$ method. The sequences of each primer are:
miR-499a-5p: F 5'-GCCCTGTCCCCTGTGCCTT-3',
R 5'-AAACATCACTGCAAGTCTT-3';
TMEM189: F 5'-GACCTACTTCTGCATCACCACAGTT-3',
R 5'-CGTTGTTGTCCAAGCATTCG-3';
GAPDH: F 5'-ACAGTCAGCCGCATCTTCTT-3',
R 5'-AAATGAGCCCCAGCCTTCTC-3'.

Western blot. Cells were lysed using RIPA buffer to extract total protein. The protein concentration was detected by BCA method. Total proteins were separated using 10–12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked with 5% nonfat milk containing 0.05% Tween-20. PVDF was blocked with different primary antibodies overnight at 4°C and secondary antibodies for 2 h at room temperature. Bands were detected using

the ECL Western blotting kit (Thermo Fisher). The luminosity of the bands was quantified using the ImageJ analysis system.

Statistical analysis. We used GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) for statistical analysis in this study. All experiments were conducted in triplicate, and the data are presented as the mean ± SD. Comparisons between groups were made using Student's t test, and multiple comparisons were made using a one-way analysis of variance, followed by Turkey's test. For all comparisons, p<0.05 was considered statistically significant.

Results

miR-499a-5p inhibited breast cancer cell growth. To determine the role of miR-499a-5p in breast cancer, we first determined the level of miR-499a-5p in breast cancer cells. The mRNA level of miR-499a-5p was down-regulated in breast cancer cells compared with breast cancer cells (Fig. 1A). Next, we verified the effect of miR-499a-5p on the growth of MDA-MB-231 cells. The overexpression and underexpression of miR-499a-5p were efficient (Fig. 1B). After the overexpression of miR-499a-5p, the viability and cell invasion ability of MDA-MB-231 cells were decreased. After the low expression of miR-499a-5p, the viability and invasion ability of MDA-MB-231 cells were enhanced (Fig. 1C and D). These data suggest that miR-499a-5p may have an important role in breast cancer progression.

miR-499a-5p directly targeted and negatively regulated TMEM189. We predicted the downstream targets of miR-499a-5p by Targetscan software (https://www.targetscan.
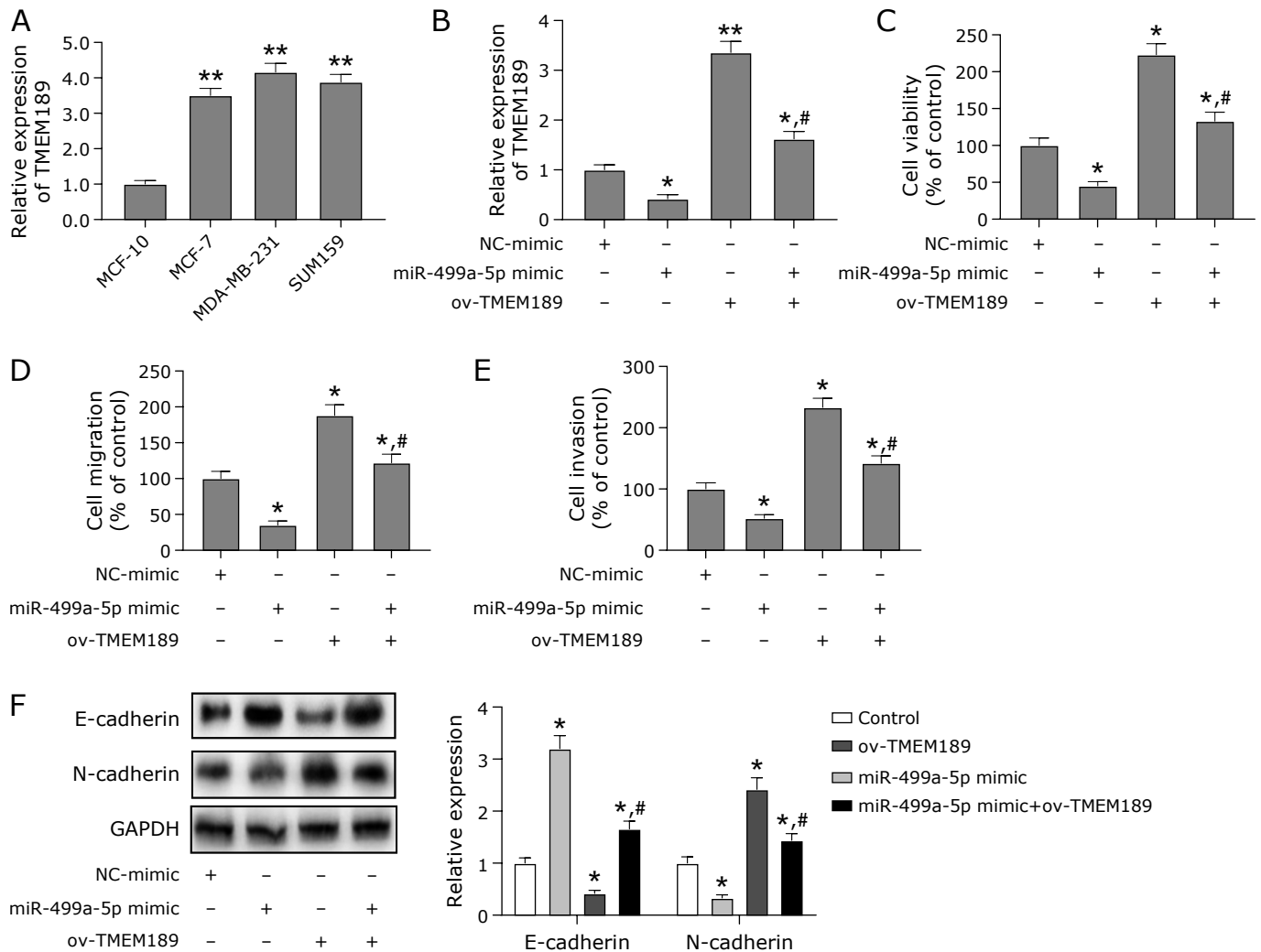
Fig. 3. miR-499a-5p inhibited breast cancer cell growth by downregulating TMEM189. (A) RT-qPCR was used to detect the mRNA and protein levels of TMEM189 in breast cells (MCF-10A) and breast cancer cells (MCF-7, MDA-MB-231, and SUM159). (B) Efficiency of overexpressing miR-499a-5p and TMEM189. (C) Cell viability was detected by CCK-8. (D) The migration ability of cells in each group was tested by wound healing assay. (E) The invasive ability of cells in each group was determined by transwell assay. (F) Protein expression levels of epithelial-mesenchymal transition (EMT)-related proteins (E-cadherin and N-cadherin). *$p<0.05$, **$p<0.01$, #$p<0.05$.

org/vert_71/). TMEM189 had a binding site for miR-499a-5p (Fig. 2A). The dual luciferase reporter gene assay showed that the miR-499a-5p mimic significantly reduced the luciferase activity of TMEM189-WT cells, while the miR-499a-5p mimic had no significant effect on the luciferase activity of TMEM189-MUT cells (Fig. 2B). This result suggested that miR-499a-5p has a physical interaction with TMEM189. After the transfection of miR-499a-5p mimics into MDA-MB-231 cells, the protein level of TMEM189 was significantly inhibited. In contrast, the inhibition of miR-499a-5p significantly increased the protein level of TMEM189 (Fig. 2C and D). These results suggest that miR-499a-5p directly targets and negatively regulates TMEM189.

miR-499a-5p inhibited breast cancer cell growth by downregulating TMEM189. Next, we examined the expression level of TMEM189 in MDA-MB-231 cells. Both mRNA and protein levels of TMEM189 were up-regulated in breast cancer cells compared with breast cancer cells (Fig. 3A). To further explore whether miR-499a-5p affects the growth of breast cancer cells by regulating TMEM189, we overexpressed miR-499a-5p and TMEM189 with good transfection efficiency (Fig. 3B). The

overexpression of miR-499a-5p inhibited the viability, cell migration and invasion ability of breast cancer cells (Fig. 3C–E). However, the overexpression of TMEM189 promoted the growth of breast cancer cells and reversed the inhibitory effect of miR-499a-5p mimic on the cells (Fig. 3C–E). These results suggest that miR-499a-5p inhibited breast cancer cell growth by downregulating TMEM189. E-cadherin and N-cadherin are markers of epithelial mesenchymal transformation (EMT), which is related to the increased risk of cancer metastasis.[18] Therefore, we detected the expression of these two proteins. We found that miR-499a-5p also increased the expression level of E-cadherin protein and decreased the protein expression level of N-cadherin by downregulating TMEM189 (Fig. 3F).

Low expression of TMEM189 induced ferroptosis in breast cancer cells. Ferroptosis is one of the effective ways to induce tumour cell death.[19] This experiment continued to explore the relationship between TMEM189 and ferroptosis. Compared with the Erastin + si-RNA group, the ferroptosis process of cells was promoted to a greater extent after knockdown of TMEM189. It was manifested as decreased GSH levels, and increased ROS level and MDA levels. In addition, the low expression of
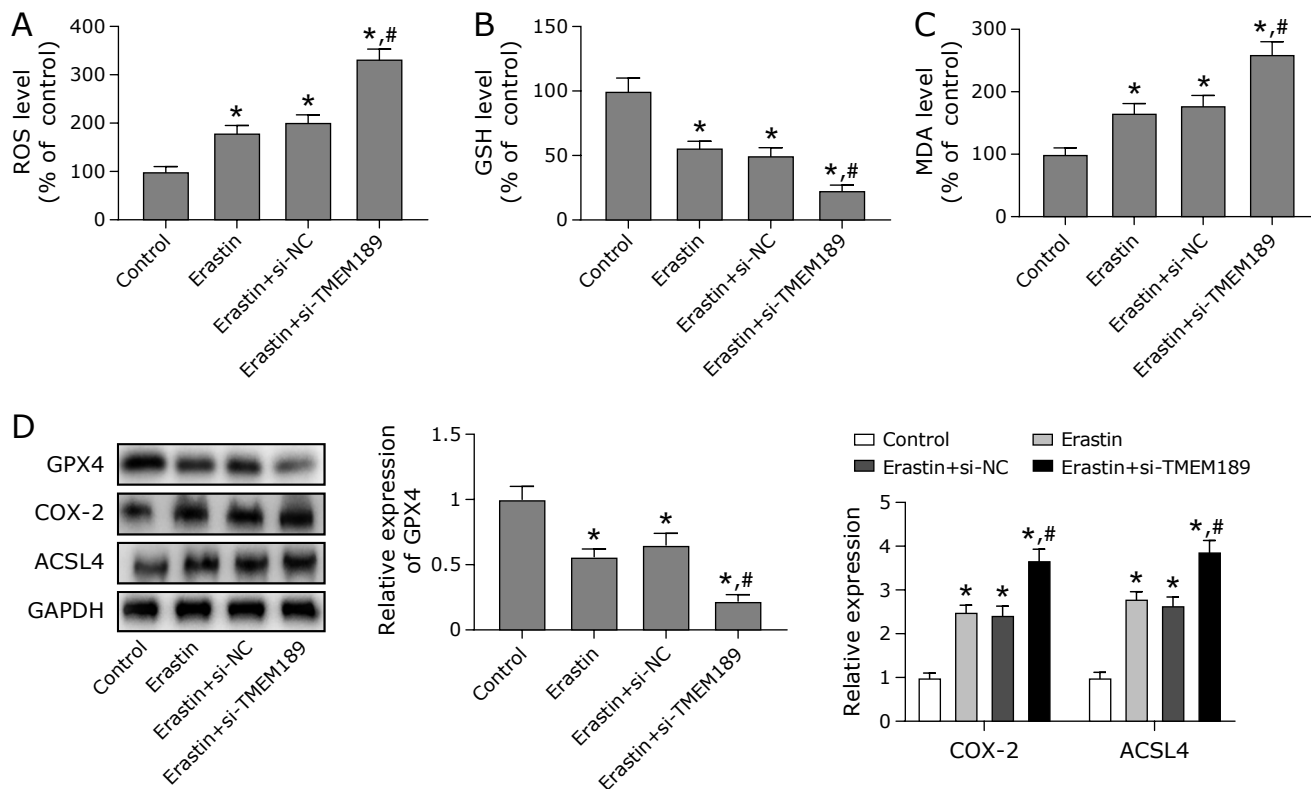
Fig. 4. Low expression of TMEM189 induced ferroptosis in breast cancer cells. MDA-MB-231 cells were transfected with si-RNA, si-TMEM189, and treated with a certain concentration of Erastin for 24 h. (A) Detection of ROS levels in each group of cells. (B) The level of GSH was detected using a kit. (C) The level of lipid peroxidation was detected using a malondialdehyde (MDA) kit. (D) Western blot was used to tested the protein expression levels of ferroptosis-related factors (COX-2, GPX4, and ACSL4). *$p < 0.05$, #$p < 0.05$.

TMEM189 also down-regulated the protein level of GPX4 and up-regulated the levels of COX-2 and ACSL4 (Fig. 4). The above results indicated that the low expression of TMEM189 promotes ferroptosis in breast cancer cells.

Low expression of TMEM189 inhibited breast cancer development through the ferroptosis pathway. Next, we treated cells which poorly expressed TMEM189 with ferroptosis inhibition (ferrostatin-1). Compared with the si-NC group, the knockdown of TMEM189 resulted in the decreased viability, migration ability and invasion ability of MDA-MB-231 cells. This was reversed after the administration of ferrostatin-1 (Fig. 5). Ferrostatin-1 significantly inhibited ferroptosis promotion and growth inhibition by si-TMEM189. In addition, the results showed that the ferrostatin-1 reversed the reduction of viability, migration and invasion of MDA-MB-231 cells caused by overexpression of miR-499a-5p (Supplemental Fig. 1*).

Discussion

Accumulating evidence suggests that miRNAs can regulate human tumour development by mediating the expression of their targets and may serve as future targets for gene therapy.[20,21] In cancer, miRNA overexpression or downregulation affects the metabolic state, tumor progression and metastasis of tumour cells by altering internal cellular pathways.[22] Studies have found that the abnormal expression of miRNAs in epigenetics is related to the occurrence and development of breast cancer.[23–25] The role of miR-499a-5p in multiple cancers has been demonstrated; however, its specific role in breast cancer is unclear. In the present study, we investigated the mechanism of action of miR-499a-5p in breast cancer cells.

Our study found that miR-499a-5p was down-regulated in MDA-MB-231 breast cancer cells. The overexpression of miR-499a-5p reduced the cell viability, migration and invasion ability of MDA-MB-231. The underexpression of miR-499a-5p promoted the growth of breast cancer cells. E-cadherin and N-cadherin are markers of epithelial mesenchymal transition (EMT). The change of cadherin expression is called "cadherin conversion". The down-regulation of E-cadherin expression is a sign of EMT and also a sign of tumor cell metastasis.[18] In this study, miR-499a-5p increased the expression of E-cadherin and decreased the expression of N-cadherin by downregulating TMEM189. These results suggested that miR-499a-5p may act as a tumour suppressor in breast cancer development, inhibiting the metastasis of breast cancer cells. In previous reports, we found that miR-499a-5p plays a dual role in cancer. miR-499a-5p was significantly down-regulated in endometrial and cervical cancer cells, and inhibited the proliferation and metastasis of cancer cells.[8,9] On the other hand, studies have shown that miRNA-499a-5p exhibits the role of a tumour-promoting factor. miRNA-499a-5p has been reported to promote lung cancer cell proliferation, migration and epithelial-mesenchymal transition (EMT) *in vitro* and tumour growth *in vivo*.[26] miRNA-499a-5p also promotes 5-fluorouracil resistance and cell proliferation by targeting PTEN to activate the PI3K/Akt signalling pathway in pancreatic cancer.[27] miR-499a-5p exhibits different roles in different tumours, possibly because it acts on different molecular targets. This issue still needs further research. Taken together, our results suggested that miR-499a-5p is able to inhibit the over-proliferation of breast cancer cells and has a tumour suppressor effect.

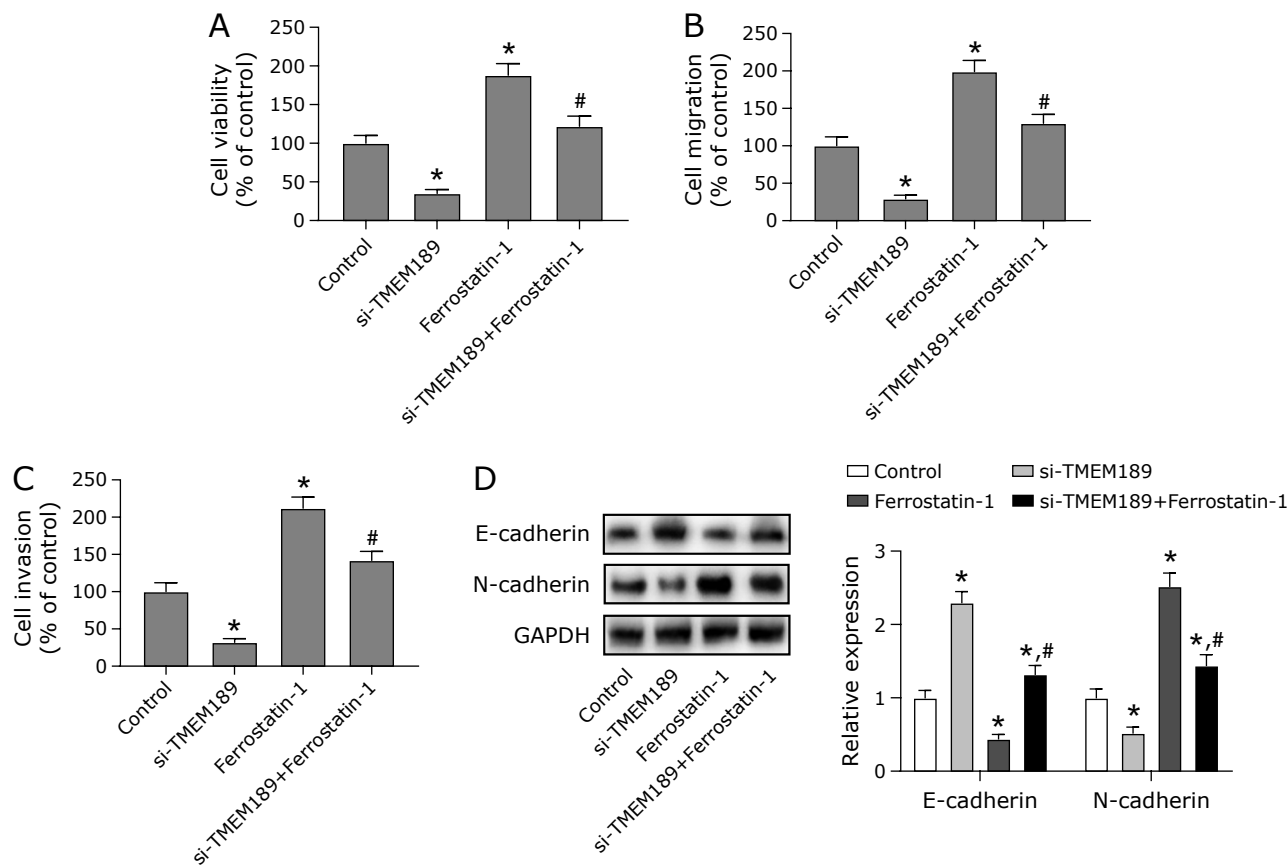Subsequently, TMEM189 was identified as a downstream

Fig. 5. Low expression of TMEM189 inhibited breast cancer development through the ferroptosis pathway. MDA-MB-231 cells were transfected with si-RNA and si-TMEM189, and cells were treated with a certain concentration of ferrostatin-1 for 24 h. (A) Cell viability was measured by CCK-8. (B) The migration ability of cells in each group was detected by wound healing assay. (C) The invasive ability of cells in each group was determined by transwell assay. (D) Protein expression levels of epithelial-mesenchymal transition (EMT)-related proteins (E-cadherin and N-cadherin). $*p<0.05$, $\#p<0.05$.

target of miR-499a-5p, and its level was upregulated in breast cancer cells. miR-499a-5p inhibited the growth of breast cancer cells by down-regulating the expression of TMEM189. It has been reported that TMEM189 is highly expressed in various human cancers, and high levels of TMEM189 promote tumour progression.[17] It has been reported that TMEM189 mediates iron ptosis of breast cancer cells by inhibiting autophagy, thereby reducing the proliferation of breast cancer cells and inhibiting tumor growth.[28] This is consistent with our results. The tumour-promoting activity of TMEM189 is inseparable from its modulating activity of plasmalogen. TMEM189 is generally thought to be involved in lipid desaturation and encodes the plasma phenethanolamine desaturase which is necessary for vinyl ether bond formation.[29] TMEM189-deficient mice exhibited a deficiency of plasma ethanolamine desaturase activity and plasmalogen.[11] Studies have shown that plasmalogens can stimulate cell growth, regulate cell survival and tumour growth.[30] Higher levels of plasmalogen are associated with an increased risk of breast cancer.[31] Therefore, we speculate that TMEM189, which has a plasmalogen-regulated effect, may affect breast cancer progression. This study is the first to demonstrate that low expression of TMEM189 can inhibit the viability, migration and invasion of breast cancer cells.

Increasing studies have shown that ferroptosis is involved in tumour progression. The induction of ferroptosis not only promotes tumour cell death, but also increases the anticancer activity of chemotherapeutic drugs.[32] Similarly, in breast cancer research, inhibition of the ferroptosis pathway promotes the migration and invasion of breast cancer cells and tumour growth *in vivo*.[33,34] The induction of ferroptosis in breast cancer cells can prevent further tumour development.[35,36] Therefore, enhancing ferroptosis in breast cancer cells is considered a promising strategy for the treatment of breast cancer. Recent studies have shown that the FAR1-ether lipid-TMEM189 axis is an important pathway driving ferroptosis susceptibility. TMEM189 inhibits ferroptosis in tumr cells, and the knockdown of TMEM189 promotes ferroptosis.[17] This provides direction for the research of TMEM189 in breast cancer. In the present study, we demonstrated that the poor of TMEM189 inhibited the proliferation, migration and invasion of breast cancer cells by promoting the ferroptosis pathway. Therefore, TMEM189 can be regarded as a tumour-promoting factor.

In conclusion, we demonstrate for the first time that miR-499a-5p inhibits breast cancer cell growth by promoting ferroptosis by downregulating TMEM189 expression. miR-499a-5p and TMEM189 may be new biomarker molecules for breast cancer, which will provide a preliminary theoretical basis for the clinical treatment of breast cancer.

Author Contributions

Acknowledgments

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest

No potential conflicts of interest were disclosed.

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