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LncRNA-MALAT1 promotes triple-negative breast cancer progression and function as ceRNA to target REEP5 by sponging miR-106a-5p

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Abstract

Axillary lymph node metastasis (ALNM) in triple negative breast cancer (TNBC) will lead to poor prognosis. Recent studies have shown that long non-coding RNAs (lncRNAs) were involved in the progression of tumors. This study aimed to explore the role and mechanism of lncRNA-MALAT1 in the progression of TNBC and its relationship with ALNM. MALAT1 is highly expressed in TNBC cells lines, tumor tissues and serum, and it is positively correlated with the degree of ALNM. In addition, MALAT1 can act as a competitive endogenous RNA (ceRNA) that regulates cellular biological behavior by competitively binding to miR-106a-5p with REEP5. In conclusion, our results show that MALAT1 could function as ceRNA promote the proliferation, invasion and metastasis of TNBC cells through MALAT1/miR-106a-5p/REEP5 axis, which is expected to provide new ideas for the diagnosis of TNBC in clinic.

Keywords Triple negative breast cancer (TNBC), Long non-coding RNA (lncRNA), Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), Competing endogenous RNA (ceRNA), Biomarkers, Prognosis

Introduction

The global cancer burden data in 2022 shows that the incidence of breast cancer is 2.31 million, accounting for 11.6% of malignancy, which is the second highest incidence cancer in the world [1]. Breast cancer is divided into four subtypes: Luminal A, Luminal B, Her-2 positive, and triple-negative [2]. Triple-negative breast cancer (TNBC) refers to estrogen receptor (ER), progesterone receptor (PR) and Her-2 receptor all negative, accounting for 10%–20% of breast cancer. Compared with other subtypes, TNBC has a higher degree of malignancy and poorer treatment outcomes [3–6]. The metastatic status of axillary lymph nodes (ALNs) is an important factor affecting the prognosis of TNBC patients, and the ALNs is the most easily metastatic site for TNBC patients [7]. Hence, understanding the molecular mechanisms associated with TNBC occurrence is of vital significance.

In recent years, with the continuous development and maturity of sequencing technology, molecular markers for cancer diagnosis have emerged continuously [8]. Long non-coding RNAs (lncRNAs) have become a hot spot in this field. LncRNAs were defined as a type of RNA more than 200 nucleotides in length and have no capacity of

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protein coding [9]. A growing number of evidence has proved that the dysregulated expression of lncRNAs was involved in the occurrence, development, and metastasis of tumors, including TNBC [10]. Competing endogenous RNA (ceRNA) hypothesis suggests that lncRNA can "neutralize" miRNAs through complementary pairing, thereby restoring the function of target RNA molecules [such as messenger RNA (mRNA)] [11]. Numerous studies have shown that lncRNA-WEE2-AS1, lncRNA-ASMTL-AS1 and lncRNA-MALAT1 can function as competing endogenous RNAs (ceRNAs) to regulated the expression of specific genes through sponge microRNAs (miRNAs), and thus influenced the malignant phenotypes of TNBC cells [12–14]. A deeper understanding of the expression pattern and mechanisms of lncRNAs is expected to provide new biomarkers for the diagnosis of TNBC.

miRNA is a single-stranded RNA molecule that is 20–23 nucleotides long and plays vital roles in many biological processes. These molecules typically reduce the translation and stability of messenger RNAs [15]. miRNA could combine with the complementary sequence of the 3′-untranslated region (3′-UTR) of the target gene mRNA, leading to mRNA degradation and/or translation blockade [16]. miR-106a-5p has been studied in many cancers including ovarian cancer, colorectal cancer, breast cancer [17–19]. However, miR-106a-5p and its interaction with lncRNA MALAT1 in TNBC are still largely unknown.

Receptor expression-enhancing protein (REEP) is an evolutionarily conserved protein family, which includes the REEP1-4 and REEP5-6 subfamilies [20]. They are involved in numerous pathological and physiological processes such as ER morphogenesis and remodeling, microtubule cytoskeleton regulation, and the development and progression of cancer [21–23]. For instance, a study by Park et al. revealed that REEP5 and REEP6 proteins are capable of refining CXCR1-mediated cellular responses, significantly promoting the proliferation and migration of lung cancer cells, thereby facilitating the initiation and progression of lung cancer [24]. Besides, Isyraqiah et al. have found that REEP5 was high expression in gastric cancer cells, and it could promote the proliferation of cancer cells [25]. However, the correlation of REEP5 and lncRNAs in TNBC has not been well characterized.

Table 1 The primer sequences for RT-qPCR

Homo-MALAT1-F	CTCAAATCTTTCCACACGC
Homo-MALAT1-R	ACGGAGAACAACTCGCATC
Homo-REEP5-F	CTTCATCGCTCTTGGTGTC
Homo-REEP5-R	TCAGCCACTGGGTATCATC
hsa-miR-106a-5p-RT Primer	GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACC TACCT
hsa-miR-106a-5p-F	AAGCGGAAAAAGTGCTTACAGT
hsa-miR-106a-5p-R	CAGTGCAGGGTCCGAGGT

RT-qPCR real-time quantitative PCR, *MALAT1* Metastasis-associated lung adenocarcinoma transcript-1, *REEP5* Receptor expression-enhancing protein 5

Table 2 Comparison of MALAT1 expression between four subtypes of breast cancer with and without lymph node metastasis

Subtypes	Mean		FC	*p*
	Without lymph node metastasis	With lymph node metastasis		
TNBC	82.0645	86.3944	1.0528	0.0109
Luminal A	90.9534	90.6887	0.9971	0.5545
Luminal B	89.8829	91.1699	1.0143	0.5994
Her-2	89.6226	87.0583	0.9714	0.6725

Significance of MALAT1 expression level difference among subgroups of breast cancer with or without lymph node metastasis by independent sample *t*-test

MALAT1 metastasis-associated lung adenocarcinoma transcript-1, *TNBC* triple-negative breast cancer, *FC* fold change

In our study, we identified the expression profile of lncRNA MALAT1 in TNBC and elucidated its role and possible mechanisms in the occurrence and development of TNBC. Our study demonstrated that lncRNA MALAT1 is highly expressed in TNBC and it is positively correlated with axillary lymph node metastasis. Specifically, MALAT1 can act as a ceRNA by competitively absorbing miR-106a-5p to target REEP5, thereby promoting the proliferation, invasion, and migration abilities of TNBC cells. Our research findings indicate that MALAT1 is crucial in the progression of TNBC, which may serve as a potential biomarker for the diagnosis of TNBC.

Materials and methods

Bioinformatics analysis

UCSC xena database (https://xenabrowser.net/datapages/) and starbase database (http://starbase.sysu.edu.cn/index.php) contains RNA sequencing data for multiple types of cancer. Based on the above database, the RNA sequences and clinical features of the TCGA BRCA dataset were downloaded and analyzed.

Cell lines and cell culture

MDA-MB-231(Beina Biology, BNCC337894), MDA-MB-468 and MCF-10A (Donated by Dr. Wu Qin) were cultured in DMEM/F12 medium (Gibco, USA, 11320033) supplemented with 10% Fetal Bovine Serum (Excell Bio, China, FSP500) and 100 U/ml penicillin/streptomycin (Gibco, USA, 15070063), and maintained in a constant temperature cell incubator at 37 ℃ and 5% CO_2.

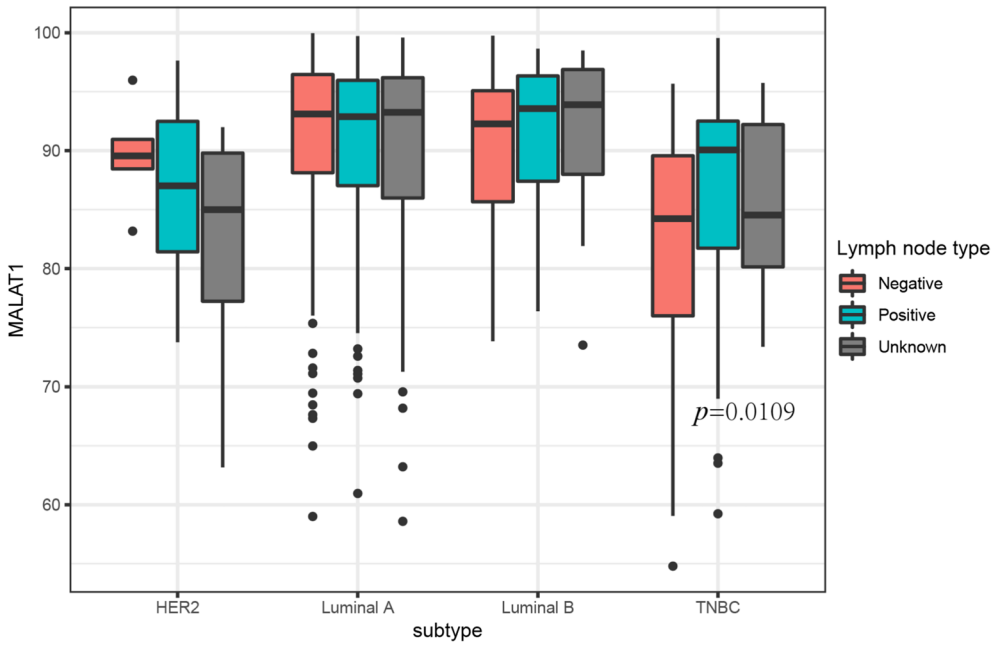

Fig. 1 Comparison of MALAT1 expression between four subtypes of breast cancer with and without lymph node metastasis. The statistical results are reflected in Table 2, which shows that the expression level of MALAT1 is significantly higher only in the TNBC group with lymph node metastasis than in the group without lymph node metastasis ($p = 0.0109$), while there is no statistical significance in the remaining subtypes. *MALAT1* metastasis-associated lung adenocarcinoma transcript-1, *TNBC* triple-negative breast cancer, *Negative* without lymph node metastasis, *Positive* with lymph node metastasis

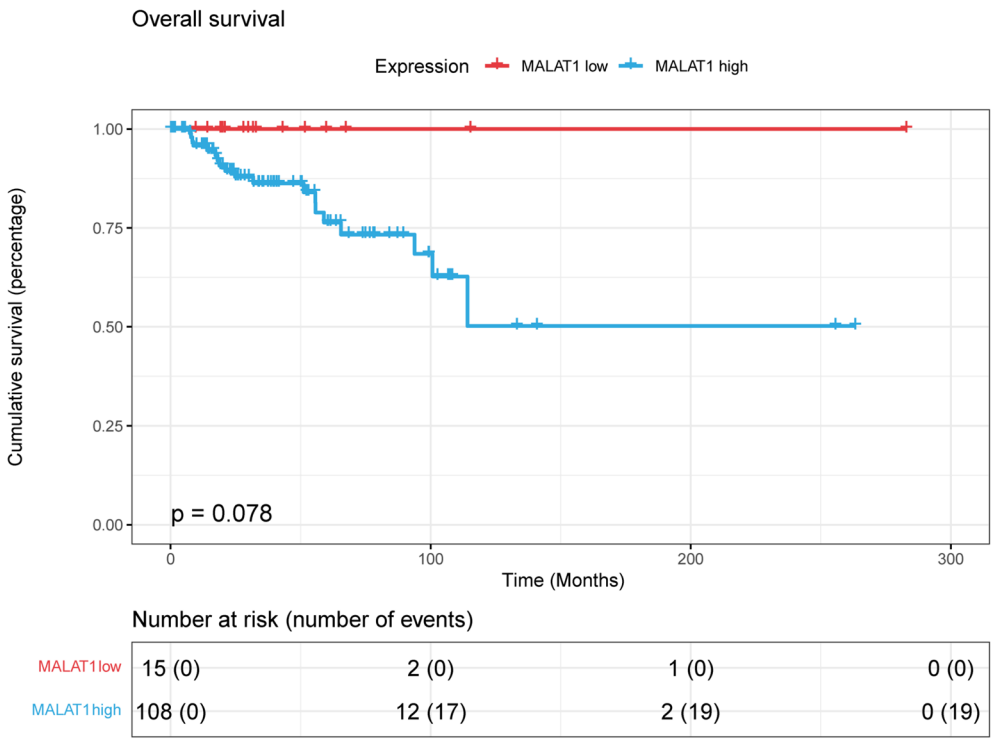

Fig. 2 The overall survival K–M curve of MALAT1 in TNBC. Number at risk: the number of patients who are still under observation and alive at the follow-up times of 0, 100, 200, and 300 months; Number of events: The cumulative number of deaths at the follow-up times of 0, 100, 200, and 300 months

Table 3 miRNAs significantly correlated with MALAT1 in TNBC

miRNAs	PCC	p
hsa-miR-145-5p	0.389158988	0.001134777
hsa-miR-106a-5p	−0.372734167	0.001894387
hsa-miR-204-5p	0.356852776	0.003034143
hsa-miR-338-3p	−0.338183572	0.005125473
hsa-miR-320c	0.332038825	0.006050064
hsa-miR-30b-5p	0.306104913	0.011761739
hsa-miR-378c	0.264095527	0.030808804
hsa-miR-346	0.263935622	0.030914036
hsa-miR-876-5p	−0.257518593	0.035392707
hsa-miR-26b-5p	0.257385148	0.035491297
hsa-miR-92b-3p	−0.255036612	0.037264201
hsa-miR-374a-5p	0.252463045	0.039290908
hsa-miR-499a-5p	0.249791293	0.041490791

MALAT1 metastasis-associated lung adenocarcinoma transcript-1, *TNBC* triple-negative breast cancer, *PCC* Pearson correlation coefficient

Cell transfection

MALAT1 shRNA (L10287, Changsha, China) and REEP5 shRNA (L10286, Changsha, China) were provided by Ruiying biological company to knockdown MALAT1 and REEP5 genes. The general biology company provided miR-106a-5p mimic and miR-106a-5p inhibitor (Anhui, China, RX052580) for miR-106a-5p gene overexpression and knockdown. Transfer the above-mentioned substances into cells according to the Exercise Transfer Agent manual. After 12 h of transfection, replace the culture medium and culture for 24 h before collecting cells.

Quantitative RT-PCR analysis

Extract total RNA from cells according to the experimental steps in the instructions of the RNA extraction kit (Generay, Shanghai, China, GK3016); reverse transcription of each group of total RNA was performed using reverse transcription kit (Vazyme, Nanjing, China, R212-02). RT-PCR was performed on a real-time fluorescence quantitative PCR instrument (DWB, Beijing, China, Accurate96×4). β-Actin serves as an internal reference gene for REEP5 and MALAT1; U6 serves as an internal reference gene for miR-106a-5p. Calculate the relative expression level of genes using the $2^{-\triangle\triangle Ct}$ method. The primer sequence for each molecule can be found in Table 1.

Western blot

Use 1 ml Western and IP cell lysate (Beyotime, China, P0013) to extract the total protein in the cell, then use BCS protein concentration test kit (Beyotime, China, P00125) to determine the protein concentration, use polyacrylamide gel electrophoresis to separate 30 ug total protein, and transfer to PVDF membrane with 200 mA constant current (Merck Millipore Ltd, Germany, IPVH00010). Subsequently, block with TBST solution containing 5% skim milk (1 h, room temperature) and incubate the PVDF membrane with REEP5 monoclonal antibody (abcom, England, Ab186755) overnight at 4 °C. Afterwards, we rinsed the membrane three times with TBST (10 min each time) and incubated them with Goat anti-Rabbit IgG (Lianke Biology, China, GAR0072) for 1 h. Then we use TBST to wash the membrane 3 times (10 min each time). Finally, X-ray development was performed using a highly sensitive ECL chemiluminescence detection kit (Beyotime, China, P0018S).

Transwell assay

Take logarithmic growth stage cells, digest with 0.25% Trypsin + 0.02% EDTA, then centrifuge and count; inoculate $1*10^5$ cells per well into the upper chamber of 24-well Transwell (Thermofish, USA, 140629), with a total volume of 200 µl. Add 600 µl 10% serum culture medium and 5% CO_2 to the lower chamber; Incubate and cultivate in a 37 ℃ incubator. After 24 h, PBS was cleaned once, fixed with 4% paraformaldehyde for 10 min and then stained with 0.1% crystal violet at room temperature for 10 min. After rinsing under sterile water, remove it and randomly select three fields for cell counting.

CCK-8 assay

Add 100 µl of CCK-8 working solution (10% CCK-8 solution + 90% DME/F12) to a 96-well plate (NEST, China, 100722BL1012B) and incubate in an incubator for 1 h; further determine the absorbance value at 450 nm using an enzyme-linked immunosorbent assay (PERLONG, Beijing, China, DNM-9602G).

Wound healing assay

Use a marker pen to draw six parallel straight lines on the back of the bottom of the six-hole plate. The cells in the logarithmic growth phase were digested and counted with 0.25% Trypsin + 0.02% EDTA. $5*10^5$ cells per well were inoculated into a six-well plate, and the corresponding 10% FBS medium was added. The total volume of each well was 2 ml, incubated at 37 ℃ in a 5% CO_2 incubator. After the cells adhere to the wall, use the 200 µl pipette tip to scratch perpendicular to the parallel line, separate the single cell layer from the middle to both sides, discard the culture medium, rinse 3 times with PBS, add serum-free culture medium, observe each group under a microscope, and select the migration situation of three areas after 24 h and take photos.

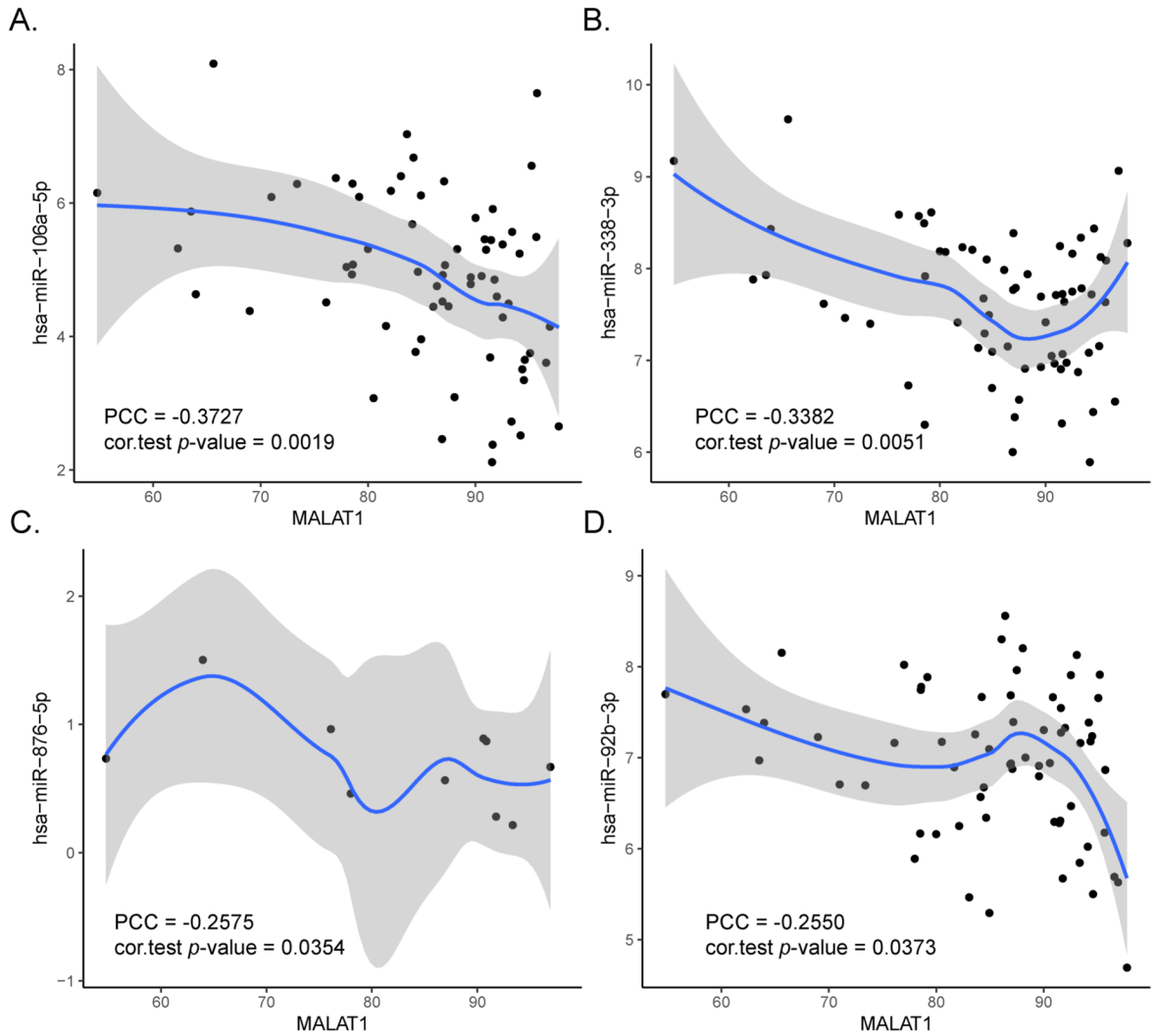

Fig. 3 Four miRNAs that significantly negatively correlated with MALAT1 in TNBC. **A** The PCC between miR-106a-5p and MALAT1 is -0.3727, $p = 0.0019$; **B** The PCC between miR-338-3p and MALAT1 is -0.3382, $p = 0.0051$; **C** The PCC between miR-876-5p and MALAT1 is −0.2575, $p = 0.0354$; **D** The PCC between miR-92b-3p and MALAT1 is −0.2550, $p = 0.0373$; *PCC* Pearson correlation coefficient

Dual-luciferase reporter assay

Conduct dual luciferase reporter gene experiments to investigate the binding relationship between miR-106a-5p and REEP5 (the REEP5 3′UTR sequence is provided in the supplementary documents). Blow and detach 293 T cells (Beina Bio, BNCC353535) adhering to the wall in the six-well plate well, centrifuge at 1500 r/min for 5 min, discard the supernatant, wash with PBS, centrifuge again, discard the supernatant, add 500 μl reporter gene cell lysate, mix well, lysis on ice for 5 min, 12,000 r/min, and centrifuge for 5 min. The supernatant is the sample solution to be tested. Take 100 μl sample solution to be tested and add it to the 96-well ELISA plate. Each group is made into 3 wells and then add 100 μl luciferase detection reagent. After mixing well, let it stand at room temperature in dark for 5 min, and measure the fluorescence intensity of firefly luciferase at 560 nm using a multifunctional fluorescence enzyme-linked immunosorbent assay (Thermofish, USA, varioskan LUX). Add 100 μl sea kidney luciferase detection working solution (sea kidney luciferase detection buffer: sea kidney luciferase detection substrate = 9:1) to various sample wells, and let it stand at room temperature in dark for 5 min. Measure the fluorescence intensity of sea kidney luciferase at 465 nm using a multifunctional fluorescence enzyme-linked immunosorbent assay.

Clinical samples

This study was approved by the Medical Ethics Committee of Zhejiang Cancer Hospital (Ethics Number: IRB-2022-143). Collect tumor and adjacent noncancerous

Table 4 mRNAs significantly positively correlated with MALAT1 in TNBC

Genes	Lymph node metastasis		FC	*p* (wilcox.test)	PCC	*p* (cor.test)
	Negative	Positive				
TP53INP1	67.6773	72.9449	1.0778	0.013	0.4695	0.0001
REEP5	90.595	92.827	1.0246	0.013	0.4524	0.0001
PAPD4	66.0185	69.1494	1.0474	0.0289	0.4241	0.0003
TOB1	77.7206	82.9948	1.0679	0.0235	0.4186	0.0004
NPHP3	45.8724	49.2936	1.0746	0.04	0.394	0.001
LMBR1L	48.6376	51.0633	1.0499	0.0406	0.3419	0.0046
ZNF721	53.88	57.805	1.0728	0.022	0.3125	0.01
DNAJC27	35.1336	37.1523	1.0575	0.0267	0.3089	0.011
GNB4	87.5355	84.4191	0.9644	0.0499	0.2983	0.0142
RNF103	72.7671	77.0609	1.059	0.0083	0.2942	0.0157
PCGF3	76.538	79.9039	1.044	0.0308	0.2905	0.0171
C5orf45	33.4958	36.3164	1.0842	0.015	0.2804	0.0215
PPARG	34.5026	38.9113	1.1278	0.0158	0.268	0.0284
C18orf1	72.0301	66.644	0.9252	0.0406	0.252	0.0397

MALAT1 metastasis-associated lung adenocarcinoma transcript-1, *TNBC* triple-negative breast cancer, *FC* fold change, *PCC* Pearson correlation coefficient

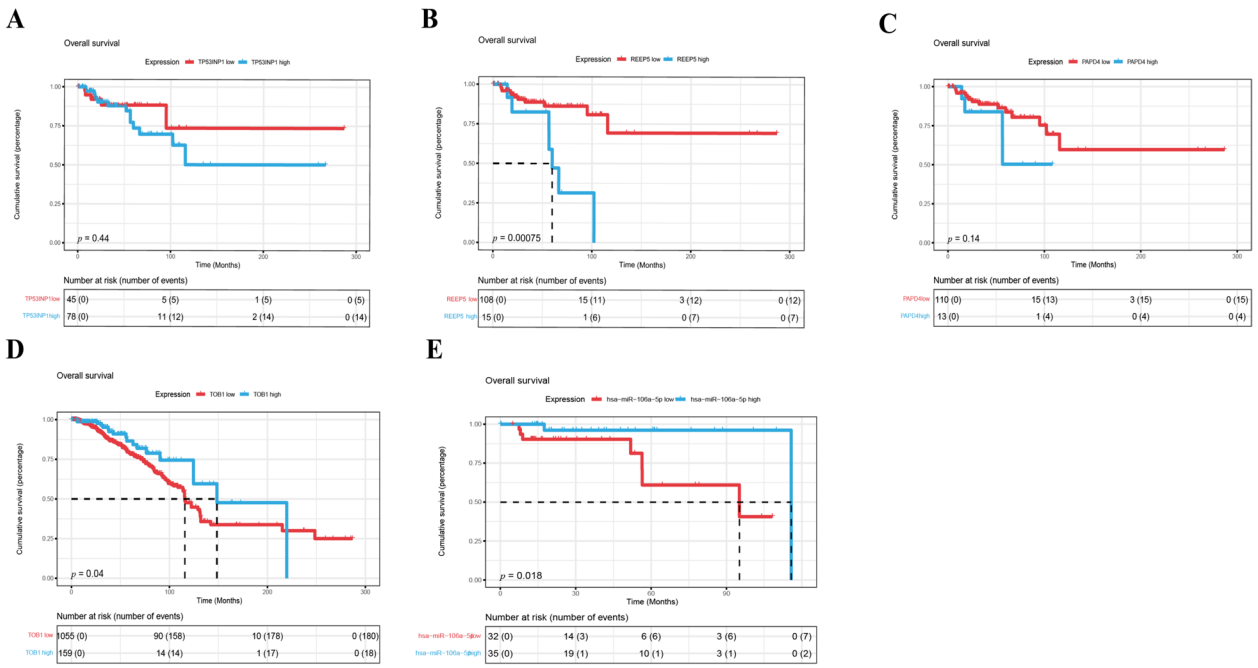

Fig. 4 Overall survival K-M curve of TP53INP1, REEP5, PAPD4, TOB1, and miR-106a-5p in TNBC. **A** There was no significant difference in OS between the high and low expression groups of TP53 ($p = 0.44$); **B** The group with high expression of REEP5 had a worse overall survival (OS) compared to the group with low expression ($p = 0.00075$); **C** There was no significant difference in OS between the high and low expression groups of PAPD4 ($p = 0.14$); **D** The group with low expression of TOB1 had a worse overall survival (OS) compared to the group with high expression ($p = 0.04$); **E** The group with low expression of miR-106a-5p had a worse overall survival (OS) compared to the group with high expression ($p = 0.018$); OS: Overall survival

breast tissue from 50 cases of TNBC patients, and collect serum from corresponding patients (20 healthy women as control group). The number of TNBC patients with no lymph node metastasis, 1–3 lymph node metastasis, and 4 or more lymph node metastases were 20, 20, and 10, respectively. All patients were diagnosed with invasive ductal carcinoma, and did not receive radiotherapy, chemotherapy, or other treatment before diagnosis.

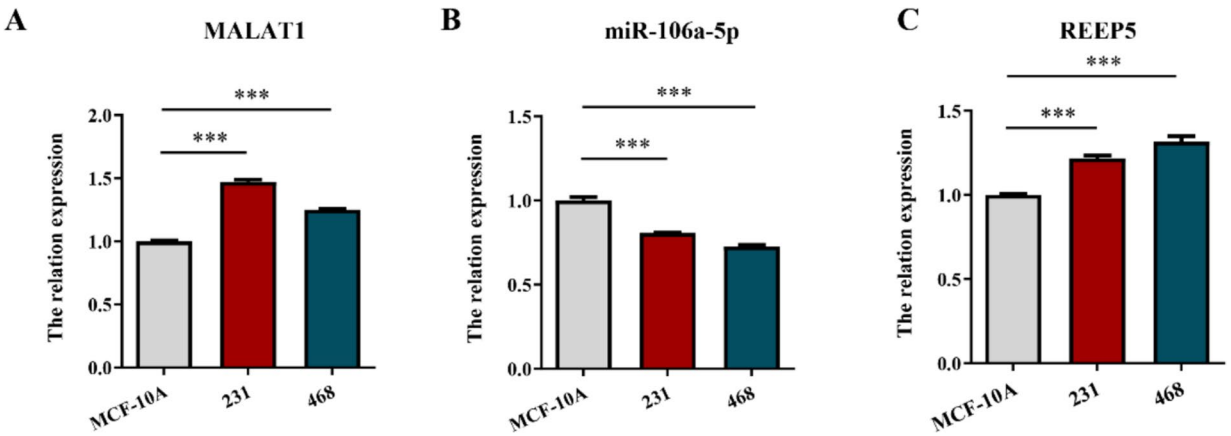

Fig. 5 Expression characteristics of target genes in TNBC cell lines. **A** The expression of MALAT1 in MCF-10A, MDA-MB-231, and MDA-MB-468 cells; **B** the expression of miR-106a-5p in MCF-10A, MDA-MB-231, and MDA-MB-468 cells; **C** the expression of REEP5 in MCF-10A, MDA-MB-231, and MDA-MB-468 cells; 231: MDA-MB-231; 468: MDA-MB-468; ***: $p < 0.001$

Immunohistochemistry (IHC)

IHC was performed using the two-step method kit from Beijing Zhongshan Biotechnology Co., Ltd. The REEP5 antibody was purchased from Abcam company, and the second antibody was purchased from Beijing Zhongshan Biotechnology. IHC staining is positive for yellow or yellowish brown, and nuclear staining is blue. Immune response score (ISR) was scored according to the percentage of positive cells (PP) [0 (0%), 1+ (≤25%), 2+ (26% −50%), 3+ (51% −75%), and 4+ (>75%)] and staining intensity (SI) [0 (No staining), 1+ (light yellow), 2+ (brown yellow), 3+ (brown)]. ISR refers to the product of SI and PP. Taking the median expression as the cutoff point, the high expression of REEP5 is determined when the ISR ≥ 8 and the low expression is determined when the ISR < 8.

Statistical analysis

All data are presented as $M \pm SD$ from at least three independent experiments. The software SPSS 25.0 and Prism 8.0 was used for statistical analysis. Statistical significance of differences between two groups was evaluated using Student's t test, and one-way analysis of variance (ANOVA) was used to determine the significance of differences among multiple groups. The differences with $p < 0.05$ were considered statistically significant.

Results

MALAT1/miR-106a-5p/REEP5 axis promotes the progression of TNBC (bioinformatics)

To explore the relationship between MALAT1 and lymph node metastasis in TNBC patients, the results showed that MALAT1 was significantly overexpressed in TNBC patients with lymph node metastasis (FC = 1.0528, $p = 0.0109$), and this relationship only existed in TNBC, which has not been reported in previous studies (Table 2, Fig. 1). Further conducted survival analysis on 123 TNBC patients with recorded prognosis data, we found that the cumulative number of deaths in the group with low MALAT1 expression was 0, while the cumulative number of deaths in the group with high MALAT1 expression was 19, indicating that high MALAT1 expression is a factor leading to poor overall survival (OS) (Fig. 2).

Initially, to identify miRNAs that have a targeting relationship with MALAT1, we discovered 13 significantly associated miRNAs through the Starbase database. Given that the ceRNA regulatory relationship requires the expression levels of lncRNA and miRNA to be inversely correlated, we found that 4 miRNAs met this criterion: hsa-miR-106a-5p, hsa-miR-338-3p, hsa-miR-876-5p, and hsa-miR-92b-3p. (Table 3, Fig. 3). Subsequently, we continued to use the Starbase database to collect mRNAs that exhibited a positive correlation with MALAT1 expression and a negative correlation with miRNA expression. By incorporating the factor of lymph node metastasis, we identified 14 genes with significantly differential expression. Further combining prognostic analysis, we found that only REEP5 conformed to the ceRNA regulatory mechanism of MALAT1 (Table 4), REEP5 showed a significant correlation with patient prognosis, the expression of upstream target gene miR-106a-5p of REEP5 is positively correlated with the prognosis of TNBC patients, which is consistent with expectations. MALAT1/ miR-106a-5p/REEP5 ceRNA axis was ultimately identified (Fig. 4).

These findings suggested that lncRNA MALAT1 might play an important role in the regulation of the

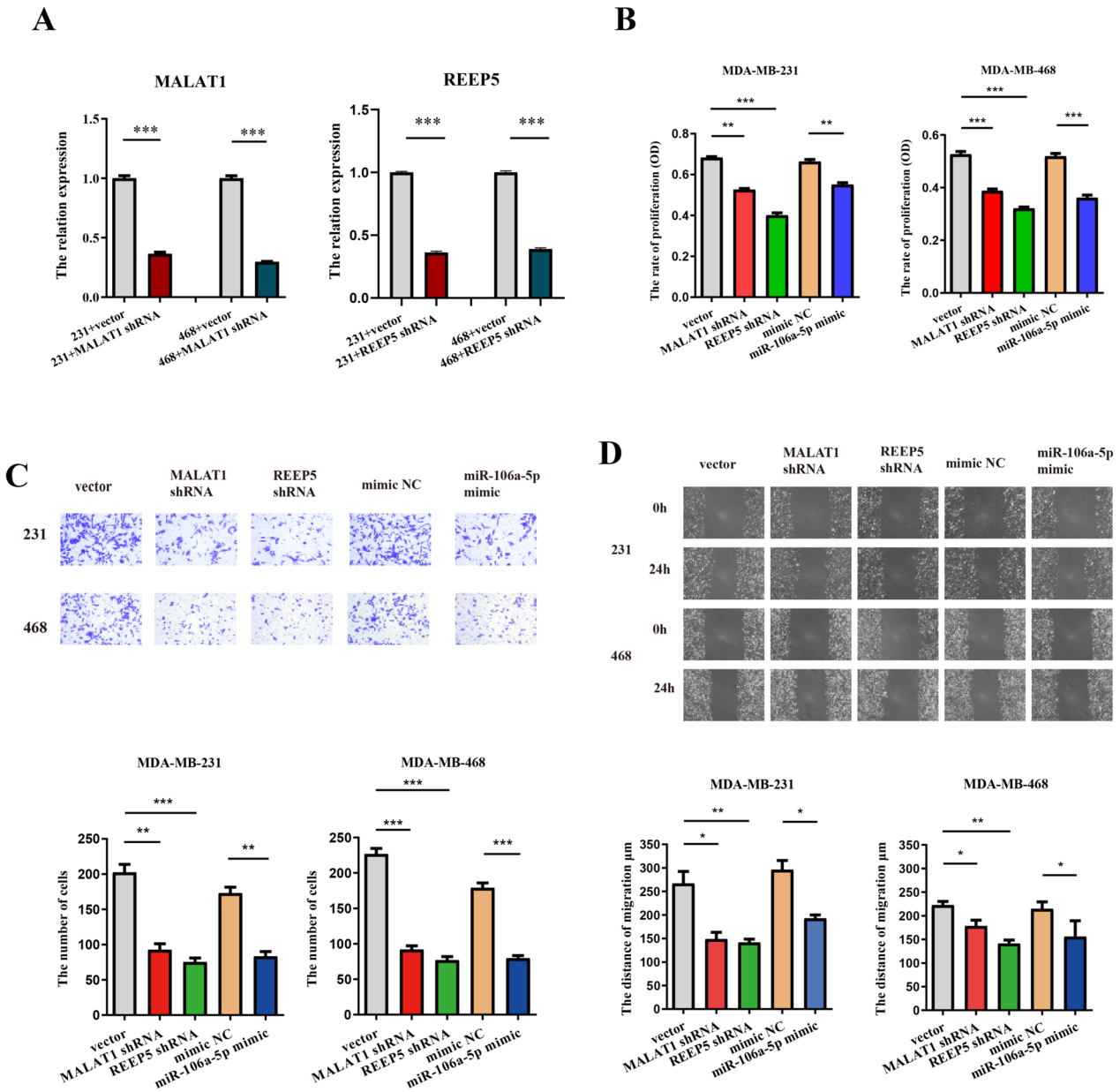

Fig. 6 The effect of target genes on the progression of TNBC cells. **A** The expression levels of MALAT1/REEP5 in TNBC cells can be significantly suppressed by MALAT1 shRNA/REEP5 shRNA; **B** proliferation experiment. **C** invasion experiment (Image of cell invasion under a 200× microscope). **D** migration experiment. OD: optical density; MALAT1: metastasis-associated lung adenocarcinoma transcript 1; REEP5: receptor expression-enhancing protein 5; NC: normal control; 231: MDA-MB-231; 468: MDA-MB-468. **: $p < 0.01$; ***: $p < 0.001$

lymph node metastasis of TNBC via MALAT1/miR-106a-5p/REEP5 ceRNA axis.

Expression of MALAT1, miR-106a-5p, and REEP5 in TNBC cells

To explore the expression of MALAT1, miR-106a-5p, and REEP5 genes in TNBC cells, RT-qPCR results showed that MALAT1 and REEP5 were remarkable overexpressed in TNBC cell (Fig. 5A, C); On the contrary, miR-106a-5p was significantly downregulated (Fig. 5B). These findings suggested that MALAT1, miR-106a-5p, and REEP5 might play an important role in the regulation of the carcinogenesis and progression of TNBC.

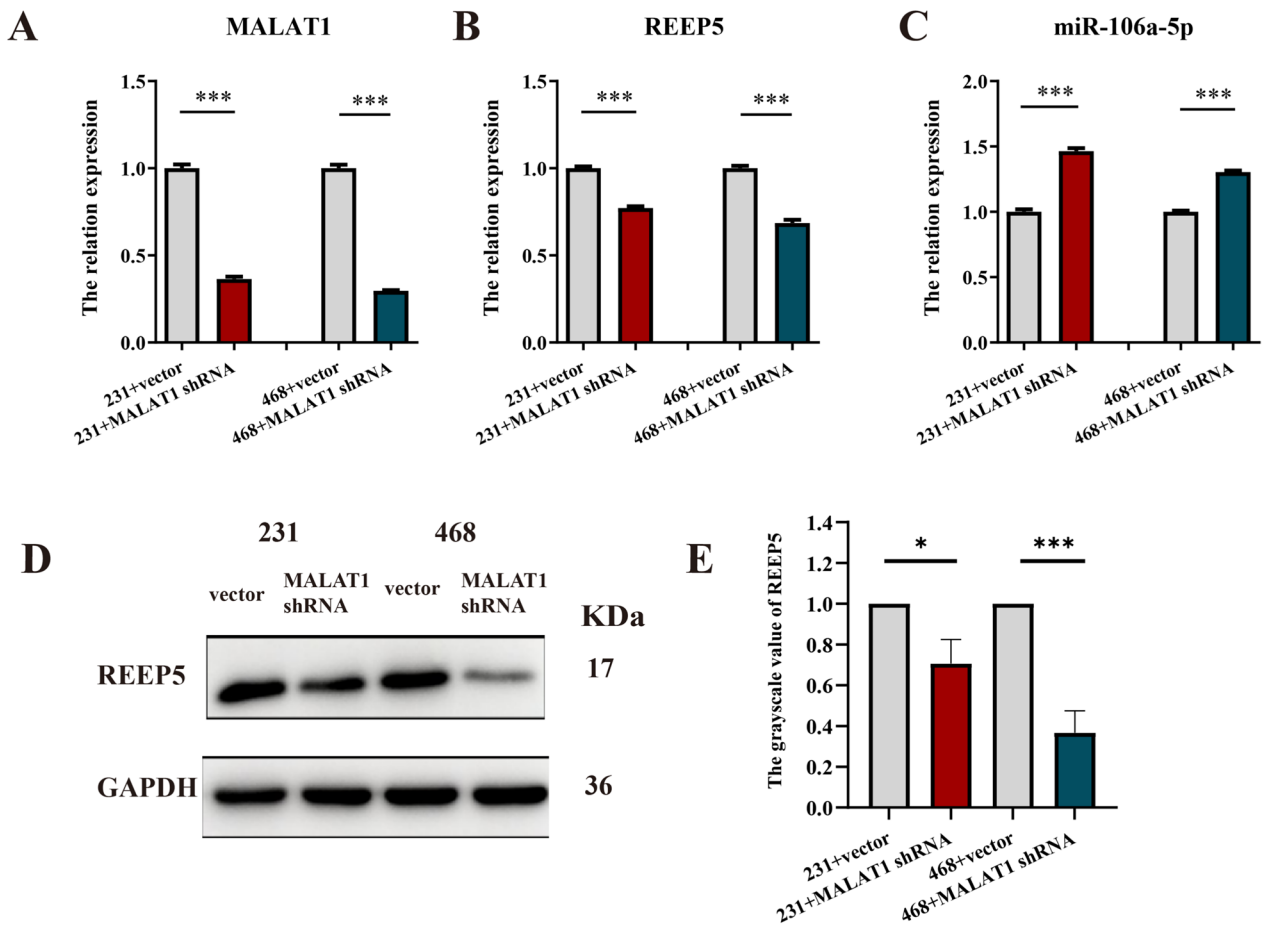

Fig. 7 MALAT1 affect the expression of REEP5 and miR-106a-5p. **A** The effect of MALAT1shRNA on the expression of MALAT1 in TNBC cells. **B**, **C** After transfection with MALAT1 shRNA, the changes in the expression of REEP5 and miR-106a-5p. **D**, **E** Changes in REEP5 protein expression levels after transfection with MALAT1 shRNA. ***: $p < 0.001$. 231: MDA-MB-231; 468: MDA-MB-468

MALAT1, miR-106a-5p, and REEP5 genes affect the progression of TNBC cells

As we all known that the enhancement of proliferation, invasion, and migration ability contributed to tumor progression. As we demonstrated that MALAT1 and REEP5 were highly expressed in TNBC cell, while miR-106a-5p was lowly expressed, we further investigate whether those genes were involved in the progression of TNBC cells. We downregulated and upregulated genes by using shRNA and mimics and the expression of genes were confirmed by RT-qPCR. We found that knocking down MALAT1 or REEP5 significantly reduced the proliferation, invasion, and migration abilities of TNBC cells compared with the control group; besides, when miR-106a-5p is overexpressed, the aforementioned abilities significantly decrease. In brief, our findings showed that MALAT1, miR-106a-5p, and REEP5 can regulate the ability of proliferation, invasion, and migration in TNBC (Fig. 6).

MALAT1 can exert carcinogenic effects in TNBC through ceRNA mechanism

MALAT1 affects the expression of REEP5 and miR-106a-5p

Previous research has confirmed that MALAT1, miR-106a-5p, and REEP5 play important roles in the occurrence and development of TNBC. To further explore their mechanisms of action, our experiments showed that the down-expression of MALAT1 significantly decreased REEP5 gene and protein level in both cell lines, while increased miR-106a-5p level (Fig. 7). With all the above results, we indicated that LncRNA MALAT1 could affect the expression of REEP5 and miR-106a-5p.

miR-106a-5p can regulate the expression of REEP5

For the sake of understanding the role of miR-106a-5p, we conducted miR-106a-5p overexpression and knockdown experiments. The results showed that when transfected with miR-106a-5p mimetics, the expression level of miR-106a-5p in TNBC cells could be significantly increased, while REEP5 gene and protein

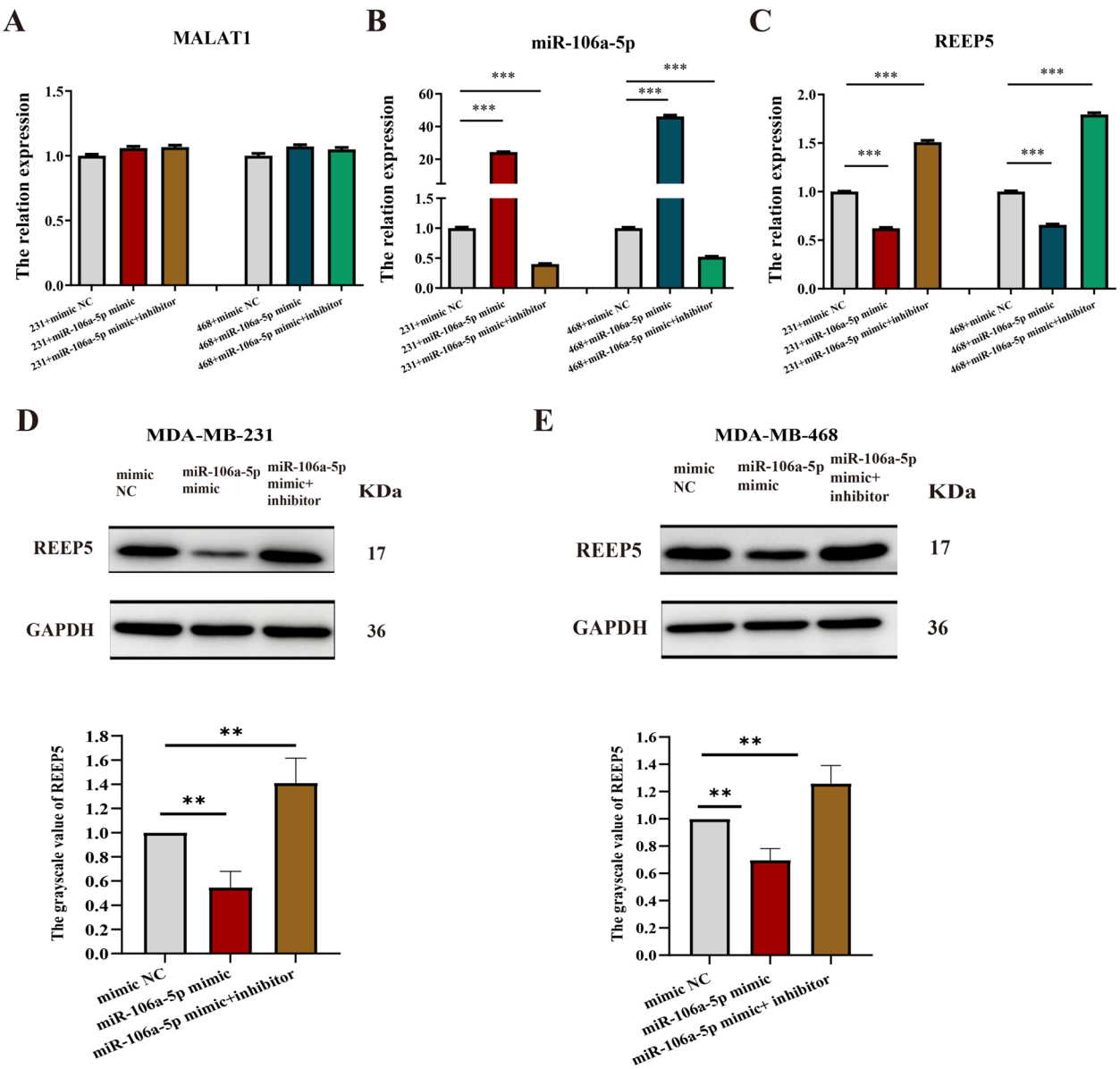

Fig. 8 miR-106a-5p can regulate the expression of REEP5. **A** miR-106a-5p has no effect on MALAT1. **B** miR-106a-5p mimic and inhibitor could significantly affect the expression level of miR-106a-5p. **C** miR-106a-5p can regulate the expression level of REEP5 gene. **D**, **E** miR-106a-5p can regulate the expression level of REEP5 protein. **: $p < 0.01$; ***: $p < 0.001$

were significantly decreased (compared with the control group); however, there was no significantly change in MALAT1 (Fig. 6). Subsequently, by knocking down the expression of miR-106a-5p, we found that only REEP5 expression changed, with significantly increased gene and protein levels (Fig. 8). Therefore, we demonstrated that miR-106a-5p could regulate the expression of REEP5, but has no regulatory effect on MALAT1.

miR-106a-5p can bind to REEP5-3'UTR

To explore whether there is a direct interaction between miR-106a-5p and REEP5, we performed dual reporter luciferase assay. As shown in Fig. 9, with the increasing amount of transfected miR-106a-5p mimics, luciferase activities of REEP5-3'UTR was significantly decreased (Fig. 9). It indicates that miR-106a-5p can target the 3'UTR region of REEP5.

Taken together, based on the above results and bioinformatics analysis, we can conclude that there is a ceRNA

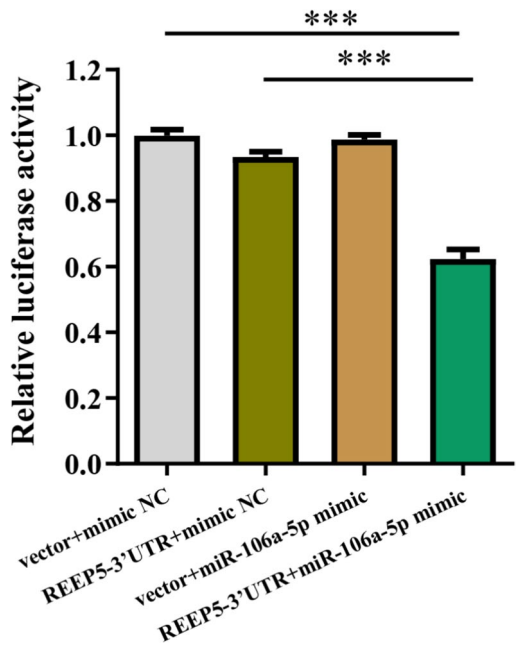

Fig. 9 Dual reporter luciferase assay. In 293T cells, after co-transfecting with miR-106a-5p mimic and the REEP5-3′UTR plasmid, the fluorescence intensity of the REEP5-3′UTR plasmid significantly decreased ($p < 0.05$)

mechanism between MALAT1, miR-106a-5p, and REEP5, which plays a carcinogenic role in TNBC.

Expression of MALAT1, miR-106a-5p, and REEP5 in primary breast tumor tissue and serum of TNBC patients

To verify whether the expression of MALAT1, miR-106a-5p, and REEP5 in clinical samples is consistent with cytological experiments, we used RT-qPCR to detect the expression level of target genes in the tumor tissues of TNBC patients, and took the corresponding normal breast tissues adjacent to the cancer of each sample as the control. As shown in Fig. 10, in three different groups, the expression levels of MALAT1 and REEP5 genes in primary breast cancer tumor tissue were significantly up-regulated compared with the control group, while miR-106a-5p showed a significant downward trend. Consistent with this, we also observed a similar expression pattern when testing serum samples from patients and 20 healthy female control samples: MALAT1 and REEP5 were highly expressed in patient serum, while miR-106a-5p was significantly downregulated. The analysis of variance revealed that the expression levels of MALAT1 and REEP5 genes increased with the degree of lymph node metastasis, while miR-106a-5p showed the opposite trend (Fig. 10, 11). IHC also showed significant differences in REEP5 protein expression levels among the three groups, the pairwise analysis results showed a statistically significant difference ($p = 0.043$) between the group without lymph node metastasis and the group with 4 or more lymph nodes metastasis, and a statistically significant difference ($p = 0.006$) between the group with 1–3 lymph nodes metastasis and the group with 4 or more lymph nodes metastasis; While, there was no statistical difference between the group without lymph node metastasis and the group with 1–3 lymph node metastases ($p = 0.329$). (Table 5, Fig. 12).

Discussion

Breast cancer is a malignant tumor with strong heterogeneity, especially in the subtype of TNBC, which has a higher degree of malignancy [8]. TNBC lack the expression of both ER and PR, and do not have amplification of the ERBB2 gene, lacking effective treatment methods [26, 27]. Compared with other subtypes of breast cancer, the overall survival (OS) of TNBC patients is shorter, it has approximately 46% of TNBC patients will have distant metastasis, and the mortality rate is 40% within the first 5 years after diagnosis [28]. The axillary lymph nodes are the most prone area to metastasis, and as the number of axillary lymph node metastases (ALNM) increases, the prognosis also deteriorates [29]. Thus, it is urgent to find new treatment methods from the mechanism of TNBC occurrence and development.

LncRNAs are emerging regulatory factors that can participate in cellular gene expression, which were considered as "garbage" previously [30, 31]. LncRNA plays an important role in regulating the transcription and translation of metabolism related genes, serving as bait, scaffold, competitive endogenous RNA (ceRNA), and ultimately leading to cancer metabolic reprogramming [32]. LncRNA, as endogenous competitive RNA (ceRNA), can affect the expression of miRNAs. miRNA can regulate the expression of target genes by binding to mRNA's 3′UTR [33, 34]. Previous studies have shown that lncRNA HOXA-AS2 could interact with miR-106a-5p to regulate the expression level of SCN3A [35]. In addition, Han Yan et al. found that MALAT1 could act as a competing endogenous RNA (ceRNA) for miR-15a/16 to regulate VEGFA expression in multiple myeloma (MM), thereby affecting tumor angiogenesis [36]. Our study showed that the expression level of MALAT1 was significantly increased in TNBC cells, tumor tissues, and serum. In addition, MALAT1 is positively correlated with the degree of ALNM in TNBC. What's more, we have discovered a MALAT1/miR-106a-5p/REEP5 axis, which MALAT1 can competitively bind miR-106a-5p

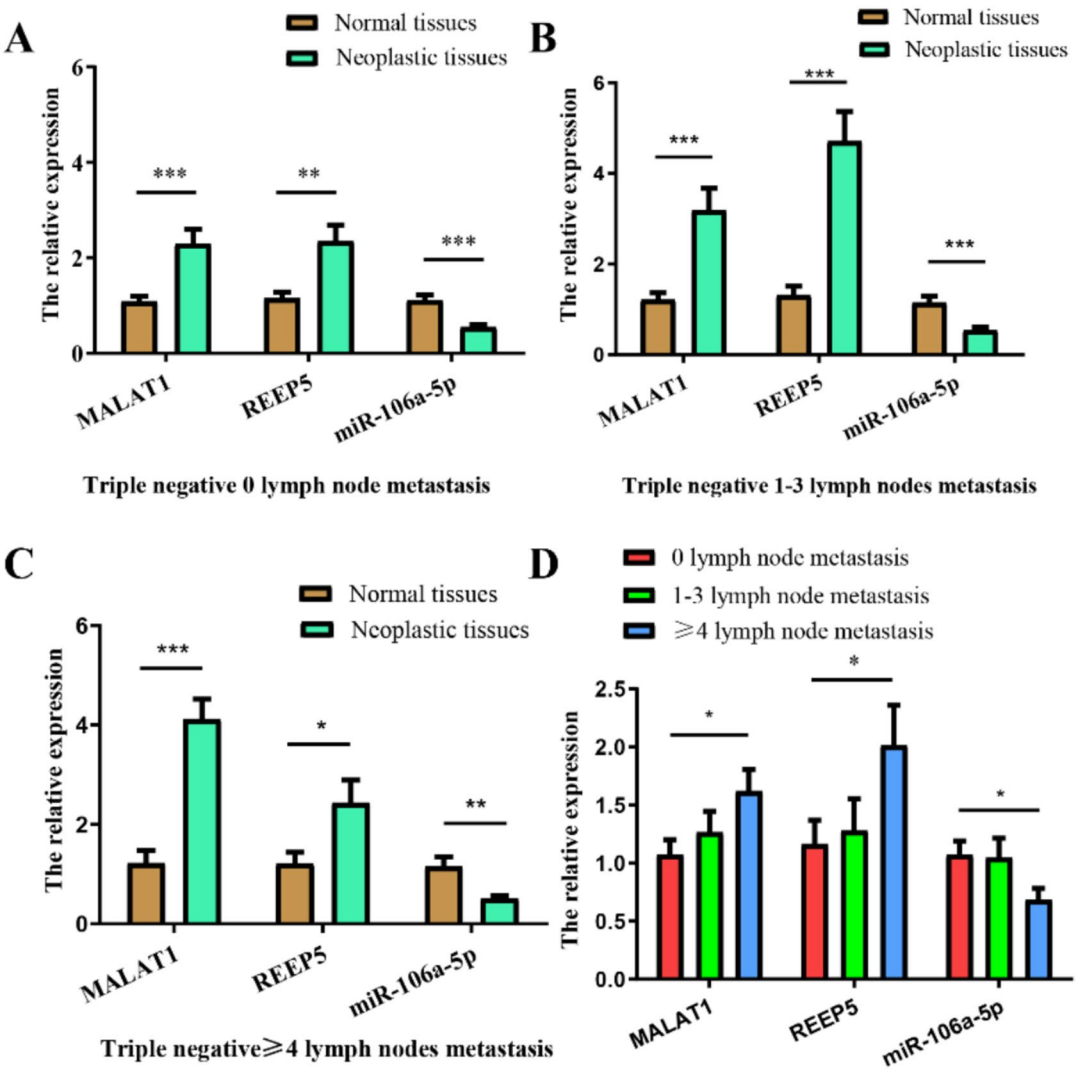

Fig. 10 Expression of MATAL1, miR-106a-5p, and REEP5 in primary breast tumor tissue and adjacent normal tissue. **A** Comparison of target gene expression in TNBC patients without lymph node metastasis; **B** comparison of target gene expression in TNBC patients with 1–3 lymph node metastasis; **C** comparison of target gene expression in TNBC patients with ≥ 4 lymph node metastasis; **D** the relationship between the expression level of target genes and the degree of lymph node metastasis. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

with REEP5, leading to REEP5 overexpression, thereby enhance the ability of proliferation, invasion, and migration of TNBC cells. The newly identified of the ceRNA mechanism provides novel insight for diagnosis of TNBC.

MALAT1 is widely expressed and highly conserved in mammalian cells, with a length of approximately 8000 nt. Zuo et al. studied 43 cases of TNBC tumor tissue and paired adjacent cancer tissues, found that the expression level of MALAT1 was significantly increased in tumor tissues; What's more, they also verified MALAT1 could promote the progression of TNBC cells [37]. The above research results were coincided with our research findings. Besides, we also found that MALAT1 is positively correlated with the degree of ALNM. It demonstrates that MALAT1 is an oncogene with potential diagnostic value for TNBC.

As another type of non-coding RNA molecule, miRNAs are also involved in the occurrence and development of many tumors [38]. miR-106a-5p is involved in the occurrence of various malignant tumors. For example, Silva et al. found significantly differential expression of the miR-106a-5p between colorectal cancer patients and normal patients through sequencing analysis, and the result was further validated in colorectal tumor tissue. They also invented a predictive model, which the

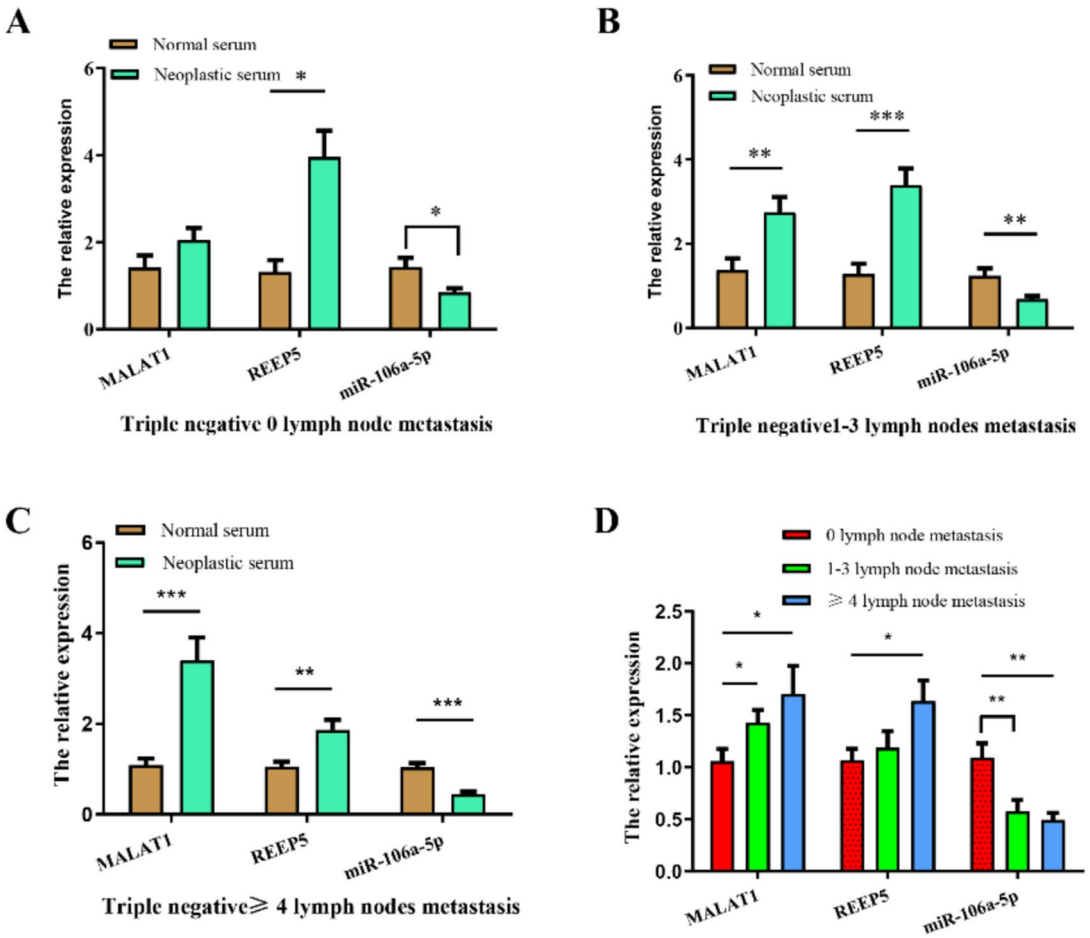

Fig. 11 Expression of MATAL1, miR-106a-5p, and REEP5 in serum. **A** Comparison of the expression levels of target genes in the serum of TNBC patients without lymph node metastasis and healthy women; **B** comparison of the expression levels of target genes in the serum of TNBC patients with 1–3 lymph node metastasis and healthy women; **C** comparison of the expression levels of target genes in the serum of TNBC patients with ≥ 4 lymph node metastasis and healthy women; **D** the relationship between the expression level of target genes and the degree of lymph node metastasis. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

accuracy, sensitivity, specificity, and AUC of miR-106a-5p in diagnosing colorectal cancer (CRC) were 76.5%, 59.4%, 86.8%, and 0.716 on the validation set. Therefore, miR-106a-5p could be used as a non-invasive method for early diagnosis of CRC [39]. In addition, Zhang et al., have confirmed that miR-106a-5p could inhibit the occurrence and development of breast cancer cells as a tumor suppressor gene. It could be combined by circRHOT1 to inhibit iron death through STAT3 signaling pathway. When miR-106a-5p expression was up-regulated, it would induce circRHOT1 deletion-like changes, and eventually lead to the decline of breast cancer cell proliferation and apoptosis [19]. Similarly, Du et al., found that over-expression of miR-106a-5p could inhibit the proliferation, migration, invasion, and epithelial mesenchymal transformation (EMT) in breast cancer cells. It could be regulated by the upstream gene VCAN-AS1, which can act as a competitive endogenous RNA (ceRNA) for miR-106a-5p to activate STAT3/HIF-1α signal pathways exert biological functions [40]. Our research results showed that the expression of miR-106a-5p significantly decreased in TNBC tumor tissue and serum.

Table 5 Differences in expression levels of REEP5 in different lymph node metastasis states

Grade*	Number	REEP5 ($X \pm S$)	F	*p*
0	20	8.2 ± 3.4	4.184	0.021
1–3	20	7.2 ± 3.2		
≥ 4	10	10.6 ± 1.6		

* Degree of TNBC lymph node metastasis; 0: No lymph node metastasis; 1–3: 1–3 lymph node metastases; ≥ 4: 4 or more lymph node metastases; *X*: Mean. *S*: standard deviation

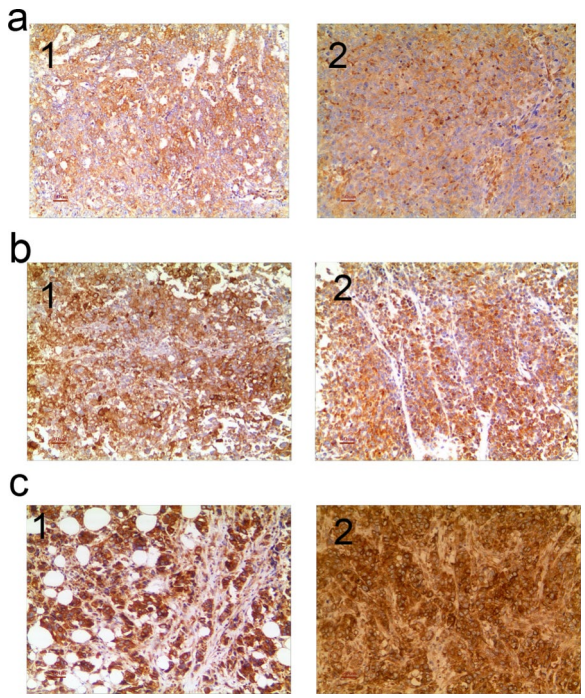

Fig. 12 Immunohistochemical images of TNBC breast tumor tissue (under 100× microscope). **a** No lymph node metastasis group; **b** 1–3 lymph node metastasis group; **c** 4 or more lymph node metastasis group

More importantly, the results of cytological experiments showed that miR-106a-5p could inhibit the proliferation, migration, and invasion of TNBC cells. These findings confirm the anti-tumor effect of miR-106a-5p in TNBC, these results were consistent with the reports from other researchers.

Receiver expression-enhancing proteins (REEPs) are a family of evolutionarily conserved proteins that play a crucial role in the structural formation and functional development of the endoplasmic reticulum [20]. REEPs are also involved in the occurrence and development of many diseases, including nervous system diseases, diabetes, and cancer [21–23]. REEP5, which is mainly distributed in the myocardium, endocrine tissues, kidneys, and proximal digestive tract, is composed of an N-terminal cytoplasmic domain, a C-terminal cytoplasmic domain, and four TMSs [41, 42]. CXC motif chemokine receptor 1(CXCR1) and CXCR2 are members of a class of GPCRs that bind to interleukin-8 (IL-8). Some studies suggested that the IL-8/CXCR1/2 signaling pathway can promote the proliferation and metastasis of various tumor cells [43]. Singh et al. found that IL-8 and its homologous receptors CXCR1 and CXCR2 played a very important role in regulating the activity of breast cancer stem-like cells. They could promote the occurrence and development of breast cancer by activating EGFR/Her2 pathway in Her2-positive breast cancer cells [44]. In addition, Park et al. found that REEP5 could specifically bind to the N-terminal region of CXCR1, thereby promoting the activation of the IL-8/CXCR1 signaling pathway and promoting the growth and migration of lung cancer cells [24, 45]. Besides, Isyraqiah et al. found that REEP5 was high expression in gastric cancer cells, and it could promote the proliferation of cancer cells [25]. The above studies indicated that REEP5 has the effect of promoting the occurrence and development of various cancer cells. However, the role of REEP5 in TNBC is still unclear. Our research revealed that REEP5 is highly expressed in TNBC and could promote the proliferation, invasion, and migration of TNBC cells. The role of REEP5 in the MALAT1/miR-106a-5p/REEP 5 axis oncogenic mechanism has been demonstrated, revealing potential diagnosis role in TNBC.

In this study, we found that the expression of miR-106a-5p increased and REEP5 decreased by knocking down MALAT1. In addition, the expression of REEP5 can be inhibited by upregulating the miR-106a-5p, further silence the miR-106a-5p can restore the expression of REEP5, but does not affect the expression of MALAT1. Further integration of dual reporter luciferase assay and bioinformatics analysis, we concluded that miR-106a-5p is a downstream gene of MALAT1, regulated by MALAT1, and its function depends on the REEP5. The specific mechanism of action can be found in Fig. 13.

We reanalyzed the downstream mechanism of the MALAT1/miR-106a-5p/REEP5 ceRNA signaling pathway through the STRING database and TCGA BRCA dataset, and found 123 genes that have a co-expression relationship with REEP5. Through functional enrichment analysis combined with the endoplasmic reticulum function of REEP5, it was found that the ability of downstream proteoglycans has changed. We will conduct a study on the relationship between TNBC lymph node metastasis and proteoglycan ability in the future (Fig. 14, Table 6).

Conclusion

In summary, our study demonstrates that LncRNA MALAT1 was overexpressed in TNBC, and it could promote the progression of TNBC through MALAT1/miR-106a-5p/REEP5 ceRNA axis. This study provides new theoretical basis for the diagnosis and treatment of TNBC.

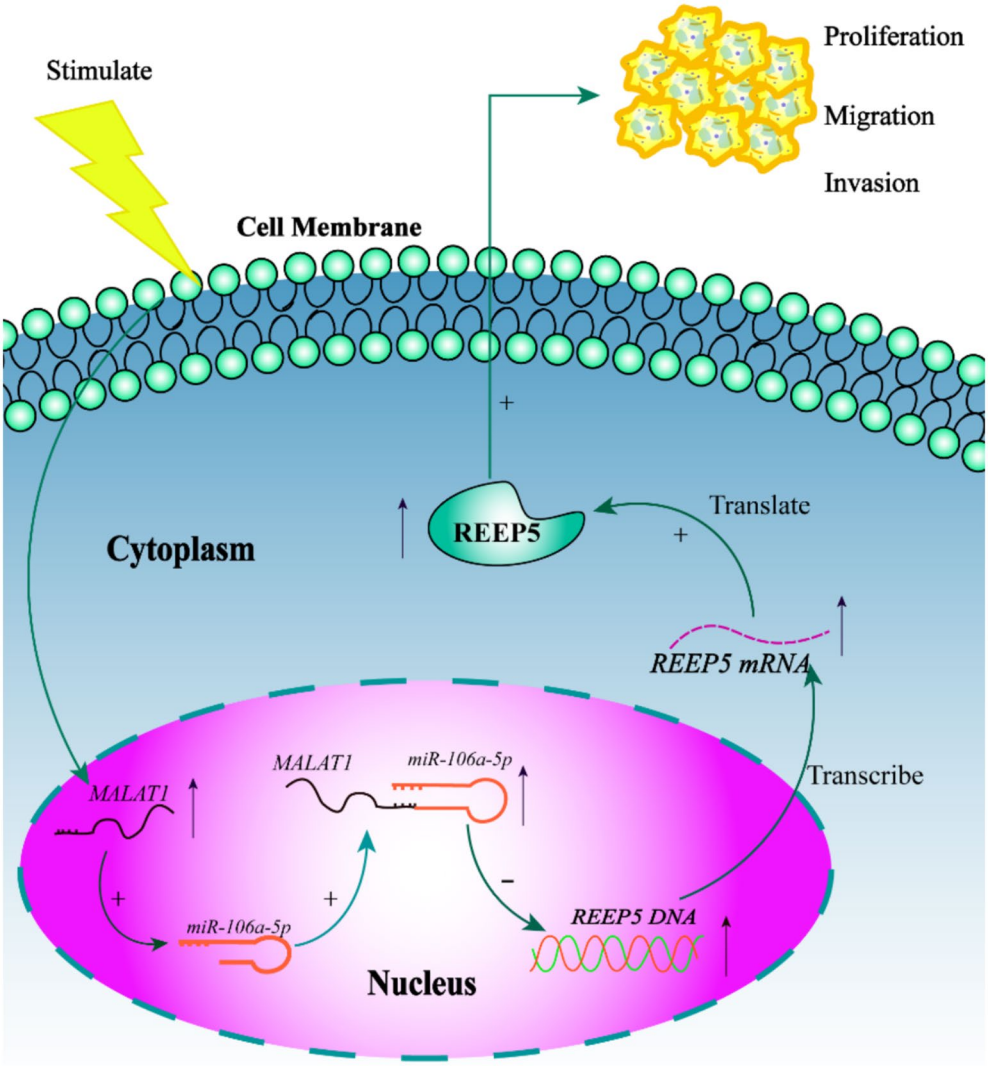

Fig. 13 Mechanism diagram of MALAT1, miR-106a-5p, and REEP5 related ceRNA. When external stimuli cause an increase in the expression of MALAT1 in cells, it can enhance its binding to miR-106a-5p, leading to a decrease in the binding of REEP5 gene to miR-106a-5p, an increase in the expression levels of REEP5 gene and protein, and further promoting the proliferation, invasion, and migration of cancer cells

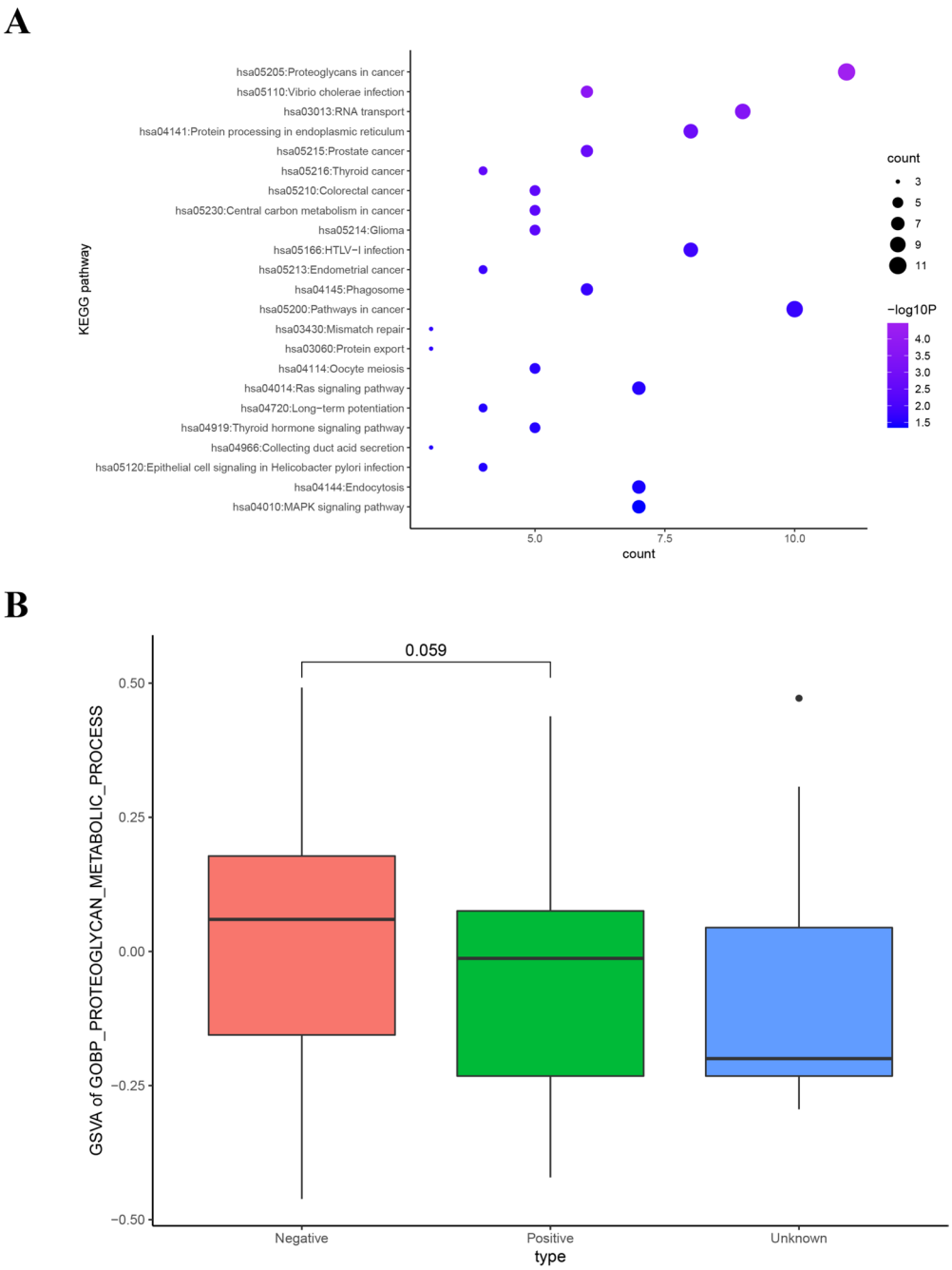

Fig. 14 Bioinformatics analysis of downstream REEP5

Table 6 123 genes interacting with REEP5 protein participate in the KEGG pathway

Term	Count	*p*
hsa05205: Proteoglycans in cancer	11	3.39E−05
hsa05110: Vibrio cholerae infection	6	1.81E−04
hsa03013: RNA transport	9	3.61E−04
hsa04141: Protein processing in endoplasmic reticulum	8	0.001690577
hsa05215: Prostate cancer	6	0.002049582
hsa05216: Thyroid cancer	4	0.003176407
hsa05210: Colorectal cancer	5	0.003703354
hsa05230: Central carbon metabolism in cancer	5	0.004153412
hsa05214: Glioma	5	0.004391736
hsa05166: HTLV-I infection	8	0.015308919
hsa05213: Endometrial cancer	4	0.016229593
hsa04145: Phagosome	6	0.018955454
hsa05200: Pathways in cancer	10	0.018980459
hsa03430: Mismatch repair	3	0.0231066
hsă0: Protein export	3	0.0231066
hsa04114: Oocyte meiosis	5	0.027298599
hsa04014: Ras signaling pathway	7	0.028562063
hsa04720: Long-term potentiation	4	0.030351209
hsa04919: Thyroid hormone signaling pathway	5	0.030566352
hsa04966: Collecting duct acid secretion	3	0.031222017
hsa05120: Epithelial cell signaling in Helicobacter pylori infection	4	0.031544433
hsa04144: Endocytosis	7	0.037420578
hsa04010: MAPK signaling pathway	7	0.045661823

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40001-025-02420-x.

Supplementary Material 1.

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Not applicable.

Author contributions
ZXP conceived and supervised the project. YHJ, ZXP, YQH and YXF designed the experiments, YQH performed the experiments, analysis, and wrote the manuscript with input from all authors. FYQ, FWL, MJF, XN, LQ, and YQJ helped with breast cancer sample collection and immunohistochemistry analysis. All authors reviewed the manuscript.

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Availability of data and materials
The data that support the findings of this study are available on request from the corresponding author, Xi-ping Zhang, upon reasonable request. TCGA BRCA dataset from the UCSC Xena database (https://xenabrowser.net/datapages/). miRNA and mRNA are searched from starbase database (http://starbase.sysu.edu.cn/index.php). Clinical and cellular experimental data are not publicly available yet, but can be available from the corresponding author, Dr. Xi-ping Zhang (email address: zxp99688@sina.com).

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Ethics Committee of Zhejiang Cancer Hospital (Ethics Number: IRB-2022-143). Additional informed consent was obtained from all patients for which identifying information is included in this article.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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