



Research on correlations of miR-585 expression with progression and prognosis of triple-negative breast cancer

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Abstract

Triple-negative breast cancer is a special type of breast cancer, characterized by younger onset age, shorter survival period, higher malignant degree, higher mortality, recurrence and metastasis. Triple-negative breast cancer is more harmful to women's life and health, compared with other types of breast cancer. This paper mainly studied the role of miR-585 in triple-negative breast cancer. Real-time quantitative PCR was used to detect the expression of miR-585 in triple-negative breast cancer cell lines and tissues. Kaplan–Meier curve and Cox proportional hazards model analysis were used to investigate the prognostic value of miR-585 in triple-negative breast cancer. CCK-8 and Transwell assays were used to detect cell proliferation, invasion and migration. miR-585 was significantly down-regulated in triple-negative breast cancer cells and tissues. The low expression of miR-585 has been shown to be significantly associated with poor prognosis in triple-negative breast cancer patients. Abnormally low expression of miR-585 can promote cell proliferation, migration and invasion. Overall, abnormally low expression of miR-585 is associated with prognosis and progression of triple-negative breast cancer. miR-585 may serve as a prognostic biomarker for patients with triple-negative breast cancer and it is expected to be a new method and strategy for the treatment of triple-negative breast cancer.

Keywords miR-585 · Triple-negative breast cancer · Correlations · Progression · Prognosis

Introduction

As one of the most common malignancies in women worldwide, breast cancer is considered to be the leading cause of cancer death [1]. Triple-negative breast cancer (TNBC), as a subtype of breast cancer, is characterized by the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) as negative [2, 3]. TNBC accounts for about 15–20% of all breast cancers. As a subtype of breast cancer with rapid proliferation and high invasion, it is prone to early recurrence and metastasis and has a poor prognosis [4, 5]. Due to the high heterogeneity of TNBC at the molecular level, the prognosis of patients with different molecular subtypes is significantly different. At present, TNBC still lacks specific biomarkers and therapeutic targets [6, 7].

MicroRNA (miRNA) is a kind of non-coding single-stranded RNA with a length of about 21–25 nt [8, 9]. It promotes the degradation of target mRNA or inhibits protein translation by complementary pairing with target mRNA completely or partially, and participates in a series of important processes in the life process [10, 11]. Studies have shown that more than 50% of miRNA genes are located in tumorigenesis-related regions or "fragile regions," amplification regions, heterozygous loss regions, or rupture translocations of chromosomes, indicating that miRNAs are involved in tumorigenesis in the form of oncogenes or tumor suppressor factors, for example, miR-186 [12], miR-7 [13], miR-296 [14]. A previous study by Hiroko Toda and co-workers investigated the miRNA expression level of TNBC by clinical specimens, they founded a total of 104 miRNAs in TNBC tissue, of which 56 were upregulated and 48 were down-regulated. Among the down-regulated miRNAs, miR-585 ranked among the top in terms of down-regulation amplitude [15]. However, their study only analyzed the inhibitory effect of miR-204 on breast cancer cells and did not conduct relevant studies on miR-585.

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In the present study, we first analyzed the expression levels of miR-585 in TNBC tissues and cell lines, then studied the influence of the abnormal expression of miR-585 on the overall survival and prognosis of TNBC patients, and finally further clarified the important role of miR-585 in TNBC through cell proliferation, migration and invasion assays.

Materials and methods

Patients and tissue samples

The TNBC cell tissue and paired adjacent normal tissues were collected from 102 patients with TNBC treated in The First Affiliated Hospital, College of Medicine, Zhejiang University from 2012 to 2015. All patients underwent surgery. The tissue samples were confirmed by pathological examination, and none patients received radiation, chemotherapy or other treatment before surgery. After removing the specimen, immediately store the tissue in liquid nitrogen at -80°C for later use. The ethics committee of The First Affiliated Hospital, College of Medicine, Zhejiang University approved this study. All patients were followed for 5 years for further survival analysis.

Cell culture and transfection

TNBC cell lines MDA-MB-453, MDA-MB-231, HCC1937, BT-579 and human normal breast epithelial cell line MCF-10A were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). TNBC cell lines and human normal breast epithelial cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, USA) at 37°C and 5% CO_2 , respectively. Cells were transfected when the cells grew approximately 50–70%. miR-585 mimic, mimic negative control (mimic NC), miR-585 inhibitor, or inhibitor NC were transfected into cells according to the Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) manufacturer's instructions. Untreated cells were used as control.

RNA extraction and quantitative real-time PCR

The total RNA was extracted by the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). After quantifying the RNA by using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, USA), cDNA was synthesized by the reverse transcription method using a TaqMan miRNA reverse transcription kit (Thermo Fisher Scientific), and the specific operation was carried out according to the kit instructions. Real-time quantitative PCR (qRT-PCR) analysis was detected using SYBR-Green I Master mix kit (Invitrogen;

Thermo Fisher Scientific, Waltham, USA) with an Applied Biosystems ABI 7500 PCR System. The relative quantification of miR-585 was determined by the $2^{-\Delta\Delta\text{CT}}$ method, and repeat 3 times.

Cell proliferation assay

We utilized the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) assays to determine the TNBC cell proliferative ability. The transfected MDA-MB-231 cells and BT-549 cells (2×10^3 cells/well) were inoculated into 96-well plates and measured every 24 h according to the manufacturer's instructions. 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well and incubated at 37°C in 5% CO_2 for 2 h. The absorbance value was determined at the wavelength of 450 nm.

Cell migration and invasion assays

Transwell assay was implemented using 24-well transwell plates. The upper chamber without Matrigel (BD Biosciences, San Jose, CA, USA) was used for migration assay, and the invasion assay was prepared Matrigel on the upper chambers. Serum-free culture medium containing 5×10^4 transfected cells was added into the upper chamber, and the lower chambers added the medium containing 15% FBS. Next, stained with crystal violet for 20 min and 5 random fields were randomly selected for counting and statistics in each well.

Statistical analysis

Data processing was mainly performed using SPSS 20.0 software (IBM, Armonk, NY, USA) and GraphPad 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The Student t test and a one-way analysis of variance were used for comparison between two and three groups, respectively. The relationship between miR-585 expression and survival rate was analyzed by Kaplan–Meier and log-rank methods. Statistically significant was defined as *P* value less than 0.05.

Results

Expression of miR-585 in TNBC tissues and cell lines

For the investigation of the feasible participation of miR-585 in TNBC initiation and progression, we analyzed miR-585 expression in TNBC tissues and adjacent normal tissues using the qRT-PCR assay. The results showed that the expression level of miR-585 in TNBC tissues was significantly lower than that in adjacent normal tissues ($P < 0.001$, Fig. 1a). The miR-585 expression in TNBC cell lines

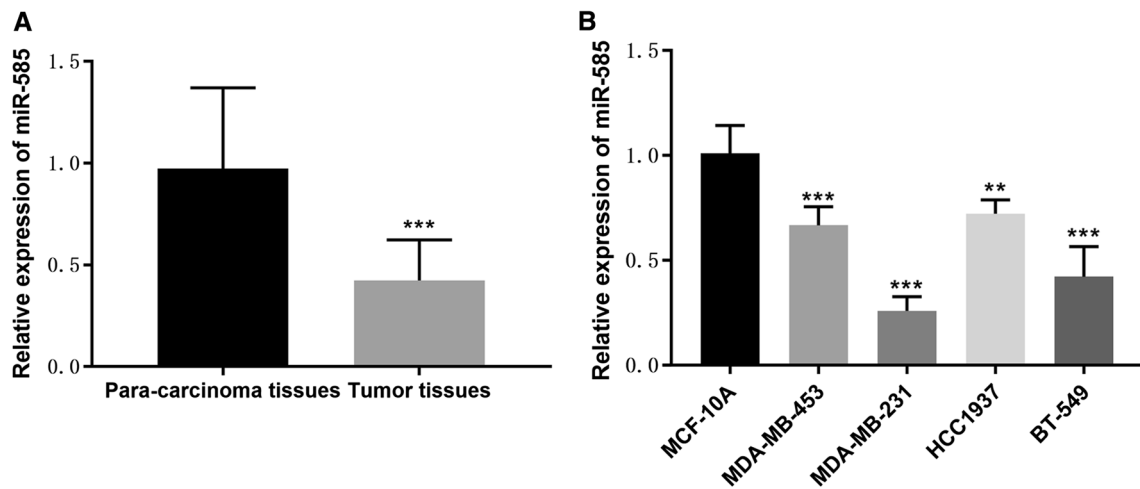


Fig. 1 The expression of miR-585 was decreased in TNBC tissues and cell lines. **a** The expression levels of miR-585 in TNBC tissue samples and para-carcinoma tissues. *** $P < 0.001$. **b** The expression levels of miR-585 in 4 TNBC cell lines. *** $P < 0.001$

(MDA-MB-453, MDA-MB-231, HCC1937, BT-549) and normal breast epithelial cell line (MCF-10A) was further detected. Similar to the results above, the expression of miR-585 in four TNBC cell lines was also lower than that in the normal cell line ($P < 0.001$, Fig. 1b).

Relationship between clinicopathological characteristics and miR-585 expression in TNBC patients

According to the relative average expression level of miR-585 in TNBC tissues, TNBC patients were divided into a low miR-585 expression group ($n = 56$) and a high miR-585 expression group ($n = 46$). Then, the relationship between

clinicopathological characteristics and miR-585 expression in TNBC patients was investigated. Table 1 showed that the expression levels of miR-585 were significantly correlated with lymph node metastasis ($P = 0.029$) and TNM stage ($P = 0.009$), but miR-585 was not associated with other clinicopathological characteristics.

Down-regulation of miR-585 is associated with poor prognosis in TNBC patients

Kaplan–Meier curves and log-rank test were used to analyze the relationship between the down-regulation of miR-585 and poor prognosis in TNBC patients. As shown in Fig. 2, the total survival time of TNBC patients was

Table 1 Association of miR-585 expression and clinicopathological parameters of TNBC

Clinical characteristics	Cases ($n = 102$)	Tissue miR-585 expression		P value
		Low ($n = 56$)	High ($n = 46$)	
<i>Age (years)</i>				0.888
≤ 60	54	30	24	
> 60	48	26	22	
<i>Tumor size (cm)</i>				0.906
< 5	45	25	20	
≥ 5	57	31	26	
<i>Tumor grade</i>				0.120
I–II	49	23	26	
III	53	33	20	
<i>Lymph node metastasis</i>				0.029
Negative	66	31	35	
Positive	36	25	11	
<i>TNM stage</i>				0.009
I–II	76	36	40	
III–IV	26	20	6	

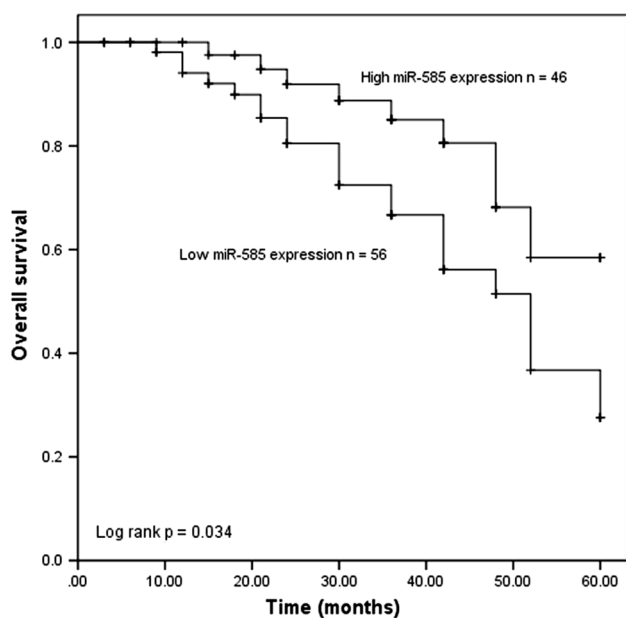


Fig. 2 Kaplan–Meier survival curve in relation to the miR-585 expression level in patients with TNBC cancer. (log-rank test $P=0.020$)

Table 2 Multivariate Cox analysis of factors for survival of TNBC patients

Variables	Multivariate Cox analysis		
	HR	95%CI	<i>P</i> value
miR-585	2.734	1.158–6.455	0.022
Age	1.357	0.618–2.982	0.447
Tumor size	1.423	0.661–3.063	0.367
Tumor grade	1.977	0.892–4.382	0.093
Lymph node metastasis	2.719	1.040–7.111	0.041
TNM stage	0.469	0.224–0.982	0.045

significantly shorter in the low-expression group of miR-585 compared with the high-expression group ($P < 0.05$). Table 2 was the Cox regression analysis, and it was used to study the independent risk factors of overall survival. The results showed that miR-585 expression (HR = 2.734, 95%CI: 1.158–6.455, $P = 0.022$), lymph node metastasis (HR = 2.791, 95%CI: 1.040–7.111, $P = 0.041$) and TNM stage (HR = 0.469, 95%CI: 0.224–0.982, $P = 0.045$) were associated with overall survival and can be the independent prognostic factors for TNBC.

Down-regulation of miR-585 promotes TNBC cell proliferation, migration and invasion

To examine the biological effects of miR-585 on TNBC cells, MDA-MB-231 and BT549 cells were transfected with

miR-585 mimic, mimic NC, miR-585 inhibitor and inhibitor NC. Then, we did the cell proliferation, invasion and migration assays. The qRT-PCR result shows that miR-585 mimics can upregulate miR-585 expression, and the miR-585 inhibitor can downregulate the miR-522 expression, while mimic NC and inhibitor NC do not affect the miR-585 expression ($P < 0.01$, Fig. 3a). Then, we utilized CCK8 assays to explore the proliferative ability changes of the TNBC cells, the results showed that up-regulation of miR-585 significantly suppressed the cells proliferation, while down-regulation of miR-585 can promote the cell proliferation ($P < 0.05$, Fig. 3b). Transwell assays were used to check the changes of the cell migratory and invasive capacities. Upregulated expression of miR-585 significantly reduced the number of migratory and invasive TNBC cells, while down-regulated miR-585 expression significantly improved the migration and invasion ability of TNBC cells, compared with control ($P < 0.01$, Fig. 4a–b).

Discussion

TNBC has the characteristics of high recurrence rate, easy metastasis, short survival time and poor prognosis, compared with other types of breast cancer [16–18]. Since TNBC does not express hormone receptors or HER2, endocrine therapy and HER2-targeted therapy have little effect on TNBC [19]. In view of the high heterogeneity of TNBC, the main research direction is to conduct a detailed study on this type of breast cancer and design targeted therapy drugs for cancer cells, so as to improve the overall cure rate of TNBC [17, 20]. At present, some latent sub-targets of TNBC have been discovered, but the research results are not ideal. Some studies have confirmed that RNA molecular variation has an important impact on the prognosis and treatment strategy of TNBC [21, 22]. In particular, the research on the fractal biology of the regulation and control of miRNA-related target genes has opened up new ideas for improving the accuracy of TNBC diagnosis and therapeutic targeting [23].

In TNBC, many miRNAs have been confirmed to be related to the prognosis of patients and play a role as prognostic biomarkers, for instance, miR-34a [24], miR-221/222 [25] and miR-200 [26]. A recent study demonstrated that miR-205-5p can inhibit the growth of breast cancer cells and reduce the risk of tumor metastasis, especially TNBC [27]. miR-585 is a less studied miRNA. As far as we know, this is the first time that miR-585 has been confirmed to play an important role in TNBC. miR-585 has been shown to have an abnormal expression in TNBC, and the correlation between miR-585 expression and the progression and prognosis of TNBC was investigated.

miR-585 may play the role of the tumor suppressor in TNBC. The above inferences can be drawn from the

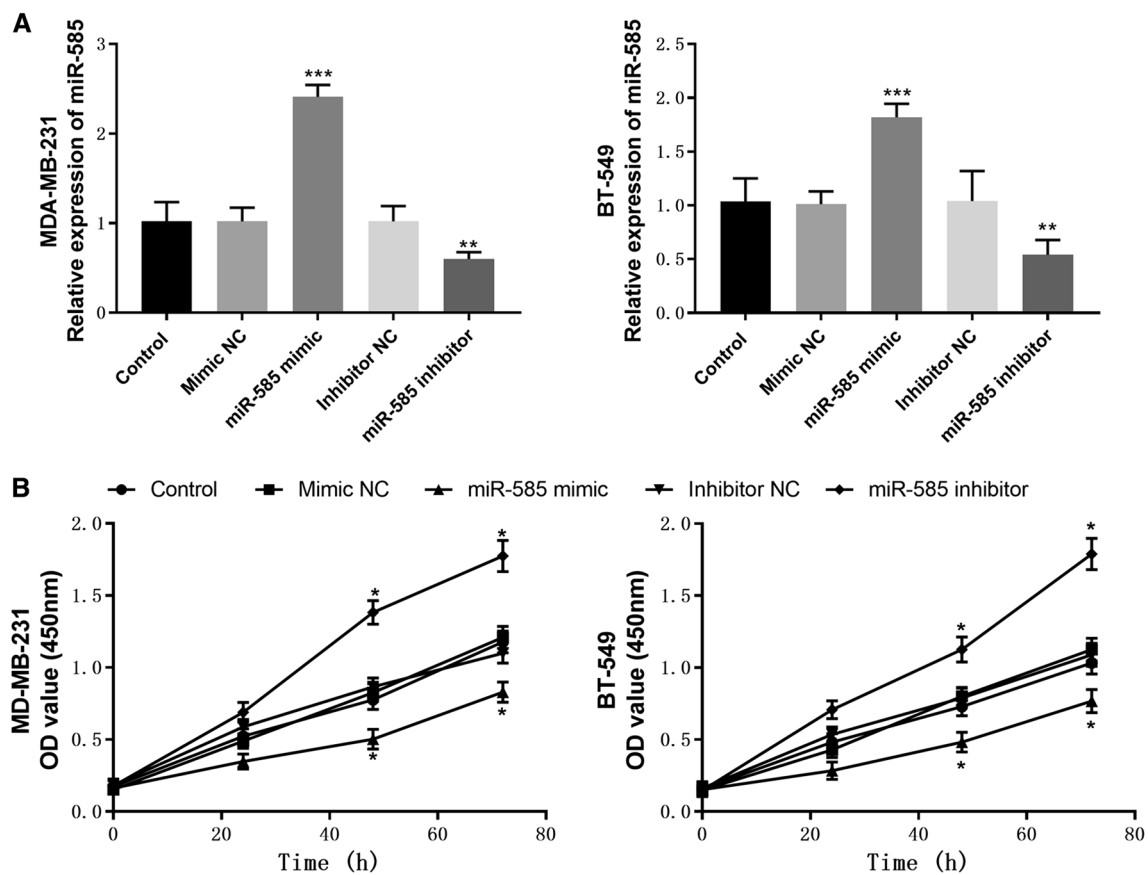


Fig. 3 Effects of miR-585 expression levels on proliferation in MDA-MB-231 and BT-549 cells. **a** The expression level of miR-585 was analyzed by qRT-PCR after transient transfection with miR-

585 mimic/inhibitor (or mimic/inhibitor NC). **b** The CCK-8 assay was performed to study cell proliferation. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$

following aspects. First of all, miR-585 expression was significantly down-regulated in TNBC tissues and cell lines. The abnormally low expression of miR-585 was significantly associated with lymph node metastasis and TNM stage in TNBC patients. In addition, the patients with down-expression of miR-585 might have a poor prognosis. In the end, we examined the effects of miR-585 on the biological behavior of TNBC by CCK-8 and Transwell assays. The results showed that low expression of miR-585 promoted cell proliferation, migration and invasion, while high expression of miR-585 decreased these behaviors. Overall, miR-585 inhibited TNBC occurrence and development, it was also closely related to the prognosis of patients, suggesting that miR-585 may be a tumor suppressor and promising prognosis biomarker in TNBC.

Previous studies have reported abnormal expression of miR-585 in other cancers and correlated with the progression of other cancers. In non-small-cell lung cancer, the expression of miR-585 was down-regulated, and down-regulated miR-585 can inhibit the proliferation, migration and invasion of TNBC cells. In addition, they also proved that miR-585 directly targeted the 3'-untranslated region (UTR)

of hSMG-1 gene [28]. In gastric cancer, the expression of miR-585 in gastric cancer tissues and cell lines was also showed a downward trend, and the expression level of miR-585 has been shown to be related to the degree of infiltration, TNM stage, lymph node infiltration and poor prognosis. Meanwhile, low expression miR-585 promoted the proliferation and migration of gastric cancer, and MAPK1 was a direct and functional target of miR-585 [29]. In colon cancer, miR-585 acts as a tumor suppressor and inhibits cell growth and proliferation in colon cancer by targeting PSME3 [30]. These studies were consistent with our results above, and considering that miR-585 is abnormally low expressed in different cancers, we deemed that miR-585 may be a tumor suppressor. In this paper, we have some shortcomings. For example, we did not study the target gene of miR-585 in TNBC, and we will conduct research and analysis on this in future work.

Collectively, this study showed that the abnormally low expression of miR-585 in TNBC tissues and cell lines, and the patients with low expression of miR-585 have a poor prognosis and a short survival time. Low expression of miR-585 could enhance the number of proliferation, migration

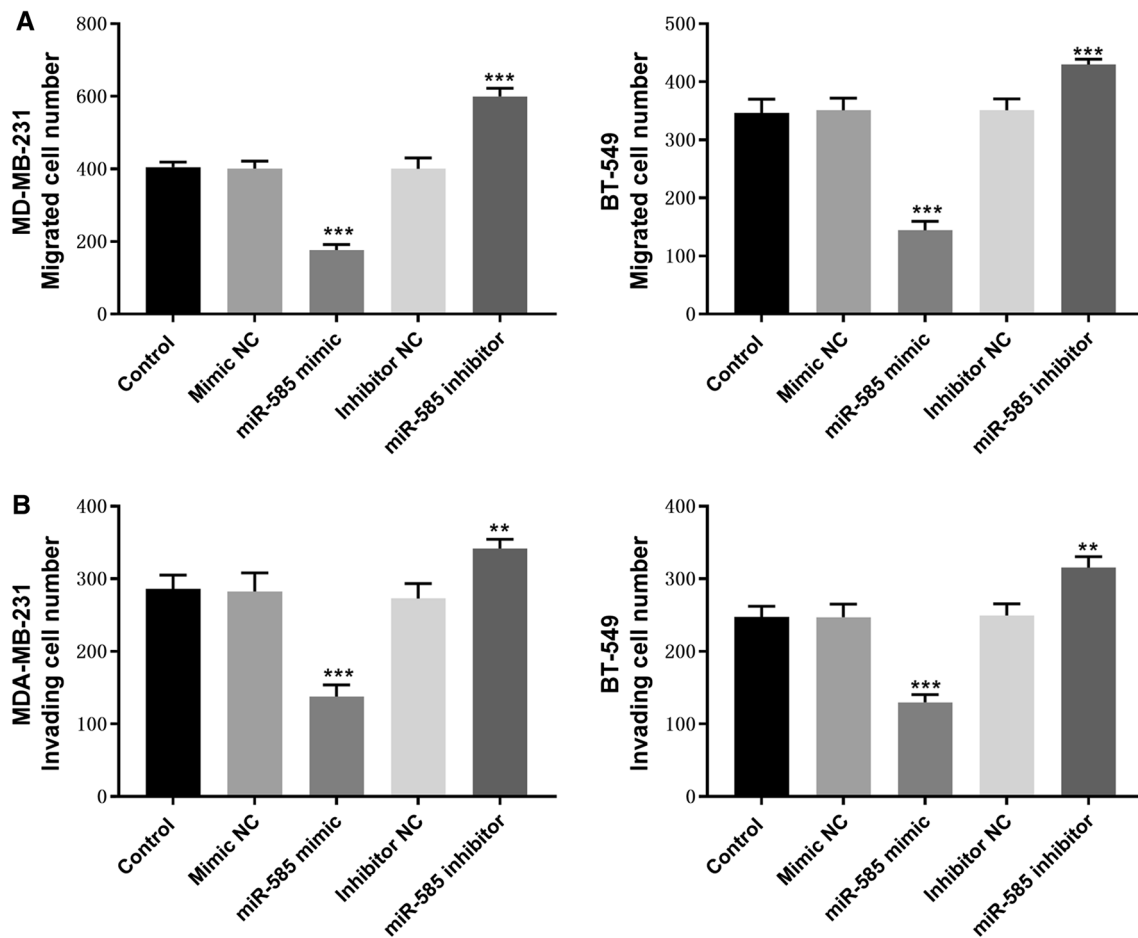


Fig. 4 Effects of miR-585 on cell migratory and invasive abilities in MDA-MB-231 and BT-549 cells. **a** Cell migration and **b** invasion numbers were assessed with Transwell assay. ** $P < 0.01$, *** $P < 0.001$

and invasion of TNBC cells. Although the function of miR-585 in TNBC is not fully understood, our findings provide a new potential prognostic marker for TNBC. This supplies a new idea for the treatment and prognosis of TNBC.

Authors' contributions MY and PF contributed to the study conception and design. Material preparation, data collection and analysis were performed by MY, SW, LC and BW. The first draft of the manuscript was written by MY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to the content of this article.

Availability of data and material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval The ethics committee of The First Affiliated Hospital, College of Medicine, Zhejiang University approved this study.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication The participant has consented to the submission of the case report to the journal.

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