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MiR-98-5p regulates proliferation and metastasis of MCF-7 breast cancer cells by targeting Gab2

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Abstract. – OBJECTIVE: The aim of this study was to explore the mechanism of miR-98-5p in influencing the malignant proliferation and metastasis capacities of breast cancer cells.

PATIENTS AND METHODS: Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression level of miR-98-5p and GRB2-associated-binding protein 2 (Gab2) in breast c r samples and cells. On-line target gene tion software and Dual-Luciferase report s-say were used to predict and verify the t t genes of miR-98-5p, respectively. Cell prol ation was measured by MTT (3-(4,5-dimethyl iazol-2-yl)-2,5-diphenyl tetrazo mide) as say. Meanwhile, migration an vas bilities, as well as the changes of helial- senchymal transition (EMT) aft nsfecti de-tected by transwell as ay We say, respectively.

RESULTS: Com d with nt non-tumor tissues and 10A cells, t pression level of miR-9 or tissues d MCF-7 cells was sig ficantly ined, whereas Gab2 was mark up-regulat esides, Gab2 was predicte a target gene 3-98-5p. Subsequent erime s indicated hat the proliferation gration asion and EMT of MCF-7 cells tra ed miR-98 5p were significantly inhibite er, up ulation of Gab2 attenu-d the ical tion of miR-98-5p on ma- t abi east cancer cells.

CLUSI We showed that miR-98-5p se as anti-oncogene in breast cancer, which a new therapeutic target for its atment.

Words:
st cancer, MiR-98-5p, GRB2-associated-binding protein 2 (Gab2), Metastasis, Epithelial-mesenchymal transition (EMT).

Introduction

ast cancer is one of e most common ma- ant tumors in emales, accounting for 7-10% l malignanc Meanwhile, it is one of the diseases t tening female health[1,2]. Huge prog n h en made in operation and radio-che py techniques for patients with breast cancer. However, due to tumor metastasis, gnosis of some patients is poor, and the rate is relatively high[3-5]. Therefore, it is necessary to search the possible mechanism of migration and invasion of breast cancer and to explore intervention measures, to improve the prognosis of breast cancer patients. Micro-ribonucleic acid (miRNA) is a kind of non-protein-coding small-molecule RNA widely existing in the human body. MiRNA is involved in a series of vital biological activities by regulating post-transcriptional expression of downstream target genes, such as cell proliferation, apoptosis and angiogenesis[6-9]. Studies in China and foreign countries have demonstrated that miRNA not only plays an important role in normal vital activities, but is also closely related to some pathological processes[10-12]. Approximately 50% of miRNAs in the body are located in tumor-associated regions or fragile sites. This indicates that the abnormal expression of miRNAs is closely related to the occurrence and development of human tumors. According to relevant reports[13-16], miRNA can be involved in gene expression and protein translation that severed as a switch. Moreover, it participates in multiple signal transduction pathways, eventually playing an important role in the occurrence and development of malignant tumors. Due to qualitative and quantitative changes in miRNAs

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during formation, their roles are entirely different in the occurrence and development of tumors. Some miRNAs serve as tumor suppressor genes that inhibit the occurrence and development of tumor[17-19]. However, some others act as oncogenes that promote the occurrence and development of tumor[20-22]. Therefore, expressions of different miRNAs in different tumor tissues or cells can serve as a diagnostic tool and prognostic marker for tumor. Furthermore, they can also become reliable targets for gene therapy. MiR-98-5p is a member of the let-7 miRNA family. By serving as an oncogene or a tumor suppressor gene, miR-98-5p is abnormally expressed in a variety of tumors, such as hepatocellular carcinoma and epithelial ovarian cancer[23,24]. However, the exact role of miR-98-5p in the development of breast cancer has not been fully elucidated yet. Therefore, the aim of this study was to investigate the correlation between miR-98-5p and breast cancer, and to explore the possible underlying mechanism.

Patients and Methods

Research Subjects and Cell Lines

From September 2015 to July 2017, a to[illegible] of 50 breast cancer patients who received treat[illegible] in the Breast Surgery Department of our hosp[illegible] were enrolled in this study. Ca[illegible]ue spec[illegible]mens and corresponding par[illegible]cen[illegible] normal tissue specimens were col[illegible]d. All [illegible]ale patients were diagnosed and [illegible]rmed [illegible]cer in the Pathology D[illegible]ar[illegible]ur hosp[illegible]l. No patient received [illegible]otherap[illegible]d chemotherapy before the op[illegible]n. Cancer t[illegible] specimens and para-carci[illegible]l tissue spe[illegible]ens were quickly cryop[illegible]erved [illegible]uid nitrogen after resection. F[illegible] cancer tiss[illegible]within 0.5 h after resectio[illegible]ere taken as canc[illegible]ssue specimens. Mea[illegible]e, nor[illegible] breast tissues that were more tha[illegible]m aw[illegible]rom cancer tissues within 0.5 h after [illegible]were ta[illegible]as para-carcinoma normal tissu[illegible]imen[illegible]his study was approved by [illegible]ics C[illegible] of Taizhou Hospital Affiliated [illegible]e Nanji[illegible]niversity of Chinese Medicine. Si[illegible]formed consents were obtained from all [illegible]efore the study. Breast cancer cell [illegible]e MCF-7 and normal breast epithelial cell line [illegible]E-10A were purchased from Shanghai Bioleaf B[illegible]nology Co., Ltd. (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) in a 5% CO_2, 37°C incubator. The cells in the logarithmic growth period were used for subsequent experiments.

Cell Transfection

MiR-98-5p mimics and si-Gab[illegible]re synthesized and transfected into breast c[illegible]cell line (MCF-7). The biological fu[illegible]ion of [illegible]98-5p was analyzed. Three gr[illegible] were est[illegible] to study the relevance[illegible]ween [illegible]R-98-5p MCF-7 cells, includi[illegible]C gro[illegible]egative control), miR-98-5p m[illegible]mi[illegible]ells tra[illegible]cted with miR-98-5p[illegible]mics), [illegible]mimi[illegible] Gab2 group (cells t[illegible]ected with[illegible]98-5p mimics and si-Gab2[illegible]e stuff wa[illegible]hased from RiboBio [illegible]ang2[illegible] China). Cell transfection was performed acco[illegible] to the instructions of Lip[illegible]ne RNAiM[illegible] (Life Technologies, T[illegible]o Fisher Scientific, [illegible]altham, MA, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total [illegible]l tissues and cells was extracted using the TRIzol method (Invitrogen, Carlsbad, [illegible]SA). The extracted RNA was then reverse [illegible]ed into complementary deoxyribose nucleic acid (cDNA) in strict accordance with miScript Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). The expression levels of miR-98-5p and Gab2 were detected *via* qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an internal reference. The relative expression levels of miR-98-5p and Gab2 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated 3 times in each group. Primer sequences used in this study were as follows: Gab2, F: 5'- CACCGCCTTCCCTTGTTTGGCAAAGC-3', R: 5'-GAACTTTGCCAAACAAGGGAAGGC-3'; miR-98-5p, F: 5'-CACCGCAGAAGCGGCACTTTATAAGCGAACT-3', R: 5'-TTATAAAGTGCCGCTTCTGCTTATAAGTTCGC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Luciferase Reporter Gene Assay

The 3'-untranslated region (3'-UTR) (Wt-Gab2-3'-UTR) of wild-type Gab2 and the 3'-UTR of mutant-type Gab2 (Mut-Gab2-3'-UTR) were co-transfected with empty plasmid and miR-98-5p overexpression plasmid into MCF-7 cells, re-

spectively. The cells were then cultured for 48 h, followed by detection using the Dual-Luciferase reporter gene assay kit. The cells were washed with PBS 3 times, lysed with PLB and shaken on a shaking table for 30 min. The cell lysis solution was mixed and blown evenly with LARII. The intensity of firefly Luciferase reaction was measured. After adding Stop & Glo Reagent (Madison, WI, USA), the intensity of Renilla Luciferase reaction was detected.

Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) lysate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was employed to extract total protein in transfected MCF-7 cells. The extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, St. Louis, MO, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After that, the membranes were blocked with 5% milk and incubated with primary antibodies of Gab2, E-cadherin, N-cadherin, Vimentin and GADPH [diluted at 1:1000, (Cell Signaling Technology (CST) Inc., Danvers, MA, USA)] at 4°C overnight. Subsequently, the membranes were incubated with the corresponding secondary antibodies (CST, Inc., Danvers, MA, USA) at room temperature for 2 h. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) method and analyzed using ImageJ software. GADPH was used as internal control. The relative changes in protein expression were calculated.

Cell Proliferation

2 h after transfection, MCF-7 cells were inoculated into 96-well culture plates at a density of 7000 cells/well. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 15 μL MTT reagent (500 μg/mL) was added into each well at 24, 72 and 96 h, respectively. After culturing for another 2 h, shaken horizontally for 10 min, the absorbance was measured using an enzyme-linked spectrophotometer, followed by zero setting using blank wells. Three replicates were set for each group.

Transwell Migration and Invasion Assay

For cell migration: transwell lower chamber was supplemented with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 15% FBS as a migration-inducing factor. Cells (5×10^4/well) were added to the upper chamber and maintained in a 37°C incubator. After 16 h of culture, the upper chamber was removed, washed with phosphate-buffered saline (PBS) and fixed with 95% anhydrous ethanol. Subsequently, the upper chamber was stained with 0.1% crystal violet at room temperature for 20 min. After drying, five fields were randomly selected for each sample, the number of migrated cells was counted under an inverted microscope (×200). For cell invasion, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was diluted to a final concentration of 1 mg/mL with 4°C pre-cooled serum-free DMEM. 50 μL of diluted Matrigel was added vertically to the center of the upper chamber, followed by incubation at 37°C for 0.5 h to form gelatinous gel. Meanwhile, the culture medium containing 15% FBS was added to the lower chamber. A total of 5×10^4 cells were added to the upper chamber and cultured for 36 h. The remaining steps were the same as the transwell migration assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Independent-samples *t*-test was used to compare the difference between the two groups. One-way analysis of variance was adopted to compare the difference among different groups, followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

Abnormal Expression of MiR-98-5p and Gab2 in Clinical Cases and Cells

The expression level of miR-98-5p in breast cancer tissues and adjacent normal tissues was determined by qRT-PCR. The results suggested that the miR-98-5p expression in cancer tissues was remarkably lower than that of adjacent normal tissues (Figure 1A). At the cellular level, similar results were obtained. The expression level of miR-98-5p was significantly decreased in breast cancer cells (MCF-7) when compared with normal breast epithelial cells (MCF-10A) (Figure 1B). These data demonstrated that miR-98-5p might play a regulatory role in breast cancer.

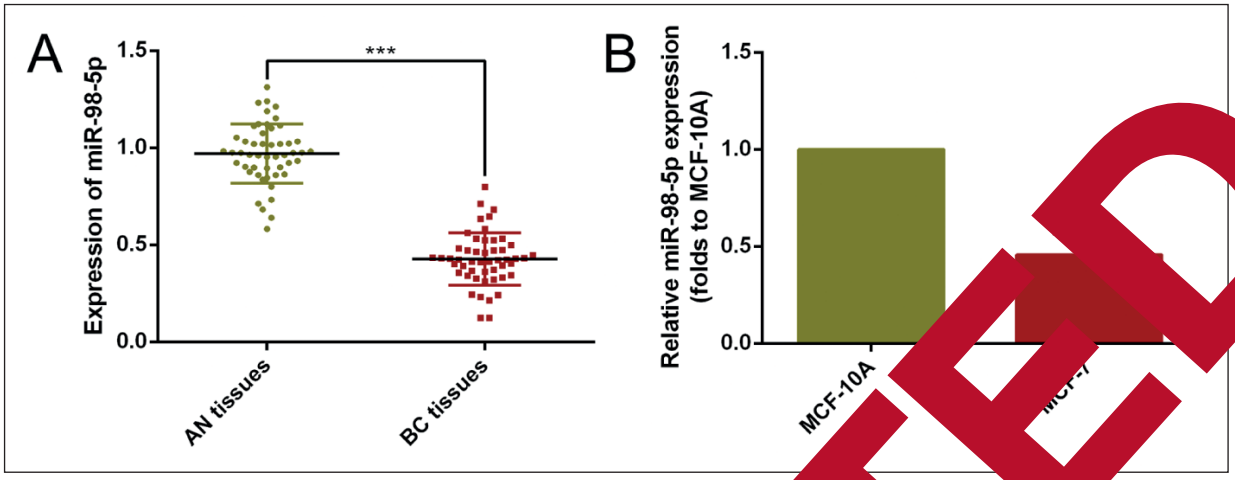

Figure 1. Expression of miR-98-5p in breast cancer tissue samples and cells. *A*, in the expression of miR-98-5p in breast cancer tissues and adjacent normal tissues (***$p < 0.001$ compared with adjacent normal tissue). ***B***, the expression of miR-98-5p in breast cell line (MCF-7) and normal breast epithelial cell line (MCF-10A) (* compared with MCF-10A).

Detection of Transfection Efficiency

Transfection efficiency was then detected by qRT-PCR. After transfection with mimics, the expression of miR-98-5p in MCF-7 cells was markedly increased. This confirmed that miR-98-5p mimics could effectively up-regulate expression of miR-98-5p in breast cancer cells (Figure 2).

Gab2 Was a Direct Target of miR-98-5p

MicroRNA target gene prediction software manifested that miR-98-5p could bind to the 3'-UTR of Gab2 (Figure 3). In addition, measuring the expression of miR-98-5p and Gab2 in breast cancer tissues and cells, we found that their expressions were relative (Figure 1A-1B, Figure 3A-3B). Luciferase reporter gene assay suggested that after co-transfection with miR-98-5p mimics and pmirGLO-Gab2 3'-UTR plasmids in MCF-7 cells, the Luciferase activity was significantly decreased. However, no significant change was found in the luciferase activity after transfection with pmirGLO-Gab2 3' UTR (mut) plasmid. These results suggested that miR-98-5p could complementarily bind to the 3'-UTR "seed region" of Gab2, further confirming that Gab2 was a potential target gene of miR-98-5p (Figure 3C). Western blotting assay indicated that the protein expression of Gab2 was remarkably declined after the up-regulation of miR-98-5p in MCF-7 cells (Figure 3D). The regulating effects were confirmed. We thought that Gab2 was a functional target gene of miR-98-5p during the progression of breast cancer.

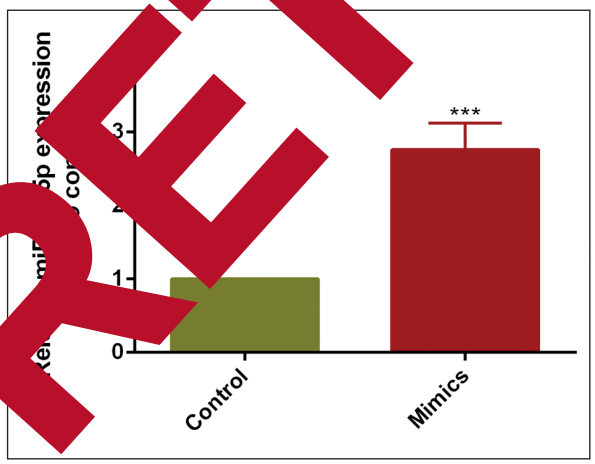

Figure 2. Transfection efficiency detected by qRT-PCR. (***$p < 0.001$).

MiR-98-5p Inhibited Proliferation of Breast Cancer Cells

MTT results showed that 24 h after miR-98-5p mimics transfection, the absorbance of breast cancer MCF-7 cells was significantly reduced and the relative cell viability was decreased. Meanwhile, the proliferation rate significantly slowed down. However, after overexpression of Gab2 in MCF-7 cells, the absorbance of MCF-7 cells was markedly increased, and the relative viability was higher. Furthermore, the proliferation rate was significantly accelerated (Figure 4).

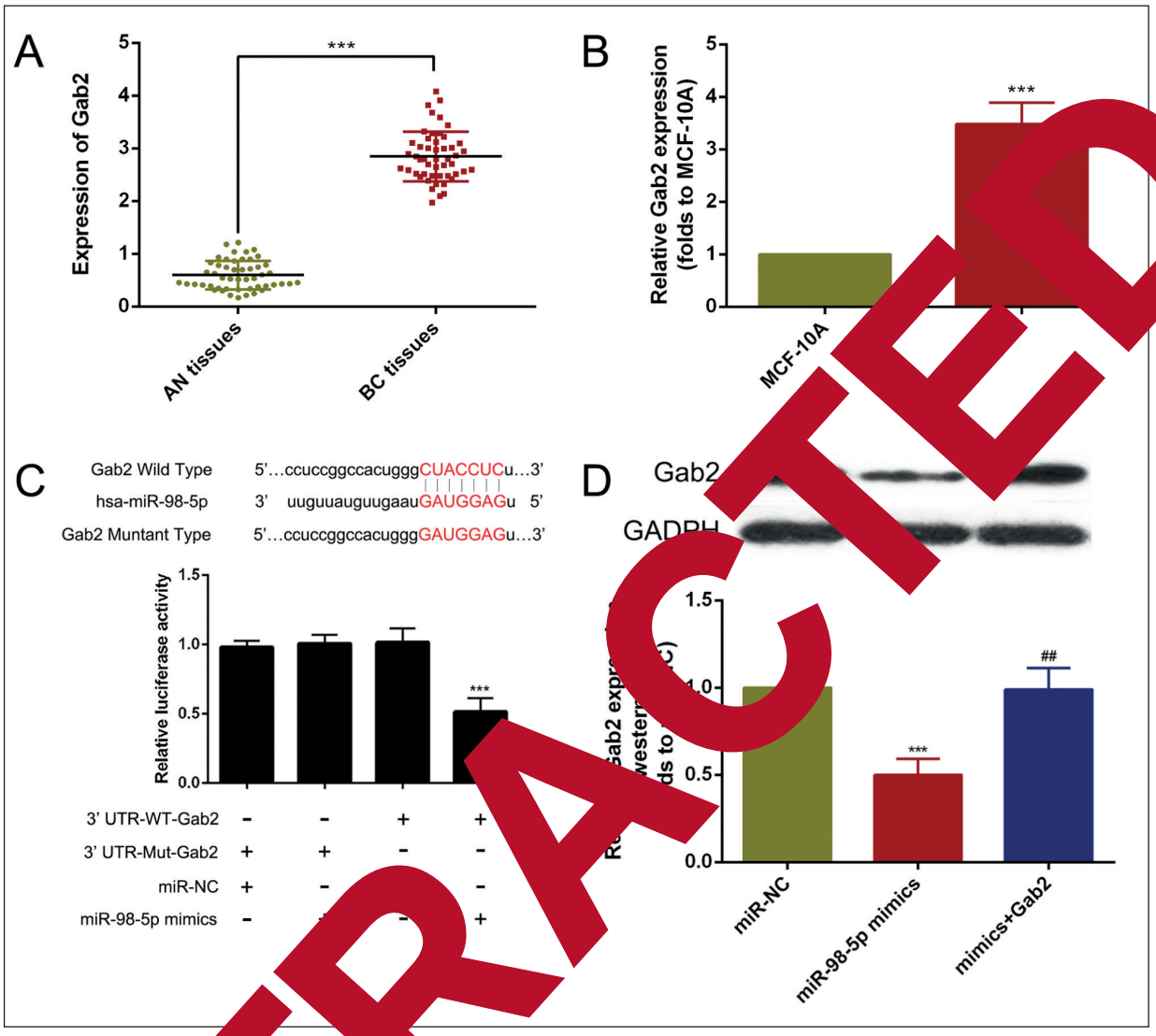

Figure 3. Gab2 was ...ct and funct... ...rget of miR-98-5p. ***A-B,*** The expressions of miR-98-5p in breast cancer tissue samples and cells. (...001). ***C,*** Diagra... ...tative miR-98-5p binding sites of Gab2 and relative activities of Luciferase reporters (***p ...1) ***D*** ...rn blot indic...d that miR-98-5p significantly decreased the protein expression level of Gab2 (***$p < 0.001$ ...C group; ...01 *vs.* Mimics group).

MiR...5p In...ited Invasion and M...ion ...CF-7 Cells

Mi... ...d inv... are the two most im... ...tant ... for ...rmal metastasis of tumor ... The ... EMT related proteins can refl... the me... ...ic ability of tumor cells[25]. In th... ...transwell assay indicated that the mi... ...vasion abilities of MCF-7 cells were ...gnificantly restricted by up-regulation of miR-...p (Figure 5C). Subsequently, we detected the p... expression levels of EMT markers after overexpression of miR-98-5p in MCF-7 cells by Western blot. As expected, the protein expression of E-cadherin was increased, whereas the protein expressions of N-cadherin and Vimentin were remarkably decreased (Figure 5A, 5B). Interestingly, the addition of Gab2 resulted in significantly enhanced malignant metastasis of MCF-7 cells.

Discussion

Breast cancer is a malignant tumor that seriously threatens human health. Despite continuous improvement in treatment, the incidence rate of breast cancer remains high[26,27]. Studies have demonstrated that migration and invasion are key factors leading to the death of patients with

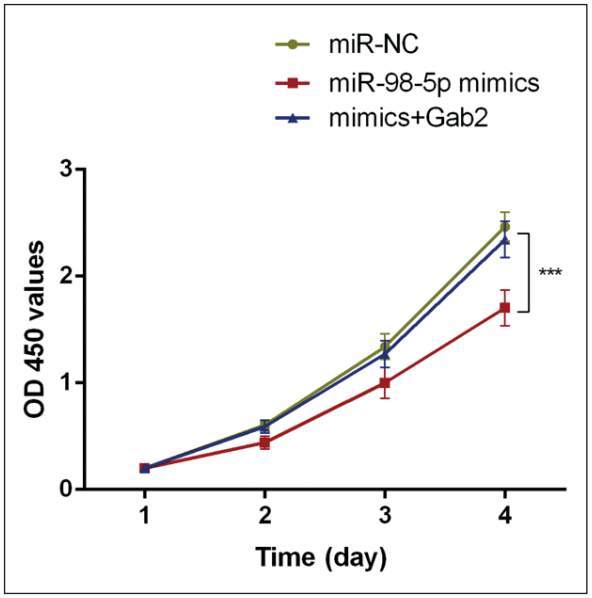

Figure 4. MiR-98-5p decreased proliferation of MCF-7 cells. Cell proliferation was detected by MTT assay (***$p < 0.001$).

breast cancer. In recent years, researches o[n] the mechanism of breast cancer have attracte[d] attention. Multiple studies have focused o[n] w[ays] to control the proliferation and metastas[is] of breast cancer and how to prevent and treat bre[ast] cancer. Meanwhile, researcher[s] have paid muc[h] attention to improving the s[urvival] and life quality of patients. Ther[efore], it is extremely important to study the m[olecular] m[echanism] of breast cancer. Tumor migra[tion] and invasio[n] refer to the processes [of] tumor [cells] invade from the primary site [to] the surroundi[ng] matrix, and to reach other [tissues] through lymph[atic] and/or blood circulation for [further] metastasis. Migration and invasion are gr[eat] obstacles to the treatment of breast cancer p[atients], which may even easily lead to poor prognosis. The occurrence of migration and invasion involves a variety of [complex] and sig[nal] transduction pathways. Recently, the role of miRNAs has attracted increasingly [much] attention from scholars at home and abroad. MiRNA regulates its target genes mainly by binding to the complementary region [of] mRNA 3'UTR, thereby inhibiting translation [or] leading to degradation. Screening the target genes for miR-98-5p is crucial for clarifying its mechanism in the occurrence of breast cancer. Meanwhile, it is of great significance in exploring new therapeutic targets. In this study, the Luciferase reporter gene assay revealed that Gab2 was a target gene of miR-98-5p. The overexpression of miR-98-5p could significantly inhibit the expression of Gab2. These results further indicated that miR-98-5p could regulate the expression of Gab2 in a target. Grb2-associated binder family protein (Gab) is a kind of highly-conserved scaffolding protein during evolution. Gab2 is a member of the Gab family encoded by the Gab2 (11q13) gene. This region is generally highly expressed in breast cancer. About 10-15% of patients with breast cancer have chromosomal amplification in this segment[29,30]. Gab2 is composed of the following structures: N-terminus of PH domain, the central part of proline-rich domain (PRD) and C-terminus containing multiple tyrosine residues[31]. The role of Gab2 in the occurrence and development of malignant tumors has aroused widespread concern. Researches have demonstrated that Gab2 is involved in regulating the proliferation, migration and invasion of various tumors[28,33-35]. It has been found that Gab2 plays a critical role in the occurrence and development of breast cancer[29-31,36,37]. In this work, our results suggested that the expression level of Gab2 was significantly increased in breast cancer specimens and cells, which was consistent with previous reports. The mechanism of miRNA involved in tumor invasion and migration is complex. However, EMT in tumors is considered the most important and indispensable process currently[38,39]. EMT refers to the process that epithelium-derived tumor cells lose epithelial phenotype while obtaining mesenchymal phenotype, thereby gaining the capacity of invasion and migration. It is the first and crucial step in metastasis of tumor cells[40]. EMT is mainly manifested as dissociation of epithelial cells, loss of cell adhesion, rearrangement of cytoskeletal accompanied by decreased epithelial cell markers (E-cadherin) and increased mesenchymal cell markers (N-cadherin and Vimentin), and gained metastasis capacity cells[41]. In this work, we analyzed the effects of miR-98-5p upregulation in breast cancer cell line MCF-7. The results found that up-regulation of miR-98-5p significantly inhibited the proliferation, whereas reduced the migration and invasive abilities of breast cancer cells. However, the overexpression of Gab2 in cells could effectively reverse the above-mentioned phenomenon induced by miR-98-5p. This suggested that low expression of miR-98-5p in breast cancer might regulate the abnormal cell proliferation, increase cell metastasis capacity

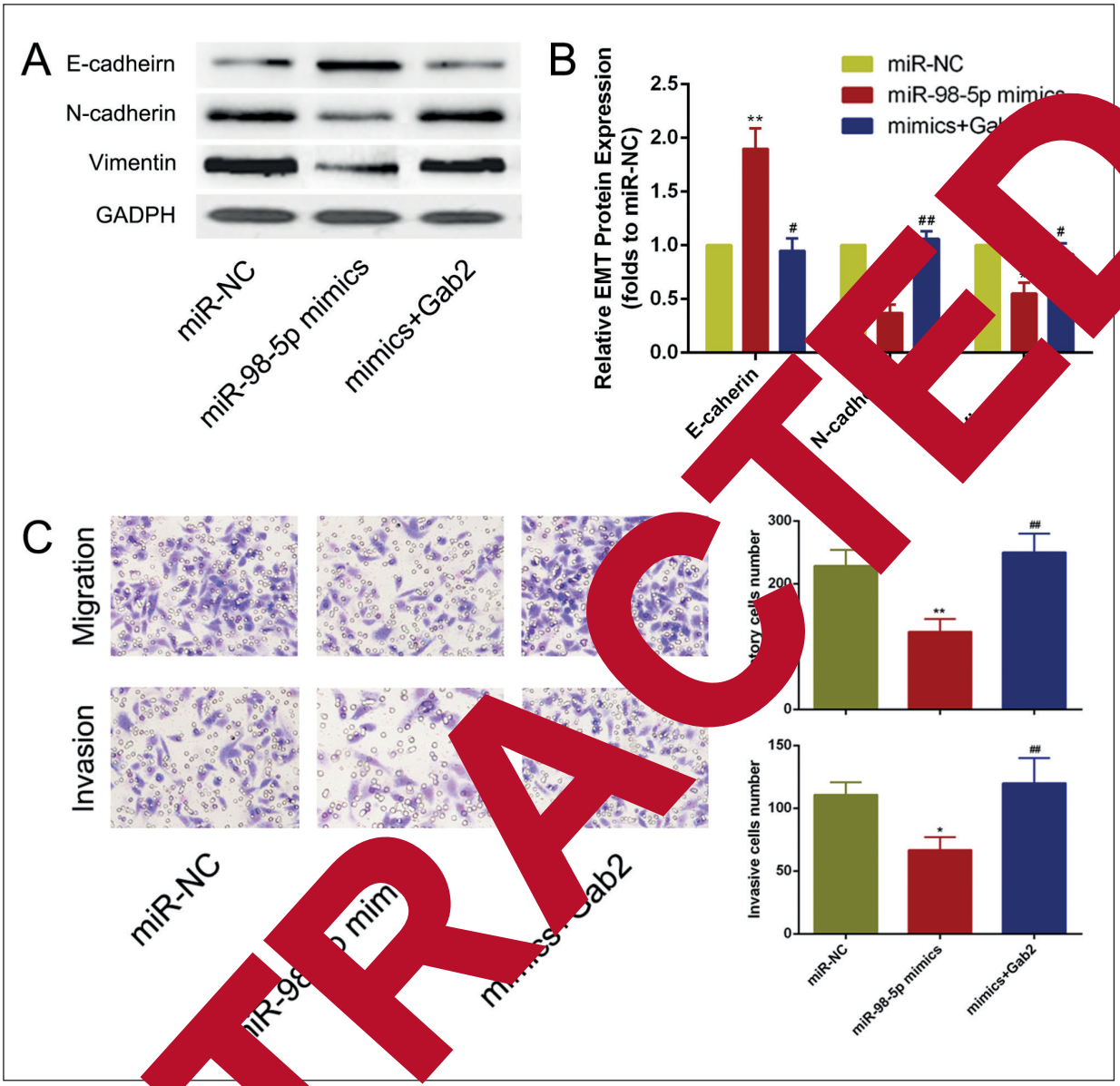

Figure 5. ...98-5p/Gab2 ax... ...ited invasion and migration of MCF-7 cells. ***A-B,*** The protein expressions of epithelial-mesench... transition (EMT) m... after transfection with mimics or si-Gab2 were detected by Western blot. ***C,*** The invasi... migra... abilities dete...ed by transwell assay. All data were presented as means ± standard deviations. (*$p <$ 0.05... < 0.01 ... group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Mimics group).

...tivate... ...rrence of EMT through by reg...ing Ga... Our findings might provide ne... for elucidating the pathogenesis of ...

cells. Meanwhile, we found that miR-98-5p affected the occurrence of EMT by targeted binding to Gab2. Our study might suggest a new basis for further studies on the mechanism of breast cancer.

Conclusions

We indicated that miR-98-5p regulates the proliferation and metastasis of breast cancer

Conflict of Interest

The Authors declare that they have no conflict of interests.

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