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MiR-92b inhibited cells EMT by targeting Gabra3 and predicted prognosis of triple negative breast cancer patients

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Abstract. – OBJECTIVE: Triple negative breast cancer (TNBC) is a subtype of breast cancer without the three markers, which has a poor prognosis than other types. Recently, studies have identified that microRNA-92b (miR-92b) acted as potential oncogene in tumor progression, however, the biological roles of miR-92b in TNBC remain unknown. The purpose of this study was to investigate the functions of miR-92b and verify its effect on the regulation of Gabra3 in TNBC.

MATERIALS AND METHODS: Dual-Luciferase reporter assay was recruited to confirm whether miR-92b directly binds to the 3'-untranslated region (3'-UTR) of Gabra3 mRNA in TNBC. Transwell assay was employed to analyze the capacities of migration and invasion. Western blot was applied to evaluate the expression of the special proteins that including Gabra3, epithelial-mesenchymal transition (EMT) markers and GAPDH.

RESULTS: We demonstrated that miR-92b was remarkably low expressed in TNBC tissues and cell lines, and particularly in inhibiting the migration, invasion and EMT of TNBC cells. On the contrary, Gabra3 was significantly overexpressed in TNBC tissues and cell lines in comparison with the corresponding paracancerous tissues and the normal breast epithelial cell line. The expression of miR-92b had a negative correlation with the expression of Gabra3 in TNBC tissues. Downregulation of Gabra3 could inhibit the migration, invasion and EMT of TNBC cells. MiR-92b mediated the expression of Gabra3 through directly binding the 3'-UTR of Gabra3 mRNA. In addition, low expression of miR-92b or overexpression of Gabra3 predicted poor prognosis of TNBC patients.

CONCLUSIONS: MiR-92b inhibited the migration and invasion-mediated EMT through directly targeting to the 3'-UTR of Gabra3 mRNA in triple negative breast cancer. The newly identified miR-92b/Gabra3 axis may make it to be a new target for clinical diagnosis and treatment of TNBC.

Key Words:
MiR-92b, EMT, Triple negative breast cancer, Prognosis.

Introduction

Breast cancer (BC) is one of the most widespread malignant tumor in female, with approximately 1.67 million cases diagnosed worldwide each year[1]. Triple negative breast cancer (TNBC) is a subtype of breast cancer without the three markers that were estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2)[2,3]. Due to the early recurrence, quick progress and poor prognosis, TNBC become a new hot spot of research in recent years[4]. Therefore, it may provide potential therapeutic value for TNBC to investigate underling molecular biomarkers.

MicroRNAs (miRNAs), a class of endogenous small noncoding RNAs with a length of 19-24 nucleotides, play a crucial role in gene silencing through directly binding to the 3'-untranslated region (3'-UTR) of target mRNAs at post-transcriptional level[5]. Growing evidence[6-9] has indicated that multiple miRNAs, acting as gene-expression regulators, are usually involved in the biological processes of breast cancer including: miR-26b, miR-1271, miR-124 and miR-130a. MiR-92b has been reported to be ectopic expression in many cancers that including breast cancer[10]. Zhao et al[11] have illuminated that miR-92b inhibited the epithelial-mesenchymal transition-induced migration and invasion of nasopharyngeal cancer cell [11]. Similarly, miR-92b could eliminate proliferation, migration, invasion and promote the apoptosis of glioma cell

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[12]. However, miR-92b could improve the abilities of epithelial-mesenchymal transition (EMT), migration and invasion in bladder cancer and also in hepatocellular carcinoma[13,14]. Therefore, the focus of this research is to explore the molecular mechanism of miR-92b in breast cancer.

GABA type A receptor α3 (Gabra3), a subunit of the gamma-aminobutyric acid reporter (GABA A), could mediate inhibitory synaptic transmission in the mature central nervous system[15,16]. Based on its high variability in function and expression, Gabra3 was connected with the onset and progression of several diseases including lung adenocarcinoma, hepatocellular carcinoma, thyrotoxic periodic paralysis and epilepsy[15,17-19]. In hepatocellular carcinoma, Liu et al[15] discovered that GABA promoted tumor growth through overexpressed Gabra3[15]. Liu et al[17] also showed that Gabra3 promoted lymphatic metastasis through mediating the upregulation of matrix metalloproteinases in lung adenocarcinoma [17]. However, Gumireddy et al[20] have determined that Gabra3 could activate the AKT pathway to promote migration, invasion and metastasis in breast cancer. Long et al[21] have illuminated that miR-92b acted as a tumor suppressor through directly targeting to Gabra3 in pancreatic cancer. Thus, we strongly believe that miR-92b could inhibit the EMT by directly targeting to Gabra3 in TNBC cells.

Patients and Methods

Patients and Clinical Samples

A collection of 51 TNBC patients were included in this study from the Central Hospital of Wuhan during 2014 to 2016, and obtained 51 pairs of cancer tissues and corresponding paracancerous tissues. Before surgery, all the patients did not receive radiotherapy or chemotherapy. Surgically excised tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C. The clinicopathological characteristics of all patients and their connection with the expression of miR-92b were described in Table I. All the specimens of this study obtained the informed consent from patients and were approved by the Ethical Committee of The Central Hospital of Wuhan.

Cell Lines and Cell Culture

The normal breast epithelial cell line MCF-10A and two TNBC cell lines (BT549 and MDA-MB-231) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Table I. miR-92b expression and clinicopathological features in 51 triple negative breast cancer.

Clinicopathological features	Cases (n=51)	miR-92b expression		*p*-value*
		22 High (%)	29 Low (%)	
Age (years)				
≤ 50	23	9 (39.1)	14 (60.9)	0.601
> 50	28	13 (46.4)	15 (53.6)	
Tumor size (mm)				
≤ 5.0	27	15 (55.6)	12 (44.4)	0.058
> 5.0	24	7 (29.2)	17 (70.8)	
TNM stage				
I-II	23	14 (60.9)	9 (39.1)	0.020*
III-IV	28	8 (28.6)	20 (71.4)	
Lymph-node metastasis				
0-2	24	14 (58.3)	10 (41.7)	0.039*
> 2	27	8 (29.6)	19 (70.4)	
Metastasis				
Present	21	12 (57.1)	9 (42.9)	0.091
Absent	30	10 (33.3)	20 (66.7)	
Gabra3				
Negative	24	14 (58.3)	10 (41.7)	0.039*
Positive	27	8 (29.6)	19 (70.4)	

**p*-values are calculated with Chi-square test.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) from TNBC clinical specimens and cells. To quantify the miR-92b and Gabra3, the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the TaqMan Human MRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) were applied to perform the PCR amplification. The relative levels of miR-92b and Gabra3 were normalized by U6 small nuclear RNA (U6) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each reaction was performed in triplicate and analysis was performed in accordance with the $2^{-\Delta\Delta Cq}$ method. The sequences of the primers were as follows: miR-92b forward 5'-GGGGCAGTTATTGCACTTGTC-3' and reverse 5'-CCAGTGCAGGGTCCGAGGTA-3'; U6 forward 5'-GTGCTCGCTTCGGCAGCACATATAC-3' and reverse 5'-AAAAATATGGAACGCTCACGAATTTG-3'; Gabra3 forward 5'-TCGGTCTCTCCAAGTTTGTGC-3' and reverse 5'-TTCCGTTGTCCACCAATCTGA-3'; GAPDH forward 5'- ATGGGGAAGGTGAAGGTCGG-3' and reverse 5'- GACGGTGCCATGGAATTTGC-3'.

Protein Extraction and Western Blotting

We extracted the total proteins from tissues and cells utilized the Radioimmunoprecipitation Assay (RIPA) Lysis Buffer, which supplemented with phenylmethyl sulfonyl fluoride (PMSF; Beyotime, Shanghai, China). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were utilized to separate the cellular proteins, and then the blots were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Followed, the primary antibodies were employed to incubate the blots at 4°C overnight. The primary antibodies were against Gabra3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-Cadherin (1:1000, Abcam, Cambridge, MA, USA), N-Cadherin (1:1000, Abcam, Cambridge, MA, USA), Vimentin (1:1000, Abcam, Cambridge, MA, USA) and GAPDH (1:2000, Cell Signaling Technologies, Danvers, MA, USA). After washed with Tris buffered saline-Tween (TBST) three times, the membranes were incubated with rabbit (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse reddish peroxidase (HRP)-conjugated secondary antibody with (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, then the protein signal were detected using the Bio-Rad Gel imaging system (Hercules, CA, USA).

Transwell Assay

200 μL MDA-MB-231 seeded cells were re-suspended in serum-free medium and into transwell inserts covered with or without Matrigel (Clontech, Mountain View, CA, USA). Meanwhile, 500 μL Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 15% fetal bovine serum (FBS), which acted as an inducer, was added into the lower chamber. After cultured 48 h, the cells still on the upper surface of the member were wiped out used a cotton swab. The migrated or invaded TNBC cells, which were removed to the lower surface of the member, were fixed and stained with methanol and 0.1% crystal violet in order.

Transfection

To overexpress or knockdown of miR-92b, miRNA vectors, including the miR-92b mimic and the miR-92b inhibitor (anti-miR-92b) that were purchased from GenePharma (Guangzhou, China), were transfected into MDA-MB-231 cells. The Gabra3 overexpressing plasmid (pcDNA3.1-Gabra3) and interferential plasmid (siRNA-Gabra3) were employed to over or interfere the expression of Gabra3 in MDA-MB-231 cells. MDA-MB-231 cells were seeded into 6-well plate and cultured at 37°C for overnight. Before transfection, the vectors were mixed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) and wrapped by Lipofectamine 2000 according to the recommended instructions; next, the mixture was added into each cell of the 6-well plate. The cells were harvested after 48 h of transfection.

Plasmid Construction and Luciferase Reporter Assay

Gabra3 has a putative binding site at 1771-1778 on mRNA 3'-UTR, predicted by TargetScan (http://www.targetscan.org/vert_71/). To verify that Gabra3 was a direct target of miR-92b, the putative binding sequences were mutated from CCGUCCC to GGCAGGG. After that, both wild type and mutant sequences were inserted into the pmirGlo vector (Promega, Madison, WI, USA), and constructed the Gabra3-pmirGlo-WT vector (WT) and Gabra3-pmirGlo-MUT vector (MUT), respectively. MiR-92b mimic or negative control vector and WT or MUT were co-transfected into TNBC cell line MDA-MB-231 and then harvested the cells after cultured for 48 hours. According to the manufacturer's protocols, the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) was used to the Luciferase activity with Renilla as internal reference.

Statistical Analysis

Statistical analyses were performed utilized Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism 6.0 (La Jolla, CA, USA). Data were presented as the mean ± SD from at least three independent experiments. Student *t*-test was performed to analyze the differences between two groups. One-way ANOVA were performed to analyze the differences among more groups, followed by Post-Hoc Test LSD (Least Significant Difference). Pearson's correlation analysis was employed to analyze the connection between the expression of miR-92b and Gabra3. Kaplan-Meier method and the log-rank test were applied to evaluate the survival of TNBC patients. The association between the expression of miR-92b and clinicopathological factors was assessed using Chi-square test. $p<0.05$ was considered to be statistically significant.

Results

The Expression of miR-92b in TNBC Tissues and Cell Lines

qRT-PCR was performed to evaluate the expression of miR-92b in tissues and cell lines. We verified that miR-92b was dramatically down-regulated ($p<0.0001$) in triple negative breast cancer tissues compared to the corresponding paracancerous tissues (Figure 1A). Apart from tissues samples, we also assessed the expression of miR-92b in two cell lines, and the results showed that miR-92b expression was decreased in TNBC cell lines BT549 ($p=0.0084$) and MDA-MB-231 ($p=0.0007$) as compared with the normal breast epithelial cell line MCF-10A (Figure 1B).

miR-92b Suppressed the Migration and Invasion-Mediated EMT of TNBC Cells

To verify the impacts of miR-92b on the migration and invasion, the miR-92b mimic and the miR-92b inhibitor were utilized to overexpress ($p=0.0008$) or knockdown ($p=0.0059$) miR-92b in MDA-MB-231 cells, and the transfection efficiencies were showed in Figure 2A. Transwell assay were evaluated by migratory and invasive abilities. Not unfortunately, the miR-92b mimic reduced both number of migration ($p=0.0006$) and invasion ($p=0.0002$), while miR-92b inhibitor markedly increased the number of migrated ($p=0.0047$) and invaded ($p=0.0028$) MDA-MB-231 cells (Figure 2B). To evaluate the potential role of miR-92b in EMT process, Western blot was used to assess the expression of EMT markers including the epithelial marker E-cadherin, mesenchymal markers N-cadherin and Vimentin. And we discovered that overexpression of miR-92b increased the expression of E-cadherin, while the expression of N-cadherin and Vimentin was decreased in MDA-MB-231 cells. On the contrary, knockdown of miR-92b reduced the expression of E-cadherin, whereas the expression of N-cadherin and Vimentin was increased in MDA-MB-231 cells (Figure 2C).

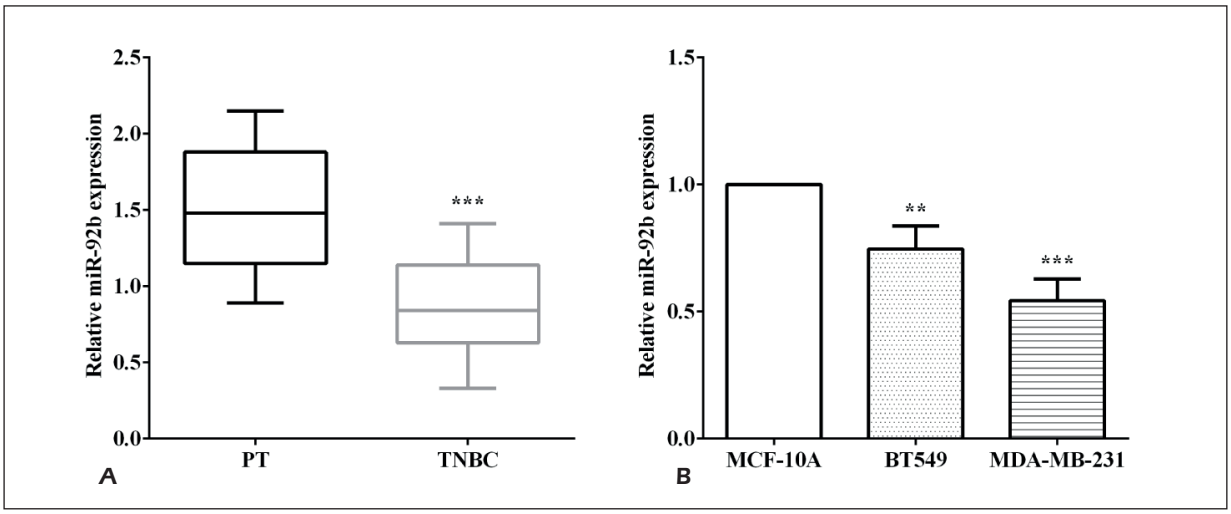

Figure 1. The expression of miR-92b in TNBC tissues and cell lines. **A**, qRT-PCR was verified that miR-92b was down-regulated in TNBC compared to the corresponding paracancerous tissues. **B**, The expression of miR-92b was lower in TNBC cell lines BT549 and MDA-MB-231 than that of normal breast epithelial cell line MCF-10A.

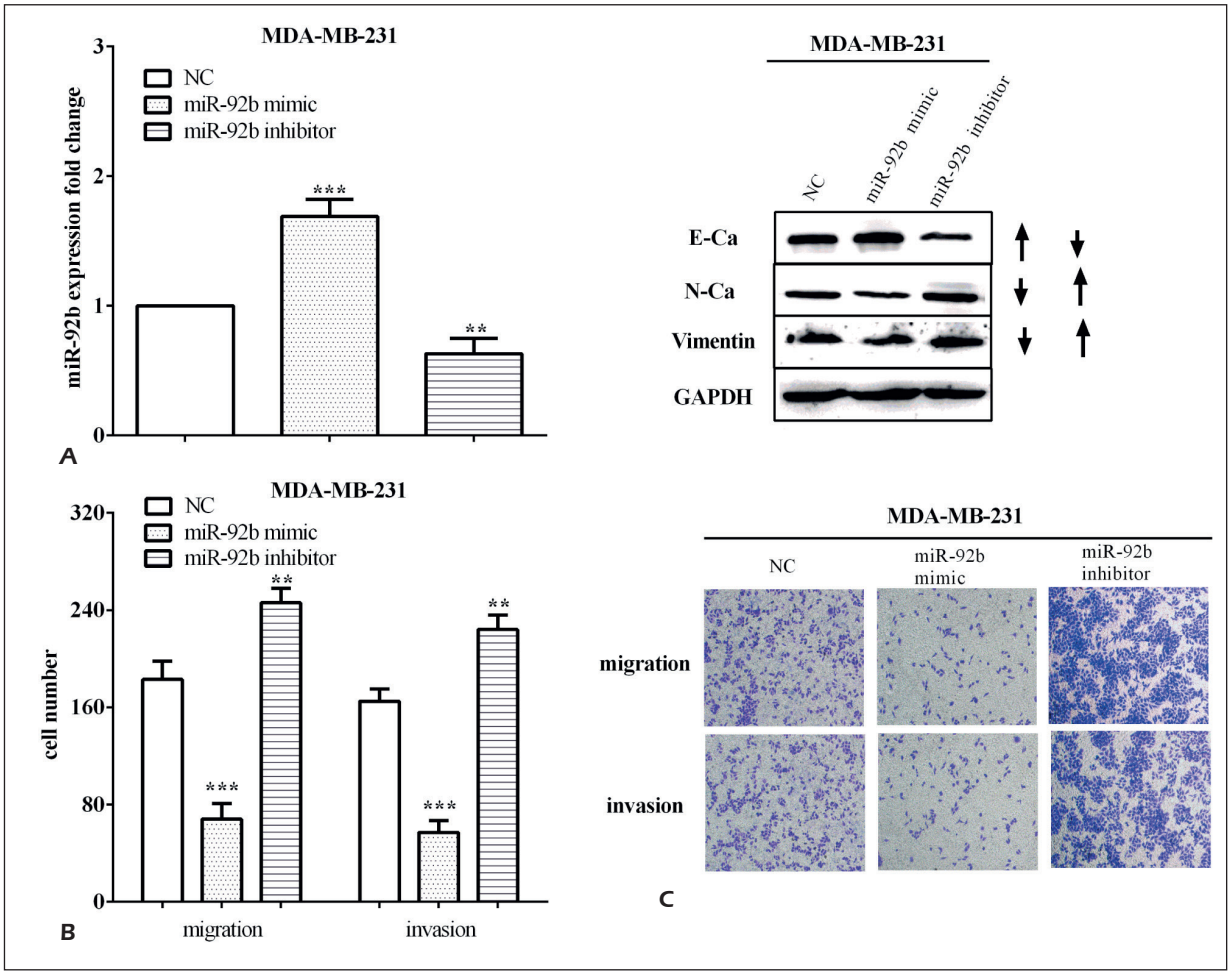

Figure 2. MiR-92b suppressed the migration and invasion-mediated EMT of TNBC cells. **A**, The transfection efficiency of overexpressing or interfering of miR-92b in MDA-MB-231 cells. **A**, Transwell assay revealed the abilities of migration and invasion MDA-MB-231 cells. **C**, MiR-92b inhibited the EMT of MDA-MB-231 cells.

The Expression of Gabra3 and the Correlation with miR-92b in TNBC Tissues

To explore the expression of Gabra3, the mRNA level of Gabra3 were measured by qRT-PCR in TNBC tissues. The expression of Gabra3 was higher (p<0.0001) in TNBC tissues than that of corresponding paracancerous tissues (Figure 3A). Thus, we analyzed the connection between the expression of miR-92b and Gabra3, and discovered that the expression of miR-92b had a negative correlation (p<0.0001, r=-0.8180) with Gabra3 expression in TNBC tissues (Figure 3B). What's more, the Gabra3 was found to be upregulate in TNBC cell lines BT549 (p=0.0018) and MDA-MB-231 (p=0.0007) in comparison with the normal breast epithelial cell line MCF-10A (Figure 3C).

Knockdown of Gabra3 Inhibited the Migration and Invasion-Mediated EMT

To explore the biological function of Gabra3 in TNBC, siRNA-Gabra3 plasmid was transfected into MDA-MB-231 cells. Both qRT-PCR and Western blot were used to assess the efficiency of downregulating Gabra3 by using siRNA-Gabra3 (p=0.0040) (Figure 4A and 4B). We discovered that knockdown of Gabra3 remarkably inhibited the migration (p=0.0012) and invasion (p=0.0008) of MA-MB-231 cells (Figure 4C). Moreover, downregulation of Gabra3 caused rise of the expression of epithelial marker E-cadherin and reduction of the expression of mesenchymal markers N-cadherin and Vimentin in MDA-MB-231 cells (Figure 4D).

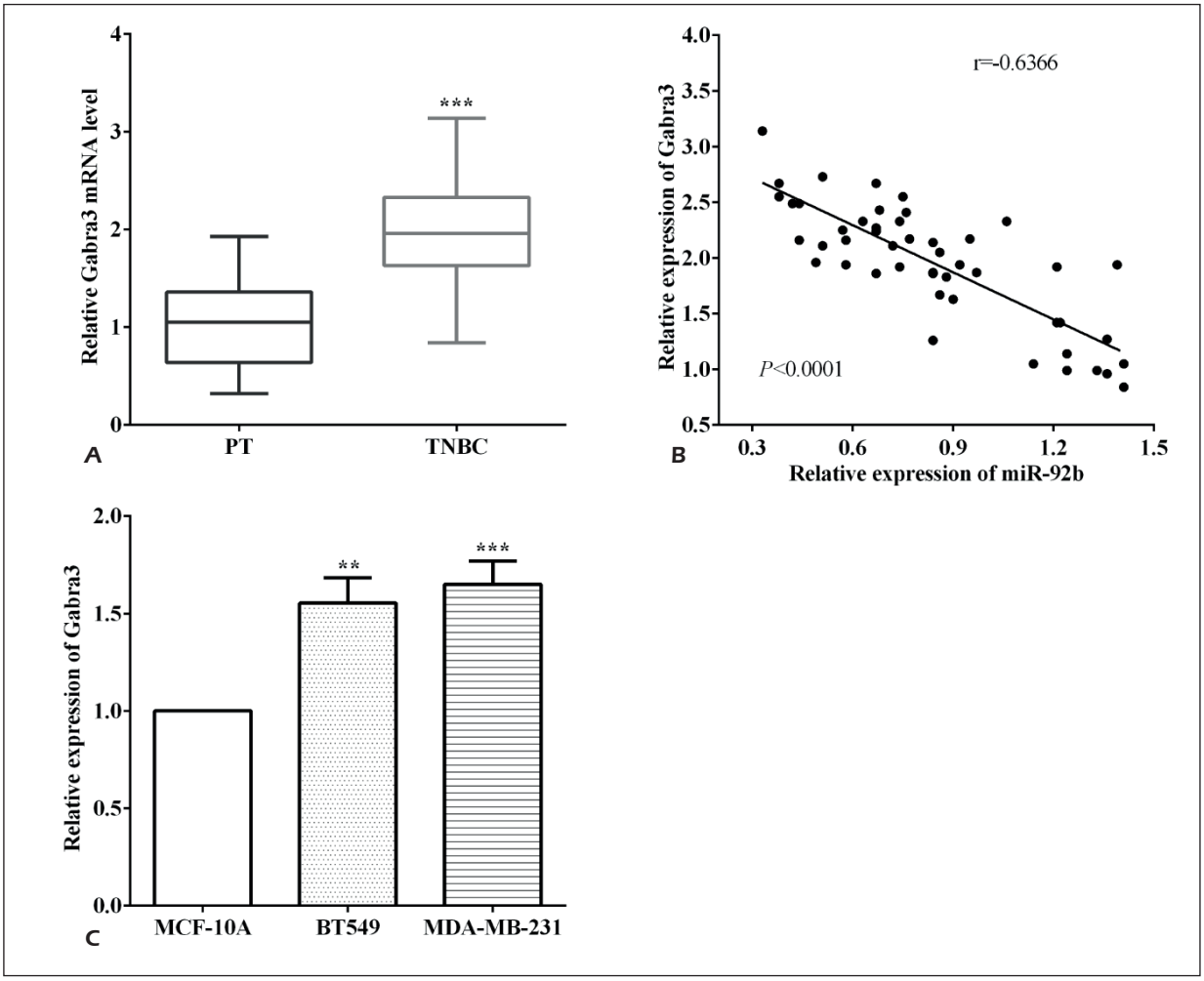

Figure 3. The expression of Gabra3 and the correlation with miR-92b in TNBC tissues. **A**, The expression of Gabra3 was higher in TNBC tissues than that in corresponding paracancerous tissues. **B**, The expression of miR-92b had a negative correlation with Gabra3 in TNBC tissues. **C**, Gabra3 was upregulated in TNBC cell lines BT549 and MA-MB-231 *vs.* normal breast epithelial cell line MCF-10A.

miR-92b Targeted to Gabra3 and Regulated the Expression of Gabra3 in TNBC Cells

To explore the molecular mechanism of how miR-92b regulated the EMT of TNBC cells, bioinformatics analysis software TargetScan was utilized to search for the potential targets of miR-92b. Gabra3 was found to contain a putative binding site of miR-92b that is located at 1771-1778 in mRNA 3'-UTR. Luciferase assay was performed to verify whether Gabra3 is a direct target of miR-92b. Before the experiment, the binding sequences on mRNA 3'-UTR of Gabra3 were mutated from CCGUCCC (WT) to GGCAGGG (MUT) (Figure 5A). The Luciferase activity of wild-type Gabra3 3'-UTR was reduced (p=0.0013) by the miR-92b mimic, whereas it had no influence (p=0.5034) on MUT (Figure 5B). In addition, qRT-PCR was performed to verify that miR-92b mediated the expression of Gabra3. As expected, the expression of Gabra3 was suppressed by the miR-92b mimic (p=0.0023), while it was increased by the miR-92b inhibitor (p=0.0016) in MDA-MB-231 cells (Figure 5C).

Identification of miR-92b and Gabra3 Associated with Poor Survival in Triple Negative Breast Cancer

According to the median value of miR-92b expression, we divided all the patients into two groups (miR-92b(-) and miR-92b(+)), and there were 22 and 29 patients, respectively. We discovered that the expression of miR-92b has a negative correlation with the TNM stage, lymph-node me-

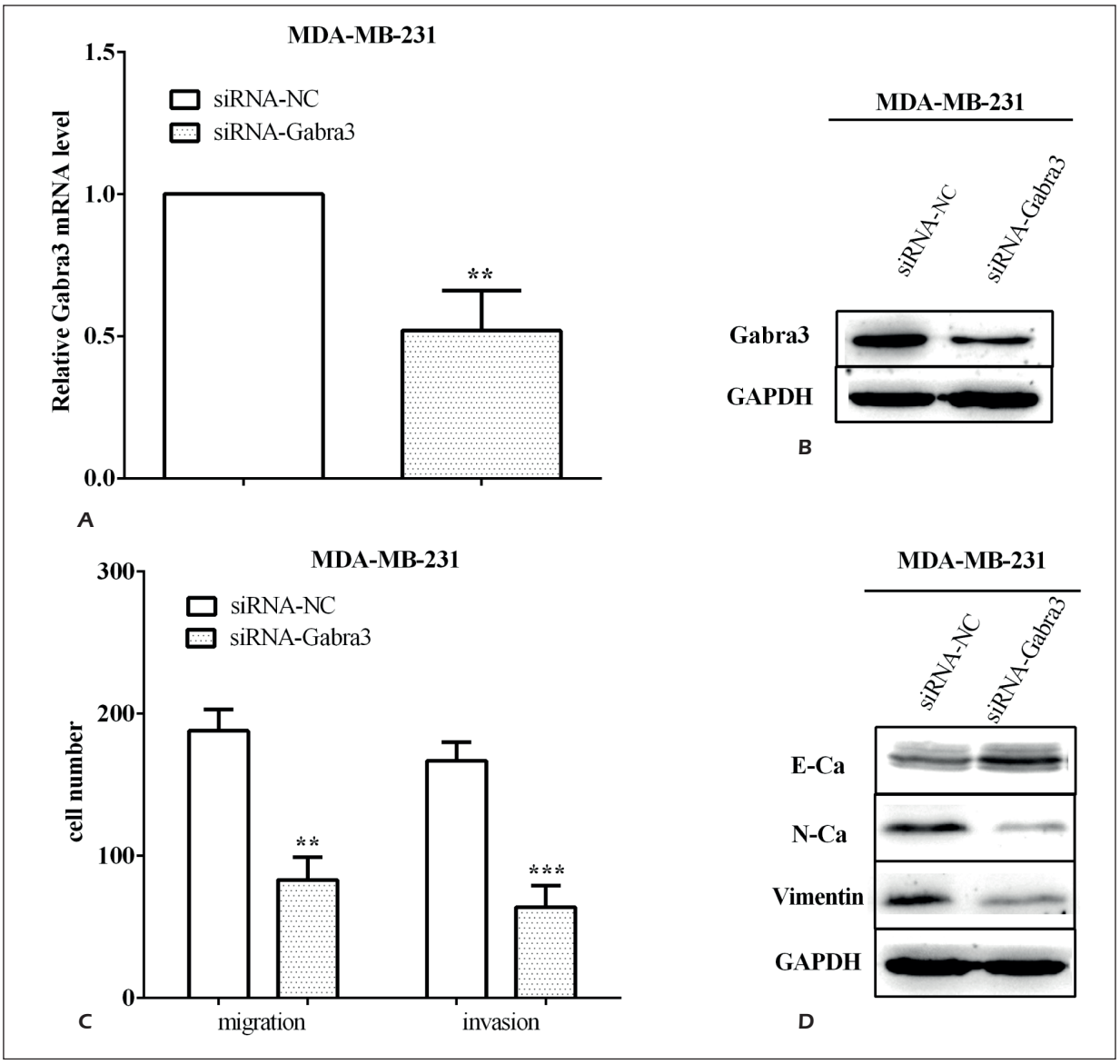

Figure 4. Knockdown of Gabra3 inhibited the EMT of MA-MB-231 cells. ***A*** and ***B***, Both qRT-PCR and Western blot illuminated the efficiency of transfecting with siRNA-Gabra3 in MA-MB-231 cells. ***C***, Knockdown of Gabra3 remarkably inhibited the migration and invasion in MA-MB-231 cells. ***D***, Downregulation Gabra3 suppressed the EMT in MDA-MB-231 cells.

tastasis and the expression of Gabra3. However, it had a tendency to associate with tumor size and metastasis (Table I). Further, Kaplan-Meier method illuminated that it had a worse overall survival (OS) of TNBC in miR-92b(-) than in miR-92b(+) (p=0.0220) (Figure 6A). On the other hand, the patients were separated into two groups (Gabra3(+) and Gabra3(-)), which were 24 and 27 patients, respectively. And we showed that Gabra3(+) predicted poor prognosis in TNBC patients, indicating that miR-92b(-) or Gabra3(+) predicted poor prognosis of TNBC (p=0.0371) (Figure 6B).

Discussion

Triple negative breast cancer is a subtype of breast cancer without ER, PR and Her2[2,3]. Recently, due to early recurrence and lower prognosis, the outcomes of patients with TNBC remain unsatisfactory[4]. Therefore, it is essential to discover novel biomarkers to help early diagnosis and improve prognosis. MicroRNAs have been reported to act as gene-expression regulators, participated in the gene expression and mRNA degradation at post-transcriptional level[5]. MiR-

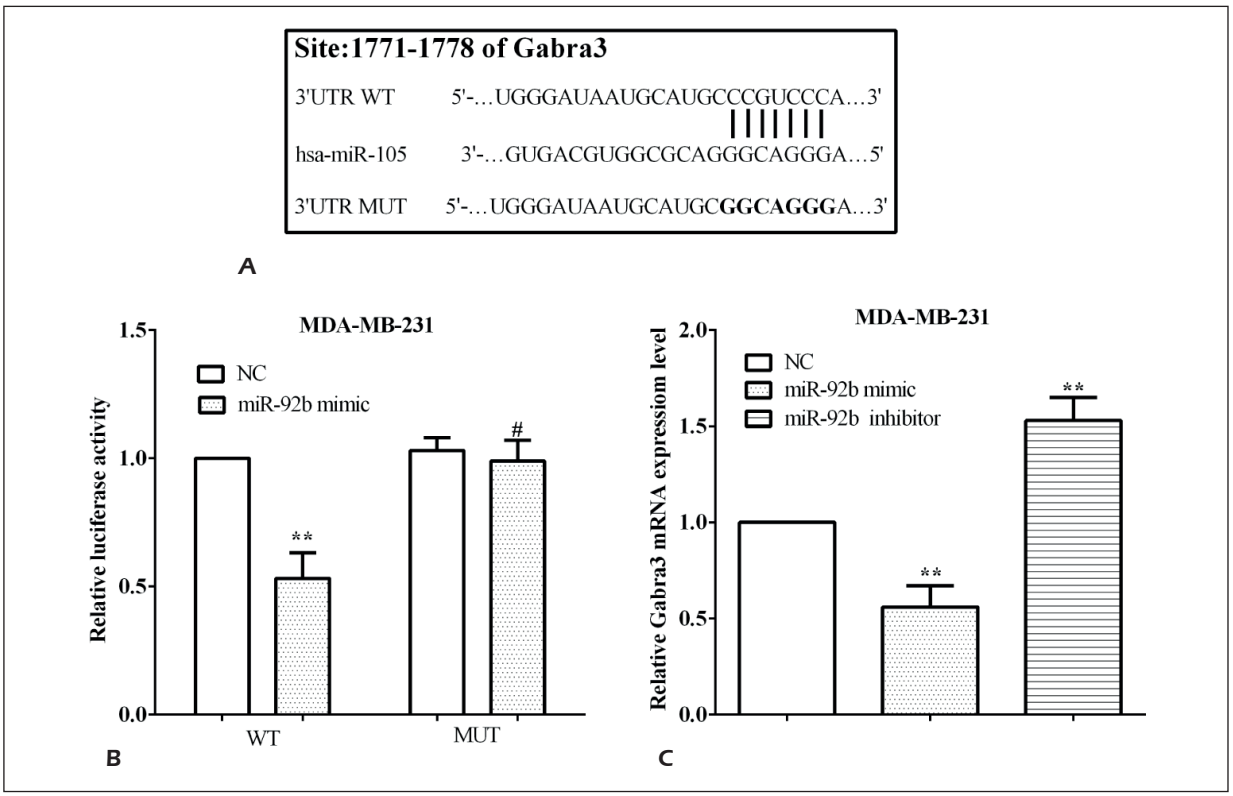

Figure 5. MiR-92b targeted to Gabra3 and regulated Gabra3 expression in TNBC cells. **A**, The putative binding site of miR-92b on mRNA 3'-UTR. **B**, The luciferase activity of wild-type Gabra3 3'-UTR was reduced by the miR-92b mimic, whereas had no influence on MUT. **C**, The miR-92b inhibited the expression of Gabra3 in MDA-MB-231 cells.

92b has been reported to be abnormal expression in many cancers that including in breast cancer[10]. MiR-92b inhibited cellular migration and invasion, and EMT of nasopharyngeal cancer[11]. Similarly, miR-92b has been found to be inhibited the proliferation, migration and invasion through PTEN/Akt signaling pathway in glioma[12]. Our results were consistent with the previous findings that miR-92b was significantly downregulated in TNBC tissues and cell lines BT549 and MDA-

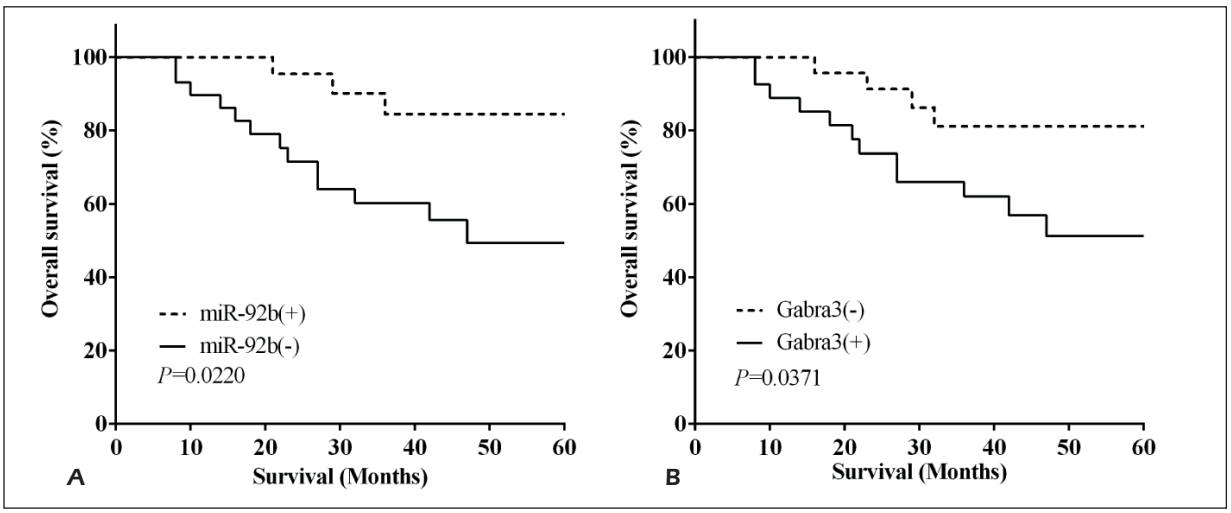

Figure 6. Identification of miR-92b and Gabra3 associated with poor survival in TNBC. **A**, miR-92b(-) was associated with worse outcome of TNBC patients. **B**, Gabra3(+) predicted poor prognosis in TNBC patients.

MB-231 compared to the paracancerous tissues and the normal cell line. MiR-92b was found to be inhibited the migration an invasion-mediated EMT of MDA-MB-231 cells. What's more, we first proposed that downregulation of miR-92b was connected with worse prognosis in breast cancer. In addition, the miR-92b has inverse correlation with TNM stage, lymph-node metastasis and the expression of Gabra3, while it had a tendency to associate with the tumor size and metastasis, which maybe because of the sample size was small. Nevertheless, our findings were opposite to those in bladder cancer and hepatocellular carcinoma that miR-92b could promote the EMT, migration and invasion[13,14]. We hypothesized that miR-92b varies widely in different cancers. Gabra3 is a subunit of GABA, which is upregulated and associated with pathological process in several diseases[15,17-19]. Liu et al[17] discovered that Gabra3 promoted lymphatic metastasis in lung adenocarcinoma through mediating upregulation of matrix metalloproteinases. We found that Gabra3 was upregulated in TNBC tissues and cell lines. More importantly, we first propose that the expression of Gabra3 has a negative relationship with miR-92b and that it was a direct target gene of miR-92b in TNBC, consistently with the findings of Long et al[21] in pancreatic cancer. Similarly to Gumireddy et al[20], we verified that knockdown of Gabra3 could suppress migration and invasion-mediated EMT of breast cancer. In addition, it's the first time we have proposed that overexpression of Gabra3 predicted poor prognosis in patients with TNBC.

Conclusions

MiR-92b was low expressed in TNBC tissues and cell lines, and inhibited the migration, invasion-mediated EMT in TNBC cells. Gabra3 was overexpressed in TNBC tissues and cells in comparison with the corresponding normal tissues and normal cells. The expression of miR-92b had an inverse connection with the expression of Gabra3 in TNBC tissues. Downregulation of Gabra3 could inhibit the migration, invasion-mediated EMT in TNBC cells. MiR-92b mediated the expression of Gabra3 through directly binding to the 3'-UTR of Gabra3 mRNA. In addition, low expression of miR-92b or overexpression of Gabra3 was associated with poor prognosis of patients with TNBC.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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