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MiR-15b facilitates breast cancer progression *via* repressing tumor suppressor PAQR3

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Abstract. – OBJECTIVE: Recently, breast cancer (BC) has become a common tumor that threatens the physical and mental health of women. Microribonucleic acids (miRNAs) have been chosen as a study object because of their roles in various cancers, including BC. Here, we mainly study the role of miR-15b in BC progression and its underlying mechanism.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the level of MiR-15b expression in 60 pairs of BC tissues and para-cancerous specimens, and the relationship between MiR-15b level and clinical features of BC patient prognosis was analyzed. MiR-15b and PAQR3 level in BC tissues and cells was tested by Western blot.

RESULTS: The results showed that miR-15b expression was higher and PAQR3 level was lower in BC. The identification of PAQR3 as a target of miR-15b in BC was carried out by Luciferase reporter assay and the results stated that the Luciferase activity was reduced by miR-15b mimic, indicating PAQR3 being a target of miR-15b in BC. Transwell assay was used for examining BC cell migration and invasion and found that miR-15b could promote BC cell migration and invasion, while the effect of PAQR3 was inhibition. Furthermore, PAQR3 could reverse the promotion effect of miR-15b on BC cell migratory and invasive ability. In addition, miR-15b expression was negatively correlated with PAQR3 performed by regression analysis.

CONCLUSIONS: Our data stated that miR-15b could facilitate BC progression *via* repressing tumor suppressor PAQR3, indicating that miR-15b/PAQR3 axis provided a therapeutic target for treating BC.

Key Words:
MiR-15b, Breast cancer, Migration, Invasion, PAQR3.

Introduction

Breast cancer (BC) is a malignant tumor that occurs in the breast gland epithelium. Women account for 99% of BC and only 1% of men[1]. With the deepening understanding of BC biology, the current treatment of BC is a variety of methods including surgery, radiotherapy, chemotherapy, endocrine therapy, biological target therapy and other means. However, understanding the basic mechanism of BC progression and search for a new therapeutic method is very important due to the recurrence and metastasis of BC[2].

Microribonucleic acids (miRNAs) have been proved to involve in various cancers progression and are the hot spot of research at present. MiRNAs were associated with BC development by targeting mRNA, including regulating cell aggressiveness, migration, invasion and apoptosis[3-5]. The over-expression of miR-630 suppressed cell proliferation by blocking cell cycle progression in BC cells by targeting BMI1[6]. Sui et al[7] reported that miR-133a functioned as a tumor suppressor in BC and inhibited cell progression *via* targeting LASP1. Furthermore, miR-429 and miR-200b were proved to curb BC cell motility and growth by regulating LIMK1[8]. In addition, it was reported that miR-15b was upregulated in BC[9]. However, the role and potential mechanism of miR-15b in regulating BC were rarely reported.

PAQR3 (progestin and adipoQ receptor family member 3) has been shown to have anti-cancer activity in multiple tumors. Lower expression of PAQR3 was found in prostate cancer and involved in cell progression[10]. Re-expression of PAQR3 inhibited esophageal cancer cell

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proliferation and aggressive phenotype[11,12], also similar to gastric cancer[13]. In addition, Yu et al[14] reported that PAQR3 was a target of miR-543 in regulating hepatocellular carcinoma cell proliferation and PAQR3 was regulated by miR-137 in promoting bladder cancer cell proliferative and invasive ability[15]. Furthermore, Zhao et al[16] proved that miR-15b targeted PAQR3 to promote gastric cancer metastasis. However, whether PAQR3 was targeted by miR-15b in regulating BC cell migration and invasion has not been reported.

In our study, we examined the role and mechanism of miR-15b in BC. The results demonstrated that miR-15b was over-expressed, while PAQR3 was lower-expressed in BC. PAQR3 was confirmed as a target of miR-15b in BC. Moreover, miR-15b mimic facilitated the migratory and invasive ability of BC cell by targeting PAQR3, which provides a potential target for treating BC.

Patients and Methods

Breast Cancer (BC) Samples

We chose sixty patients who underwent surgery at Hospital from November 2013 to March 2016 and collected their tissue samples after breast resection. The patients should not have a history of cancer and did not have radiotherapy or chemotherapy before surgery. Signed informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Cancer Institute and Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. All BC tissues were immediately frozen in a -80°C refrigerator.

Cell Lines and Cell Culture

BC cell lines (MDA-MB-231, BT474, SKBR3 and MCF7) and human mammary epithelial cell line MCF-10A were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C under the atmosphere containing 5% CO_2.

Cell Transfection

We transfected miR-15b mimic or inhibitor which purchased from GenePharma (Shanghai, China) into MDA-MB-231 cells to over-express or silence miR-15b and we transfected progestin and adipoQ receptor family member 3 (PAQR3) vector into MDA-MB-231 cells to over-express PAQR3. Then, we added all the cells into 24-well plates and cultured in medium and the transfections were performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) for 48 h.

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was carried out to extract total RNA from BC cells and tissues. The messenger ribonucleic acid (mRNA) expression was quantified by Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). As follows were the sequences of the primers: miR-15b-F: TGGAATTGA CTTGGACCATAATAGA; miR-15b-R: AATAGTTGCTGTATCCCT. PAQR3-F: CTC AAGGACAACCCGTACATCAC; PAQR3-R: AAACTTTTGATACACAGCCTGGA C. U6-F: GCTTCGGCAGCACATATACTAAAAT; U6-R: CGCTTCACGAATTTGC GTGTCAT. β-actin-F: GATCATTGCTCCTCCTGAGC; β-actin-R: ACTCCTGCTTG CTGATCCAC. U6 and β-actin were used as internal controls. The $2^{-\Delta\Delta CT}$ method was used to detect the relative expression of miR-15b and PAQR3.

Western Blotting

Radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) buffer was added to the BC tissues and cells to extract total proteins and we measured the protein concentration by bicinchoninic acid (BCA) kit (Applygen, Beijing, China). 50 μg of protein samples were added in and separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) when the total proteins were at the same level, then transferred to nitrocellulose filter membrane (NC) (Millipore, Billerica, MA, USA). Subsequently, we blocked the membranes with skim milk (5%-10%) dissolved by 0.1% Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 2 h and then added the primary antibodies (PAQR3, 1:1000; β-actin, 1:1000; Abcam, Cambridge, MA, USA) to incubate at 4°C overnight. After the membranes were washed 3 times, the secondary antibodies were added in and incubated for 2 h at room temperature. Finally, the enhanced chemiluminescence

kit (ECL; Millipore, Billerica, MA, USA) were used to detect the signals. β-actin acted as a loading control.

Cell Migration Assay

For migration assay, a transwell chamber with 8 μm pore size polycarbonic membrane (Costar, Coppell, TX, USA) was first placed into the 24-well plates to separate the top and the lower chambers. 2×10^5 BC cells in medium without serum were seeded into the top chamber, and Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) medium supplemented with 10% FBS was added into the lower chamber. Then, we cultured the BC cells for 48 h at 37°C and the migratory cells in lower chamber from upper chamber were fixed with 100% methanol, stained with 0.1% crystal violet, imaged and counted.

Cell Invasion Assay

For invasion assays, the same numbers of BC cells in medium without serum were seeded into the top chamber with the filter coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The RPMI-1640 medium containing 20% FBS was added into the lower chamber. After BC cells were cultured for 48 h, the invaded cells from upper chamber were fixed, stained and imaged.

Luciferase Reporter Assay

The Luciferase reporter assay was performed using MDA-MB-231 cells. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to con-transfect miR-15b mimic or control mimic with 3'-UTR of wild or mutated PAQR3. The Dual-Luciferase Reporter System (Promega, Madison, WI, USA) was used to measure the Luciferase activity.

Statistical Analysis

Experiments were carried out at least three times and the results were displayed as Mean ± SD (Standard Deviation). The differences between the two groups were compared using Student's *t*-test in Statistical Product and Service Solutions (SPSS; SPSS Inc., Chicago, IL, USA). The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Statistical analysis and graph presentations were performed by SPSS v.19.0 software and GraphPad Prism 5 software (Version X; La Jolla, CA, USA). Statistically significant difference was considered as $p<0.05$.

Results

Upregulation of MiR-15b and Downregulation of PAQR3 in BC

Abnormal expression of miR-15b could play an inhibitory effect or promotion effect on BC development. Therefore, we first tested miR-15b expression in BC tissues and cells by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). As we saw in Figure 1A, miR-15b expression was raised in BC tumors than normal. Next, we detected the miR-15b expression in various stages of BC and the results indicated that miR-15b expression was higher in stage III/IV than stage I/II, and upregulated in all cancer stages compared to normal (Figure 1B). Subsequently, we tested miR-15b expression in BC cells and found that its expression was increased in all BC cell lines compared to the normal MCF10A cells (Figure 1C). PAQR3 was proved as a tumor suppressor in many cancers and functioned as a target of miR-15b in gastric cancer. Here, we first investigated PAQR3 expression in BC tissues and cells to find its effect on regulating BC. The results of Western blot and qRT-PCR all showed the decreased expression of PAQR3 in BC tissues and cells, which was opposite to miR-15b (Figure 1D, 1E). We then detected whether there was a connection between miR-15b and PAQR3, the regression analysis showed that they were negatively correlated (Figure 1F).

MiR-15b's Promotion Effect on BC Cell Migration and Invasion

We explored the role of miR-15b in BC cell migration and invasion was inhibited or stimulative. Firstly, miR-15b mimic or inhibitor was transfected into MDA-MB-231 cells to overexpress or silence miR-15b. The successful transfection of miR-15b was seen in Figure 2A. Subsequently, we used the transwell assay to measure miR-15b effect on cell migration and invasion. As Figure 2B showed, there was a markedly increased cell migration by re-expression of miR-15b in MDA-MB-231 cells, whereas decreased by inhibiting miR-15b. For invasion assay, it was similar to cell migration (Figure 2C).

Confirmation of PAQR3 as a Target of MiR-15b in BC

We further detected whether PAQR3 was regulated by miR-15b in BC cells. We used TargetScanHuman 7.1 to confirm preliminary the

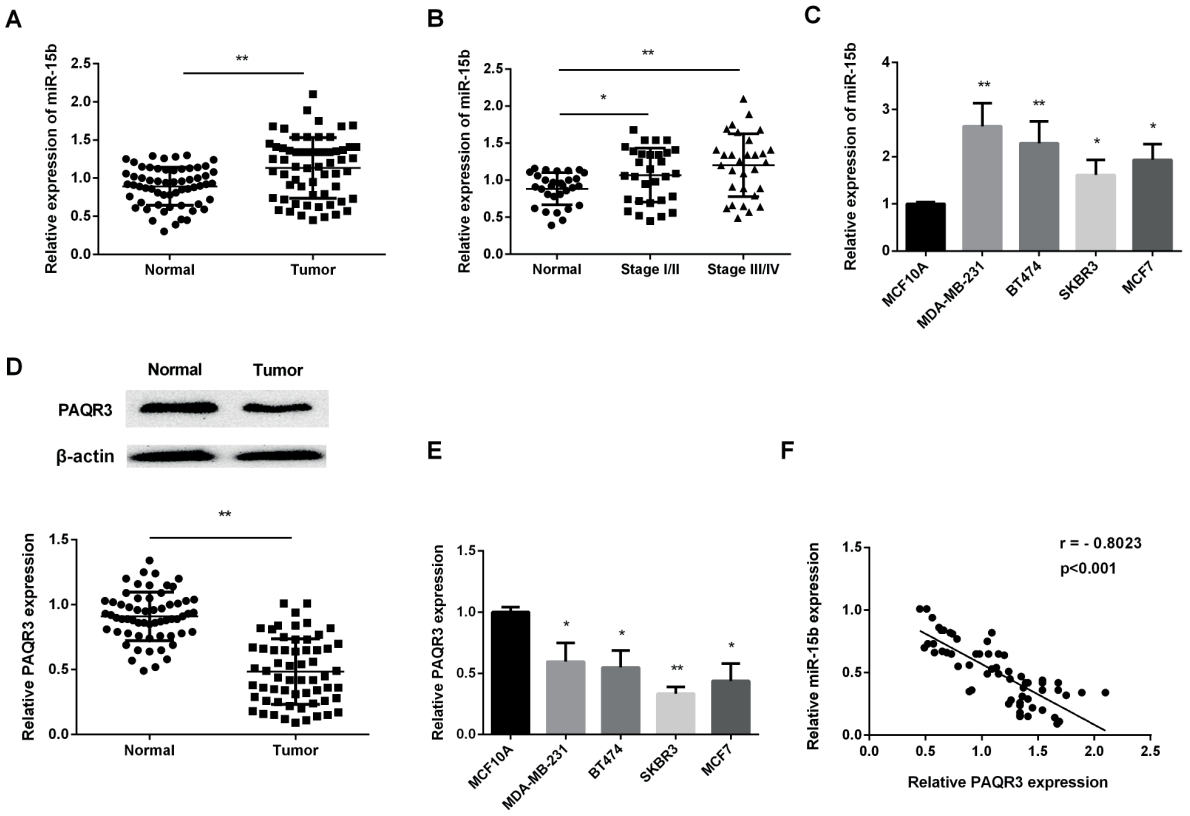

Figure 1. High miR-15b expression and low PAQR3 expression in BC samples and cells. **A,** Detection of miR-15b expression in BC samples by QRT-PCR. **B,** Detection of miR-15b expression in different stage of BC tumors by QRT-PCR. **C,** Detection of miR-15b expression in BC cells by QRT-PCR. **D,** Measurement of PAQR3 level in BC tissues by Western blot or QRT-PCR. **E,** Measurement of PAQR3 level in BC cells by qRT-PCR. **F,** Negatively correlation of miR-15b and PAQR3 by regression analysis (r= -0.8023, p<0.001). *p<0.05, **p<0.01.

hypothesis that PAQR3 was a target of miR-15b. As the color map showed in Figure 3A, the binding sites of PAQR3 3'UTR with miR-15b were at position 2608-2614. Then, we used the Dual-Luciferase reporter assay to further confirm this hypothesis in MDA-MB-231 cells. The results stated that the relative PAQR3 Luciferase activity was reduced by miR-15b mimic in WT, while there were no significant differences in MUT (Figure 3B). Based on the results, we could confirm that PAQR3 was a target of miR-15b in BC. Secondly, we examined whether changing the expression of miR-15b could affect PAQR3 expression by QRT-PCR and Western blot. The results showed that the overexpression of miR-15b inhibited PAQR3 expression, while the knockdown of miR-15b made PAQR3 expression raised (Figure 3C-3D).

Overexpression of PAQR3 Inhibited BC Cell Migration and Invasion

We investigated PAQR3's role in BC progression. We over-expressed PAQR3 using by PAQR3 vector due to its lower expression in BC. Subsequently, Western blot and QRT-PCR were used to detect the relative PAQR3 expression in BC cells respectively. As we expected, PAQR3 expression was markedly increased when raised PAQR3 expression in BC cells (Figure 4A). The results in Figure 4B stated that BC cell migration detected by transwell assay was decreased when over-expressed PAQR3 expression and similar results were seen in cell invasion (Figure 4C).

The Role of PAQR3 in BC Cell Regulated by MiR-15b

As the effect of miR-15b and PAQR3 on BC cell migration and invasion was opposite, we

examined whether PAQR3 could affect the role of miR-15b in BC progression. The transwell assay was carried out to examine cell migration and invasion in MDA-MB-231 cells after overexpression of miR-15b or both miR-15b and PAQR3. As we showed above, the miR-15b mimic group showed increased cell migration. However, the overexpression of both miR-15b and PAQR3 could attenuate cell migration in comparison with the overexpression of miR-15b alone (Figure 5A), suggesting that PAQR3 could reverse the promotion effect of miR-15b on BC cell migration. In addition, the results in Figure 5B showed that the relative cell invasion in BC cells was increased by miR-15b mimic. However, re-expression of both miR-15b and PAQR3 reduced cell invasion in comparison with cells over-expression of miR-15b alone, suggesting that PAQR3 could reverse miR-15b promotion effect on BC cell invasion. In short, miR-15b could facilitate BC cells migration and invasion by targeting PAQR3.

Discussion

The results in our study stated that miR-15b was over-expressed in BC and PAQR3 was lower expressed in BC. As an oncogene in BC, miR-15b facilitated cell migration and invasion. Furthermore, miR-15b was proved to target PAQR3 to promote BC cell invasion and migration. Synchronously, PAQR3 could reverse miR-15b promotion effect on BC cell invasion and migration.

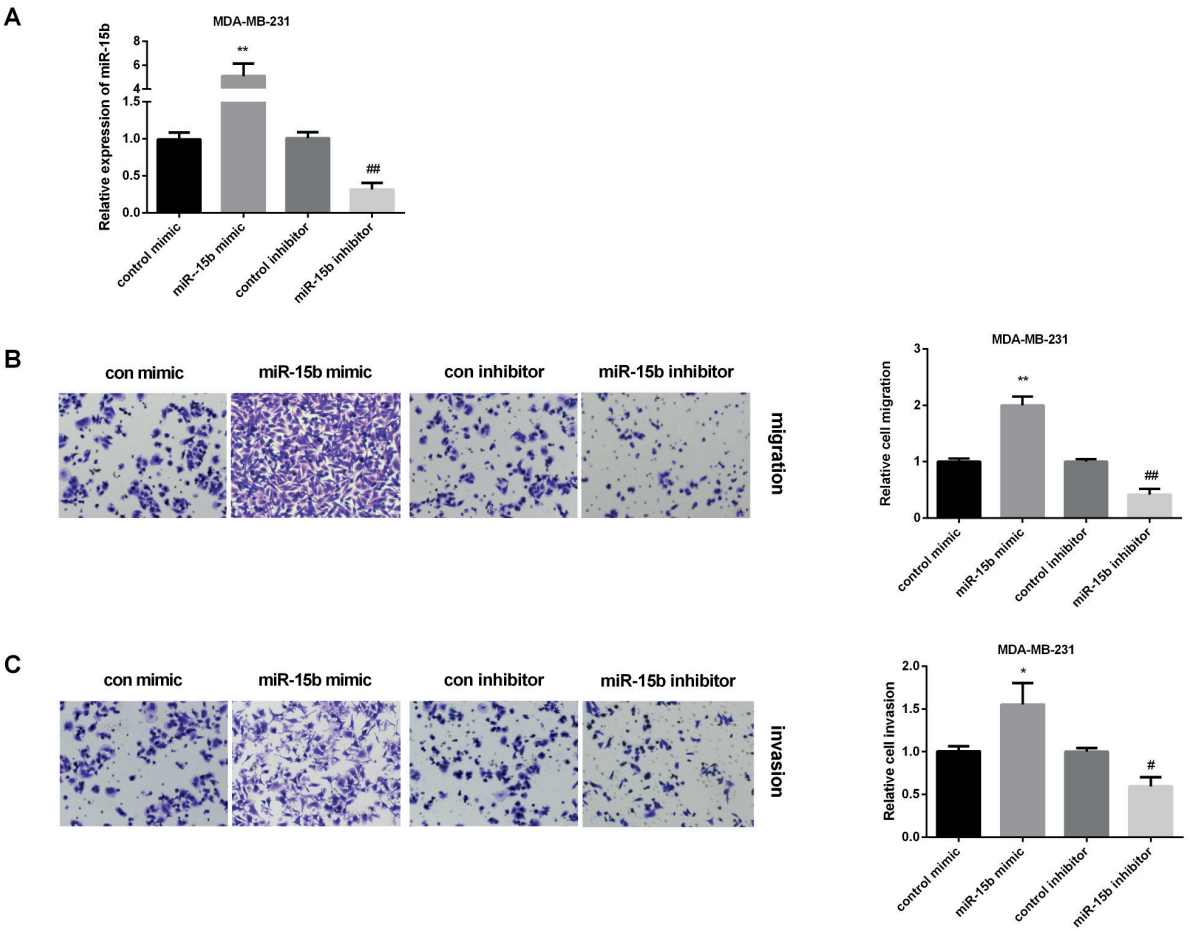

Figure 2. The promotion effect of miR-15b on BC cell migration and invasion. **A,** MiR-15b expression examined by QRT-PCR in MDA-MB-231 cells after over-expression or silencing miR-15b. **B,** Cell migration measured by transwell assay in MDA-MB-231 cells after over-expression or silencing miR-15b (magnification: 40×). **C,** Cell invasion measured by transwell assay in MDA-MB-231 cells after over-expression or silencing miR-15b (magnification: 40×). $^{*}p<0.05$, $^{**}p<0.01$; $^{\#}p<0.05$, $^{\#\#}p<0.01$.

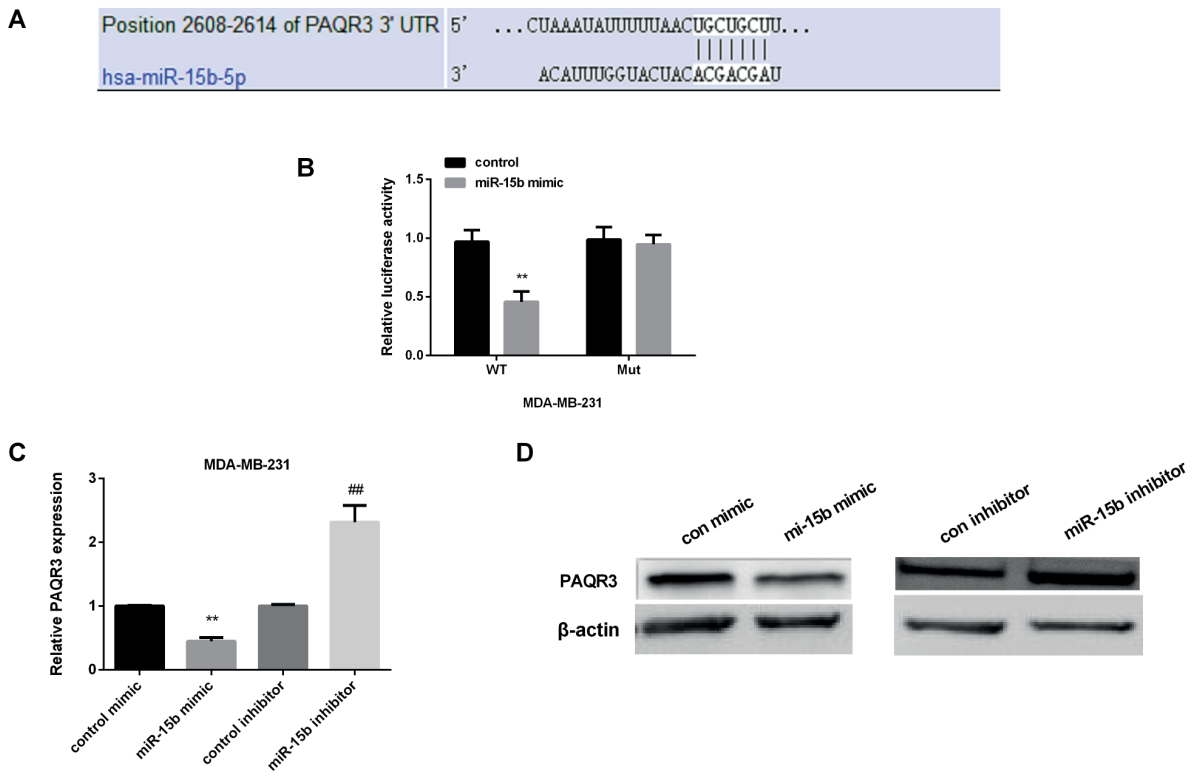

Figure 3. Identification of PAQR3 as a target of miR-15b. **A,** Mapping the binding sites of miR-15b and PAQR3. **B,** Detection of the Luciferase activity of PAQR3 in MSA-MB-231 cells transfected with miR-15b mimic or control mimic. **C,** Detection of PAQR3 mRNA expression in MDA-MB-231 cells treated with miR-15b mimic or inhibitor. **D,** Detection of PAQR3 protein level in MDA-MB-231 cells treated with miR-15b mimic or inhibitor. $^{**}p<0.01$; $^{\#\#}p<0.01$.

Therefore, our work demonstrated that the potential mechanism of miR-15b regulating in BC was by targeting PAQR3, suggesting that miR-15b/PAQR3 axis might offer an effective treatment for BC patients.

There was increasing evidence that miRNAs played key roles in various cancers' biological and pathological processes as oncogenes or tumor suppressors by regulating their target genes. These included regulating BC cell proliferation, migration and invasion[16]. It has been proved that miR-34, miR-150, miR-630, miR-133a functioned as a tumor suppressor in BC[5,6,18,19], while miR-1246, miR-200, miR-27a acted as a tumor promoter[4,20,21]. Our study showed that miR-15b played promotion effect on BC as an oncogene, which is consistent with the reports that miR-15b was overexpressed in BC cells[22].

It's well known that the mechanism of miR-15b in regulating breast cancer was by targeting multiple mRNA genes. It was reported that miR-15b regulated breast cancer progression by targeting MTSS1[23]. Kratassiouk et al[24] reported that miR-15b regulated BC cell cycle by targeting CPEB1. Liu et al[25] found that miR-15b played an important role in BC *via* regulating Smurf2. However, Zhao et al[16] reported that miR-15b could facilitate the migration of gastric cancer cell by targeting PAQR3. Based on the above researches, our work first showed that miR-15b promoted BC cell invasion and migration *via* regulating PAQR3, which is in line with our speculation that PAQR3 might be a target of miR-15b in regulating BC development.

PAQR3 was proved to have anti-cancer activity in multiple tumors and targeted by various miRNAs, such as in esophageal cells[12], prostate cancer[26], colorectal cancer[27]. Xiu et al[15] showed that PAQR3 was targeted by miR-137 in up-regulating the proliferation of bladder cancer cell, and was proved to be targeted by miR-543 in regulating hepatocellular carcinoma development[14]. In our study, we stated that PAQR3 expression was lower in BC and overexpression of PAQR3 suppressed cell migration and inva-

sion. Our results were consistent with previous studies that PAQR3 acted as tumor suppressors in BC[28,29]. We also demonstrated that PAQR3 could reverse miR-15b promotion effect on BC development.

Conclusions

Briefly results showed that miR-15b mimic promoted BC progression while PAQR3 inhibited its progression. It's the first time we proved

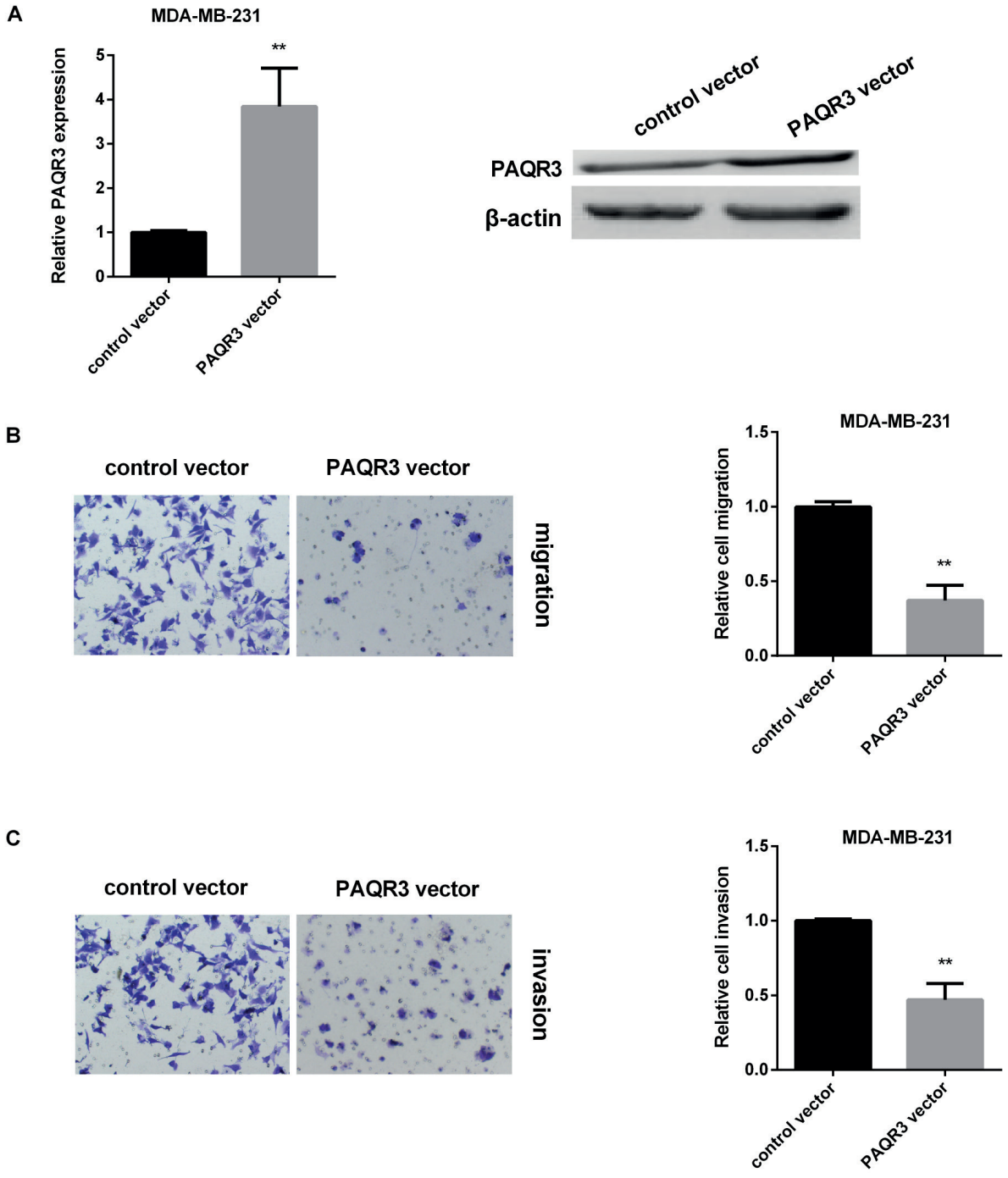

Figure 4. The inhibition effect of PAQR3 on BC cell migration and invasion. **A,** Measurement of PAQR3 mRNA and protein expression in BC cells when overexpression of PAQR3. **B,** Measurement of cell migration in BC cells when overexpression of PAQR3 (magnification: 40×). **C,** Cell invasion tested in BC cells when overexpression of PAQR3 (magnification: 40×). $^{**}p<0.01$.

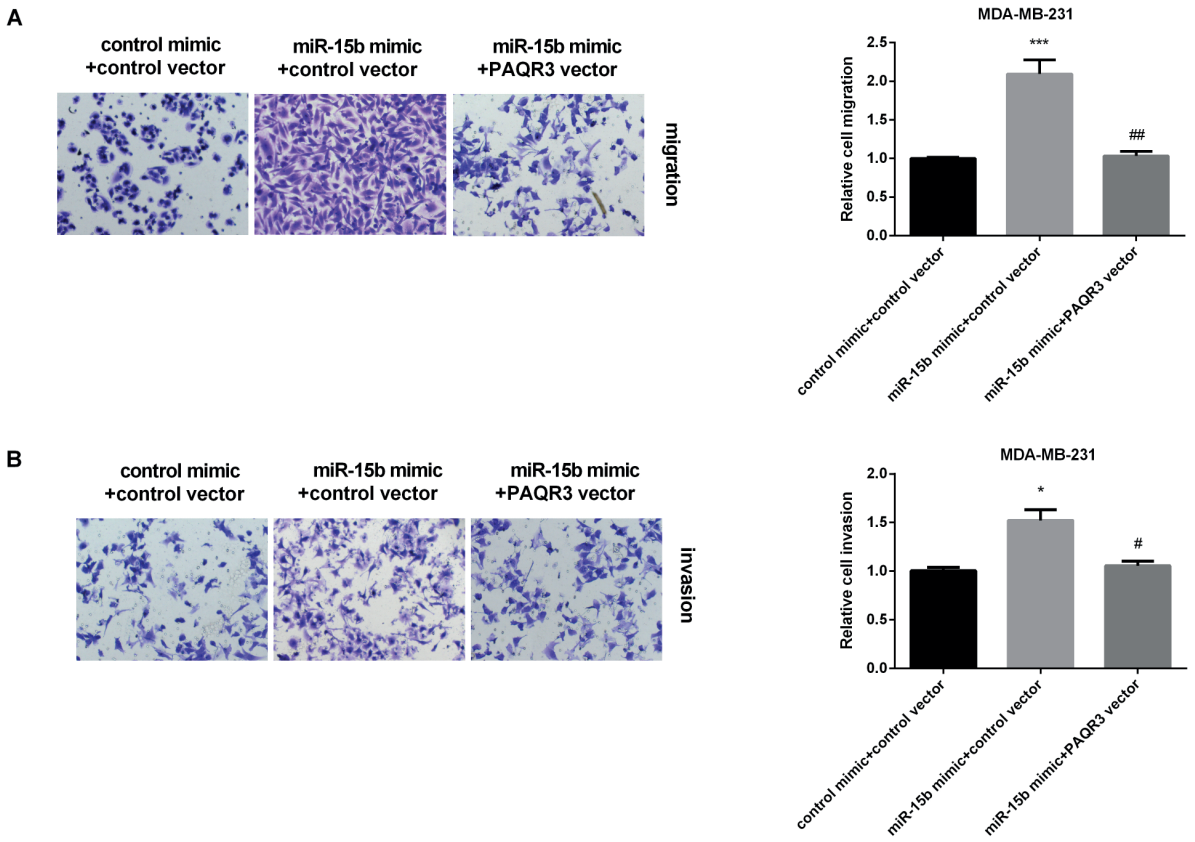

Figure 5. The role of PAQR3 in BC cell migration and invasion regulated by miR-15b. **A,** Measurement of cell migration in BC cells treated with miR-15mimic or both miR-15 mimic and PAQR3 (magnification: 40×). **B,** Measurement of the invasion of BC cells treated with miR-15mimic or both miR-15 mimic and PAQR3 (magnification: 40×). *p<0.05, ***p<0.001; $^{\#}p$<0.05, $^{\#\#}p$<0.01.

that PAQR3 was a specific target of miR-15b in BC and PAQR3 could partially attenuate miR-15b promotion effect in BC, indicating that miR-15b/PAQR3 axis might be a potential application in BC diagnosis and therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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