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MicroRNA-328-3p inhibits the tumorigenesis of bladder cancer through targeting ITGA5 and inactivating PI3K/AKT pathway

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Abstract. – OBJECTIVE: Previous studies have shown that microRNA-328-3p (miR-328-3p) is involved in tumorigenesis of many human cancers. However, the specific function of miR-328-3p remains unclear in bladder cancer (BC). Therefore, this research was designed to investigate the role of miR-328-3p in BC.

PATIENTS AND METHODS: Expressions of miR-328-3p and integrin α5 (ITGA5) were measured by quantitative Real-time polymerase chain reaction (qRT-PCR). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays were used to explore the function of miR-328-3p in BC. The expression of the corresponding genes was observed *via* Western blot and immunocytochemical assays. The dual luciferase assay was applied to verify the relationship between miR-328-3p and ITGA5. Tumor growth was measured *via* xenograft tumor formation assay.

RESULTS: Downregulation of miR-328-3p was identified in BC tissues, which predicted poor prognosis in BC patients. Moreover, miR-328-3p suppressed cell proliferation, migration and invasion in BC through targeting ITGA5. Furthermore, miR-328-3p inhibited epithelial-mesenchymal transition (EMT) and inactivated PI3K/AKT pathway in BC. Besides that, miR-328-3p was found to inhibit tumor growth of BC.

CONCLUSIONS: MiR-328-3p inhibited tumorigenesis of BC through targeting ITGA5 and inactivating the PI3K/AKT pathway.

Key Words:
miR-328-3p, ITGA5, Bladder cancer, PI3K/AKT pathway.

Introduction

Bladder cancer (BC) is the most common tumor in the urinary system, and most BC patients are middle-aged men[1]. In China, BC accounts for 3.2% of all malignant tumors, with a male to female ratio of 4:1[2]. Although there are many reasons for tumorigenesis of BC, one of the most important and often overlooked reason is smoking. Smoking causes 50-60% of BC patients in developed countries[3]. Although the situation of BC is not encouraging, there are many ways to treat BC. And the most mainstream therapy is still surgery. However, patients with BC at advanced stage cannot undergo surgery. Adjuvant therapy such as radiotherapy and chemotherapy can be used for palliative therapy of BC[4]. In addition, about 70% of BC patients will relapse after transurethral resection. And the recurrence rate can be reduced to 25-40% after intravesical perfusion with BCG or chemotherapy[5]. The 5-year survival rate of BC patients after total cystectomy is 60-70%[6]. The key to improving prognosis is early diagnosis and treatment. Therefore, it is necessary to develop effective markers for the diagnosis and treatment of BC. Recently, it has been reported that many microRNAs (miRNAs) are involved in the pathogenesis of human cancers, in which miRNAs can function as oncogenes and tumor suppressors[7]. Chen et al[8] demonstrated that miRNAs could be used as biomarkers for the diagnosis of BC. For example, miR-576-3p was identified as a novel marker associated with poor clinical outcome in BC[9]. Cheng et al[10] reported that miR-200c promoted cell migration and invasion by directly targeting RECK in BC. Nan et al[11] found that miR-4295 promoted cell growth in BC by targeting BTG1. In contrast, miR-1-3p inhibited proliferation and invasion of BC cells by suppressing CCL2 expression[12]. Besides that, miR-223-3p inhibited mi-

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gration and invasion of BC cells by modulating NCOA11[13]. However, the function of miR-328-3p has not been found in BC until now. Previous studies have shown that miR-328 exhibited different effects in some human cancers. MiR-328 had been found to be associated with NSCLC brain metastasis and mediate NSCLC cell migration[14]. Saberi et al[15] proposed that miR-328 acted as an oncogene in human invasive breast carcinoma. And Luo et al[16] indicated that miR-328 enhanced cell motility through post-transcriptional regulation of PTPRJ in human hepatocellular carcinoma. In contrast, Wang et al[17] found that miR-328 inhibited cell proliferation and tumorigenesis by targeting TCF7L2 in cervical cancer. In addition, miR-328 was also found to inhibit renal tubular cell epithelial-mesenchymal transition (EMT) by targeting CD44 in pressure-induced renal fibrosis[18]. The opposite result of miR-328 prompted us to explore its role in BC.

Integrin α5 (ITGA5) is a member of Integrins family that mediates the interactions between cells and cells or extracellular matrix and signal transduction[19]. Abnormal expression of ITGA5 had been identified in acute myeloid leukemia[20]. Furthermore, upregulation of ITGA5 had been reported to induce invasion and EMT[21]. In addition, Murillo et al[22] proposed that inhibition of ITGA5 integrin decreased phosphatidylinositol 3-kinase (PI3K) activation in human colon cancers. Moreover, activation of the PI3K/AKT (protein kinase B) pathway had been implicated in carcinogenesis of BC[23]. And miR-143 and miR-145 induced synergistic inhibition of human BC cell growth by regulating the PI3K/AKT signaling pathway[24]. These studies implied that ITGA5 might be involved in tumorigenesis of BC. In the current study, the potential functions of miR-328-3p were investigated in BC. The relationship between ITGA5 and miR-328-3p was confirmed in BC cells. Our results will improve the efficiency of BC diagnosis and contribute to the clinical application of BC.

Patients and Methods

Clinical Tissues

28 pairs of BC tissues and adjacent normal samples were acquired from the Yinzhou Hospital Affiliated to Medical College of Ningbo University. All participants provided written informed consents prior to the study. The experiment was approved by the Institutional Ethics Committee of Yinzhou Hospital Affiliated to Medical College of Ningbo University. All BC patients did not receive any treatment prior to surgery. Finally, these tissues were frozen in liquid nitrogen and then stored in a -80°C refrigerator for further experiments.

BC Cells Culture

The normal urinary epithelial cell SV-HUC-1 and 5637, T24, J82 BC cell lines were used for this experiment. These cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Next, these cells were seeded in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and incubated at 37°C with 5% CO_2.

Cell Transfection

The miR-328-3p mimic or inhibitor, miR-328-3p plasmid and negative control (NC) were obtained from Ribobio (Guangzhou, China). Next, they were transferred into T24 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufactures' protocols.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the standard method. And the synthesis of the complementary deoxyribose nucleic acid (cDNA) was performed using the First Stand cDNA Synthesis Kit (Tiangen Biotechnology, Beijing, China). Quantitative RT-PCR was performed on ABI 7300 Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-328-3p and ITGA5. Finally, their expressions were calculated using the $2^{-\Delta\Delta ct}$ method. The sequences of primers were listed as follows: miR-328 forward primer 5'-CTGGCCCTCTCTGCCC-3', miR-328 reverse primer 5'-GTGCAGGGTCCGAGGT-3'; U6-forward primer 5'-TTCCTCCGCAAGGATGACACGC-3', U6-reverse primer 5'-GTGCAGGGTCCGAGGT-3'; ITGA5 forward primer 5'-CTACAATGATGTGGCCATCG-3', ITGA5 reverse primer 5'-GGATATCCATTGCCATCCAG-3'; GAPDH-forward primer 5'-CACCATCTCTCCAGAAGTGGAC-3', GAPDH-reverse primer 5'-GGCGTCATCAAAGTTCTGCCAAC-3'.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 assay was performed to measure cell proliferation based on the manufacturer's instructions. Next, 3×10^4 T24 cells were plated and incubated in 96-well plates for 0, 24, 48 and 72 h. They were placed in an incubator with 5% CO_2 at 37°C. Next, each well was added with 10 μL of CCK-8 reagents for 2 h (Dojindo, Kumamoto, Japan). Finally, they were detected using a microplate reader (Molecular Devices) at an absorbance of 450 nm.

Transwell Assays

Transwell chambers (8-μm pore size membranes) were used for cell migration and invasion assays. Lower chamber was added with 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2. Next, the upper chamber with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell invasion. And cell migration assay was performed without matrigel. Afterwards, 3×10^4 T24 cells were cultured in the upper chamber with serum-free medium. After 24 h, the migrated or invading cells were fixed with methanol and stained with crystal violet. Finally, we counted the number of removed cells using a microscope.

Dual Luciferase Assay

The wild or mutant 3'-UTR of ITGA5 was inserted into the pmirGLO luciferase vector (Promega, Madison, WI, USA) for luciferase reporter experiments. Next, wild or mutant 3'-UTR of ITGA5 and miR-328-3p mimics were transfected into T24 cells. Finally, Dual Luciferase Assay System (Promega, Madison, WI, USA) was applied to analyze luciferase activity.

Immunocytochemical Assay

The section of BC tissues were dewaxed, hydrated, and washed twice with phosphate-buffered saline (PBS) for 5 min. After blocking with 5% goat serum (diluted in PBS), we incubated the cells with ITGA5 (cytomembrane) antibody at 37°C for 1-2 h. We then washed them three times with PBS for 5 min. Subsequently, we incubated them with the corresponding secondary antibody for 1 h at 37°C. After washing 3 times with PBS, a mixture of diaminobenzidine (DAB) (Solarbio, Beijing, China) was used for color development of this section. The section was washed, counterstained, dehydrated, transparentized and fixed. Images were captured using a microscope.

Western Blot Analysis

Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the protein was transferred into polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) and incubated with 5% non-fat milk in at room temperature. We then incubated the membrane overnight at 4°C with ITGA5, EMT markers (E-cadherin, N-cadherin, Vimentin), PI3K/AKT markers (AKT, p-AKT) and GAPDH primary antibodies (1:1000; Abcam, Cambridge, MA, USA). After washing, they were incubated with the corresponding secondary antibodies. Finally, protein expression levels were measured by enhanced chemiluminescence (ECL, Rockford, IL, USA).

Xenograft Tumor Formation Assay

We purchased nude mice (6 weeks old) from the Model Animal Research Center at Nanjing University (Nanjing, China). All animal experiments were approved by the Animal Care and Use Committee of Medical College of Ningbo University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. First, 4×10^6 T24 cells with miR-328-3p plasmid or negative control were injected into the right flank of nude mice. Tumor volume was observed every 4 days. After 4 weeks, the mice were sacrificed and the tumors were used for further studies.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and Graphpad Prism 6 (La Jolla, CA, USA). Data were shown as mean ± SD (standard deviation). Differences between two groups were calculated through χ^2 test. Comparisons between groups were analyzed using One-way analysis of variance (ANOVA) method followed by Post-Hoc Test (Least Significant Difference). Kaplan-Meier analysis with log-rank test was applied to measure the survival rate of BC patients. Significant differences were defined as $p<0.05$.

Results

Downregulation of miR-328-3p Was Identified in BC Tissues

The expression of miR-328-3p was first detected in BC tissues through qRT-PCR assay.

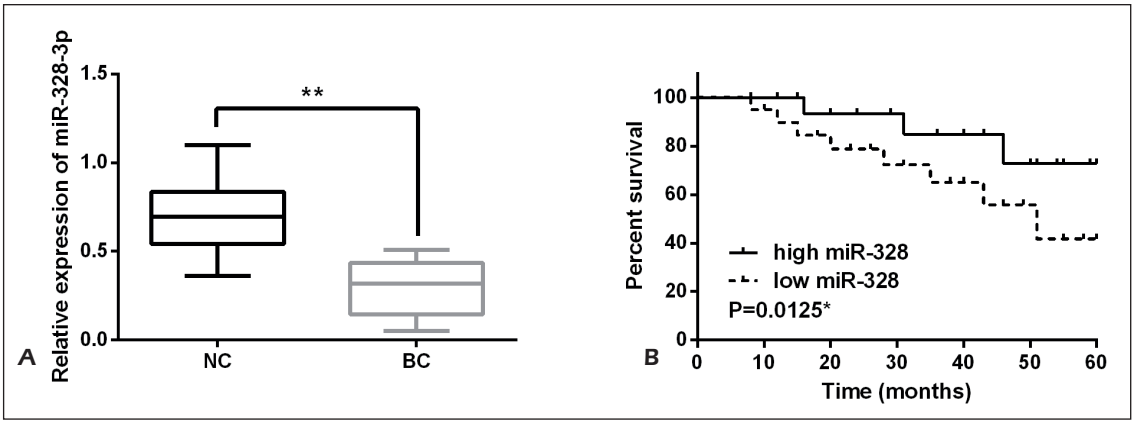

Figure 1. Downregulation of miR-328-3p was identified in BC tissues. **A**, The mRNA expressions of miR-328-3p in BC tissues **B**, Lower miR-328-3p expression was related to shorter overall survival in BC patients. *p<0.05, **p<0.01.

We found that miR-328-3p was downregulated in BC tissues compared to normal tissues (Figure 1A). As shown in Table I, the downregulation of miR-328-3p was found to be associated with TNM stage (p=0.007) and lymph node metastasis (p=0.029). These results indicated that miR-328-3p was involved in tumorigenesis of BC. Moreover, we also found that miR-328-3p could predict the prognosis of BC patients. And high miR-328-3p expression had a longer overall survival in BC patients (p=0.0125, Figure 1B). Therefore, miR-328-3p was deduced to be a valuable biomarker for BC.

MiR-328-3p Suppressed Cell Proliferation, Migration and Invasion in BC

Next, miR-328-3p expression was observed in the 5637, T24, J82 and SV-HUC-1 cell lines to explore its function in BC. Similarly, downregulation of miR-328-3p was also identified in 5637, T24, J82 cells compared to SV-HUC-1 cells (Figure 2A). MiR-328-3p mimics or inhibitor was then transfected into T24 cells. The results of qRT-PCR experiment showed that miR-328-3p expression was enhanced by transfection of miR-328-3p mimics and blocked by miR-328-3p inhibitor (Figure 2B). Functionally, CCK-8 as-

Table I. Relationship between miR-328-3p expression and their clinic-pathological characteristics of BC patients.

Characteristics features	Cases	miR-328-3p		*p*-value
		High	Low	
Age (years)				0.08
≥ 60	15	5	10	
< 60	13	5	8	
Gender				0.221
Male	18	7	11	
Female	10	3	7	
Tumor size				0.233
<3 cm	16	4	12	
≥3 cm	12	6	6	
TNM stage				0.007*
I-II	22	8	14	
III-IV	6	2	4	
Lymph node metastasis				0.029*
No	24	7	17	
Yes	4	3	1	

Statistical analyses were performed by the χ^2 test. *p<0.05 was considered significant.

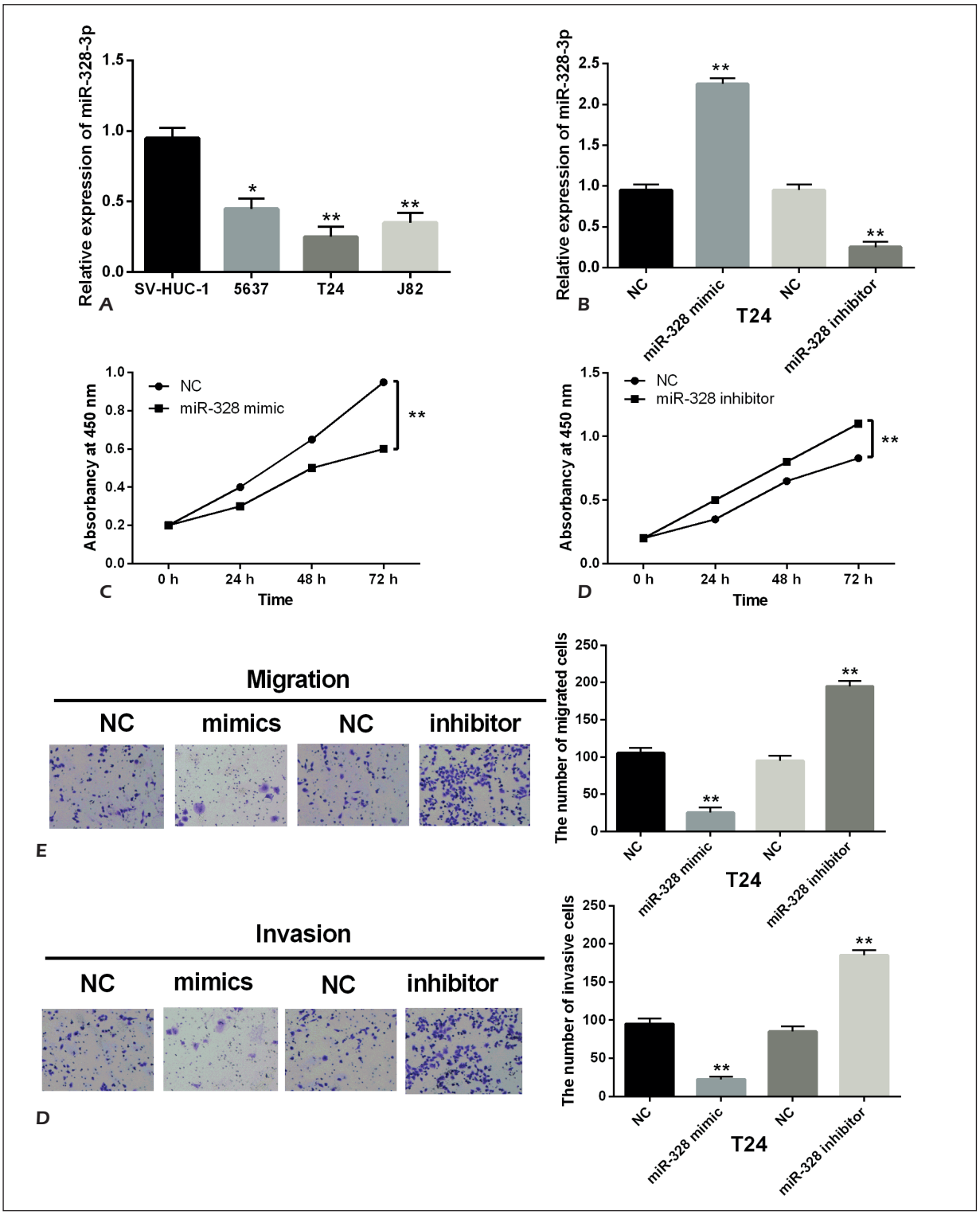

Figure 2. MiR-328-3p suppressed cell proliferation, migration and invasion in BC. **A**, The mRNA expressions of miR-328-3p in 5637, T24, J82 and SV-HUC-1 cell lines B, MiR-328-3p expression in T24 cells with miR-328-3p mimics or inhibitor **C-D**, Cell proliferation in cells containing miR-328-3p mimics or inhibitor. **E-F**, Cell migration and invasion analysis in cells containing miR-328-3p mimics or inhibitor was detected. $^{**}p<0.01$.

say showed that overexpression of miR-328-3p suppressed proliferation of T24 cells, whereas knockdown of miR-328-3p had an opposite effect on cell proliferation in BC (Figure 2C, 2D). Consistently, the same effects of miR-328-3p on cell migration and invasion were also identified in BC (Figure 2E, 2F). Hence, miR-328-3p was considered to play an inhibitory role in the pathogenesis of BC.

ITGA5 Was a Direct Target of miR-328-3p in BC Cells

Further, we investigated target genes of miR-328-3p to further illuminate its regulatory mechanism in BC using TargetScan (http://www.targetscan.org/). We found that miR-328-3p had binding sites with the 3'-UTR of ITGA5 (Figure 3A). To verify this prediction, luciferase reporter assay was performed. The results showed that miR-328-3p mimics reduced the luciferase activ-

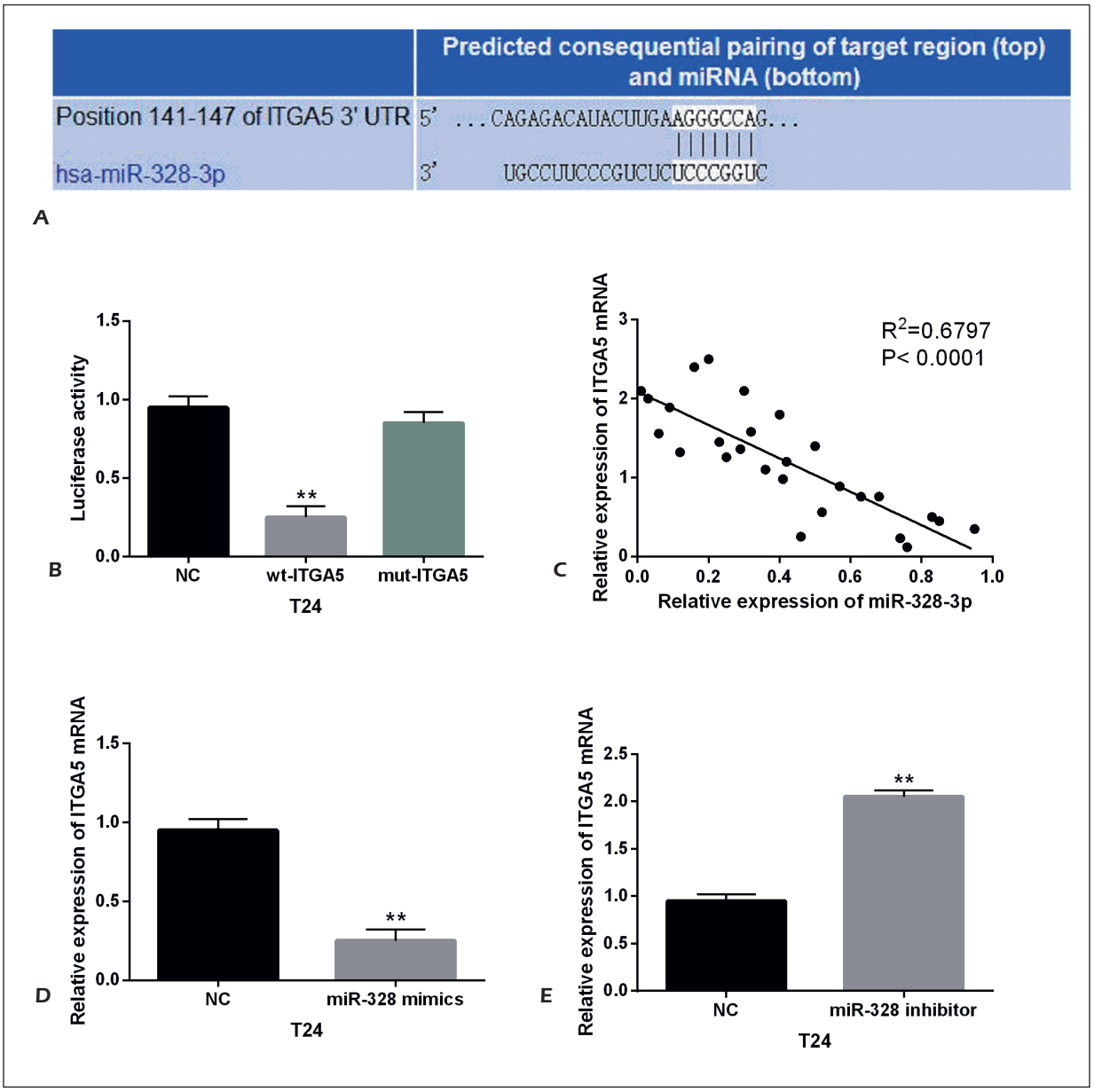

Figure 3. ITGA5 was a direct target of miR-328-3p in BC cells. **A**, The binding sites between miR-328-3p and ITGA5 **B**, Luciferase reporter assay **C**, The correlation between miR-328-3p and ITGA5 **D-E**, The expression of ITGA5 in T24 cells containing miR-328-3p mimics or inhibitor **$p<0.01$.

ity of wt-ITGA5. However, the luciferase activity of mut-ITGA5 was not affected by miR-328-3p mimics (Figure 3B). Then, ITGA5 expression was found to have a negative correlation with miR-328-3p in BC tissues (p<0.0001, R^2=0.6797; Figure 3C). Moreover, ITGA5 was downregulated in T24 cells with miR-328-3p mimics (Figure 3D) and upregulated in T24 cells with miR-328-3p inhibitor (Figure 3E). In brief, miR-328-3p directly targeted ITGA5 and negatively regulated its expression in BC.

ITGA5 Was Upregulated in BC Tissues

Next, the alternation of ITGA5 expression was measured in BC tissues. The results of IHC revealed a positive detection of ITGA5 protein expression in the cytomembrane of BC cells (Figure 4A). In addition, we found that ITGA5 was significantly upregulated in BC tissues compared with adjacent normal tissues (Figure 4B). Furthermore, high ITGA5 expression was found to have a shorter overall survival in BC patients (p=0.0487, Figure 4C), suggesting that ITGA5 may be involved in the development and prognosis of BC.

MiR-328-3p Suppressed EMT and Inactivated PI3K/AKT Pathway in BC

In addition, we investigated the effects of miR-328-3p on EMT and PI3K/AKT pathway in BC to further confirm its function. First, we found that overexpression of miR-328-3p suppressed N-cadherin and Vimentin expressions and promoted the expression of E-cadherin (Figure 5A). In contrast, downregulation of miR-328-3p had an opposite effect on the expression of these markers (Figure 5B). Thus, we considered that overexpression of miR-328-3p inhibited the metastasis of BC cells by regulating EMT. Furthermore, upregulation of miR-328-3p obviously repressed p-AKT expression, and downregulation of miR-328-3p promoted p-AKT expression in T24 cells (Figure 5A, 5B). However, the expression of AKT was not affected by them. These findings further confirm the suppressive effect of miR-328-3p in BC.

MiR-328-3p Inhibited the Tumor Growth of BC

Finally, we subcutaneously injected T24 cells with miR-328-3p stable transfection plasmid or miR-NC into nude mice. The tumor volume of BC was smaller in nude mice with miR-328-3p plasmid than that of miR-NC (Figure 6A). Similarly, the growth rate of BC tumors was lower in nude mice with miR-328-3p plasmid than that of miR-NC (Figure 6B). In conclusion, miR-328-3p inhibited tumor growth of BC.

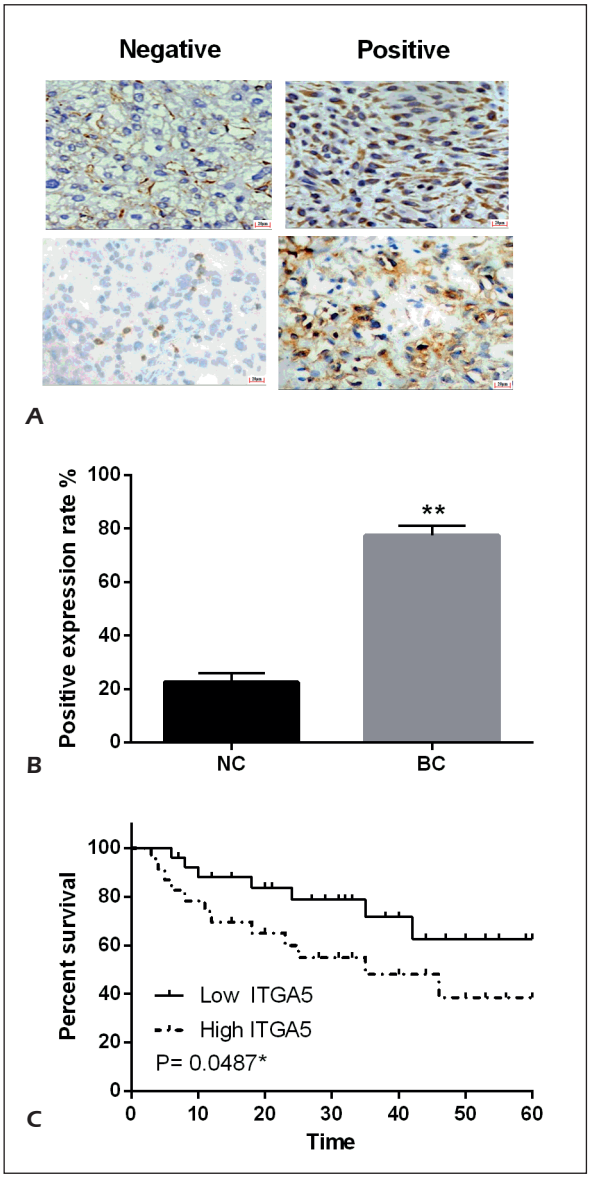

Figure 4. ITGA5 was upregulated in BC tissues. **A-B**, The protein expression of ITGA5 in BC tissues detected by immunohistochemistry **C**, High ITGA5 expression was related to shorter overall survival in BC patients. **p<0.01.

Discussion

A growing number of studies have demonstrated that many miRNAs are involved in the tumorigenesis and progression of BC, such as miR-192[25], miR-429[26] and miR-495[27]. In this study, we

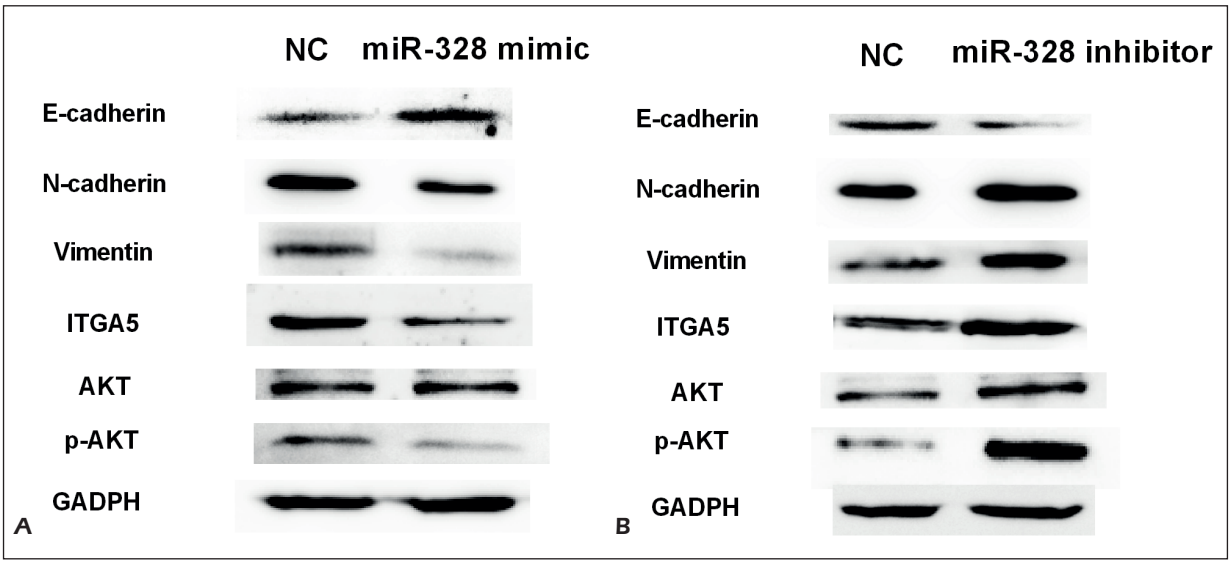

Figure 5. MiR-328-3p suppressed EMT and inactivated PI3K/AKT pathway in BC. **A-B**, Western blot analysis of E-cadherin, N-cadherin, Vimentin, AKT and p-AKT in T24 cells with miR-328-3p mimics or inhibitor.

investigated the role of miR-328-3p in the pathogenesis of BC. First, downregulation of miR-328-3p was identified in BC tissues, which predicted a poor prognosis in BC patients. Moreover, miR-328-3p was found to play an inhibitory effect on the progression of BC. These findings will help improve the diagnosis, prognosis and treatment of BC. Previous studies had reported that miR-328 was downregulated in glioblastoma and predicted worse survival of glioblastoma patients[28]. In addition, Liu *et al*[29] also reported that the low expression of miR-328 was also associated with poor prognosis in patients with acute myeloid leukemia. Besides that, we found that overexpression of miR-328-3p suppressed cell proliferation, migration and invasion in BC. Consistent with our findings, miR-328 was also identified to inhibit proliferation of human melanoma cells by targeting TGFB2[30]. And Lin *et al*[31] found that miR-328 inhibited migration and EMT in nasopharyngeal carcinoma cells by targeting CD44. Here, miR-328-3p suppressed EMT in BC to regulate cell metastasis. It was also found that miR-328-3p inhibit tumor growth of BC, which was not reported in previous studies.

Furthermore, miR-328-3p directly targeted ITGA5 in this study. We found that the expression of miR-328-3p and ITGA5 had an inverse relationship in BC. Similarly, ITGA5 has been demonstrated in previous studies as a downstream target of miRNAs, such as miR-26a[32], miR-128[33] and miR-148b[34]. Yoo *et al*[35] found that miR-330-

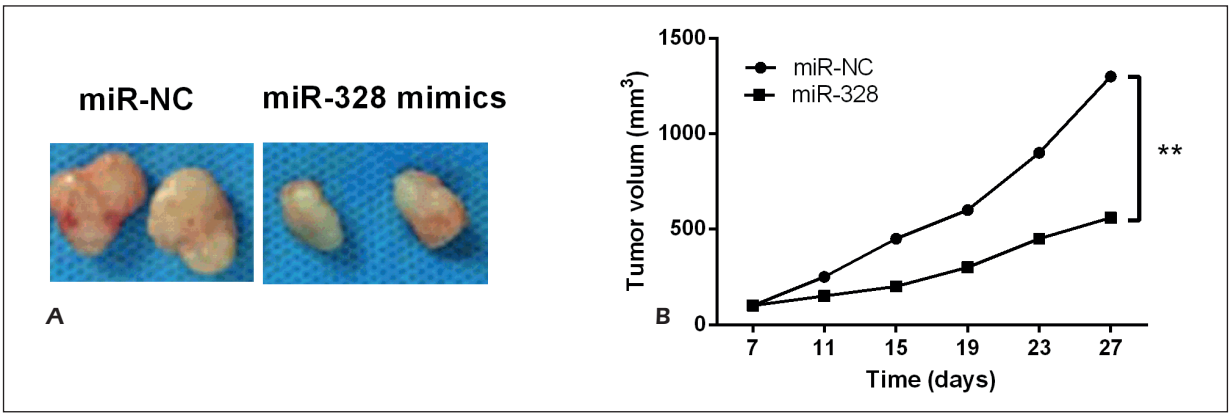

Figure 6. MiR-328-3p inhibited tumor growth of BC. **A**, Photographs of BC tumor tissue in miR-328-3p and miR-NC groups **B**, Growth rate for the tumor volumes of BC was examined every 4 days. $^{**}p<0.01$.

5p negatively regulated the expression of ITGA5 in human colorectal cancer, which was consistent with our results. Moreover, miR-328-3p was found to inhibit tumorigenesis of BC through targeting ITGA5 in this study. Same as our conclusion, Xu *et al*[36] reported that miR-31 functioned as a tumor suppressor in urothelial BC by targeting ITGA5. Besides that, we found that miR-328-3p inhibited the development of BC by targeting ITGA5 and inactivating the PI3K/AKT pathway in current research. Consistently, Zhang *et al*[37] indicated that upregulation of miR-31 targeting ITGA5 suppressed cell invasion and metastasis by indirectly regulating the PI3K/AKT pathway in human gastric cancer. The PI3K/AKT signaling pathway had been reported to be involved in the molecular mechanism of BC[38]. In this study, we found that miR-328-3p was inversely associated with activation of the PI3K/AKT pathway in BC. Moreover, miR-328-3p suppressed the proliferation and metastasis of BC cells through negatively regulating PI3K/AKT signaling pathway. Hou *et al*[39] also demonstrated that CLCA4 inhibited cell proliferation, migration, and invasion through inactivation of PI3K/AKT pathway in BC. Briefly, miR-328-3p functioned as an inhibitory miRNA through blocking the PI3K/AKT pathway.

Conclusions

Downregulation of miR-328-3p firstly was observed in BC, which predicted poor prognosis in BC patients. Moreover, miR-328-3p inhibited tumorigenesis of BC through targeting ITGA5 and inactivating the PI3K/AKT pathway. Although we have investigated some functions of miR-328-3p in BC, more details on miR-328-3p function are still needed in BC.

Competing interests

The authors declare that they have no competing interests.

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