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ORIGINAL ARTICLE

Biological functions of miR-363-3p/BTG2 in the metastasis of bladder cancer

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Summary

Purpose: *This study aimed to detect the differential expression of microRNA-363-3p (miR-363-3p) in bladder cancer (BC) samples and to explore its influence on metastasis of BC cells.*

Methods: *Expression level of miR-363-3p in 70 cases of BC tissues and paracancerous tissues was detected. After establishing miR-363-3p overexpression model in 253j and RT4 cells, their migratory ability was assessed by Transwell and wound healing assay. The interaction between miR-363-3p and its downstream gene was predicted online and further confirmed by luciferase assay. Their involvement in regulating metastasis of BC cells was finally explored.*

Results: *MiR-363-3p was downregulated in BC tissues compared with that in the paracancerous tissues. Overexpression of miR-363-3p markedly weakened migratory ability in BC cells. BTG2 was the downstream gene binding miR-363-3p. In addition, overexpression of BTG2 reversed the inhibitory effect of miR-363-3p on BC cell migration.*

Conclusions: *MiR-363-3p is lowly expressed in BC samples. It weakens in vitro migratory ability in BC cells through downregulating BTG2.*

Key words: *MiR-363-3p, BTG2, bladder cancer, metastasis*

Introduction

Bladder cancer (BC) accounts for 1-2% of all malignant tumors. It is reported that in 2018, the incidence of BC ranked 4th in male solid tumors and 10th in females worldwide. Globally, the number of newly diagnosed BC patients in 2018 reached 429,800 and the its death number was up to 165,100 [1,2]. In China, the incidence and mortality of BC are in the first place, which are seriously on the rise annually [3,4]. Histologically, more than 90% of BC cases are transitional cell carcinoma, including 75-85% of non-muscle invasive bladder cancer (NMIBC) and 15-25% of muscular invasive bladder cancer (MIBC) [5,6]. BC is featured by multi-center foci, frequent recurrence and significant malignancy and invasiveness following tumor relapse [7,8]. Within 5 years of treatment in NMIBC patients, about 30-80% of them develop recurrence for at least once, and 10-45% patients deteriorate into MIBC [8]. The pathogenesis of BC remains unclear. It is generally considered that BC is a multi-factor and multi-gene disease that involves the accumulation of gene mutations and external carcinogenic environment [9,10]. Currently, cystoscopy is the golden standard for the diagnosis of BC. Surgery and chemotherapy/radiotherapy are preferred to BC. However, adverse events of chemotherapy and radiotherapy are serious. A great number of BC patients lose the surgical opportunity owing to the advanced stage [11,12]. It is necessary to search sensitive and effective hallmarks for BC. Biologically targeted therapies for BC that are less invasive, cheap and effective are urgently required [10].

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MicroRNAs (miRs) are single-strand, non-coding RNAs with 18-22nt long. A miR usually targets one or multiple mRNAs and thus induces their degradation or translation inhibition. Through post-transcriptional regulation on target mRNAs, miRs are extensively involved in biological activities [13-15]. Abundant evidences has shown that miRs are related to tumorigenesis-associated genes [16-18]. Differentially expressed miRs in BC samples may exert as proto-oncogenes or tumor suppressors [19,20]. Previous studies have shown that miR-363-3p participates in cell phenotypes and tumor cell behaviors [21,22]. Here, we evaluated the possible role of miR-363-3p in BC cell phenotypes and its molecular mechanisms on regulating the metastasis of BC. Our findings may provide a new therapeutic target for BC.

Methods

BC patients and sample collection

A total of 22 pairs of BC and paracancerous tissues were collected from BC patients undergoing surgery. They were pathologically confirmed by H&E staining and stored at-80°C. None of patients had preoperative chemotherapy/radiotherapy. This study got approval by the Ethics Committee of Yantaishan Hospital (09-CN-SDYT-YHD-9043801) and signed written informed consent forms were obtained from the patients.

Cell lines and reagents

Human BC cell lines (T24, 253j, 5637, J82, RT4, UMUC3) and the human bladder epithelial cell line (SV-HUC-1) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were applied in culture medium. Cell passage was conducted when cells were grown to 80-90% confluence using trypsin (Gibco, Rockville, MD, USA).

Transfection

Cells were cultured to 50% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for determination of transfection efficacy and functional experiments.

Transwell migration assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μL of suspension (1×10^5 cells/mL) was applied in the upper layer of the chamber with 700 μL of DMEM medium containing 20% FBS in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample.

Wound healing assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH) or U6. MiR-363-3p: forward: 5'-GCCGAGAATTGCACGGTAT-3', reverse: 5'-CTCAACTGGTGTCGTGGA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse:

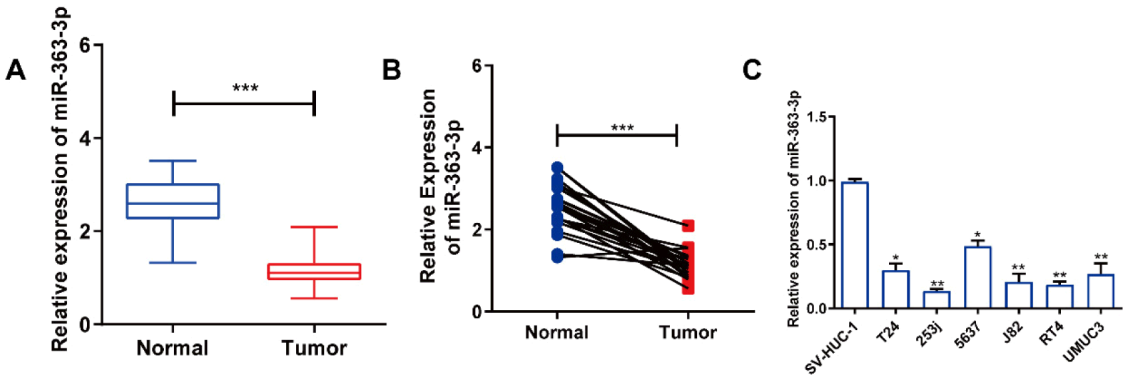

Figure 1. Downregulation of miR-363-3p in BC samples. **A** and **B:** miR-363-3p was downregulated in BC tissues compared with that in paracancer tissues. **C:** BC cell lines exhibited significantly lower expression of miR-363-3p compared with human bladder epithelial cells (SV-HUC-1). Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

5'-ACGCTTCACGAATTTGCGT-3'; BTG2: forward: 5'-CATCATCAGCAGGGTGGC-3', reverse: 5'-CCCAATGCGGTAGGACAC-3'; GAPDH: forward: 5'-GCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ACGACCAAATCCGTTGACTC-3'.

Western blot

Cells or tissues were lysed on ice for isolating proteins. After detection of protein concentration by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). They were subsequently loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase assay

Wild-type or mutant-type BTG2 vector was constructed by inserting predicted sequences or mutant ones into the pmiR vector. 253j and RT4 cells were pre-inoculated in 24-well plates, which were co-transfected with NC mimic/miR-363-3p mimic and BTG2-WT/BTG2-MUT. After 48 h cell culture, they were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the t-test. Chi-square test was conducted for analyzing the relationship between miR-363-3p level and clinical data of BC patients. Pearson's correlation test was applied for evaluating the relationship between two genes. Kaplan-Meier curves were depicted for survival analysis. $P<0.05$ was considered as statistically significant.

Results

Downregulation of miR-363-3p in BC samples

We detected miR-363-3p levels in 70 pairs of BC and paracancer tissues by qRT-PCR. It was

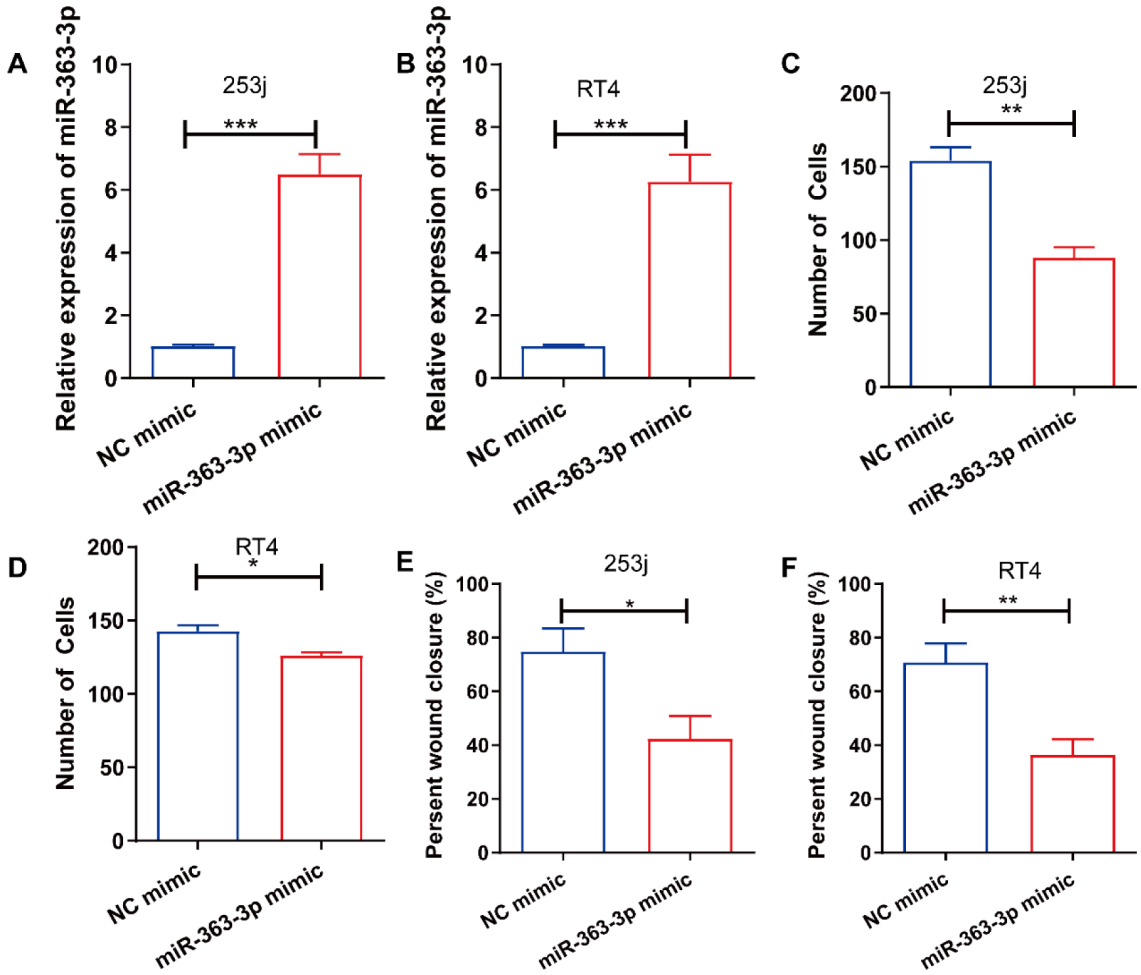

Figure 2. Overexpression of miR-363-3p weakened migratory ability in BC. **A** and **B:** Transfection efficacy of miR-363-3p mimic in 253j and RT4 cells. **C** and **D:** Migration in 253j and RT4 cells transfected with NC mimic or miR-363-3p mimic. E and F: Wound healing percentage in 253j and RT4 cells transfected with NC mimic or miR-363-3p mimic. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

found that miR-363-3p was downregulated in BC tissues (Figure 1A, 1B). Compared with human bladder epithelial cells, miR-363-3p was identically downregulated in BC cell lines (Figure 1C).

Overexpression of miR-363-3p weakened migratory ability in BC

We constructed miR-363-3p overexpression model in 253j and RT4 cells by transfection of miR-363-3p mimic (Figure 2A and 2B). Compared with those transfected with NC mimic, migratory cell number was lower in BC cells transfected with miR-363-3p mimic (Figure 2C and 2D). Besides, lower wound closure percentage was observed in BC cells overexpressing miR-363-3p (Figure 2E and 2F). It is suggested that miR-363-3p markedly weakened migratory ability in BC cells.

A negative interaction between miR-363-3p and BTG2

Interestingly, protein level of BTG2 was downregulated in 253j and RT4 cells overexpressing miR-363-3p (Figure 3A). Bioinformatics prediction revealed the predicted consequential pairing of target region in BTG2 3'UTR and miR-363-3p (Figure 3B). Overexpression of miR-363-3p largely decreased luciferase activity in the wild-type

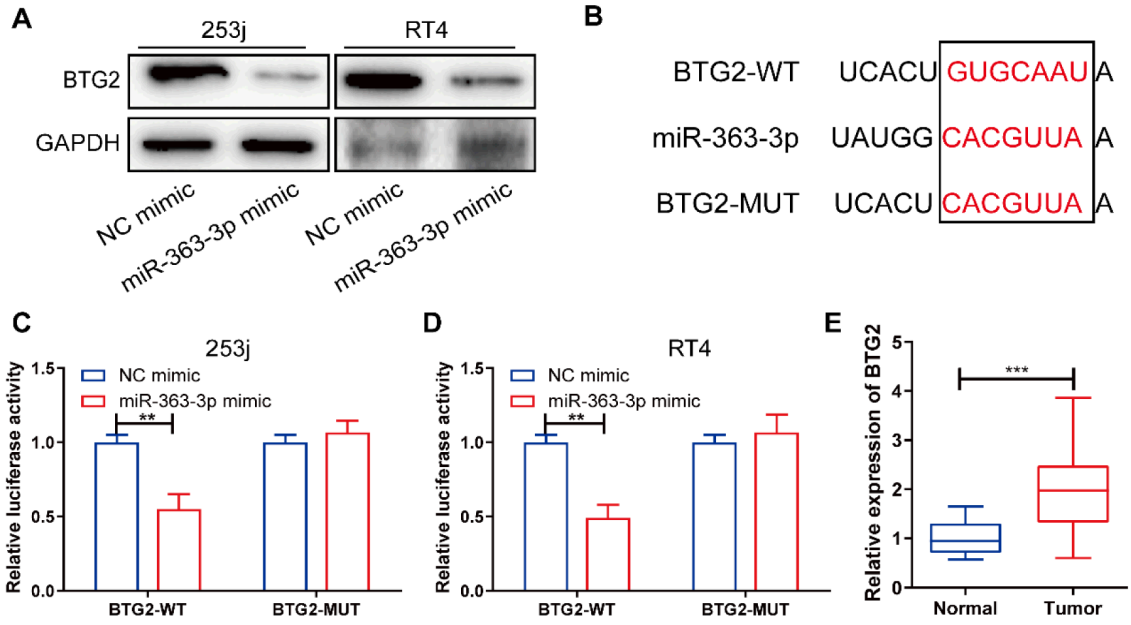

Figure 3. A negative interaction between miR-363-3p and BTG2. **A:** Protein level of BTG2 in 253j and RT4 cells transfected with NC mimic or miR-363-3p mimic. **B, C** and **D:** Luciferase activity in 253j and RT4 cells co-transfected with NC mimic/miR-363-3p mimic and BTG2-WT/BTG2-MUT. **E:** Differential expression of BTG2 in BC and paracancer tissues. Data were expressed as mean±SD. **p<0.01, ***p<0.001.

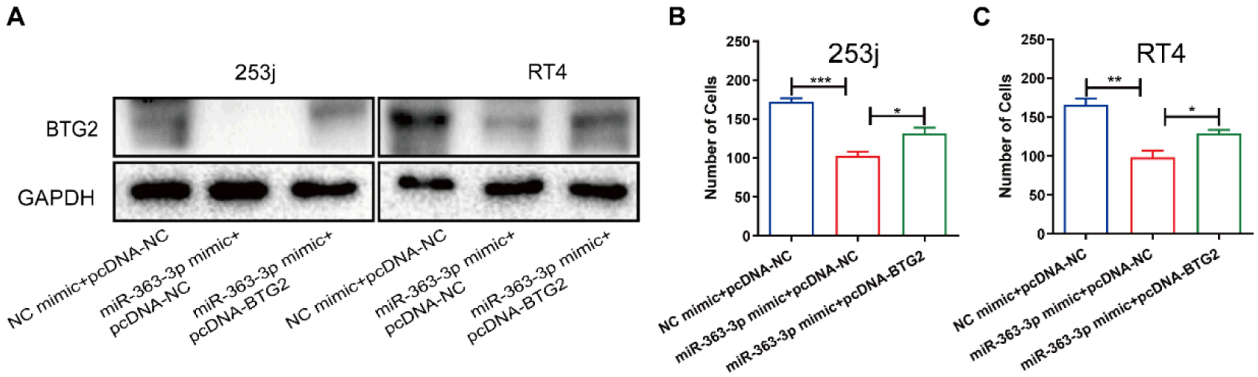

Figure 4. Overexpression of BTG2 abolished the inhibitory effect of miR-363-3p on BC metastasis. **A:** BTG2 level in 253j and RT4 cells co-transfected with NC mimic+pcDNA-NC, miR-363-3p mimic+ pcDNA-NC or miR-363-3p mimic+pcDNA-BTG2. **B** and **C:** Migration in 253j and RT4 cells co-transfected with NC mimic+NC, miR-363-3p mimic+NC or miR-363-3p mimic+pcDNA-BTG2. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

BTG2 vector, verifying that BTG2 was the downstream target binding miR-363-3p (Figure 3C and 3D). Compared with paracancer tissues, BTG2 was highly expressed in BC tissues (Figure 3E).

Overexpression of BTG2 abolished the inhibitory effect of miR-363-3p on BC metastasis

Subsequently, miR-363-3p and BTG2 co-overexpression model was constructed to further analyze the biological functions of BTG2 in BC. Transfection efficacy of pcDNA-BTG2 was first tested (Figure 4A). Compared with 253j and RT4 cells overexpressing miR-363-3p, those co-overexpressing miR-363-3p and BTG2 presented more migratory cells (Figure 4B, 4C). Collectively, the inhibitory effect of miR-363-3p on BC cell migration was abolished by co-overexpression of BTG2.

Discussion

Tumor biological behaviors are determined by changes in single or multiple genes. The occurrence and development of tumors are closely related to the activation of oncogenes and/or the inactivation of tumor suppressors [16,17]. More and more fundamental researches on BC have focused on tumor-associated genes and their encoded products. They can be utilized as biological hallmarks to detect BC as early as possible, thus enhancing the therapeutic outcomes [7-9]. Genetic factors leading to the occurrence and progression of BC remain largely unclear. It is of great significance to analyze the molecular mechanisms of BC and to improve the screening and treatment efficacy [10-12].

Through complementary base pairing, miRs can regulate the transcription of almost all human genomes [13-15]. MiRs are vital factors involved in the maintenance of tumor microenvironment, which are distributed in the tumor-associated genomic region [15-17]. BC-associated miRs have been previously reported [19,20] and have shown to possess diagnostic and prognostic potentials in BC [18-20]. Through microarray analysis and literature review, miR-363-3p is identified to be related to the development of osteosarcoma and colorectal cancer [21,22]. In this article, we collected 22 pairs of BC and paracancer tissues. It was discovered that miR-363-3p was downregulated in BC tissues. We thereafter speculated that miR-363-3p may be a tumor suppressor involved in the development of BC. Subsequently, miR-363-3p overexpression model was established by transfection of miR-363-3p mimic in 253j and RT4 cells. Both transwell and wound healing assay results uncovered that overexpression of miR-363-3p remarkably inhibited the migratory ability in BC.

A complicated network involving miRs and their targets controls vital life activities [14-16]. If a miR and its corresponding target mRNA are bound in an open reading frame in a completely complementary manner, the mRNA will be specifically degraded. The binding in the mRNA 3'UTR only results in the mRNA translation without influencing its stability [15,16]. We have confirmed that BTG2 was the target binding miR-363-3p. Converse to miR-363-3p, BTG2 was upregulated in BC tissues. Notably, overexpression of BTG2 markedly abolished the role of miR-363-3p in suppressing the migratory ability in BC cells. Collectively, we have demonstrated that miR-363-3p/BTG2 axis was beneficial to the diagnosis, treatment and evaluation of BC.

Conclusions

MiR-363-3p is lowly expressed in BC samples. It weakens *in vitro* the migratory ability in BC cells through downregulating BTG2.

Conflict of interests

The authors declare no conflict of interests.

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