MiR-194-5p inhibits cell migration and invasion in bladder cancer by targeting E2F3

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Summary

Purpose: MicroRNA (miR)-194-5p is downregulated in bladder cancer (BC), but its role in BC has not been determined mechanistically.

Methods: The expression levels of miR-194-5p and E2F transcription factor 3 (E2F3) were determined by means of quantitative reverse transcription and polymerase chain reaction in BC specimens. In addition, T24 BC cells were transfected with a miR-194-5p mimic, a miR-194-5p inhibitor, or E2F3 small interfering (si)RNA, and the level of E2F3 protein expressed by these cells was assessed by western blotting. A dual luciferase reporter assay was applied to verify the binding site between miR-194-5p and the 3’ untranslated region of E2F3. Transwell assays were performed to examine cell migration and invasion.

Results: Dysregulation of miR-194-5p in BC was closely associated with node metastasis and differentiation. In BC specimens and cell lines, miR-194-5p mRNA was downregulated, while E2F3 mRNA was upregulated. Overexpression of miR-194-5p suppressed the expression of E2F3 mRNA and protein. By regulating E2F3, miR-194-5p inhibited cell migration and invasion in BC. Treatment of BC cells with E2F3 siRNA had the same effect as did overexpression of miR-194-5p.

Conclusions: MiR-194-5p directly targets E2F3 and inhibits cell migration and invasion in BC.

Key words: bladder cancer, E2F3, invasion, migration, miR-194-5p

Introduction

Bladder cancer (BC) occurs in the mucosa of the bladder and is among the top 10 most common malignant tumors in humans [1,2]. BC can occur at any age -including childhood- but its incidence increases with age, with the highest incidence rate occurring in patients 50 to 70 years old. The incidence of BC in males is 3 to 4 times higher than in females [3,4]. In recent decades, the 5-year overall survival rate has increased to approximately 75% [5]. However, effective treatments still are unavailable for patients diagnosed with advanced BC. Therefore, early diagnosis and treatment of this disease is crucial.

The development of targeted therapies, involving genomic and proteomic techniques, is considered to be the next generation in cancer research.
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[6]. Specifically, miRs have emerged as important targets because these molecules can function as oncogenes or tumor suppressors by regulating cancer tumorigenesis and metastasis [7,8]. Investigators have demonstrated that miR-194 is upregulated in pancreatic ductal adenocarcinoma and that this process contributes to tumor progression [9]. Downregulation of miR-194 had been found in renal childhood neoplasms [10], gastric carcinoma [11], and non-small cell lung cancer [12]. Kong et al. demonstrated that miR-194 suppresses tumor migration and invasion in prostate cancer by repressing the human nuclear distribution protein [13]. In laryngeal squamous cell carcinoma, miR-194 exerts inhibitory actions by suppressing Wee1 [14]. Zhang et al. determined that miR-194 inhibits cell proliferation and invasion by targeting RAP2B in BC [15].

The oncogene E2F transcription factor 3 (E2F3) has been shown to promote cancer progression [16,17]. Li et al. determined that miRNA-125b inhibited the progression of BC by suppressing E2F3 [18]. Moreover, patients with BC and E2F3 overexpression have a higher mortality rate than do patients with BC and low E2F3 expression [19]. In other cancers, miR128-1 and miR-564 were found to inhibit cell growth by targeting E2F3 [20,21]. However, the mechanism by which miR-194-5p regulates E2F3 in BC has not been determined.

Herein, we demonstrate that miR-194-5p expression is dysregulated in BC. We determined that miR-194-5p inhibits cell migration and invasion in BC by suppressing E2F3.

Methods

Clinical samples and cell culture

Fifty-eight BC specimens and adjacent tissues were obtained from patients who underwent surgery, and no other treatment, at the Jining First People’s Hospital. All patients gave written informed consent. Tissues were frozen in liquid nitrogen and stored in a freezer at -80°C until use. Human BC cell lines T24 and 5637, as well as a noncancerous urothelial cell line (SV-HUC-1), were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). This experiment was approved by the Institutional Ethics Committee of Jining First People’s Hospital.

Transfection

A mimic and an inhibitor of miR-194-5p as well as E2F3 small interfering (si)RNA were purchased from Ribobio (Guangzhou, China). T24 and 5637 cells were transfected with these molecules by means of Lipo-fectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions.

RNA extraction and amplification

TRIzol reagent (Invitrogen) was used to extract total RNA. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out with SYBR Green PCR Master Mix on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) to detect the expression levels of miR-194-5p and E2F3 mRNA. U6 and GAPDH served as the controls for miR-194-5p and E2F3, respectively. The levels of miR-194-5p and E2F3 were ascertained by the 2-ΔΔCt method.

Dual luciferase assay

The mutant or wild-type E2F3 3’ untranslated region (i.e., E2F3-3’UTR-Wt or E2F3-3’UTR-Mut) was inserted into the pGL3 promoter vector for luciferase reporter experiments. Cells then were transfected with the vector construct and miR-194-5p mimic using Lipofectamine 2000. Luciferase activity was determined 24 hrs posttransfection using a Dual-Luciferase Reporter Assay system (Beyotime Institute of Biotechnology, Beijing, China).

Transwell migration and invasion assay

Transwell chambers (Corning, Corning, NY) were applied to evaluate the migratory and invasive abilities of bladder cancer cells. Transfected cells were placed into the upper chamber on a noncoated membrane at a density of 5×10⁴ cells/chamber without FBS. The lower chamber was filled with 20% FBS to induce cells to migrate through the membrane. For the invasion assay, the experiment was prepared similarly except that the cells were put in the upper chamber on a coated membrane. After sufficient incubation to allow for migration/invasion, the cells were stained with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) and visualized.

Western blot

Total proteins were obtained using RIPA buffer. Proteins were separated by means of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes. Membranes were blocked with nonfat milk at room temperature and then were incubated overnight at 4°C with anti-E2F3 or anti-GAPDH antibodies. Membranes were then incubated with the corresponding secondary antibodies. Protein levels were measured by a gel imaging system (JS-780, Shanghai Peiqing, Shanghai, China).

Statistics

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad, San Diego, CA) and SPSS 17.0 (IBM, Armonk, NY). Data were presented as mean ± standard deviation (SD). Comparisons were made using the Student’s t-test and the χ² test. Statistical significance was set at p<0.05.
Results

Low expression of MiR-194-5p in BC cell lines and tissues

The mRNA expression of miR-194-5p was determined in T24 and 5637 cell lines by qRT-PCR. We found that miR-194-5p expression was lower in T24 and 5637 cells than in normal control cells (SV-HUC-1) (Figure 1A). We also applied qRT-PCR to evaluate miR-194-5p expression levels in 58 human BC specimens and in unaffected tissues. We found that miR-194-5p expression was lower in human BC samples than in normal tissues (Figure 1B) and that miR-194-5p downregulation was associated with node metastasis (Figure 1C) and differentiation (Figure 1D).

MiR-194-5p suppresses cell migration and invasion in BC

To explore the function of miR-194-5p in BC, T24 cells were transfected with a miR-194-5p mimic or a miR-194-5p inhibitor. Transfection of 5637 cells also was carried out, but 5637 cells yielded less pronounced effects. Expression of miR-194-5p by cells transfected with the miR-194-5p mimic or inhibitor was verified with qRT-PCR (Figure 2A,B). Overexpression of miR-194-5p by transfection with the mimic yielded inhibition of BC cell migration. Conversely, miR-194-5p inhibition resulted in greater migration (Figure 2C). Accordingly, the invasive abilities in cells expressing the miR-194-5p mimic were decreased substantially, whereas cells expressing the miR-194-5p inhibitor had increased invasion (Figure 2D). These results confirmed that miR-194-5p suppresses cell migration and invasion in BC.

MiR-194-5p directly targets E2F3 in BC cells

To explore the regulatory role of miR-194-5p in BC, the target genes of this miR were ascertained using TargetScan (http://www.targetscan.org). Based on conservation of its binding site,
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Figure 2. The tumor-suppressing effect of miR-194-5p in BC. A-B: T24 cells were transfected with a miR-194-5p mimic or a miR-194-5p inhibitor, and the expression of miR-194-5p was measured by qRT-PCR. C-D: Abnormal expression of miR-194-5p was associated with improved migratory and invasive capacities in T24 cells. **p<0.01. NC: negative control.

Figure 3. MiR-194-5p directly targets E2F3 in BC cells. A: Binding site conservation between the wild-type E2F3 3′-UTR and miR-194-5p. B: Results of the luciferase reporter assay. C: Expression of E2F3 protein in cells expressing the miR-194-5p mimic or inhibitor. **p<0.01. NC: negative control.
E2F3 was considered as a candidate target gene (Figure 3A). To determine whether miR-194-5p directly targets E2F3, a dual-luciferase reporter vector containing E2F3-3′UTR-Wt or E2F3-3′UTR-Mut was constructed. Expression of the wild-type 3′UTR resulted in suppression of luciferase activity, whereas the luciferase activity of cells expressing the mutant 3′UTR was similar to that of cells that did not harbor either UTR construct (Figure 3B).

We also determined the expression of E2F3 protein in T24 cells transfected with the miR-194-5p mimic or inhibitor. We found that the miR-194-5p mimic group had low E2F3 protein expression, whereas the miR-194-5p inhibitor had high E2F3 expression (Figure 3C). Hence, miR-194-5p directly targets E2F3 in BC cells.

**BC cell migration and invasion are inhibited by siE2F3**

In T24 cells expressing E2F3 siRNA, E2F3 expression was lower than in control cells, as expected (Figure 4A). Moreover, BC cell lines T24 and 5637 expressed higher endogenous levels of E2F3 than did noncancer control cells (SV-HUC-1) (Figure 4B). In the presence of E2F3 siRNA, BC cell migration and invasion was inhibited; this effect was similar to that of miR-194-5p overexpression (Figures 4C and 4D). These findings indicate that E2F3 promotes tumorigenesis in BC.

**E2F3 partially rescues miR-194–induced inhibition in BC**

The effect of E2F3 overexpression on migration and invasion of BC cells overexpressing miR-
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194-5p was examined. Specifically, T24 cells that expressed the miR-194-5p mimic were transfected with an E2F3 expression vector or negative control. We verified that E2F3 mRNA and protein levels were augmented posttransfection (Figures 5A and 5B). We then evaluated cell migration and invasion in cells overexpressing miR-194-5p with or without E2F3 overexpression (Figures 5C and 5D) and observed that E2F3 overexpression partially reverses the miR-194-5p–induced suppression of cell migration and invasion.

**Discussion**

In treatment of advanced cancers, procedures that target miRs have shown promising results [22]. Many miRs are involved in tumorigenesis and progression of BC, including miR-1-3p [23], miR-223-3p [24], miR-429 [25], and miR-497 [26]. Recently, miR-194 was shown to be downregulated in various cancers [10-12,27]. Our findings support these previous studies; we determined that miR-194-5p is downregulated in BC cells and in clinical BC specimens. A low level of miR-194-5p was associated with node metastasis and differentiation. Similarly, other researchers have shown that miR-194-5p downregulation was related to poor prognosis in laryngeal squamous cell carcinoma [14]. Overexpression of miR-194-5p yielded suppression of BC progression in terms of cell migration and invasion. We determined that this regulatory process involves direct targeting of E2F3 by miR-194-5p. E2F3 is a transcription factor that is known to regulate cell cycle progression and that promotes cell growth and survival, particularly in BC [28].

Upregulation of E2F3 in BC has been investigated previously [18]. E2F3 overexpression was found to inhibit BC cell proliferation [19]. The ex-

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Figure 5. E2F3 partially reverses the inhibitory effect of miR-194-5p in BC cells. The relative expression levels of A: E2F3 mRNA and B: E2F3 protein in cells coexpressing the miR-194-5p mimic and the E2F3 expression vector. C and D: Results of transwell assays of cell migration and invasion in which cells coexpressing the miR-194-5p mimic and the E2F3 expression vector were used. **p<0.01. NC: negative control.
pression levels of miR-200b, miR-503, and miR-449a were shown to have regulatory effects on E2F3 [29-31]. In our study, suppression of E2F3, induced by miR-194-5p overexpression, repressed cell migration and invasion in BC. Furthermore, expression of siE2F3 had the same effects as overexpression of miR-194-5p in BC. Our results strongly support the hypothesis that miR-194-5p suppresses BC by downregulating E2F3. BC cell migration and invasion can be inhibited by upregulation of miR-194-5p and consequent suppression of E2F3. Furthermore, E2F3 overexpression partially reversed the effect of miR-194 overexpression in BC. Our future work will involve exploring the functional effects of E2F3 on BC cell migration and invasion, particularly in the presence of miR-194-5p.

In this study, we verified that miR-194-5p suppresses BC cell migration and invasion by down-regulating E2F3. Our findings may have clinical implications for inhibiting tumorigenesis in BC.

Conflict of interests

The authors declare no conflict of interests.

References