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HOXA10 promotes the development of bladder cancer through regulating FOSL1

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Abstract. – OBJECTIVE: This study was aimed to investigate the expression characteristics of HOXA10 in bladder cancer (BCa), and to further study whether it can promote the development of BCa via regulating FOSL1.

PATIENTS AND METHODS: The expression of HOXA10 was examined by quantitative Real Time-PCR (qRT-PCR) in 37 pairs of tumor tissue and paracancerous specimens of BCa patients; meanwhile, in BCa cell lines, the expression of HOXA10 was also verified using qRT-PCR. Subsequently, after HOXA10 knockdown model was constructed in BCa cell lines (EJ and J82) using lentivirus transfection, transwell, as well as wound healing assays, were performed to analyze the influence of the downregulation of HOXA10 on the biological function of BCa cells. Finally, Luciferase reporting assay and cell reverse experiment were applied to explore the specific interaction between HOXA10 and FOSL1.

RESULTS: The results of qRT-PCR indicated that the expression level of HOXA10 in BCa tissue samples was remarkably higher than that in adjacent normal ones, with a statistically significant difference. At the same time, the overall survival rate of patients with high expression of HOXA10 was found to be lower than those with low expression. Meanwhile, compared with cells in sh-NC group, the metastasis ability of BCa cells in sh-HOXA10 group was remarkably weakened. In addition, it was found that the levels of FOSL1 and HOXA10 were negatively correlated in BCa tissues. The result of the Luciferase reporter gene assay revealed that HOXA10 could be targeted by FOSL1 through certain binding sites between them. In addition, HOXA10 was found to be capable of further regulating the malignant progression of BCa by modulating FOSL1.

CONCLUSIONS: HOXA10 expression is remarkably elevated in BCa tissues and cell lines, which is closely relevant to the poor prognosis of BCa patients. In addition, HOXA10 may be able to accelerate BCa metastasis *via* modulating FOSL1 expression.

Key Words:
HOXA10, FOSL1, BCa, Metastasis.

Introduction

Bladder cancer is one of the most common malignancies of the urinary system, accounting for the fifth highest incidence of malignancies in the world and the second most lethal malignancy of the urinary system[1-3]. Due to its high incidence and mortality, BCa has gradually become a research hotspot of the urinary system[4,5]. Currently, the treatment of BCa is still unable to effectively delay tumor progression and recurrence[4,5]. The most effective way to treat BCa is still surgery, which is often supplemented by adjuvant therapies, such as chemotherapy and biological targeted therapy. However, the 5-year survival rate after surgery is only 58% to 66%, and the therapeutic effect is still unsatisfactory[6,7]. Data show that nearly 50-70% of patients with superficial BCa still have recurrence after BCa resection (whether adjuvant chemotherapy is combined or not), and the cancer still progresses to invasive BCa in a considerable proportion of patients within 5 years[8]. Recurrence or metastasis of BCa may be related to complex molecular regulatory mechanisms[9,10]. Many studies have been conducted on the biological characteristics and molecular regulatory mechanism of BCa, and some progresses have been made. However, the gene regulatory network of BCa is still unclear[11,12]. Abnormal gene expression plays an important role in the occurrence and development of BCa, which has been a research hotspot[12]. Therefore,

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in-depth study of the pathogenesis and molecular regulatory mechanism of BCa and exploration of appropriate new therapeutic targets are potential directions for the diagnosis and treatment of this disease.

The HOX gene, a gene family containing a common domain consisting of 183 nucleotide sequences, is often composed of four clusters including A, B, C, and D in mammals. It has been found that HOX gene clusters (A~D) are located on chromosome 7p15, 17p21, 12q13, and 2q31 in humans, respectively, with a total of 39 genes[13,14]. HOX protein can not only activate transcription factors by combining monomer or isomeric dimer, but also dimer or trimer with cofactor to enhance or inhibit the expression of target genes, thereby playing an essential role in the development of limbs and organs of mammals[15,16]. Under normal physiological conditions, the signal mediated by HOX gene is in balance with other signals involved in regulating HOX gene[16]. However, the abnormal expression of HOX gene will break the normal physiological balance and lead to the occurrence of pathology[17,18]. As a member of the homologous box gene family, HOXA10 plays a pivotal role in regulating embryonic development and self-renewal of blood, bone, endometrial cells, and other cells[19,20]. Increasing studies[21] have shown that the abnormal expression of HOXA10 is closely related to the tissue classification, occurrence, and development of tumor.

HOXA10 is an important member of the HOX gene family and participates in the cell proliferation and differentiation during tissue development[22,23]. However, the abnormal expression of HOXA10 may be involved in the development of tumors. The expression of HOXA10 in cancer tissues reflects the basic characteristics of cancer tissues, which is of great significance for the diagnosis of early cancer and the treatment of tumors[23,24]. Although HOXA10 has been shown to promote or inhibit tumor development in some tumors, the expression and role of HOXA10 in BCa tissues or cell lines so far have not been reported. Therefore, the purpose of this study is to clarify HOXA10 expression in BCa and adjacent tissues and its correlation with the clinicopathological features of BCa patients, so as to provide a basis for further research on the occurrence and development of BCa and the molecular targets affecting the clinical prognosis of these patients.

Patients and Methods

Patients and Colon Cancer Samples

In this study, 37 pairs of specimens were selected from surgically treated BCa cases, and BCa tissues and their corresponding adjacent tissues were collected and then stored at -80°C. The collection of clinical specimens was approved by the Ethics Monitoring Committee, and patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Cell Lines and Reagents

Five human BCa cells (T24, EJ, J82, 253j, 5637) and human bladder epithelial immortalized cells (SV-HUC-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), while Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and fetal bovine serum (FBS; Gibco, Rockville, MD, USA) were purchased from American Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured in a 37°C incubator with 5% CO_2, with DMEM medium containing 10% fetal bovine serum.

Transfection

The negative control (sh-NC) and the lentivirus containing the HOXA10 knockdown sequence (sh-HOXA10) were purchased from Shanghai Jima Company. The cells were plated in 6-well plates and grown to a cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After 48h, the cells were collected for qRT-PCR analysis and cell function experiments.

Transwell Cell Migration and Invasion Assay

After transfection for 48 hours, the cells were trypsinized and resuspended in serum-free medium. After cell counting, the cell density was adjusted to 2.0×10^5/ml, and the transwell chamber containing Matrigel and no Matrigel was placed in the 24-well plate. 200 µl of the cell suspension was added in the upper chamber, while 500 µl of medium containing 10% FBS was added to the lower chamber. After incubation in an incubator at 37°C for 48 hours, the chamber was removed, fixed with 4% paraformaldehyde for 30 minutes,

and stained with crystal violet for 15 minutes. Subsequently, the cells were washed with phosphate-buffered saline (PBS), and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed in 5 randomly selected fields of view under the microscope.

Cell Wound Healing Assay

The cells after transfection for 48 hours were digested, centrifuged and resuspended in the serum-free medium to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, the cells were rinsed gently with PBS for 2-3 times, then, low-concentration serum medium (such as 1% FBS) was added in, and then, the cells were observed again after 24 hours. According to the pre-experiment of scratches, it was judged whether the cells had healing ability according to the migration area; for the scratch test, the difference in the cell healing ability was judged according to the migration area.

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

After the cells were treated accordingly, 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, real-time PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions, and the PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Three replicate wells were repeated for each sample and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: HOXA10, forward: 5'-CTCGCCCATAGACCTGTGG-3', reverse: 5'-GTTCTGCGCGAAAGAGCAC-3'; FOSL1, forward: 5'-CAGGCGGAGACTGACAAACTG-3', reverse: 5'-TCCTTCCGGGATTTTGCAGAT-3'; GAPDH, forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Il, USA). The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and later transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. The primary antibodies against HOXA10, FOSL1, and GAPDH, as well as the secondary antibodies against mouse and rabbit, were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the Luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected into EJ and J82 cells with the reporter plasmid. A specific Luciferase substrate was added, then, Luciferase reacted with the substrate to generate fluorescence, and the activity of the Luciferase was determined 48 hours later using the Promega Luciferase kit to detect the intensity of the fluorescence.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (Version X; La Jolla, CA, USA). The differences between the two groups were analyzed by using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment, and the data were expressed as average ± standard deviation (x±s). There were three levels of $p<0.05$, $p<0.01$, and $p<0.001$ at the significance level, and $p<0.05$ was considered statistically significant.

Results

HOXA10 Was Highly Expressed in BCa Tissues and Cell Lines

To determine the expression level of HOXA10 in BCa, a total of 37 pairs of BCa tumor tissues and adjacent tissues were collected. qRT-PCR results showed that the expression of HOXA10 in BCa patients was higher than that in adjacent normal tissues (Figure 1A, 1B), suggesting that HOXA10 may play a role as an oncogene in BCa. In addition, HOXA10 was remarkably expressed in BCa cell lines compared to human normal liver cell lines (LO2), and the difference

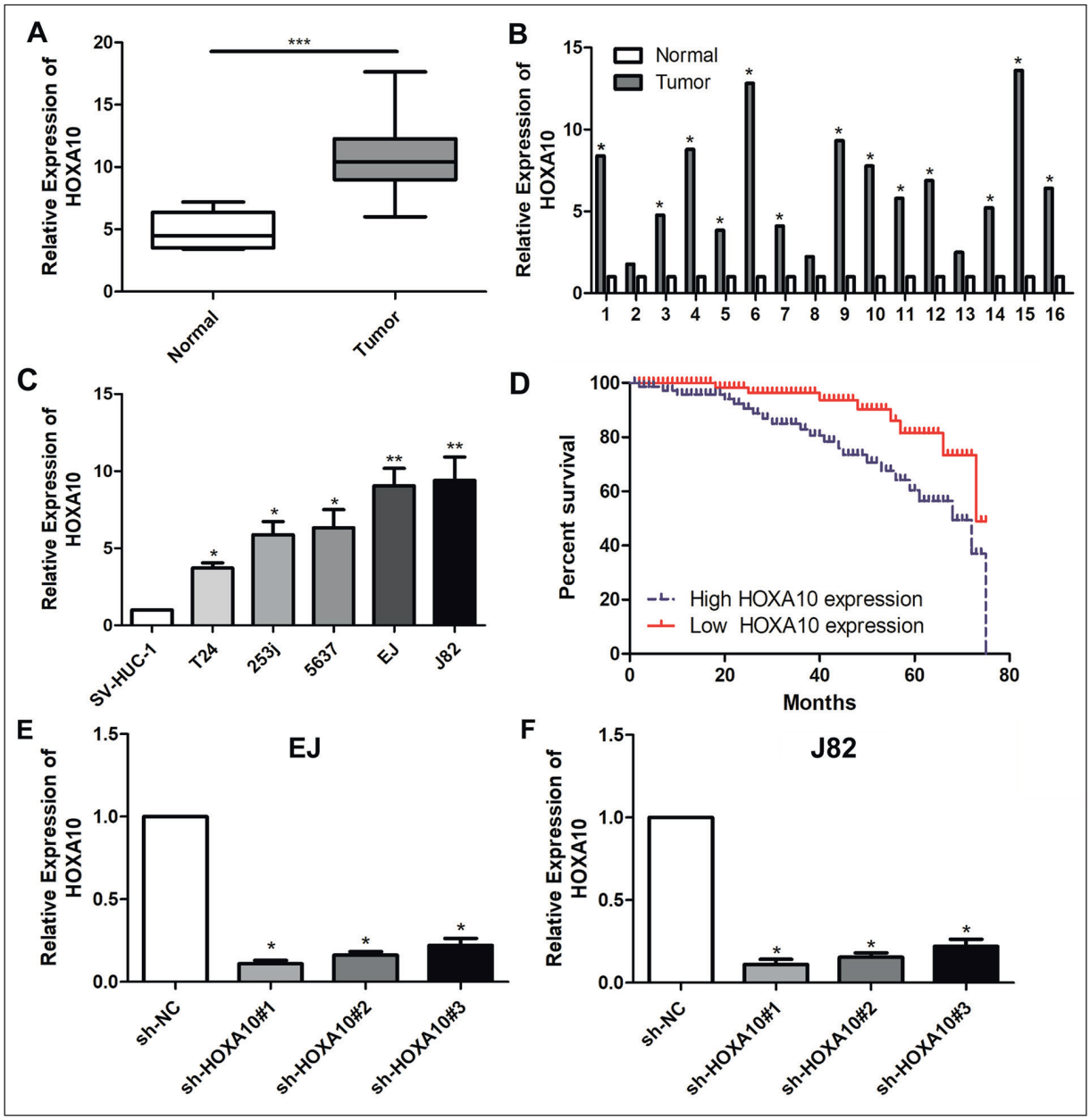

Figure 1. HOXA10 is highly expressed in bladder cancer tissues and cell lines. **A, B,** QRT-PCR was used to detect the difference in expression of HOXA10 in bladder cancer tissues and adjacent tissues. **C,** QRT-PCR was used to detect the expression level of HOXA10 in osteosarcoma cell lines. **D,** Kaplan Meier survival curve of bladder cancer patients based on HOXA10 expression; the prognosis of patients with high expression was significantly worse than that of low expression group. **E,** QRT-PCR verified the interference efficiency of HOXA10 after transfection of HOXA10 knockdown vector in EJ cell line. **F,** QRT-PCR verified the interference efficiency of HOXA10 after transfection of the HOXA10 knockdown vector in the J82 cell line. Data are mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

was statistically significant (Figure 1C). According to the mRNA expressions of HOXA10 in 37 pairs of BCa tissues and adjacent normal tissues, HOXA10 expression was divided into high expression group and low expression group, and the relationship between HOXA10 expression and the prognosis of BCa patients was analyzed. As shown in Figure 1D, high expression of HOXA10 was closely related to the poor prognosis of BCa. These results demonstrated that HOXA10 may play an oncogene role in BCa and its expression is closely correlated with the poor prognosis of BCa.

Knockdown of HOXA10 Inhibited Cell Migration and Invasion

To investigate the cytological function of HOXA10 in BCa, a knockdown HOXA10 lentiviral vector was constructed. After transfecting the HOXA10 lentiviral vector into the EJ and J82 cell lines, the qRT-PCR assay was performed to verify the interference efficiency (Figure 1E, 1F). Transwell and wound healing assays were performed to detect cell transfer after knocking down HOXA10 in EJ and J82 cell lines, respectively. The results showed that, compared with sh-NC group, the invasion and migration ability of BCa cells in the HOXA10-silencing group was remarkably reduced (Figure 2A). In addition, the migration ability of BCa cells in the HOXA10 silence group was remarkably reduced (Figure 2B). These results indicated that the knockdown of HOXA10 inhibited cell migration and invasion.

HOXA10 Bounded to FOSL1

As shown in Figure 3A, in order to further verify the targeting of FOX1 to HOXA10, a Luciferase reporter gene assay was performed. The results demonstrated that HOXA10 can be targeted by FOSL1 through this binding site. QRT-PCR experiments were performed to detect the expression of FOSL1 in 37 pairs of BCa tumor tissues and their corresponding paracancerous tissues, as well as in BCa cell lines, and the results showed that the expression level of FOSL1 was remarkably lower in BCa tissues than that in adjacent tissues (Figure 3B). According to the mRNA expression of FOSL1 in 37 pairs of BCa tissues and paracancerous tissues, FOSL1 expression was divided into high expression group and low expression group, and the relationship between FOSL1 expression and the prognosis of BCa patients was analyzed. As shown in Figure 3C, high expression of FOSL1 was closely related to poor prognosis of BCa. These results demonstrated that HOXA10 can be targeted by FOSL1 in BCa.

Interaction Between HOXA10 and FOSL1

After constructing the HOXA10 knockdown lentiviral vector in the EJ and J82 cell lines, Western Blotting results showed that the expression level of FOSL1 in the HOXA10-silencing group was remarkably increased, and the difference was statistically significant (Figure 4A, 4C). Subsequently, the FOSL1 knockdown vector in EJ and J82 cell lines was constructed, and Western Blotting results showed that the expression level of HOXA10 was remarkably increased after FOSL1 knockdown, and the difference was statistically significant (Figure 4B, 4D). In addition, the expression of HOXA10 and FOSL1 were detected by qRT-PCR, and the results showed that HOXA10 and FOSL1 were negatively correlated in BCa tissues (Figure 4E), suggesting that HOXA10 can modulate the expression of FOSL1.

FOSL1 Modulated HOXA10 Expression in BCa Cell Lines

To further explore the ways in which HOXA10 promoted the malignant progression of BCa, it was found through correlation bioinformatics analysis that there may be a relationship between HOXA10 and FOSL1. After knocking down FOSL1 in EJ and J82 cell lines that had silenced HOXA10, the expression level of HOXA10 was detected by qRT-PCR method, and it was found that FOSL1 can counteract the expression of HOXA10 (Figure 5A). Subsequently, through transwell and cell scratch experiments, FOSL1 was found to counteract the effect of HOXA10 on hepatocellular carcinoma metastasis (Figure 5B, 5C). In sum, we could conclude that HOXA10 may promote the malignant progression of BCa *via* regulating FOSL1.

Discussion

BCa is the most common malignant tumor of the urinary and reproductive system in China, which directly threatens the survival of patients or affects the quality of life[1-3]. For the treatment of BCa, early surgical resection combined with postoperative intravesical infusion chemotherapy is

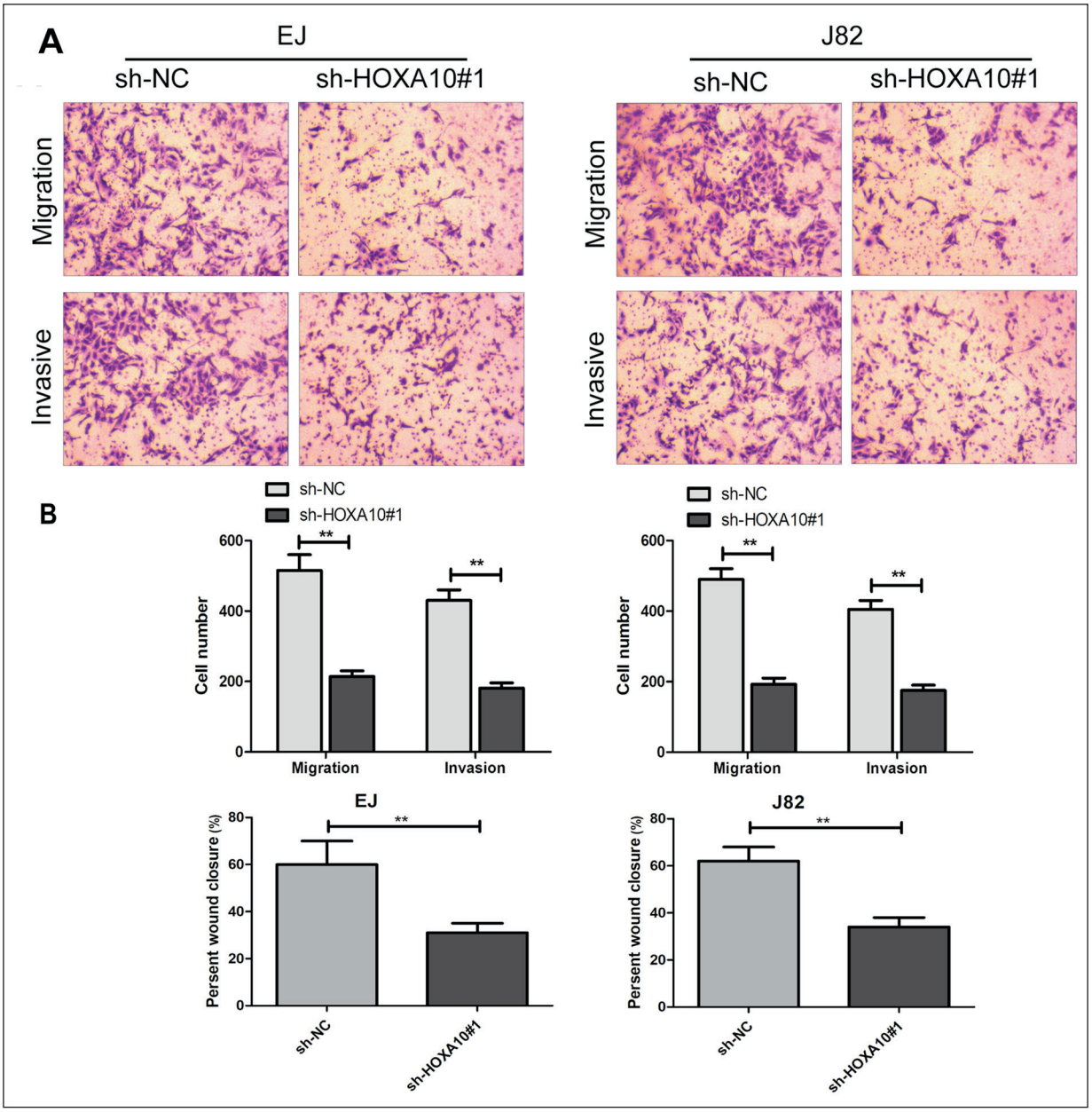

Figure 2. Silencing HOXA10 promotes invasion and migration of bladder cancer cells. **A,** Transwell assay detected the effect of silencing HOXA10 on invasion and migration of bladder cancer cells in EJ and J82 cell lines (magnification: 20x). **B,** The cell wound healing assay detected the ability of the EJ and J82 cell lines to silence HOXA10 on bladder cancer cells. Data are mean ± SD, *$p<0.05$.

recommended[6-8]. The main problems facing BCa treatment are tumor progression and recurrence. 10%-67% of patients with superficial BCa undergoing transurethral resection (TURBT) will relapse within 12 months, and eventually progress to musculocutaneous invasive BCa[8,9]. The latter has a worse prognosis and a higher mortality rate, with an interstitial phenotype, and its ability to invade and metastasis is stronger[10]. Although the application of bladder perfusion chemotherapy reduced the recurrence rate of postoperative tumors, the multi-drug resistance generated by maintaining perfusion chemotherapy reduced its efficacy[7,9]. Therefore, how to curb tumor progression and enhance its drug sensitivity are essential issues that need to be urgently solved[10-12].

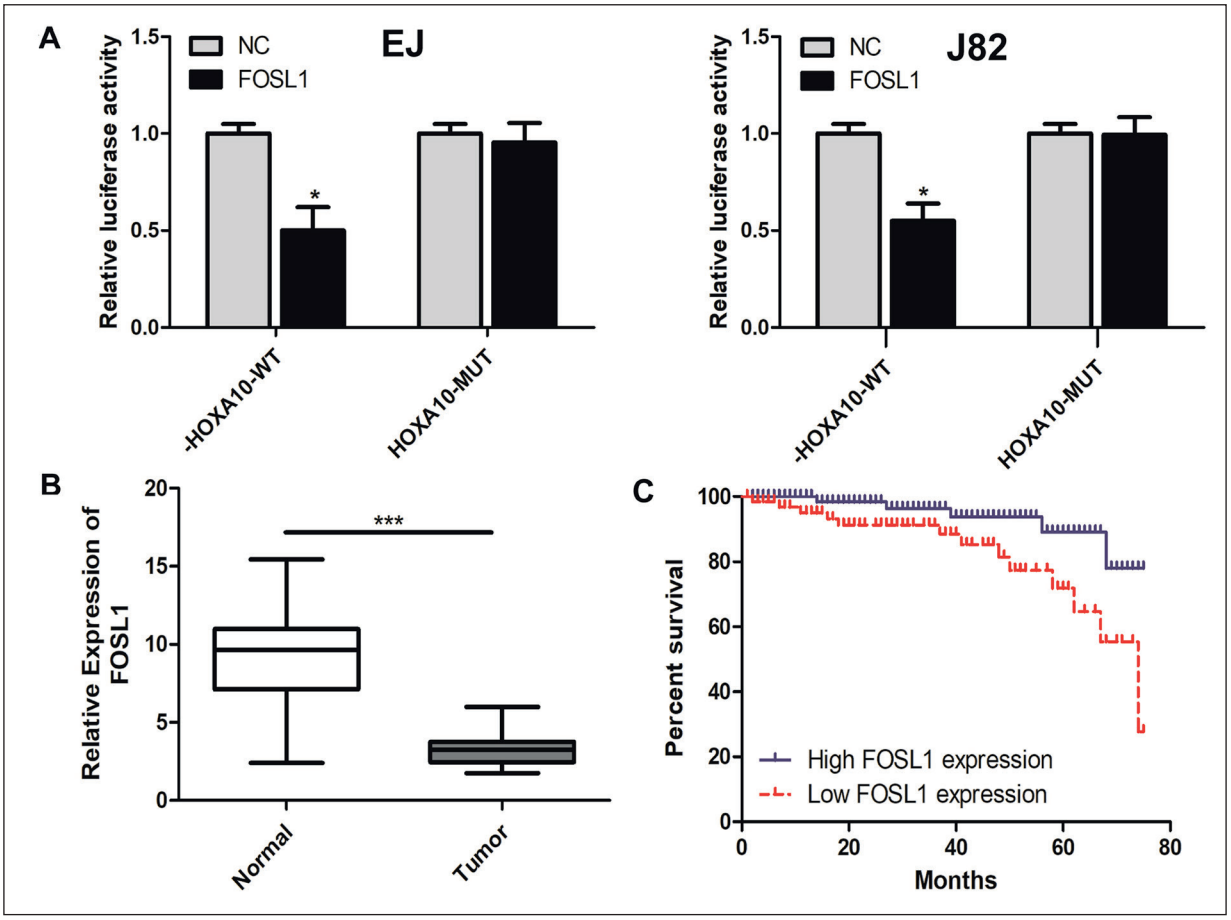

Figure 3. Direct targeting of FOSL1 by HOXA10. **A,** The Dual-Luciferase reporter gene assay verified the direct targeting of HOXA10 and FOSL1. **B,** QRT-PCR was used to detect the difference in FOSL1 expression in bladder cancer tissues and adjacent tissues. **C,** Kaplan Meier survival curve of bladder cancer patients based on FOSL1 expression was shown; the prognosis of patients with low expression was significantly worse than that of high expression group. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

HOXA10 is one of the A cluster genes of the HOX family, which is involved in regulating embryonic development and cell proliferation and differentiation[19,20]. The abnormal expression of HOXA10 has been proved by many literatures[20-22] to be closely related to the occurrence and development of tumor. Currently, the interplay between HOXA10 expression in BCa and its paracancerous tissues and its prognosis still remains elusive. In this study, through the collection of a large sample of human BCa tissue and paired normal bladder tissue samples and the detection of HOXA10 gene expression, the mRNA expression of HOXA10 gene was found to be significantly upregulated in BCa tissues, suggesting that HOXA10 gene may be a new cancer-promoting gene for BCa. In addition, further comparison of sv-huc-1 cells from normal bladder epithelial cells revealed that HOXA10 mRNA expression was significantly high in J82 and EJ cell lines, which further confirmed that HOXA10 gene may play a cancer-promoting role in BCa. Subsequently, an *in vitro* experiment was conducted in order to further explore the molecular mechanism of HOXA10 in the progression of BCa and it was proved that HOXA10 may be a disease-related gene. After synthesizing and transfecting sh-NC and silencing sh-HOXA10 sequences, the ability of BCa cells to metastasize was detected by transwell and wound healing assays, and it was found that silencing HOXA10 could inhibit the invasion and migration ability of BCa cells. These results suggested that HOXA10 may play a pivotal role in BCa, and the genetic interference or drug intervention against HOXA10 can remarkably affect the biological behaviors of BCa cells.

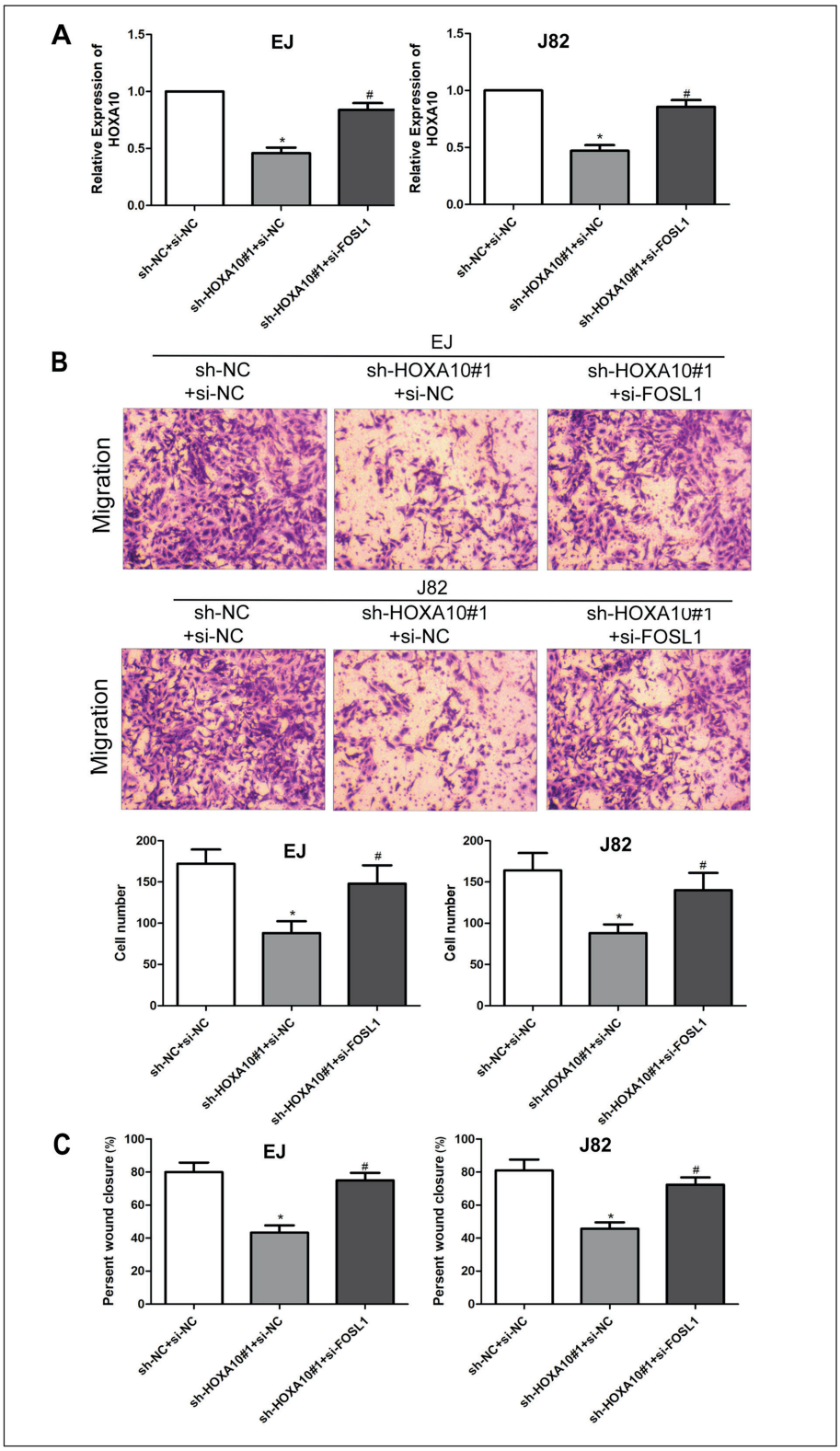

Figure 5. FOSL1 regulates the expression of HOXA10 in bladder cancer tissues and cell lines. **A,** HOXA10 expression levels in HOXA10 and FOSL1 co-transfected cell lines were detected by qRT-PCR. **B,** Transwell assay detected the invasion and migration of hepatocellular carcinoma cells after co-transfection of HOXA10 with FOSL1 (magnification: 20x). **C,** The cell wound healing was used to detect the ability of HOXA10 and FOSL1 to co-transfect and to regulate the ability of hepatocyte cancer cells to crawl. Data are mean ± SD, *p<0.05.

To further explore the role of HOXA10 in promoting the development of BCa, we used bioinformatics, Dual-Luciferase reporter gene assay, and other molecular biology experiments to verify the direct binding of HOXA10 to downstream FOSL1. Subsequently, by collecting BCa tissue samples and detecting the difference in expression of FOSL1 gene, it was found that the mRNA expression of FOSL1 gene was remarkably downregulated in BCa tissues. To further explore the regulation of HOXA10 and FOSL1 expression in BCa cell lines, the expression levels of HOXA10 and FOSL1 were found to be negatively correlated. In addition, cell reverse experiments also elucidated the effect of silencing FOSL1 on the invasiveness and migration of BCa cells after knocking down HOXA10. Based on the above results, we assume that HOXA10 might be able to serve as an oncogene to enhance the invasiveness and metastasis capability of BCa cells; meanwhile, the related-mechanism studies have demonstrated that HOXA10 comes into play by regulating FOSL1.

Conclusions

Taken together, these data showed that the expression of HOXA10 in BCa is remarkably increased, which is remarkably associated with poor prognosis of BCa. In addition, HOXA10 may promote the malignant progression of BCa *via* regulating FOSL1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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