

Long Non-Coding RNA BCAR4 Binds to miR-644a and Targets TLX1 to Promote the Progression of Bladder Cancer

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Background: Bladder cancer is a serious threat to human health. It is meaningful to study the pathogenesis of bladder cancer. Long non-coding RNAs (lncRNAs) are reported to promote or inhibit bladder cancer development. However, the role of lncRNA BCAR4 in the regulation of bladder cancer remains unclear.

Purpose: This study was to explore the role of lncRNA BCAR4 in the progression of bladder cancer cell.

Methods: RT-PCR was used to examine the expression of BCAR4 and miR-644a. CCK8 assay, colony formation assay, Transwell assay were used to detect the progression of bladder cancer cells after transfecting of indicated plasmids.

Results: The expression of BCAR4 was higher in bladder cancer cell lines than normal urothelial cell line. Moreover, the expression of BCAR4 was associated with the advanced stage and metastasis of bladder cancer. Through knockdown of BCAR4, we discovered that knockdown of BCAR4 significantly decreased the proliferation, migration and invasive abilities of bladder cancer cells. Mechanically, we showed that BCAR4 can bind to miR-644a directly and targets TLX1. Moreover, we also showed that miR-644a was also highly expressed in bladder cancer cells and inhibition of miR-644a or overexpression of TLX1 can increase the migration abilities of bladder cancer caused by knockdown of BCAR4.

Conclusion: We showed that BCAR4 sponged miR-644a to modulate the expression of TLX1 and promote bladder cancer development.

Keywords: BCAR4, miR-644a, TLX1, bladder cancer, proliferation and migration

Introduction

Bladder cancer is a malignant tumor that occurs in the bladder mucosa.¹ It is the most common malignant tumor in the urinary system and one of the ten most common tumors in the whole body.² Bladder cancer can occur at any age, even in children.³ The incidence increased with age, with a high incidence of 50 to 70 years old. The incidence of bladder cancer in males is 3 to 4 times that in females.⁴ Bladder cancer seriously affects people's normal life, not only destroys the level of metabolism, but also does great harm to the urinary system.^{5,6} Therefore, it is very important to study the pathogenesis of bladder cancer.⁷

Long non-coding RNAs (lncRNAs) play important roles in cancer.⁸ Genome association studies have found that a large number of lncRNAs are associated with various types of cancer.^{9,10} lncRNA may inhibit or promote the proliferation and metastasis of tumors. Because of the diversity and specificity of genomic expression

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of lncRNAs, lncRNAs have been identified as new tumor molecular markers and therapeutic targets.¹⁰ There are many reports that discovered lncRNAs participate in the regulation of bladder cancer.¹¹ BCAR4 was a long non-coding RNA that was identified to regulate many cancers progression.^{12–14} Wei et al reported that BCAR4 can promote the proliferation of glioma cells through EGFR/PI3K/AKT signaling.¹⁵ Moreover, Siddique H found that BCAR4 can act as a prognostic marker for colorectal cancer.¹⁶ However, the role of BCAR4 in the regulation of bladder cancer remains unclear.

MicroRNAs, referred to as miRNAs, are a class of non-coding single stranded RNA molecules with about 22 nucleotides (nt), which are cut by Dicerase from a 70nt pre-microRNAs (pre-microRNAs).^{17,18} MicroRNAs are involved in a series of important processes in life, including development, hematopoiesis, organogenesis, apoptosis and cell proliferation, and even cancer.^{19,20} For example, miR-644a was proved to regulate the progression of gastric cancer, hepatocellular carcinoma and breast cancer.^{21,22} Studies have found some microRNAs closely related to bladder cancer.^{23,24} However, the role of miR-644a in the regulation of bladder cancer remain unknown and it is not clear what specific mechanisms that these microRNAs promote or inhibit the occurrence of bladder cancer.

TLX1, also known as HOX11 was proved to be an important transcription factor involved in the regulation of embryonic spleen organogenesis.²⁵ Moreover, TLX1 was also proved to regulate the progression of many cancer and Vanden Bempt et al reported that TLX1 was participated in the regulation of T Cell Acute Lymphoblastic Leukemia.^{26,27} However, the role of TLX1 in the regulation of bladder remains elusive.

Materials and Methods

Samples

Human bladder cancer samples and adjacent healthy bladder tissues were obtained from 38 bladder cancer patients under surgery at Ruijin Hospital. All samples were kept in liquid nitrogen before use. This study was approved by the Ethics Committee of Taiyuan Second People's Hospital. All written informed consents were received from patients.

Cell Lines

Bladder cancer cell lines: UMUC3, SW780, 5637, T24 and one normal urothelial cell line: SVHUC-1 cells were

obtained from the Tumor Cell Bank of the Chinese Academy of Medical Science (Shanghai, China). The UMUC3, T24 and SVHUC-1 cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) plus 10% FBS and ampicillin and streptomycin at 37 °C and 5% CO₂. The 5637 cells and SW780 cells were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA) plus 10% FBS and ampicillin and streptomycin at 37 °C and 5% CO₂.

CCK8 Assay

CCK8 assays were performed using CCK8 detection kit (7 sea biotech, Shanghai, China) according to the instruction of the Kit.

Cell Transfection

Small interfering RNA oligonucleotides (siRNA, 5'-AUA AAAUGCCACACAACCA-3') that targeting BCAR4 and a negative control were purchased from GenePharma (Shanghai, China). miR-644a mimics (5'-AGUGUGGCU UUCUUAGAGC-3'), miR-control (5'-AUCUGCGUAAG AUUCGAGUC-3'), miR-644a inhibitors (5'- GCTCTUU GUUUGCCUCUCT-3') and control inhibitors (5'UAA CUAUACAUCGGAUU-3') were purchased from GenePharma (Shanghai, China). Cells were transfected with 100 nM miR-644a mimics, miR-control, miR-644a inhibitors or control inhibitors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Preparation and RT-PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, total RNA was subsequently reverse-transcribed into cDNAs using the PrimeScript RT reagent Kit (Promega, Madison, WI, USA) according to the protocol of the manufacturer. RT-PCR was performed using SYBR Green PCR Master Mix reagents (Takara) in the 7300 Real-Time PCR System (Applied Biosystems). The primers were listed as below:

U6 forward, 5'-CGCTTCGGCAGCATATA C-3'; U6 reverse, 5'-TTCACGAATTTGCGTGTCAT-3'; GAPDH forward, 5'-CATGAGAAGTATGACAACAGCC-3'; GAPD H reverse, 5'-AGTCCTTCCACGATAACCAAAGT-3'; TL X1 forward, 5'- GACCGGCTCCTACAACGTG-3'; TLX1 reverse, 5'- CTGCGGTTACTCTCCATCCAG-3'; BCAR4 forward, 5'- gaactgctatatgcatttgc-3'; BCAR4 reverse, 5'-

acgaattaggctcttgattg-3'; 18S forward, 5' -cagccaccgagatt gagca-3'; 18S reverse, 5'-tagtagcgacggcggtgtg-3'.

Transwell Migration and Invasion Assay

Transwell assay was performed as previously described.²⁸ Briefly, migration or invasion chamber was coated with Matrigel (BD Biosciences, Cowley, United Kingdom). Cells were then seeded into the upper chamber with serum-free DMEM medium and the lower chamber was supplemented with DMEM containing 10% FBS. After 24h incubation, cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min.

Luciferase Assay

pMIR-TLX1-3'UTR (WT or Mutant) or pMIR-BCAR4 (WT or Mutant) and miR-644a (mimics or inhibitor) were transfected into T24 or SW780 cells along with pRL-TK vectors (Promega, USA). After 24 hrs culturing, luciferase activities were measured using a dual Glo™ Luciferase Assay System (Promega) according to the manufacturer's protocols.

Statistical Analysis

A Student's *t*-test was used to analyze the differences between two groups. A one-way ANOVA followed by a Tukey's post-hoc test was used for multiple comparisons. The overall survival was analyzed by Kaplan-Meier analysis and a Log rank test. GraphPad Prism 6 software was used to analyze all results. The results were expressed as mean ± SD. *P* < 0.05 was considered to be significant.

Results

LncRNA BCAR4 Is Associated with Bladder Cancer Development

LncRNA BCAR4 was proved to participate in the regulation of many cancers. However, the function of BCAR4 in the regulation of bladder cancer progression remains unknown. To explore the role of BCAR4 in bladder cancer, we firstly examined the expression of BCAR4 in bladder cancer cell lines and normal bladder cells. Through RT-PCR assay, we found that BCAR4 was highly expressed in bladder cancer cell lines (Figure 1A). Moreover, we also

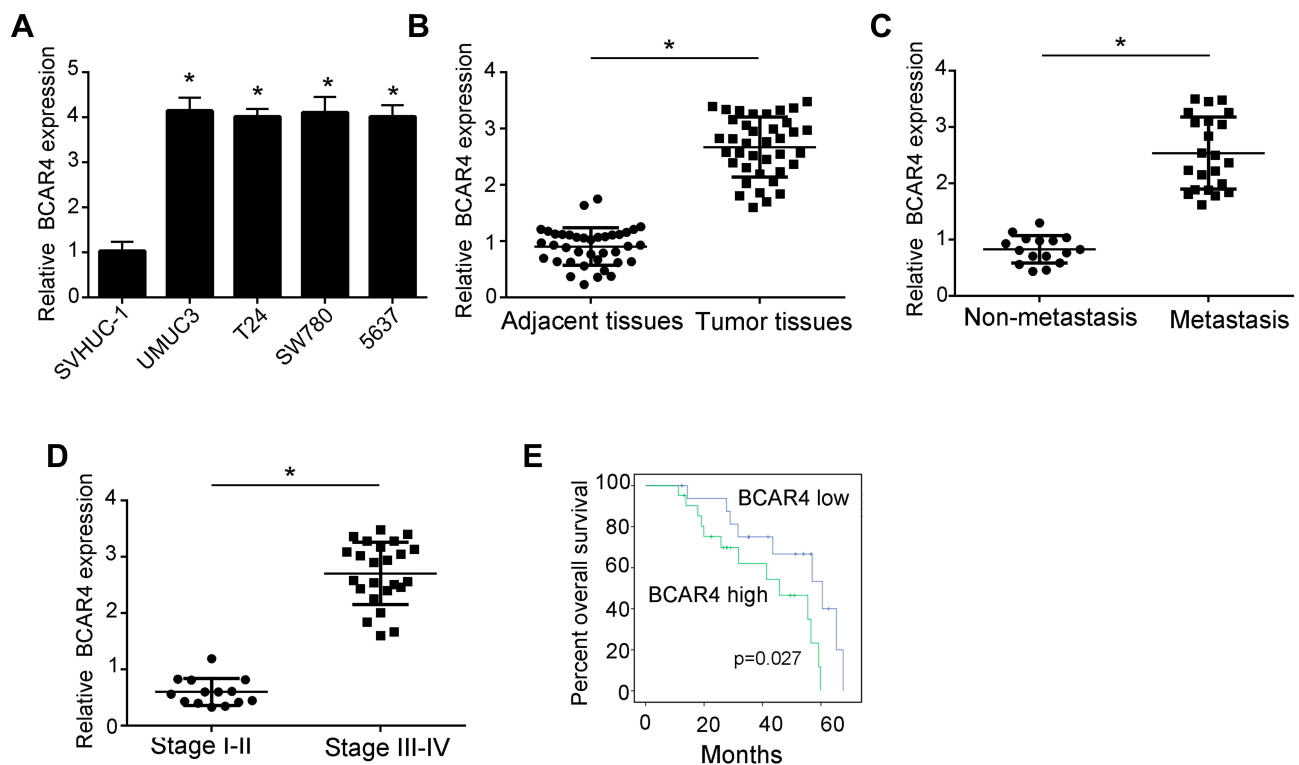


Figure 1 The expression of BCAR4 was elevated in bladder cancer cells and associated with bladder cancer progression. **(A)** Relative expression of BCAR4 in bladder cancer cell lines or normal urothelial cell line was examined by RT-PCR. Bladder cancer cell lines: UMUC3, T24, SW780, 5637. Normal urothelial cell line: SVHUC-1. Fold change was normalized to 18S. **(B)** Relative expression of BCAR4 was examined using RT-PCR in bladder cancer samples and normal bladder tissues. *n*=38. Fold change was normalized to 18S. **(C)** Relative expression of BCAR4 was examined using RT-PCR in non-metastasis bladder cancer samples (*n*=16) and metastasis bladder cancer samples (*n*=22). Fold change was normalized to 18S. **(D)** Relative expression of BCAR4 was examined using RT-PCR in III-IV stage bladder cancer samples (*n*=24) and I-II stage bladder cancer samples (*n*=14). Fold change was normalized to 18S. **(E)** Kaplan-Meier analysis were performed to analyze the overall survival of bladder cancer patients with different BCAR4 expression. Higher BCAR4 level (*n*=19) and lower BCAR4 level (*n*=19) (*P*=0.027). **P*<0.05. All experiments were repeated three times independently.

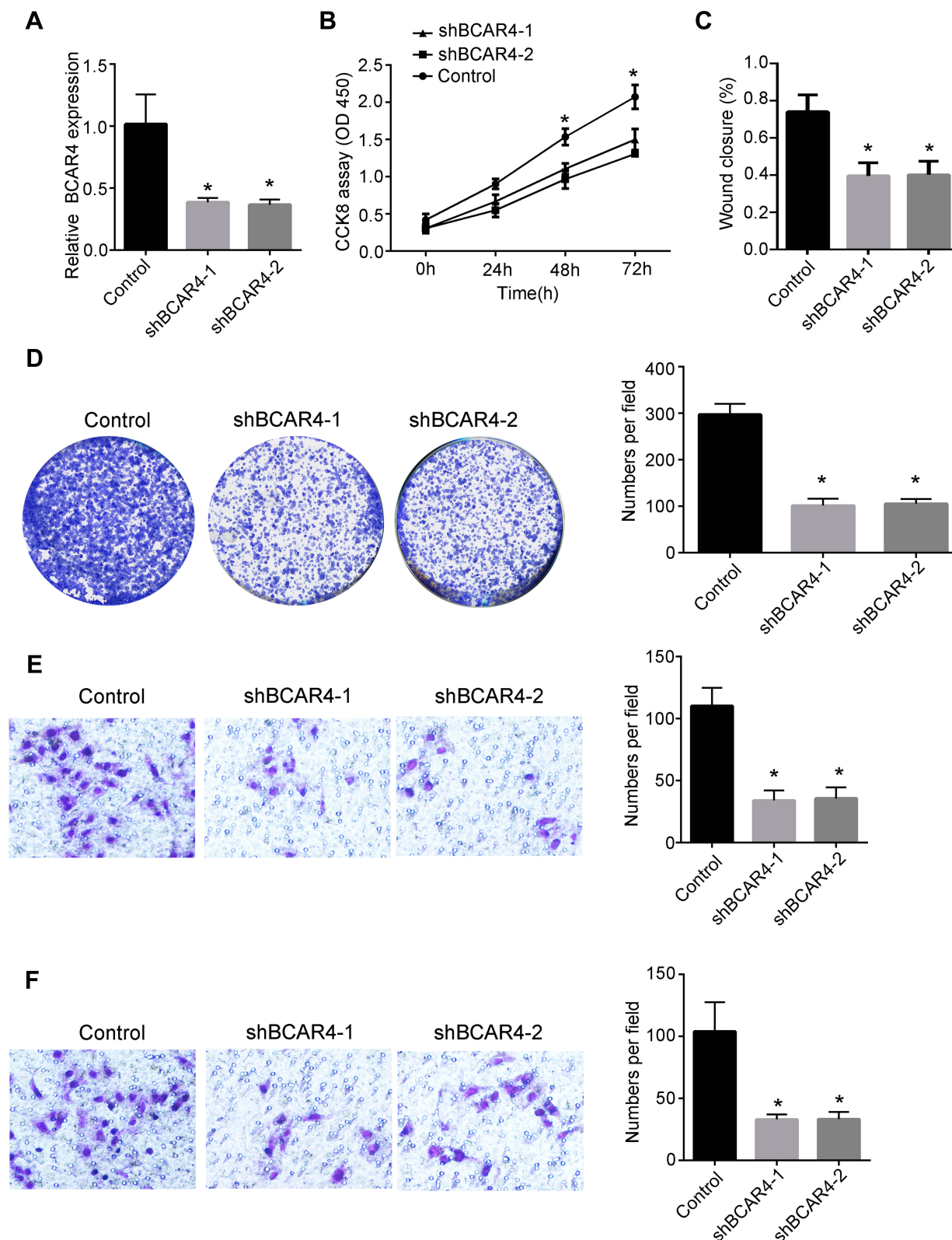


Figure 2 Knockdown of BCAR4 inhibit the progression of bladder cancer cells. **(A)** Relative expression of BCAR4 was examined using RT-PCR assay after transfecting negative control plasmid or shBCAR4 plasmids. Fold change was normalized to 18S. **(B)** CCK8 assay was performed to examine the proliferation abilities of bladder cancer cells after knockdown of BCAR4. **(C)** Wound healing assay was performed to detect the migration abilities of bladder cancer cells after knockdown of BCAR4. **(D)** Colony formation assay was performed to detect the proliferation abilities of bladder cancer cells after knockdown of BCAR4. **(E)** Transwell migration assay was used to detect the migration abilities of bladder cancer cells after transfecting negative control plasmid or shBCAR4 plasmids. **(F)** Transwell invasion assay was used to detect the invasive abilities of bladder cancer cells after transfecting negative control plasmid or shBCAR4 plasmids. * $P < 0.05$. All experiments were repeated three times independently.

collected 38 bladder cancer samples and found that the expression of BCAR4 in bladder cancer samples was higher than paired healthy bladder tissues (Figure 1B). Through RT-PCR analysis, we discovered that the expression of BCAR4 was associated with bladder cancer metastasis and advanced stages (Figure 1C and D). To explore the overall survival rate of bladder cancer patients with different BCAR4 expression, we performed Ker-Mein analysis. We divided the 38 bladder cancer patients into 2 groups based on the median expression of BCAR4. We found that lower BCAR4 expression group possessed better overall survival while higher BCAR4 expression group possessed worse overall survival (Figure 1E). Collectively, we showed that the expression of BCAR4 was associated with bladder cancer development.

Knockdown of BCAR4 Decreased Bladder Cancer Progression

To find the function of BCAR4 in the regulation of bladder cancer progression, we performed a series of functional assays. Firstly, we constructed two shRNAs specifically

targeted to BCAR4 sequence (Figure 2A). Then, we performed CCK8 assays and found that the cell viability of bladder cells were significantly decreased after knockdown of BCAR4 (Figure 2B). Moreover, colony formation assay also proved that BCAR4 knockdown decreased the proliferation of bladder cancer cells (Figure 2D). Finally, we performed transwell assays and wound healing assays to examine the migration and invasive abilities of bladder cancer cells. We found that the knockdown of BCAR4 remarkably inhibited the migration and invasive abilities of bladder cancer cells (Figure 2C, E and F). Taken together, these data showed that BCAR4 promoted the proliferation, migration and invasive of bladder cancer cells.

BCAR4 Binds to miR-644a Directly

To find the molecular mechanism that BCAR4 regulated bladder cancer development, we performed bioinformatics analysis. We found that BCAR4 can form complementary base pairing with miR-644a directly (Figure 3A). Besides, we also discovered that miR-644a was highly expressed in bladder cancer cell lines compared to normal bladder cell line

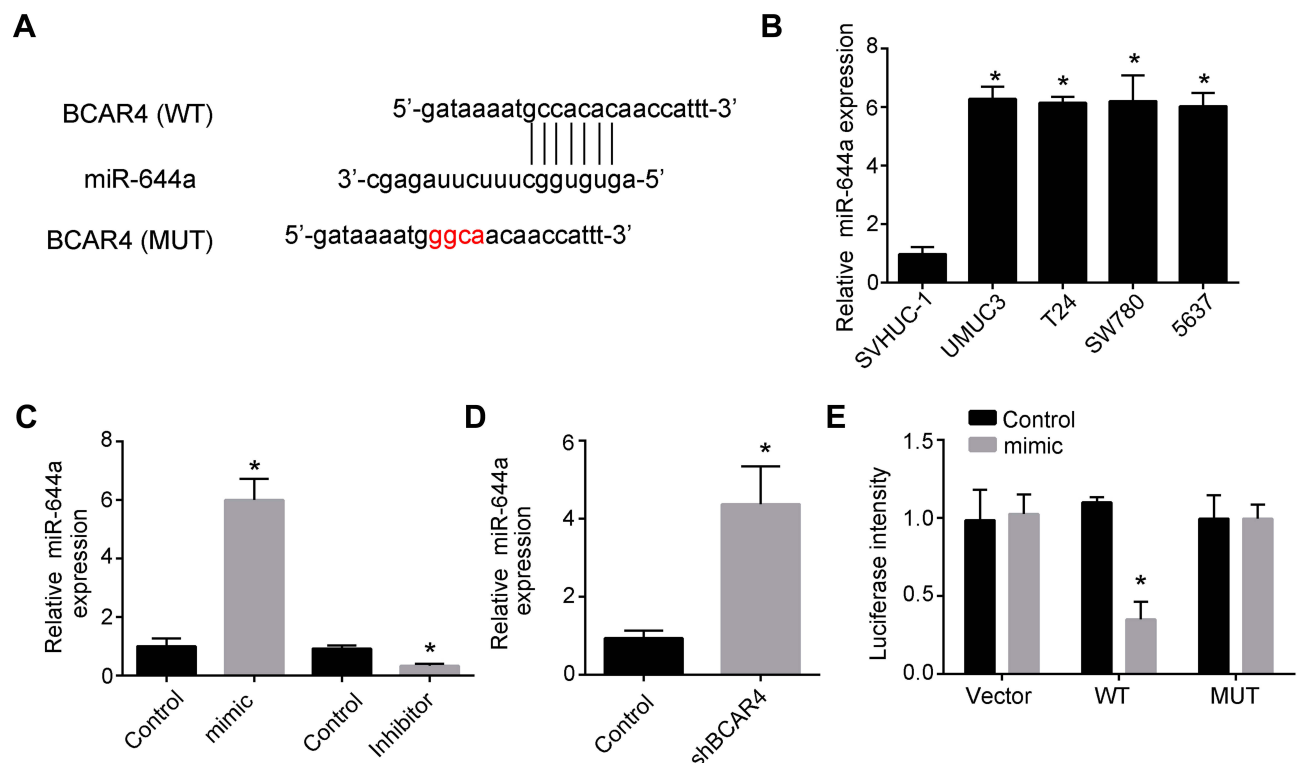


Figure 3 BCAR4 binds to miR-644a directly and regulate the expression of miR-644a. **(A)** Binding sites between BCAR4 and miR-644a. **(B)** Relative expression of miR-644a in bladder cancer cell lines or normal urothelial cell line was examined using RT-PCR assay. Bladder cancer cell lines: UMUC3, T24, SW780, 5637. Normal urothelial cell line: SVHUC-1. Fold change was normalized to 18S. **(C)** Relative expression of miR-644a was examined using RT-PCR assay after transfecting indicated plasmids. Fold change was normalized to 18S. **(D)** Relative expression of miR-644a was examined using RT-PCR assay after transfecting negative control plasmid or shBCAR4 plasmid. Fold change was normalized to 18S. **(E)** Luciferase assay was performed to detect the luciferase intensity after transfecting indicated plasmids. *P<0.05. All experiments were repeated three times independently.

(Figure 3B). To illustrate the molecular regulation between BCAR4 and miR-644a, we constructed miR-644a overexpression plasmid and miR-644a inhibitor (Figure 3C). We found that the expression of miR-644 was significantly elevated after knockdown of BCAR4 (Figure 3D). Moreover, we also performed luciferase assay. We discovered that BCAR4 can bind to miR-644a directly and overexpression of miR-644a significantly decreased the expression of BCAR4 (Figure 3E). Besides, miR-644a cannot regulate the expression of BCAR4 mutant (Figure 3E). Collectively, we showed that BCAR4 can bind to miR-644a directly and regulate the expression of miR-644a.

BCAR4 Promotes the Expression of TLX1 by Regulating the Expression of miR-644a

To find the potential gene that regulated by BCAR4 and miR-644a, we performed bioinformatics analysis again using TargetScan7 software. We showed that TLX1 was a potential target that can bind to miR-644a directly (Figure 4A). We found that the expression of TLX1 was elevated after inhibiting miR-644a expression while the expression of TLX1 was decreased after overexpression of miR-644a (Figure 4B). Through luciferase assay, we showed that miR-644a binds to WT TLX1 other than MUT TLX1 (Figure 4C). Besides, we also found that

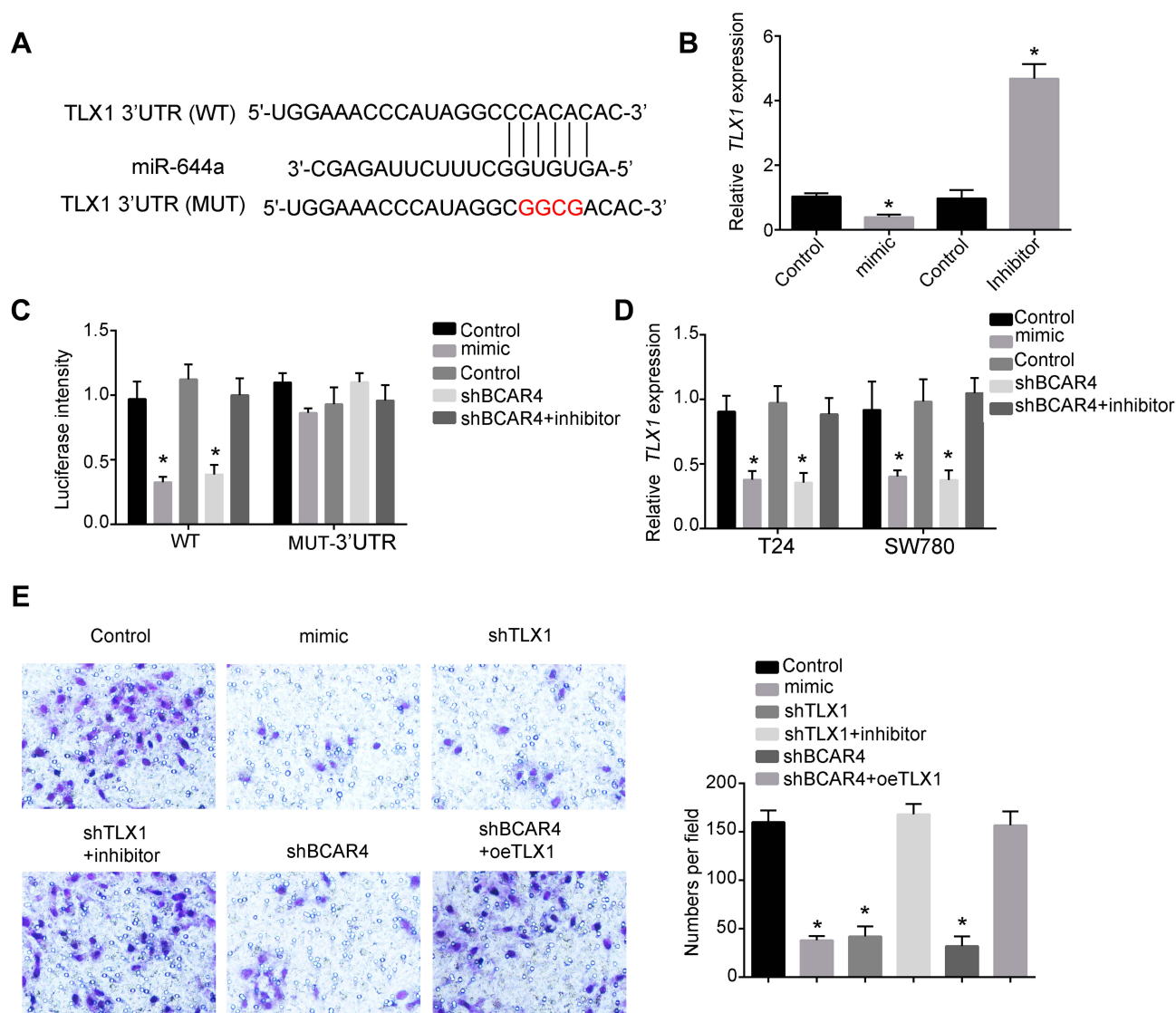


Figure 4 TLX1 was targeted by BCAR4 and miR-644a. (A) Binding sites between miR-644a and TLX1. (B) Relative expression of TLX1 was examined using RT-PCR assay after transfecting indicated plasmids. Fold change was normalized to 18S. (C) Luciferase assay was performed to detect the luciferase intensity after transfecting indicated plasmids. (D) Relative expression of TLX1 was examined using RT-PCR assay after transfecting indicated plasmids in T24 and SW780 cells. Fold change was normalized to 18S. (E) Transwell migration assay was used to detect the migration abilities of bladder cancer cells after transfecting indicated plasmids. *P<0.05. All experiments were repeated three times independently.

overexpression of miR-644a or knockdown of BCAR4 significantly decreased TLX1 expression while inhibition of miR-644a can rescue the decreased BCAR4 expression caused by knockdown of BCAR4 (Figure 4D). Consistently, the migration abilities of bladder cancer cells were decreased after knockdown of BCAR4, TLX1 or overexpression of miR-644a. And also, inhibition of miR-644a coupled with knockdown of TLX1 or overexpression of miR-644a coupled with knockdown of BCAR4 can rescue the numbers of migration bladder cancer cells (Figure 4E). Collectively, we showed that TLX1 was targeted by BCAR4 and miR-644a to regulate the migration of bladder cancer.

Discussion

In this study, we showed that the expression of BCAR4 was much higher in bladder cancer cell lines and bladder cancer samples. Knockdown of BCAR4 significantly decreased the proliferation, migration and invasion abilities of bladder cancer cells. Moreover, we discovered that BCAR4 can bind to miR-644a directly and regulate the expression of TLX1. The expression of miR-644a was also proved to be higher in bladder cancer cell lines. Besides, we showed that knockdown of BCAR4 can increase the expression of miR-644a and inhibition of miR-644a can increase the expression of TLX1. So, we found that overexpression of TLX1 significantly promote the expression of TLX1. Finally, through transwell migration assays, we showed that overexpression of TLX1 or inhibition of miR-644a increased the migration abilities of bladder cancer cells decreased by knockdown of BCAR4, TLX1 or overexpression of miR-644a.

LncRNAs were shown to play vital functions in the regulation of bladder cancer development.⁹ Pan J and colleagues found that lncRNA UCA1 can promotes the cisplatin or gemcitabine resistance in bladder cancer through modulating miR-196a-5p.²⁹ Ye T and colleagues showed that linc00346 is upregulated in bladder cancer tissues and knockdown of linc00346 inhibits bladder cancer progression.³⁰ Though many lncRNAs were proved to participate in the regulation of bladder cancer development.^{31,32} However, the function of BCAR4 in the regulation of bladder cancer have not been characterized.

TLX1 is a transcription factor that controls the cell fate specification and organ expansion during spleen development.³³ Tlx1 deletion in mice causes asplenia.³⁴ Moreover, many studies have showed that TLX1 was associated with T-cell acute lymphoblastic leukemia.^{35,36}

However, the function of TLX1 in the regulation of bladder cancer remains unknown. In our study, we firstly showed that BCAR4 and miR-644a can regulate the expression of TLX1 and the expression of TLX1 was associated with bladder cancer progression.

Disclosure

The authors report no conflicts of interest in this work.

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