miR-130b promotes bladder cancer cell proliferation, migration and invasion by targeting VGLL4

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Abstract. Bladder cancer (BCa) is the most common urological cancer, and more and more evidence suggests that microRNAs (miRNAs) play an important role in BCa pathogenesis. Aberrant miR-130b expression has been reported in several types of cancers. The aim of the present study was to elucidate the effects of miR-130b on BCa progression. miR-130b expression in BCa cell lines and tissues was detected using real-time PCR (RT-PCR), and vestigial-like protein 4 (VGLL4) expression in tissue specimens and BCa cells that had been transfected with miR-130b mimics and inhibitors was detected using western blotting. Dual-luciferase reporter assay was performed to confirm whether the VGLL4 gene is a direct target of miR-130b, and in vitro cell function testing, Cell Counting Kit-8 (CCK-8) assay, colony formation assay, wound healing and Transwell assays were performed to examine BCa cell proliferation, migration and invasion ability after the cells were transfected with miR-130b mimics and inhibitors and VGLL4 siRNA. miR-130b was found to be upregulated in BCa cells and tissues. miR-130b overexpression promoted BCa cell proliferation, migration and invasion, whereas miR-130b inhibition had the opposite effects. Dual-luciferase reporter assay confirmed that the VGLL4 gene was a direct target of miR-130b and that VGLL4 suppression was crucial for miR-130b-induced BCa cell proliferation, migration and invasion. The present study showed that miR-130b was significantly upregulated in BCa and may play an oncogenic role in BCa occurrence and development by targeting VGLL4. miR-130b may be a potential therapeutic target in the treatment of BCa.

Introduction

Bladder cancer (BCa) is the most common malignant tumor in the urinary system, and more than 300,000 new cases of the disease are diagnosed each year worldwide (1,2). BCa can be divided into the following two major types: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Patients with NMIBC have a good prognosis, and the 5-year survival rate for such patients is over 88% (3); however, ~70% of affected patients will experience disease recurrence at the same position or another bladder site (4). The 5-year survival rate for patients with MIBC is only ~60% (3,5). Hence, studies investigating the potential mechanisms underlying BCa occurrence and development are urgently needed. Recently, the results of an increasing number of studies have suggested that microRNAs (miRNAs) play an important role in BCa pathogenesis, and may thus provide clinicians with new therapeutic targets and strategies for treating the disease (6-9).

miRNAs are a class of conserved small non-coding RNAs that exert their effects by binding to the 3' untranslated regions (3'UTRs) of their target messenger RNAs (mRNAs), leading to mRNA degradation or translational inhibition and, ultimately, gene expression regulation (10). The results of previous studies indicate that miRNAs play important roles in cancer occurrence and development as oncogenes or tumor-suppressor genes (11). miRNAs can affect cell proliferation, apoptosis, invasion, metastasis and epithelial-mesenchymal transition (EMT) (12-14). miR-130b, which is located at the 22q11 locus (15), has been shown to be aberrantly expressed in some cancers, as it is overexpressed in renal cell (16) and hepatocellular carcinoma (17), and glioma (18), but is downregulated in papillary thyroid (19) and endometrial carcinoma (20), and prostate cancer (21).

In a preliminary experiment, we found that miR-130b was upregulated in BCa cells and tissues and that ectopic miR-130b expression was closely related to BCa cell proliferation, migration and invasion, indicating that miR-130b may function in BCa progression.

Using bioinformatic analysis software, we identified vestigial-like protein 4 (VGLL4) as the downstream target gene of miR-130b. VGLL proteins are newly emerging TEAD-interacting partners and transcriptional cofactors that participate in tumorigenesis. Unlike other members of the VGLL family, VGLL4 contains an extra TDU domain, which
is thought to be functionally distinct from the other functional domains of the protein, and has been identified as a transcriptional inhibitor that inhibits YAP-induced tumor growth and development in Drosophila and humans (22). The role of VGLL4 in gastric, pancreatic and lung cancer, and esophageal squamous cell carcinoma has recently attracted more attention. VGLL4 is considered a new tumor-suppressor gene (23-28).

This study showed that the VGLL4 gene was a direct target of miR-130b and that VGLL4 downregulation mediated by miR-130b played a critical role in BCa cell proliferation, migration and invasion.

Materials and methods

Cell cultures and clinical tissue specimens. The indicated human BCa cell lines (T24, 5637 and J82) and immortalized human bladder urothelial cell line (SV-HUC-1) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in HyClone™ RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Life Sciences, HyClone Laboratories, Logan, UT, USA). All cells were cultured in a humidified incubator at 37°C with 5% CO₂ and 95% air. From January 2016 to September 2016, thirty pairs of clinical tissue specimens (bladder tumor tissue specimens and adjacent non-tumor bladder tissue specimens), including 19 men and 11 women with a median age of 63 years, were obtained from patients undergoing transurethral bladder tumor resection (17 cases) or radical cystectomy (13 cases) at the Department of Urology, The First Hospital of China Medical University. All these cases were staged by the 2002 UICC TNM classification. Of these 30 cases of BCa, 17 cases were NMIBC (≥pT1), and the remaining cases were MIBC (≥pT1). The cases were also classified according to the WHO 2004 for grade, including 19-low grade papillary urothelial carcinoma, and 11-high grade papillary urothelial carcinoma. No patients had received radiotherapy, chemotherapy or adjuvant therapy before surgery. All specimens were obtained after each patient provided written informed consent to participate in this study, which was approved by the Research Ethics Committee of The First Hospital of China Medical University. Some specimens were fixed in formalin, while others were immediately placed in liquid nitrogen and stored at -80°C for further analysis.

RNA extraction and real-time PCR (RT-PCR). To detect VGLL4 mRNA, we extracted total RNA from cultured cells and frozen tissues using Invitrogen™ TRIzol™ reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. cDNA was synthesized with a PrimeScript™ RT reagent kit (Takara, according to the manufacturer's instructions). cDNA was synthesized using a miRCURY LNA™ Universal cDNA Synthesis kit II (Exiqon A/S, Vedbaek, Denmark), and RT-PCR was performed with miRCURY LNA™ ExiLENT SYBR®-Green Master Mix (Exiqon A/S), according to the manufacturer's instructions. miR-130b-3p and VGLL4 expression levels were normalized to U6 and β-actin expression levels, respectively. Relative expression levels were calculated using the 2-ΔΔCt method. The primers for VGLL4 were: 5’-GCTGTTTTCTTGTGCTAGCC-3’ (forward) and 5’-CACGGCAGGGTCTGTATTC-3’ (reverse), and the primers for β-actin were: 5’-TCACCATGGATGATGATATCGC-3’ (forward) and 5’-AGGAATCTTTCTGACCATGC-3’ (reverse). The hsa-miR-130b-3p and U6 LNA™ PCR primer sets were provided by Exiqon.

Transient transfection. T24 and 5637 cells were transfected with hsa-miR-130b-3p mimics, negative-control mimics, hsa-miR-130b-3p inhibitors, negative-control inhibitors and VGLL4 siRNA (GenePharma, Shanghai, China) using Invitrogen™ LipoFectamine™ 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The VGLL4 siRNA sense sequence was as follows: 5’-UCUGAACAGAACUGCAGA-3’; and the VGLL4 siRNA antisense sequence was as follows: 5’-AUUGGCAUGUCUUGUUCAGA-3’. The expression levels of all transfected genes were confirmed with RT-PCR or western blotting.

Luciferase assays. A total of 5637 cells were seeded in 24-well plates and incubated for 24 h before transfection. miR-130b NC/pmirGLO-VGLL4-3’UTR-WT, miR-130b mimic/pmirGLO-VGLL4-3’UTR-WT, miR-130b NC/pmirGLO-VGLL4-3’UTR-MUT and miR-130b mimic/pmirGLO-VGLL4-3’UTR-MUT (synthesized by GenePharma, Shanghai, China) were transiently co-transfected into the cells using Lipofectamine 2000. Cell lysates were gathered 48 h after transfection and assessed using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. All experiments were performed in triplicate.

Cell proliferation assay. Cell proliferation assay was conducted with the Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan). The cells were seeded in 96-well plates (3x10³ cells/well) and incubated for 24 h before transfection. Ten microliters of CCK-8 was added to every well at 1, 2, 3, 4 and 5 days after transfection. The absorbance was measured at 450 nm using a microplate reader. All experiments were performed in triplicate.

Colony formation assay. The cells (5x10² cells/well) were seeded in 6-well plates in complete growth media and incubated for 2 weeks. The cell colonies were then fixed with methanol and stained with 0.1% crystal violet before being imaged and counted (defined as ≥50 cells/colony) using an AID iSpot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). The experiment was performed three times for each cell line.

Wound healing assay. The cells were seeded in 6-well plates (5x10³ cells/well) and incubated until they reached 100% confluence. Then, the cells were wounded by a 200-µl pipette tip, and a cross-shape was generated in the monolayer. The cells were subsequently washed with PBS for cellular debris removal and cultured for 24 h. Then, the widths of wounds...
were measured under a light microscope (Olympus Corp., Tokyo, Japan). The widths between the cells which migrated the furthest on each side were measured to calculate the relative wound closure area. The experiment was performed three times for each cell line.

Invasion assay. Cell invasion assay was performed with Corning Transwell insert chambers (Corning Incorporated, New York, NY, USA) with an 8.0-µm pore size. Matrigel (Corning Incorporated) was used to coat the top chamber. A total of 2×10^4 cells were suspended in 200 µl of serum-free medium and seeded in the upper chamber, and RPMI-1640 containing 10% FBS, which was used as a chemoattractant, was added to the lower chamber. After incubating for 24 h, the cells in the upper chamber were removed with cotton swabs, and the cells that had passed through the membrane to invade the lower chamber were fixed with methanol and stained with 0.1% crystal violet before being counted under a light microscope. All experiments were performed in triplicate.

Western blot analysis. The transfected cells and frozen tissues (including the tumor and adjacent non-tumorous tissues) were lysed, and the extracted proteins were quantified by a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal quantities of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% non-fat milk at room temperature for 2 h, and then incubated with the appropriate primary antibody at 4°C overnight. Following 3 washes in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were incubated with the appropriate secondary antibody for 2 h at 37°C. Specific band signals were detected using a chemiluminescence system (Bio-Rad, Philadelphia, PA, USA) and analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA) in accordance with the manufacturer’s protocol. The primary antibody rabbit polyclonal anti-VGLL4 (1:1,000; cat. no. V2890; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and a secondary antibody to mouse IgG (1:5,000; cat. no. ab193651; Abcam, Cambridge, MA, USA) or rabbit IgG (1:5,000; cat. no. ab191866; Abcam) were used for the experiments. The protein levels were normalized to those of GAPDH (1:1,000; cat. no. ab8245; Abcam).

Statistical analysis. All data were processed with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and presented as the mean ± standard deviation (SD) of three independent experiments. Differences were evaluated and analyzed with the Student’s t-test. P-values <0.05 were considered statistically significant.

Results

miR-130b expression is upregulated in BCa. RT-PCR was used to detect miR-130b expression levels in BCa cell lines and tissues. miR-130b was found to be significantly elevated in the indicated BCa cell lines (T24, J82 and 5637) compared with the immortalized human bladder epithelial cell line (SV-HUC-1) (Fig. 1A). We also examined miR-130b expression levels in 30 BCa clinical tissue samples and matched adjacent non-tumor tissue samples. The results of the analysis showed that miR-130b expression levels in BCa tissues were significantly higher than those in matched adjacent non-tumor tissues (Fig. 1B). In addition, miR-130b expression levels in the MIBC tissues were significantly higher than those in the NMIBC tissues (Fig. 1C). These results indicated that miR-130b expression levels were upregulated in BCa, suggesting that miR-130b may play a role in promoting tumor progression in BCa.

miR-130b promotes cancer cell proliferation, migration and invasion. To further investigate the potential role of miR-130b in the promotion of tumor progression in BCa, we transfected miR-130b-3p mimics and miR-130b-3p mimic controls (as control) into the T24 and 5637 cells and confirmed miR-130b-3p overexpression by RT-PCR (data not shown). We used CCK-8 and colony formation assays to assess the effects of miR-130b on cell proliferation. The results of the CCK-8 assay indicated that miR-130b overexpression significantly increased BCa cell growth rates compared with the negative control (NC) cell growth rates (Fig. 2A). The results of the colony formation assay showed that BCa cells overexpressing miR-130b formed a higher number of colonies than the control NC cells (Fig. 2B). Additionally, the results of the wound healing assay showed that the migration ability of BCa cells overexpressing miR-130b was significantly higher than that
of the control NC cells (Fig. 2C). Furthermore, the results of the Transwell assay indicated that miR-130b overexpression caused significant increases in the invasive capabilities of BCa cells compared with that of the control NC cells (Fig. 2D). All of these results indicated that miR-130b overexpression promoted BCa cell proliferation, migration and invasion.

**miR-130b inhibition suppresses BCa proliferation, migration and invasion.** To confirm the role of miR-130b in BCa proliferation, migration and invasion, we performed loss-of-function studies by transfecting miR-130b-3p inhibitors and miR-130b-3p inhibitor controls [as control (INC)] into T24 and 5637 cells. The results of the CCK-8 and colony formation assays showed that miR-130b suppression significantly decreased the proliferative capacity of the BCa cells compared with that of the INC cells (Fig. 3A and B). Additionally, the results of the wound healing and Transwell assays indicated that miR-130b inhibition caused a significant decrease in the migratory and invasive capabilities of the BCa cells compared to those of the INC cells (Fig. 3C and D). These results indicated that miR-130b knockdown inhibited BCa cell proliferation, migration and invasion.

**VGLL4 is a direct target gene of miR-130b in BCa cells.** To further explore the molecular mechanisms underlying the effects of miR-130b in BCa, we used bioinformatic analysis software (TargetScan, miRBase) to predict the target genes of miR-130b. VGLL4 was identified as one of the potential target genes of miR-130b in BCa cells (Fig. 4F). Regarding the clinical specimens, VGLL4 protein expression levels in BCa tissues were significantly decreased compared with those in the adjacent non-tumor bladder tissues, and VGLL4 protein expression levels were lower in MIBC tissues than in NMIBC tissues (Fig. 4C). In BCa cells, VGLL4 protein expression levels were decreased in the miR-130b-overexpressing cells, while VGLL4 protein expression levels were significantly increased in the miR-130b-silenced cells (Fig. 4A and B). However, there was no significant difference in VGLL4 mRNA expression levels between the two cell types (data not shown). miR-130b expression levels were negatively correlated
with those of VGLL4 in the BCa tissue specimens (r = -0.7757, P < 0.0001) (Fig. 4D). To determine whether VGLL4 is regulated by the direct binding of miR-130b to its 3'UTR, we performed luciferase assays. PmirGLO-VGLL4-3'UTR-WT and miR-130b mimics were co-transfected into 5637 cells and caused a significant decrease in luciferase activity in those cells compared with control cells. In contrast, PmirGLO-VGLL4-3'UTR-MUT and miR-130b mimics were co-transfected into 5637 cells and caused no significant changes in luciferase activity in the treated group compared with the NC group (Fig. 4E). These results suggested that the VGLL4 gene was a direct target of miR-130b in BCa cells.

VGLL4 suppression is crucial for miR-130b-induced BCa cell proliferation, migration and invasion. To determine whether miR-130b-mediated BCa cell proliferation, migration and invasion are attributable to VGLL4, we transfected a miR-130b inhibitor/VGLL4-siRNA and miR-130b inhibitor/control vector into T24 and 5637 cells (Fig. 5A). The results of the above-mentioned assays showed that VGLL4 inhibition resulted in significantly increased BCa cell proliferation (Fig. 5B and C), migration (Fig. 5D) and invasion (Fig. 5E) ability in the cells transfected with the miR-130b inhibitor. These results showed that the inhibitory effects of the miR-130b inhibitor on BCa cell proliferation, migration and invasion were significantly reversed by VGLL4 suppression. Hence, we confirmed that miR-130b-induced cell proliferation, migration and invasion were facilitated by VGLL4 suppression.

Discussion

More and more studies have shown that aberrant miRNA expression plays an oncogenic or tumor suppressive role. The oncogenic effects are exerted by tumor suppressor gene repression, and the tumor suppressive effects are exerted by oncogene inhibition (29,30). The important roles of miRNAs in cancer occurrence and development have also been experimentally demonstrated in animal models (31). Bladder cancer (BCa) is the most common malignant tumor in the urinary system, hence, increasing numbers of studies have focused on BCa biological characteristics, occurrence and development and have elucidated the roles of miRNAs in BCa. For example, a study reported that miR-200c functions as an oncogene in BCa and that miR-200c downregulation significantly inhibits
BCa cell migration and invasion (32). Another study reported that miRNA-206 acted as a tumor suppressor in BCa by targeting YRDC; inhibiting BCa cell proliferation, colony formation, migration and invasion; and inducing cell cycle arrest at G0/G1 phase (33). Moreover, the expression levels of some miRNAs, including miR-125b, miR-30b, miR-204, miR-99a and miR-532-3p, were significantly downregulated in patient urine supernatants. These miRNAs could be used as promising diagnostic markers for the non-invasive diagnosis of BCa (34).

In the present study, the role of miR-130b in BCa was studied in further detail. The results of this study showed that miR-130b expression levels in surgically resected tumor specimens were significantly higher than those in adjacent non-tumor bladder tissue specimens. Moreover, higher miR-130b expression levels were observed in MIBC tissues than in NMIBC tissues. We also studied the effects of miR-130b gain/loss-of-function on various aspects of T24 and 5637 cell behaviors. We found that miR-130b overexpression or knockdown promoted or inhibited BCa cell proliferation, migration and invasion. Overall, we concluded that miR-130b may be an oncogene and plays an important role in the pathogenesis of BCa.

To study the detailed mechanisms underlying the function of miR-130b in BCa, we identified the downstream target gene of miR-130b using bioinformatics analysis software (miRBase, TargetScan). Bioinformatics analysis demonstrated that VGLL4 may be a direct target of miR-130b in BCa. VGLL4 is a member of the VGLL family, and VGLL proteins have recently emerged as a new TEAD-interacting partners and participate in cancer development. VGLL proteins are transcriptional cofactors. Recently, more attention has been devoted to the role of VGLL4 in cancer. VGLL4 was believed to act as a tumor suppressor gene in many types of cancer (24-26,35). The results of a previous study indicated that VGLL4 directly competes with YAP to bind TEADs and that VGLL4-mimicking peptides potently suppressed gastric cancer tumor growth in vitro and in vivo (23). Previous study demonstrated that YAP was upregulated in some BCa cell (5637) and overexpressed YAP promoted BCa cell growth and migration (36). Furthermore, the inhibition of YAP expressions significantly increased cytotoxic drug sensitivity.
Figure 5. miR-130b promotes bladder cancer (BCa) cell proliferation, migration and invasion by inhibiting VGLL4. (A) Western blotting was performed to measure VGLL4 expression levels in miR-130b-inhibitor transfected bladder cells (T24 and 5637) transfected with VGLL4-siRNA and a control vector. (B) CCK-8 assay was performed to detect the cell proliferation ability of the indicated cells. (C) Representative micrographs and quantification of crystal violet staining in the colony formation assay of the indicated cells. (D) Effects on the migration ability of the indicated cells, as demonstrated by wound healing assay; Scale bar, 100 µm. (E) Representative results of the invasion assays of the indicated cells; Scale bar, 100 µm. Data represent the mean ± SD of three independent experiments; *P<0.05, **P<0.01, ***P<0.001; #P<0.05, ##P<0.01.
and reduced the migration of chemoresistant BCa cells (37). In the present study, VGLL4 protein expression levels in BCa tissues were significantly decreased compared with those in adjacent non-tumor bladder tissues, and the inhibitory effects of the miR-130b inhibitor on BCa cell proliferation, migration and invasion were significantly reversed by VGLL4 suppression. Furthermore, we found that the VGLL4 gene is a direct target of miR-130b and that miR-130b downregulated VGLL4 expression. miR-130b overexpression and knockdown in BCa cells decreased and increased VGLL4 protein levels, respectively, but did not significantly affect VGLL4 mRNA levels. These findings indicated that miR-130b played an important role in regulating VGLL4 translation rather than VGLL4 mRNA degradation. Therefore, what is the specific role of VGLL4 in BCa is to be further confirmed in our next studies.

In summary, our results indicate that miR-130b, a potential oncogene, decreased VGLL4 expression by directly binding to the 3'UTR of VGLL4 mRNA and promoted BCa cell proliferation, migration and invasion. Thus, miR-130b/VGLL4 may be a new target for the diagnosis and treatment of BCa.

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Availability of data and materials

The datasets supporting the conclusions of this article and its additional files are available upon reasonable request.

Authors' contributions

The study was conceived by XL, CK and ZZ. Experiments were come into effect by XL. Statistical analysis was carried out by XL. Manuscript was written by XL. All authors recognized the final manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The First Hospital of China Medical University, and written informed consent was obtained from the patients in our center.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


