Original Article

MiR-506 suppresses proliferation and invasion of bladder cancer by targeting FOXQ1

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Abstract: FOXQ1 (Forkhead box protein Q1), a member of the forkhead family of transcription factors, is implicated in progression of various cancers. However, the modulation of FOXQ1 expression in human bladder cancer at post-transcriptional levels remains unclear. In this study, luciferase assay revealed that miR-506 was able to target 3’-untranslated region (3’-UTR) of FOXQ1. Furthermore, quantitative PCR and western blot assay demonstrated that miR-506 was able to suppress the expression of FOXQ1 at the levels of mRNA and protein. miR-506 expression was downregulated in bladder cancer tissue and cell lines, and overexpressed miR-506 significantly attenuated cellular proliferation and invasion. The rescue experiment confirmed that miR-506 exerted its biological functions by targeting FOXQ1. Collectively, our study demonstrates that miR-506 can impair the proliferation or metastasis of human bladder cancer cells by downregulation of FOXQ1.

Keywords: miR-506, FOXQ1, bladder cancer, proliferation, invasion

Introduction

Bladder cancer is a urological malignancy with particularly high morbidity worldwide [1]. Many molecules involved in these alterations and serve as diagnostic markers of tumor growth and disease progression. Despite improvements of diagnosis and treatment strategies, bladder cancer remains a highly prevalent and lethal malignancy [2]. Therefore, it is urgent to elucidate the underlying molecular mechanisms of osteosarcoma and find novel diagnostic and prognostic biomarkers for improving the clinical outcome of bladder cancer patients.

MicroRNA (miRNA), an abundant group of small (22-nucleotide) noncoding RNA molecules that regulate gene expression by binding to the 3’ untranslated region (UTR) of mRNA by partial sequence homology and play an important role in variety of biological processes including cell proliferation, apoptosis, invasion, migration, differentiation and so on [3-6]. Accumulated evidences prove that miRNAs function as either oncogenes or tumor suppressors and are aberrantly expressed in many human cancers [7]. miRNAs have been recognized as critical regulators in development and progression of cancer including bladder cancer [8-10]. miR-506 functions as an tumor suppressor and was downregulated in various cancers including esophageal cancer [11], colon cancer [12, 13], cervical cancer [14], liver cancer [15], gastric Cancer [16], ovarian cancer [17] and so on. However, the expression levels and mechanism of miR-506 in bladder cancer tumorigenesis is still unclear.

FOXQ1 (also known as HFH1), a member of the FOX gene family, contains the core DNA binding domain with flanking wings of FOXQ1 contribute to its sequence specificity [18, 19] FOX genes are involved in many critical biological processes such as cell cycle regulation, embryonic development, cell signaling, tissue-specific gene expression and tumorigenesis [20-22]. Recent studies have reported that FOXQ1 was involved in tumor metastasis and proliferation in gastric cancer, colorectal cancer, hepatocellular carcinoma [20, 21, 23]. Recently, Zhu et al. showed that short hairpin RNA targeting FOXQ1 inhibits invasion and metastasis via the reversal of epithelial-mesenchymal transition in bladder cancer [24]. However, the modulation of FOXQ1 expression in human bladder cancer at post-transcriptional levels remains unclear.
In our study, we identified that miR-506 was down-regulated in bladder cancer tissues and cell lines. Furthermore, miR-506 was further identified to be a tumor suppressor, as overexpressed miR-506 in bladder cancer cell lines inhibits cell proliferation and invasion by targeting FOXQ1. Thus, our data suggest important roles of miR-506 in bladder cancer pathogenesis and indicate its potential application in cancer therapy.

**Materials and methods**

**Human tissue specimens**

Twenty paired tissue specimens of bladder cancer and matched normal tissues were obtained from the First Affiliated Hospital of Nanchang University. The matched normal tissues were further confirmed by pathologist for their normal origin that they do not have tumor cells. All the tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use. The Institute Research Medical Ethics Committee of Nanchang University granted approval for this study.

**Cell culture and transfection**

Human bladder cancer cell lines (BIU-87, EJ and MGH-U1) and non-malignant bladder cell line (SV-HUC1) were maintained in Hyclon 1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 1% penicillin/streptomycin and incubated in a humidified (37°C, 5% CO₂) incubator.

Human bladder cancer cell lines BIU-87 cells were seeded in 12-well plates and incubated overnight, then transiently transfected with miR-506 mimic, miR-506 inhibitor, negative control (miR-con), and inhibitor negative control (anti-miR-con) sequences using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The full-length FOXQ1 cDNA (which included the ORF and 3’ UTR) was PCR amplified and cloned into the pcDNA3.1 vector to generate the pcDNA-FOXQ1 constructs that were used in the rescue assays. BIU-87 cells in 12-well plates were co-transfected with miR-506 mimic and the pcDNA-FOXQ1 plasmid DNA.

**Luciferase assay**

Cells at 70% confluence in a 96-well plate were transfected with 0.01 μg Renilla and 0.1 μg firefly using 0.25 μl of transfection reagent. Cell extracts were prepared 48 h after co-transfection, and the luciferase activity was detected using a Luciferase Reporter Assay System kit (Promega, Madison, WI, USA). The mean values were detected, and the firefly luciferase activity was normalized to the Renilla luciferase activity.

**qRT-PCR**

The PCR amplification for the quantification of the miR-506 and U6 was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan Human MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). The relative expression of miR-506 was shown as fold difference relative to U6. The PCR amplification for the quantification of the FOXQ1 and GAPDH mRNAs was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and a SYBR®Premix Ex Taq™ ii (Perfect Real Time) Kit (Takara Bio, Shiga, Japan).

**Western blot**

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using polyclonal (rabbit) anti-FOXQ1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto, Pierce) were used for detection.

**Cell proliferation**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide (MTT) assay was used to estimate cell viability [25]. Briefly, cells were plated at a density of 1×10⁴ cells per well in 96-well plates. After exposure to specific treatment, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C. After the removal of the medium, 150 mM DMSO solutions were added to dissolve the formazan crystals. The absorbance was read at 570 nm using a multi-well scanning spectrophotometer reader. Cells in the control group were considered 100% viable at 48 h.
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Cell invasion assay

At 48 hours after transfection, cells were seeded onto the basement Matrigel-coated membrane matrix (BD Biosciences) present in the insert of a 24-well culture plate. Fetal bovine serum was added to the lower chamber as a chemoattractant. After a further 48 hours, the noninvading cells were gently removed with a cotton swab. Invasive cells located on the lower side of the chamber were stained with Crystal Violet and counted.

Figure 1. The expressions of miR-506 in bladder cancer tissue and cell lines. A. miR-506 expression levels were examined by qRT-PCR in bladder cancer tissue (Cancer) and adjacent non-neoplastic tissues (Normal). Each bar represents the mean of three independent experiments. *P < 0.01 versus adjacent non-neoplastic tissues (Normal). B. miR-506 expression levels were examined by qRT-PCR in non-malignant bladder cell line (SV-HUC1) and three bladder cell lines (BIU-87, EJ and MGH-U1). Each bar represents the mean of three independent experiments. *P < 0.01 versus SV-HUC1 cell line.

Figure 2. miR-506 directly targeted FOXQ1. A. Representative diagram of the predicted wild-type (WT) or mutant (Mut) binding site of miR-506 in the 3'-untranslated region (UTR) of FOXQ1 mRNA. B. The luciferase reporter plasmid containing the WT or Mut FOXQ1 3'-UTR was cotransfected into BIU-87 cells with miR-506 mimics. Luciferase activity of the cells was assayed at 48 h after transfection, and the values were normalized to the normal control values. *P < 0.01 versus control.

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean ± S.D and analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using Student’s t-test and a two-tailed P < 0.05 was considered to indicate statistical significance.

Results

miR-506 expressed was downregulated in bladder cancer specimens and cell lines

To evaluate the expression of miR-506 in human bladder carcinoma, we first quantitatively analyzed the levels of reverse transcription-PCR in 20 cases of clinical bladder carcinoma tissues and matched adjacent non-neoplastic tissues. The result showed that miR-506 expression levels were generally reduced in bladder cancerous specimens, compared with corresponding adjacent non-neoplastic tissues (Figure 1A). We also determined the expression level of miR-506 in different bladder cancer cell lines by quantitative RT-PCR. Comparing with the non-malignant bladder cell line (SV-HUC1), the expression level of miR-506 was consistently down-regulated in three blad-
miR-506 suppresses proliferation and invasion of bladder cancer cell lines (BIU-87, EJ and MGH-U1) (Figure 1B).

**FOXQ1 is a direct target gene of miR-506 in bladder cancer cells**

In this study, the miRNA target prediction websites www.microRNA.org and TargetScan were used and identified a conserved miR-506-binding site in the 3’-UTR of FOXQ1 mRNA. We then cloned WT or Mut target region sequence of the FOXQ1 3’-UTR, which was inserted into a luciferase reporter vector (Figure 2A). Subsequently, these reporter vectors were cotransfected with miR-506 mimics and miR-506 mimics control (miR_con) into the bladder cancer cell line. As shown in Figure 2B, co-transfection of miR-506 mimics suppressed the luciferase activity of the reporter containing wild-type FOXQ1 3’ UTR sequence, but failed to inhibit that of mutated FOXQ1 by dual-luciferase reporter assay. These data indicate that FOXQ1 is one of the direct targets of miR-506 in bladder cancer.

**miR-506 represses FOXQ1 expression in bladder cancer cells**

Next, we analyzed the regulation of miR-506 on FOXQ1 expression. We transfected miR-506 mimics orpcDNA-FOXQ1 or cotransfected...
miR-506 suppresses proliferation and invasion of bladder cancer cells. qRT-PCR and Western blot analysis revealed FOXQ1 expression were significantly increased by transfecting with pcDNA-FOXQ1 (Figure 3A). miR-506 expression was significantly increased by transfecting with miR-506 mimics and decreased by transfecting with miR-506 inhibitors, compared with negative control group (Figure 3B). In addition, miR-506 mimics markedly increased the expression of FOXQ1 (Figure 3B), which was rescued by ectopic FOXQ1 (Figure 3C). These results demonstrated that FOXQ1 expression is regulated by miR-506 in bladder cancer cells.

MiR-506 suppresses proliferation and invasion of bladder cancer cells via FOXQ1

It has been reported that FOXQ1 is implicated in bladder cancer progression [24]. Therefore, we are interested in whether miR-506 inhibits proliferation and migration of bladder cancer cells through FOXQ1 (Figure 4). The MTT assay revealed that miR-506 could remarkably reduce migration ability of bladder cancer cells, which was reversed by ectopic FOXQ1 (Figure 4A). The transwell invasion assay manifested that cell invasion was decreased when the cells were treated with miR-506, but FOXQ1 overexpression could rescue inhibition of cell invasion mediated by miR-506 in the cells treated with both miR-506 and FOXQ1 (Figure 4B and 4C). Collectively, we conclude that miR-506 is able to suppress cell proliferation and invasion through targeting FOXQ1 in bladder cancer.

Discussion

In this study, we reported for the first time that miR-506 was markedly downregulated in bladder cancer clinical specimens and cell lines. The over-expression of miR-506 repressed the proliferation and invasion of bladder cancer cells. Moreover, we identified that FOXQ1 was identified as a new direct and functional target of miR-506 by using dual luciferase assay, and miR-506 evidently suppressed FOXQ1 mRNA and protein expression in bladder cancer cells. Overexpressed miR-506 can evidently repress the proliferation and invasion of bladder cancer cells, which was reversed by ectopic FOXQ1. Our study suggested that miR-506 acts as a
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MiRNAs are crucial in maintenance of normal cellular function, and accumulating studies showed that high abundant of dysregulated miRNAs function as tumor suppressors or oncogenes, and contribute to the initiation and progression of cancer [26]. To date, several studies have identified miR-506 dysregulated expression in various cancers, which implicated in development and progression of these cancers [13, 27]. Hua et al. found that expression of miR-506 was commonly down-regulated in breast cancer cells and breast cancer specimens, and up-regulation of miR-506 inhibited cellular proliferation, migration and invasion as well as disrupt the cell cycle of breast cancer cells [28]. miR-506 is also down-regulated in hepatocellular carcinoma, and enforced expression of miR-506 inhibits proliferation, migration and invasion in vitro, and suppresses tumor growth in vivo [29]. In our study, we identified that the expression of miR-506 was significantly downregulated in bladder cancer tissues and cell lines. Overexpressed miR-506 significantly repressed cell proliferation and invasion in bladder cancer cell lines. These data suggest miR-506 as a tumor suppressor in bladder cancer.

Foxq1, a member of the FOX gene family, has been reported to be involved in various biological processes including embryonic development, cell cycle regulation, tissue-specific gene expression, cell signaling, and tumorigenesis [30]. Recent studies have clearly showed that Foxq1 was implicated in tumor proliferation and metastasis in various cancers including breast cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, gastric cancer and glioblastoma [19-21, 23, 31-33]. Recently, high expression levels of FOXQ1 were observed in bladder cancer tissues [24]. However, the potential mechanism remains unclear. Here, we found that FOXQ1 was a direct target of miR-506, and miR-506 could regulate the expression of FOXQ1 in bladder cancer. Moreover, FOXQ1 overexpression reversed miR-506-induced repression of the proliferation and invasion of bladder cancer.

In conclusion, our results suggest that the expression of miR-506 is down-regulated in bladder cancer tissues and cell lines, and functions as a novel tumor suppressor to repress the proliferation and invasion of bladder cancer cells by targeting FOXQ1. Therefore, miR-506 may be a novel molecular therapeutic target for the treatment of bladder cancer.

Disclosure of conflict of interest

None.

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References

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