miR-96 regulates FOXO1-mediated cell apoptosis in bladder cancer

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Abstract. Transitional cell carcinoma (TCC) is one of the most common types of malignancies and a leading cause of genitourinary system cancer mortality worldwide. The tumor suppressor gene FOXO1, a member of the forkhead box O (FOXO) subfamily of transcription factors, is downregulated in a number of cancers, including TCC; however, the underlying mechanisms are poorly understood. In the present study, we used microRNA (miRNA) target prediction algorithms to identify a conserved potential miR-96 binding site in the 3'-untranslated region (3'-UTR) of FOXO1. Using quantitative real-time PCR (qRT-PCR) and northern blot analysis, we identified that miR-96 was downregulated in TCC tissues compared to normal bladder tissues (NB), suggesting that the loss of FOXO1 expression in TCC may be mediated by miR-96. To confirm this, we transfected pre-miR-96/anti-miR-96 into the T24 TCC cell line and revealed that miR-96 expression was sufficient to significantly reduce FOXO1 expression. Conversely, FOXO1 expression was not completely restored by the inhibition of miR-96 in T24 cells. Moreover, RNA silencing of FOXO1 significantly reduced miR-96 inhibitor-mediated T24 cell apoptosis. In conclusion, our study demonstrates that the miR-96 targeting of FOXO1 is upregulated in TCC; in addition, TCC tumorigenesis may be partly due to the ability of miR-96 to promote FOXO1 repression, thereby bypassing cell apoptosis controls.

Introduction

Transitional cell carcinoma (TCC), the most common histopathological type of bladder cancer, is one of the most prevalent malignancies and a leading cause of genitourinary system cancer mortality worldwide (1,2). Although a number of therapeutic strategies are available, including intravesical chemotherapy, surgery, radiation therapy and systemic chemotherapy, approximately 75% of patients with non-muscle invasive bladder cancer face a five-year survival rate of between 88-98%. Additionally, approximately 50% of all advanced patients develop subsequent metastatic disease following the first aggressive treatment (3,4). Despite technological advances, the survival rates of bladder cancer patients have not changed over the past 20 years. Therefore, it is essential to identify new anticancer agents, which may not only aid in the understanding of the molecular mechanisms of TCC, but may also improve the survival rates of TCC patients.

FOXO1, a member of the forkhead box O (FOXO) subfamily of transcription factors, functions as a tumor suppressor and regulates genes involved in the apoptotic response, cell cycle checkpoints and cellular metabolism (5). Clinical and experimental data suggests that FOXO1 is downregulated in various types of cancer, including TCC (6-8); however, the molecular mechanism resulting in FOXO1 aberrant expression is poorly understood. Acting as a master cell regulator, FOXO1 is activated through phosphorylation, acetylation or the insulin-stimulated PI3K signaling pathway (9). Recent evidence suggested that post-transcriptional regulation may be important for FOXO1 downregulation and the modulation of its activity (10).

microRNAs (miRNAs), common post-transcriptional factors, are a class of small non-coding RNAs that negatively regulate gene expression by facilitating mRNA degradation or translational inhibition. Emerging evidence suggests that downregulated miRNAs are involved in the pathogenesis of bladder cancer (11). However, to date, there have been no studies on miR-96 regulating FOXO1 expression in TCC.

In this study, we used bioinformatic technologies to identify whether miR-96 was a predicted target for the FOXO1 gene. We also investigated whether miR-96 was upregulated in TCC compared to normal bladder (NB) tissues using quantitative real-time PCR (qRT-PCR) and northern blot analysis, and explored the regulation mechanisms of miR-96 to FOXO1 by transfection and RNA interference. Our novel findings suggest

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that the ability of miR-96 to promote FOXO1 repression may play a key role in TCC tumorigenesis through bypassing cell apoptosis control.

Materials and methods

miRNA prediction. The prediction of the FOXO1 3'-untranslated region (3'-UTR) as a miRNA binding target was determined using miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl?type=miRanda), Targetscan (www.targetscan.org) and PicTar (http://pictar.mdc-berlin.de/). miRNAs that were simultaneously predicted by all three programs were selected for this study.

Cell culture. The human T24 TCC cell line was stored at Central Laboratory, School of Stomatology, China Medical University, Liaoning, China. The cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Sigma, St. Louis, MO, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2. Only cells in the log phase were selected for the following experiments.

Patients and specimens. A total of 40 bladder TCC samples, from patients who had not undergone previous chemotherapy or radiation therapy, were collected at The Affiliated Shengjing Hospital, China Medical University. Additionally, 20 NB tissues were obtained from patients with benign diseases. Informed consent was obtained prior to tissue collection and the study was approved by the local ethics committees. All specimens were frozen in liquid nitrogen immediately after resection and stored at -80˚C until use.

qRT-PCR. Transcripts were measured using a standard SYBR-Green-based Real-Time PCR assay. miRNAs were isolated using the mirVana™ miRNA Isolation kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and cDNAs were synthesized using the QuantiMir RT Kit Small RNA Quantitation System (System Biosciences, Mountain View, CA, USA). Real-time PCR was then conducted in a 30-μl reaction volume using the Applied Biosystems 7500HT PCR system with miR-96-specific primers. Amplifications were analyzed using the comparative C_T method and U6 small nuclear RNA (snRNA) was used as a reference control.

Northern blot analysis. Total RNA was isolated from each tissue using TRIzol reagent (Invitrogen Life Technologies) and Northern blotting was conducted as described by Myatt *et al* and Várallyay *et al* (6,12). The probe sequence of miR-96 was 5'-AGCAAAAATGTGCTAGTGCCAAA-3'. Following Perfect Hyb Plus hybridization at 68˚C, membranes were developed and analyzed. Northern blots hybridized with an 18S ribosomal RNA (rRNA) cDNA were used as controls.

Transfection. Appropriate cells were seeded onto 6-well plates 24 h prior to transfection using oligofectamine according to the manufacturer's instructions (Invitrogen Life Technologies). Cells were incubated with 60 nM pre-miR-96, anti-miR-96 or appropriate scramble controls (Invitrogen Life Technologies) for 4 h in Opti-MEM media prior to the addition of normal growth media. The cells were then assayed 48 h after transfection. For small interfering RNA (siRNA) transfection, 100 nM of a siRNA designed to target FOXO1 (Cat No. HSS103719) and a matching negative control oligonucleotide were used. qRT-PCR was conducted to determine the efficiency of transfection.

Western blot analysis. Transfected cells were washed with ice-cold PBS and solubilized with lysis buffer. A total of 50 μg of cell lysate was subjected to 10% SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked and hybridized with a FOXO1 primary antibody (1:1000) and a horseradish peroxidase-conjugated secondary antibody. After washing, proteins were detected using the enhanced chemiluminescence system (ECL; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) . Results were quantified by scanning densitometry using a thermal imaging system (FTI-500; Pharmacia Biotech, Sweden). β-actin was used as a housekeeping protein.

Apoptosis assays. Apoptosis was detected using the Annexin V-FITC Apoptosis Detection kit (Biosea, Beijing, China) (13). Cells incubated with Annexin V-FITC and propidium iodide (PI) were subjected to flow cytometry (Ex=488 nm; Em=635 nm) within 1 h and analyzed using CellQuest software. Annexin V-positive cells were regarded as apoptotic.

Statistical analysis. Each experiment was conducted in triplicate. Data are expressed as the mean ± standard deviation (SD) and were analyzed with SPSS version 13.0 software. Statistical significance was analyzed using the one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-96 as a putative target for FOXO1. By combining the results of miRanda, TargetScan and PicTar, we identified a panel of conserved miRNAs with the potential to target the 3'-UTR of FOXO1 transcripts. Only miR-96/site 1 (probability of conserved targeting, $P_{CT}=0.94$) (miR-96) was predicted in all three databases and was highly conserved among vertebrates; although, miR-96 has another predictive target site in the FOXO1 3'-UTR, miR-96/site 2 ($P_{CT}<0.1$; Fig. 1).

miR-96 is upregulated in TCC. In order to explore the regulation mechanisms of miR-96 to FOXO1, we first examined miR-96 expression levels in 40 TCC and 20 NB samples by qRT-PCR analysis. As shown in Fig. 2A, the levels of miR-96 were significantly upregulated in TCC compared with NB samples ($P<0.05$). We also conducted northern blot analysis to further confirm the expression difference of miR-96 in TCC and NB samples. In accordance with the qRT-PCR data, miR-96 demonstrated a higher level of expression in the TCC samples ($P<0.05$; Fig. 2B). In conjunction with the results from Kim *et al* (8) and our prediction, we speculated that the loss

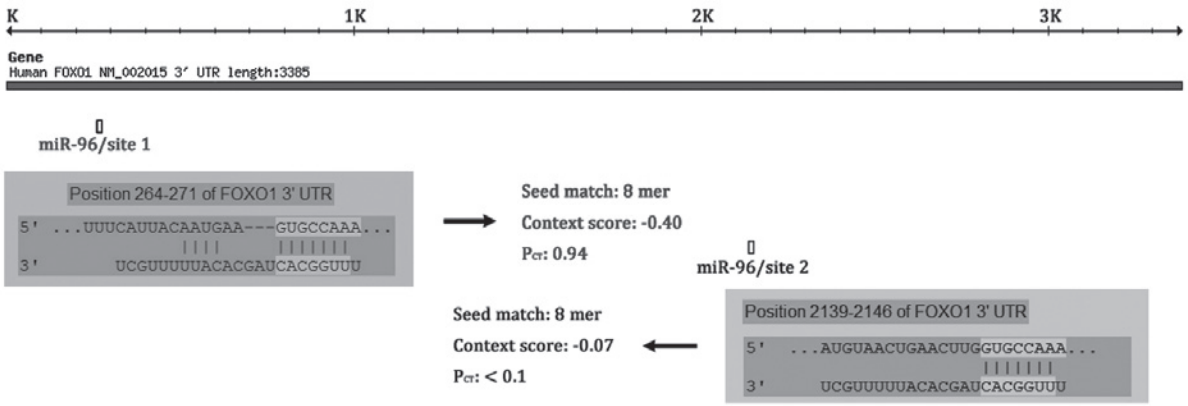

Figure 1. miR-96 is predicted to target the 3'-UTR of FOXO1 transcription. Schematic diagram revealing the location of miR-96 binding sites in the 3'-UTR of FOXO1, predicted by miRanda, TargetScan and PicTar. Seed region bindings are highlighted by the lighter shaded section. 3'-UTR, 3'-untranslated region; FOXO1, forkhead box O1.

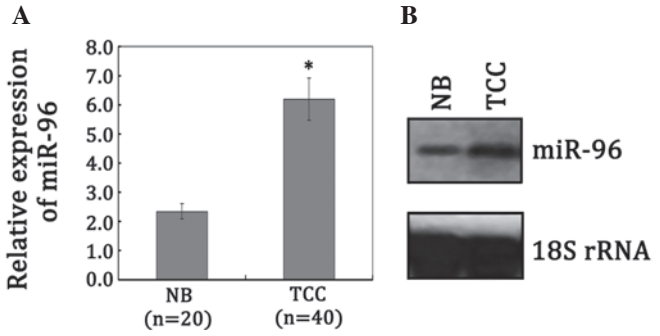

Figure 2. miR-96 is upregulated in human TCC. (A and B) reveal qRT-PCR and northern blot analysis results of miR-96 expression in 40 TCC and 20 NB specimens with U6 snRNA and 18S rRNA as reference genes, respectively. The results are shown as the mean ± SD. *P<0.05 vs. NB specimens. NB, normal bladder; TCC, transitional cell carcinoma; rRNA, ribosomal RNA; qRT-PCR, quantitative real-time polymerase chain reaction; snRNA, small nuclear RNA; SD, standard deviation.

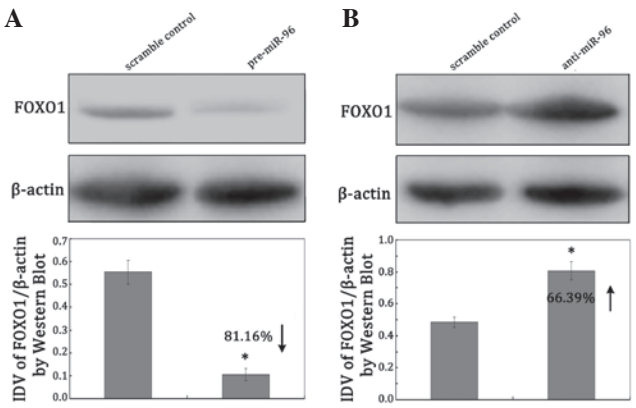

Figure 3. Effects of miR-96 on FOXO1 expression in T24 TCC cells. (A and B) reveal western blot analysis results of FOXO1 protein expression in T24 cells transfected with either pre-miR-96 and its scramble control or anti-miR-96 and its scramble control, respectively. β-actin was used as a reference control. The results are shown as the mean ± SD of three independent experiments. Quantitative analysis was conducted by measuring the IDV of protein bands. Percentage of FOXO1 expression decreased and increased by 81.16 and 66.39%, respectively, compared to the individual scramble control. *P<0.05 vs. scramble control. FOXO1, forkhead box O1; IDV, integrated density value; TCC, transitional cell carcinoma.

of FOXO1 expression upon malignant transformation is correlated with miR-96 aberrant expression in human TCC.

miR-96 represses FOXO1 expression in T24 cells. To examine the repressive potential of miR-96 on FOXO1, pre-miR-96 was transfected into T24 cells and endogenous FOXO1 expression levels were monitored using western blot analysis. Cells were also transfected with a scramble control, which had no effect on FOXO1 expression. The results revealed that overexpression of miR-96 effectively downregulated FOXO1 expression (P<0.05; Fig. 3A). In the reverse experiment, we used anti-miR-96 to silence the activity of miR-96 in T24 cells and monitored the expression of FOXO1. The results demonstrated that the transfection of miR-96 inhibitors elicited a reproducible induction of FOXO1 levels (P<0.05; Fig. 3B). In contrast, FOXO1 levels were effectively unchanged upon transfection of the anti-miR scramble control. These observations indicate that miR-96 may repress FOXO1 expression, but transfection of miR-96 inhibitors was insufficient to completely restore FOXO1 expression in T24 TCC cells.

miR-96 inhibits T24 cells apoptosis in a FOXO1-dependent manner. Based on the hypothesis that the repression of FOXO1 by miR-96 may cause human TCC cells to escape apoptosis, we next determined the effect of miR-96 repression in T24 cells. First, we conducted a cell apoptosis analysis using flow cytometry, and the results demonstrated that transfection of the anti-miR-96 was effective in inducing T24 cell apoptosis (Fig. 4A). Subsequently, in order to demonstrate the requirement of FOXO1 in anti-miR-96-induced cell apoptosis, we co-transfected anti-miR-96 together with a functional siRNA targeting FOXO1, which repressed endogenous FOXO1 levels (Fig. 4B). Under these conditions, a significant reduction in anti-miR-96-induced cell apoptosis was observed (P<0.05), which is consistent with FOXO1 induction being critical for this effect (Fig. 4C). Together, our results suggest that miR-96 may repress FOXO1 expression, thereby promoting TCC progression.

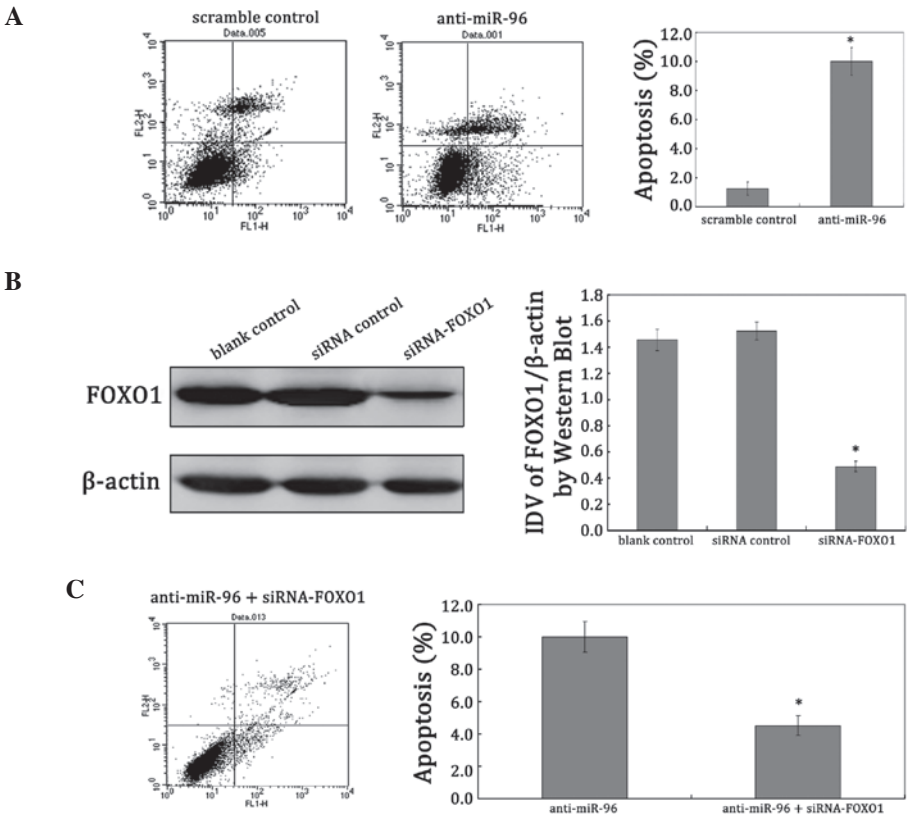

Figure 4. miR-96 inhibits T24 cell apoptosis in a FOXO1-dependent manner. (A) Apoptosis analysis of T24 cells transfected with anti-miR-96 and its scramble control. Cells located in the lower right quadrant were apoptosis positive. Histogram results are the mean of three independent experiments. *P<0.05 vs. scramble control. (B) Western blot analysis demonstrates the effect of siRNA-FOXO1 on FOXO1 expression in T24 cells. (C) Apoptosis analysis of T24 cells transfected with anti-miR-96 and siRNA-FOXO1. Histogram demonstrates that the apoptotic rate was significantly reduced compared to that in the anti-miR-96 transfection group. *P<0.05 vs. anti-miR-96. FOXO1, forkhead box protein O1; siRNA, small interfering RNA; IDV, integrated density value.

Discussion

miRNAs function as important regulators of target genes that are involved in normal development and development of diseases, including cancer (14). Various cancer types, stages or differentiation states have unique miRNA expression profiles (15-17), and in recent years, researchers have made great efforts to discover miRNAs that function as novel biomarkers for cancer diagnosis. Han *et al* (17) discovered a great number of miRNAs involved in bladder cancer and identified the miRNAs and miRNA*s that were significantly upregulated or downregulated in bladder urothelial carcinoma compared to the matched normal urothelium. hsa-miR-96 (log2 ratio=4.664328) was revealed as the most significantly upregulated miRNA. The expression of miR-96 lacks real-time PCR validation; however, in our study we used qRT-PCR, northern blot analysis and a serial assay to demonstrate that miR-96 was upregulated in human TCC and that miR-96 promoted FOXO1 repression through the bypassing of cell apoptosis control. Our results revealed that miR-96 is critical for the development of human TCC, and that miR-96 upregulation is one of the mechanisms of FOXO1 repression in TCC tumorigenesis.

miR-96, miR-183 and miR-182, located proximally in the genome, belong to the same miR-183 family. Moreover, miR-96, miR-183 and miR-182 share the same transcription start site (chr7: 129207158), suggesting that these miRNAs may be coordinately expressed and function together during tumorigenesis (18). A recent study identified that miR-96 and miR-182, which are highly expressed in Michigan Cancer Foundation 7 (MCF-7) breast cancer cells, repress the endogenous expression of FOXO1 gene and cause the oncogenic transformation of breast cells (7). It was also revealed that miR-96, miR-182 and miR-183 are overexpressed in endometrial cancer and function as an oncogene through the repression of FOXO1 expression. Subsequently, aberrant miR-183 family expression resulted in deregulated cell cycle control and impaired apoptotic responses (6). In this study, we predicted miR-182 and miR-183 binding sites in the 3'-UTR of FOXO1 transcripts using three miRNA prediction programs (miRanda, Targetscan and PicTar). In conjunction with our present findings that transfection of miR-96 inhibitors was insufficient to completely restore FOXO1 expression in T24 TCC cells, we speculated that as miR-183 family members, miR-182 and/or miR-183 may regulate FOXO1 expression, directly or indirectly, in a miR-96-dependent manner. However, this requires further study.

In addition to the miR-96 mediated repression, the FOXO1 gene may be regulated by a number of other mechanisms, including methylation and various pathways. Studies demonstrate that promoter methylation may downregulate FOXO1 gene expression in certain endometrial cancers (10).

Additionally, the upregulation of Skp2, an oncogenic subunit of the Skp1/Cul1/F-box protein ubiquitin complex, may downregulate FOXO1 protein levels by promoting ubiquitination and degradation of phosphorylated FOXO1 (19). FOXO1 activity is associated with the chemosensitization of a number of cancers and numerous studies support the notion that apoptosis escape plays an important role in the resistance of carcinomas to chemotherapy and radiotherapy (6,20). Using RNA interference and transfection technologies, our study revealed that FOXO1 downregulation may significantly reduce miR-96 mediated TCC cell apoptosis, which provided a theoretical basic for further investigation of miR-96/FOXO1 as a potential therapeutic target for TCC.

In conclusion, we identified that the miR-96 targeting of FOXO1 was upregulated in TCC; in addition, TCC tumorigenesis may be partly due to the ability of miR-96 to promote FOXO1 repression, thereby bypassing cell apoptosis controls.

Acknowledgements

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