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MiR-103/107 induces tumorigenicity in bladder cancer cell by suppressing PTEN

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Abstract. – OBJECTIVE: MiR-103/107 has been shown to be implicated in the pathogenesis of various malignant diseases. The present study was designed to analyze the expression, function and mechanism of miR-103/107 in the bladder cancer tumorigenesis.

PATIENTS AND METHODS: Bladder cancer tissues and the paired normal tissues were collected during the surgical treatment of radical cystectomy, and the expression of miR-103/107 was measured by quantitative Reverse Transcriptional Polymerase Chain Reaction (RT-PCR). After modulation of miR-103/107 level in bladder cancer cells using antagomiR or mimics, several experimental approaches such as MTT assay, flow cytometry analysis and Western blot have been applied to determine cell viability, cell cycle and protein expression, respectively. Luciferase reporter assay was performed to determine the target of miR-103/107.

RESULTS: miR-103/107 expression is upregulated in the tumor site of bladder cancer specimens, and it is positively associated with tumor stages. Inhibition of miR-103/107 by its antagomiR decreased the cell growth potential and induced cell cycle arrest. Moreover, inhibition of miR-103/107 also suppressed the PI3K/AKT signaling. Further analysis revealed that miR-103/107 directly targets the 3' untranslated region (UTR) of PTEN mRNA to promote PI3K/AKT signaling, which was corroborated by the negative correlation between miR-103/107 and PTEN in tumor specimens.

CONCLUSIONS: The oncogenic role of miR-103/107 in bladder cancer is revealed for the first time. MiR-103/107 regulates cell proliferation and PI3K/AKT signaling partially through PTEN dependent mechanism. Thus, inhibiting miR-103/107 may be a therapeutic approach for bladder cancer treatment.

Key Words:
Bladder cancer, miR-103/107, AKT, PTEN.

Introduction

Bladder cancer is a type of prevalent malignancy in the urinary epithelium[1]. The common therapies to treat bladders cancer include surgery, radiotherapy, chemotherapy and immunotherapy[2]. Although many of the cases of bladder cancer are diagnosed at an early stage and the survival rates for bladder cancer is relatively high, the recurrence of bladder cancer may lead to considerable treatment failures. The unmet demands of the treatment strategies for bladder cancer call for a deep understanding on its pathogenesis. It is widely believed that the interplay between the genetic susceptibility and risk factors such as smoking and frequent bladder infections, contributes to the onset of bladder cancer[3-5]. However, currently we only have limited understanding on the genetic and molecular sides. Over the last two decades, a class of short non-coding RNAs, namely microRNAs (miRs) has emerged as important molecular regulator in the tumorigenicity of the urinary bladder epithelium[6-8]. MicroRNAs generally have the ability to bind to the 3' untranslated region (UTR) of their potential targeting mRNAs and subsequently will suppress their expression. Numbers of microRNAs have been shown to have different expression profiles in bladder cancer samples. Large-scale expression profile studies have identified an altered expression of 261 microRNAs in bladder cancer samples, and some of them have been functionally tested[6]. Recent gene profiling data have shown that miR-103 and miR-107 are upregulated in bladder cancer[9,10]. MiR-103 and miR-107 belong to the same family and have identical seed sequence with only one-base difference. The dysregulation of miR-103/107 family has been reported to be related to colorectal cancers, heart disease, diabetes and

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obesity[11-13]. However, whether it has a regulatory role in bladder cancer remains unknown. In the present work, we reasoned that miR-103/107 may be differentially expressed in bladder cancer and may function as important regulators for the tumorigenicity of bladder cancer cells. Using multiple experimental approaches, we identified that the miR-103/107 family functions as a tumor promoting effector which suppresses PI3K/AKT signaling via direct binding with PTEN, indicative of a therapeutic potential for the inhibition of miR-103/107 in this cancer type.

Patients and Methods

Patients

Bladder cancer tissues and the paired normal tissues were collected in the radical cystectomy surgeries from patients who were diagnosed with bladder cancer. The study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University and the informed consents were obtained from the patients. The tissue specimens were examined by experienced pathologists, and tissues were immediately transferred to liquid nitrogen.

Quantitative Reverse Transcription (RT) PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from tumor tissues and bladder cancer cells, according to the instructions provided by manufacturer. The first strand cDNA was synthesized using a ReverTra Ace qPCR RT Kit from Toyobo (Tokyo, Japan). The expression of miR-103 and miR-107 was measured using SYBR Green Real-time PCR Master Mix (Toyobo, Tokyo, Japan). The primers for detection of miR-103 and miR-107 were purchased from Genepharma Co. Ltd (Shanghai, China), U6 was used as an internal control. The primers for PTEN and its internal control GAPDH were purchased from BGI (Beijing, China). The primer sequence for PTEN is F: 5'-TGGATTCGACTTAGACTTGACCT-3' R: 5'-GGTGGGTTATGGTCTTCAAAAGG-3', and the primer sequence for GAPDH is F: 5'-GGAGCGAGATCCCTCCAAAAT-3' R: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The quantitative RT-PCR program was as follows: 95°C for 10 min; 95°C for 5 s, 60°C for 20 s and 72°C for 20 s in 40 cycles, followed by an extension procedure for 5 minutes at 72°C. Three technical repeats were performed for each independent sample, and the mean values were used for quantification.

Cell Culture

Human bladder cell lines UMUC2 and 5637 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. To inhibit the expression of endogenous miR-103/107, antagomiRs for both microRNAs (Ribo Biotech, Guangzhou, China) were co-transfected. MiR-103 and miR-107 mimics were also co-transfected to overexpress them in bladder cancer cells.

Cell Viability Assay

Cell viability was measured using an MTT-based assay. Cells were plated at the concentration of 2×10^5 per ml in 24-well plates, followed by transfection of antagomiR-NC or antagomiR-103/107. The dimethyl thiazolyl diphenyl tetrazolium (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA) was added into each well according to manufacturer's instructions. After the 4-hour incubation, cells were taken out and dissolved in dimethylsulfoxide (DMSO). The absorbance values at 490 nm were obtained.

Cell Cycle Analysis

Cell cycle was determined by flow cytometry using cell cycle analysis kit (Solarbio, Beijing, China) 72 h after transfection according to the manufacturer's instructions. Cells were fixed in pre-cold 70% ethanol and stained with propidium iodide (PI). A BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for detection.

Western Blot

Protein samples were collected using Sodium Dodecyl Sulfonate (SDS) lysis buffer (Beyotime, Shanghai, China) from treated cells. 10% SDS-polyacrylate gel electrophoresis (PAGE) gel was used to separate the protein extracts according to the molecular weight. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), and blocked with 5% non-fat milk. The following primary antibodies from Cell Signaling Technology

(Danvers, MA, USA) were used for detection: p-AKT (4060), AKT (9272), p-P70S6K (9204), P70S6K (2708), p-mTOR (5536), mTOR (2983), PTEN (9188) and GAPDH (5174). The horseradish peroxidase (HRP)- secondary Goat anti-rabbit antibody was purchased from Solarbio (Beijing, China). The bands for proteins were visualized using BeyoECL-plus detection system (Beyotime, Shanghai, China).

Luciferase Reporter Assay

The wild type and Mutant 3'UTR of PTEN were generated and cloned into pmiRGLO plasmid (Promega, Madison, WI, USA) by Aiyou Biotech (Shanghai, China). The cell lines were transfected with negative control (NC) or miR-103/107 mimics with luciferase reporter for 24 h. The dual luciferase (Renilla as internal control and Firefly as reporter activity) was detected using a dual luciferase detection system (Promega, Madison, WI, USA).

Statistical Analysis

Data were expressed as means ± standard deviation (SD), $n \geq 3$ for each group. Statistical significance was determined by Student's *t*-test or one way ANOVA followed by LSD test. Correlation between miR-103/107 and PTEN was calculated by Spearman's correlation. $p < 0.05$ is used as the indication for statistical significance.

Results

MiR-103/107 Expression is Upregulated in Bladder Cancer

To determine the role of miR-103/107 in bladder cancer, we first used the quantitative RT-PCR method to analyze their expressions in cancer specimens. Bladder cancer and adjacent normal tissue specimens from 17 patients were subjected to this experiment. As shown in Figure 1A and B, both miR-103 and miR-107 have

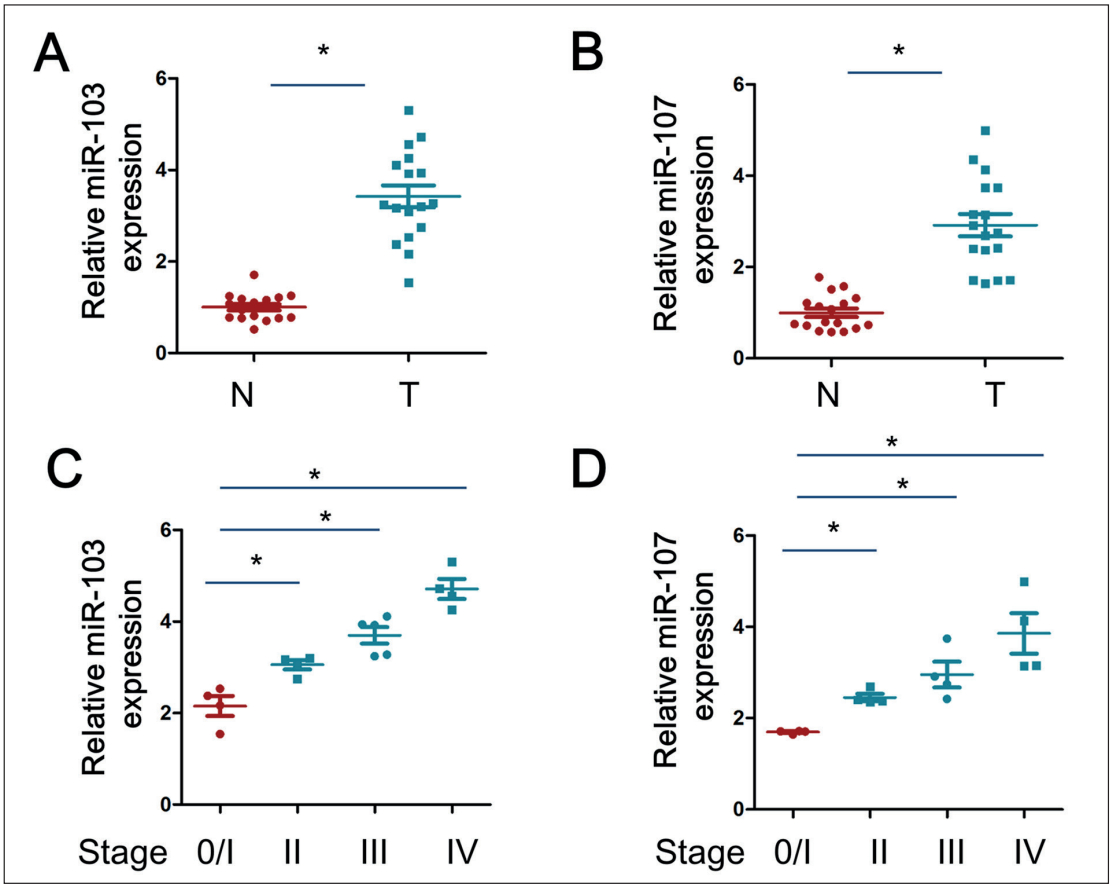

Figure 1. MiR-103/107 expression is upregulated in bladder cancer. ***(A)*** The expression of miR-103 in bladder cancer specimens (T) and adjacent normal specimens (N). ***(B)*** The expression of miR-107 in bladder cancer specimens and adjacent normal specimens. ***(C)*** The expression of miR-103 in bladder cancer specimens from different stages. ***(D)*** The expression of miR-107 in bladder cancer specimens from different stages. *$p < 0.05$.

been significantly upregulated in bladder cancer tissues compared to its paired control tissues. Surprisingly, in all the examined cancer specimens, miR-103/107 expression was positively associated with the tumor stages (Figure 1C and D). These data indicate that miR-103/107 is upregulated in bladder cancer; its association with tumor stages indicates a possible role in the progression of bladder cancer.

Inhibition of miR-103/107 Expression in Bladder Cancer Cells Suppressed Cell Proliferation and Induced Cell Cycle Arrest

To determine the biological significance of miR-103/107 upregulation, we transfected cells with antagomiR-103 and antagomiR-107 (hereafter antagomiR-103/107) to inhibit its expression in two bladder cancer cell lines, UMUC2 and 5637. As shown in Figure 2A and C, cell viability data suggest that inhibition of miR-103/107 attenuated cell growth. To further identify the cause of this attenuation, we performed cell cycle analysis using flow cytometry. In both cell lines, antagomiR-103/107 was able to increase the cell proportion in G1 phase; accordingly, cells in S and G2 phases were less in antagomiR-103/107 transfected group (Figure 2B and D). These data suggest that inhibition of miR-103/107 may suppress cell proliferation through inducing cell cycle arrest.

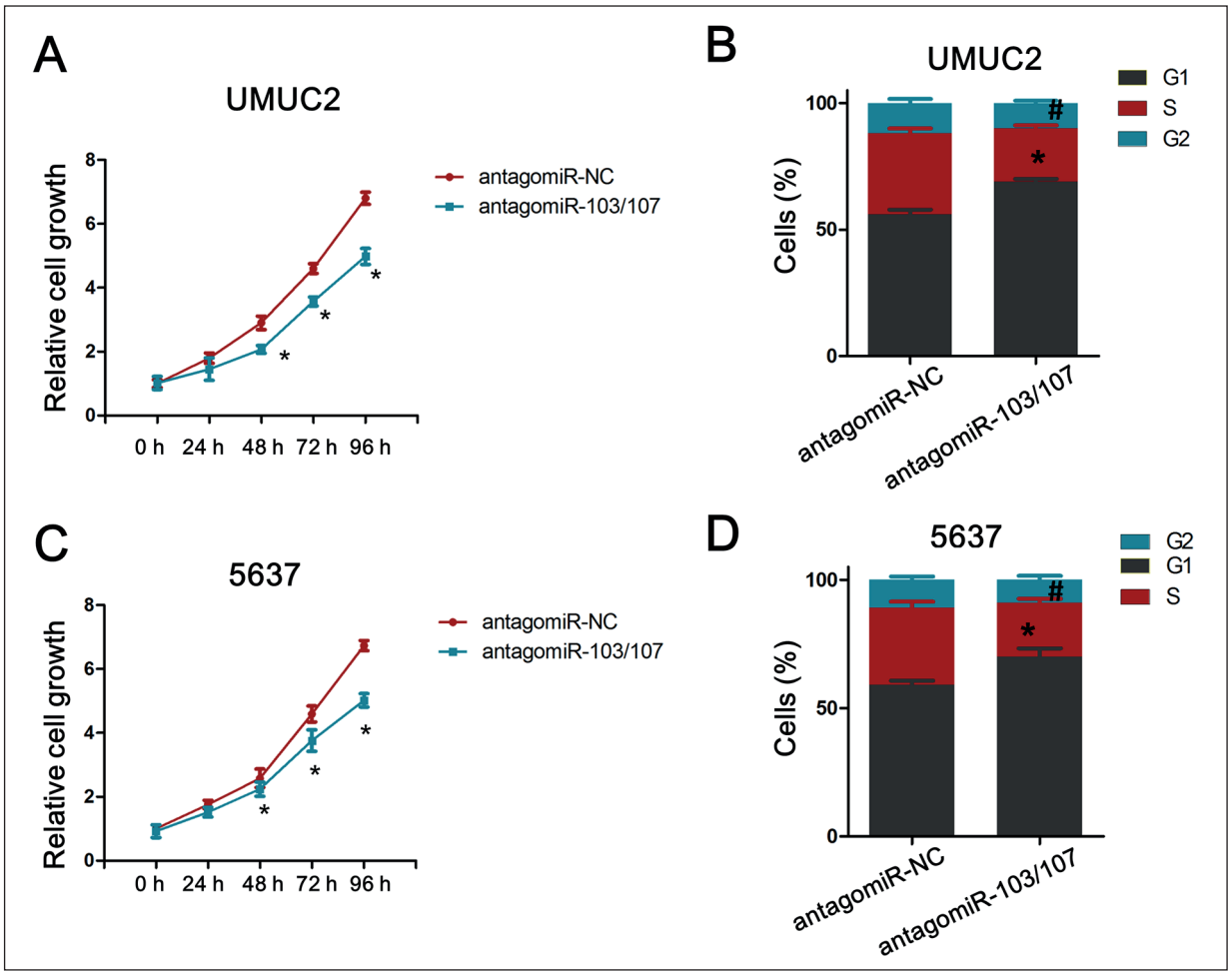

Figure 2. Inhibition of miR-103/107 expression in bladder cancer cells suppressed cell proliferation and induced cell cycle arrest. ***(A)*** The growth curve of UMUC2 cells after transfection of antagomiR-NC (negative control) or antagomiR-103/107. ***(B)*** The cell cycle distribution of UMUC2 cells after transfection of antagomiR-NC or antagomiR-103/107. ***(C)*** The growth curve of 5637 cells after transfection of antagomiR-NC or antagomiR-103/107. ***(D)*** The cell cycle distribution of 5637 cells after transfection of antagomiR-NC or antagomiR-103/107. *$p < 0.05$ *vs.* antagomiR-NC in (A) and ***(C)***; *$p < 0.05$ *vs.* antagomiR-NC G1 phase, $^{\#}p < 0.05$ *vs.* antagomiR-NC S phase in ***(B)*** and ***(D)***. NC, negative control.

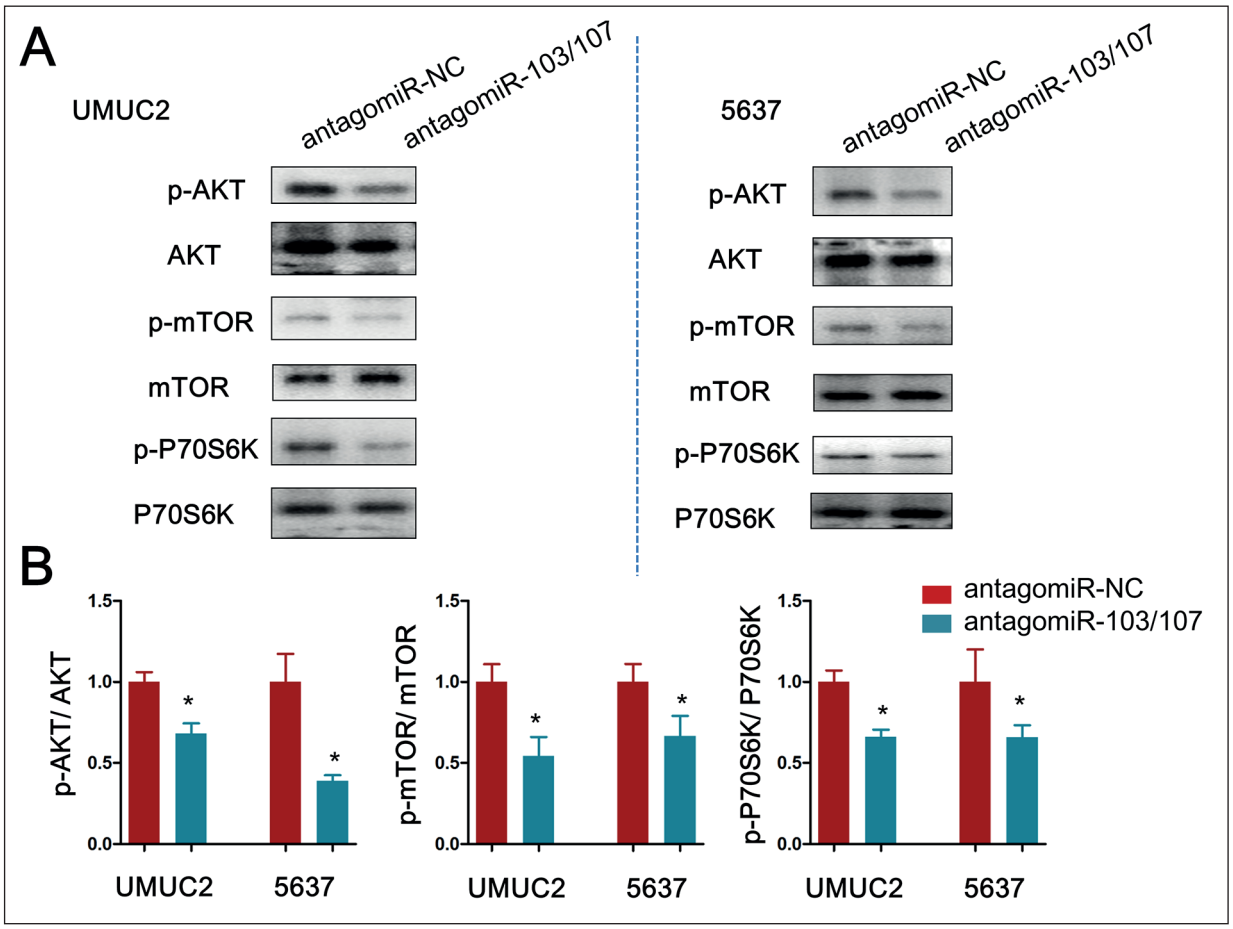

Figure 3. Inhibition of miR-103/107 expression in bladder cancer cells suppressed PI3K/AKT signaling. ***(A)*** The Western blot images for the effect of antagomiR-103/107 on AKT, mTOR and P70S6K phosphorylation in UMUC2 and 5637 cells. ***(B)*** The quantification data for the images shown in (A). *$p < 0.05$ *vs.* antagomiR-NC.

Inhibition of miR-103/107 Expression in Bladder Cancer Cells Suppressed PI3K/AKT Signaling

We next sought to determine the molecular mechanism of the action of miR-103/107 in bladder cancer cells. PI3K/AKT is central in regulating cell cycle and its activity may lead to increased tumorigenesis. Therefore, the activity of PI3K/AKT signaling was determined after transfection of antagomiR-103/107. Phosphorylation of AKT was significantly downregulated in UMUC2 and 5637 cells after miR-103/107 inhibition (Figure 3A). Phosphorylation of mTOR and p70S6K, which is controlled by AKT activity, showed a consistent downregulation (Figure 3A and B) in both cell lines. These data indicate that miR-103/107 modulates cell proliferation probably through the PI3K/AKT/mTOR cascade.

PTEN is a Direct Target of miR-103/107

To investigate the mechanism of miR-103/107 in direct regulation of PI3K/AKT signaling activity, a bioinformatics analysis was performed online (www.microrna.org). We found that miR-103 and miR-107, which have only one different base in their sequence, were predicted to bind with PTEN 3'UTR (Figure 4A). PTEN, which functions as a phosphatase, is a well-established negative regulator of PI3K/AKT signaling. In both cell lines, miR-103/107 significantly decreased luciferase reporter activity; this binding specificity was confirmed using a reporter with mutant 3'UTR (Figure 4B). Consistently, we observed that miR-103/107 downregulated PTEN protein in UMUC and 5637 cells (Figure 4C). More importantly, in tumor specimens, PTEN expression is negatively correlated with miR-103 or miR-107 (Figure 4D and E). Together, these data strongly

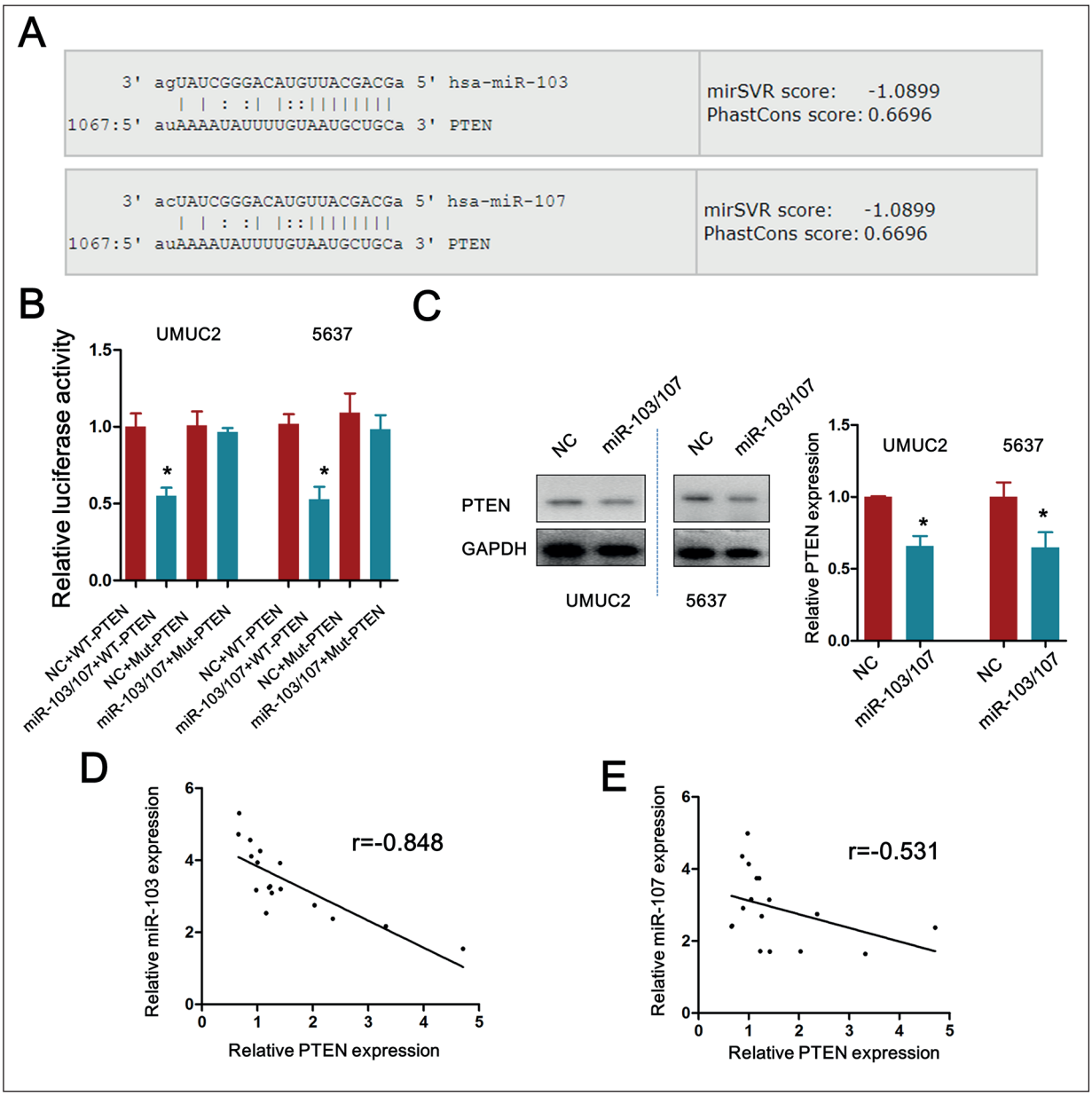

Figure 4. PTEN is a direct target of miR-103/107. ***(A)*** Illustration for the seed region-targeting site of miR-103 and miR-107 in the 3'UTR of PTEN mRNA. ***(B)*** The luciferase activity of wild type (WT) and mutant (Mut) reporter after transfection of miR-103/107 in UMUC2 and 5637 cells. ***(C)*** The effect of miR-103/107 on PTEN protein expression in UMUC2 and 5637 cells. ***(D)*** and ***(E)*** The correlation between expressions of PTEN and miR-103/miR-107 level in tumor specimens. *$p < 0.05$ *vs.* NC+WT-PTEN in (B); *$p < 0.05$ *vs.* NC in (C).

support that miR-103/107 family regulates cell proliferation and PI3K/AKT signaling activity through directly targeting PTEN.

Discussion

Understanding the molecular basis for the tumorigenicity of bladder cancer cells may be a challenging issue and will benefit for developing new drugs or therapeutic methods. Mimics or inhibitors of microRNAs can be drugable biological molecules to cure multiple diseases[14,15]. In this study, we report a novel microRNA family, miR-103/107, which functions as a promoting factor for bladder cancer cell proliferation. The expression of miR-103/107 is significantly up-regulated in bladder cancer tissues. Of note,

there is a positive correlation between tumor stages and microRNAs expression levels in cancer cells, suggesting its potential role in tumor progression. Notably, inhibition of miR-103/107 in bladder cancer cell lines significantly inhibited cell proliferation, which is likely to be caused by G1/S cell cycle arrest. On the molecular level, we found that PTEN is a direct target of miR-103/107. Forced knockdown of endogenous miR-103/107 by their antagomiRs could significantly suppress the downstream PI3K/AKT signaling. We demonstrated that miR-103/107 functions to promote tumorigenicity of bladder cancer cells, and that inhibition of its expression may be a feasible therapeutic method. MicroRNAs possess various roles in the development and progression of bladder cancers. Previous data have shown that they have been implicated in multiple fundamental cellular processes and pathways such as extracellular matrix (ECM)-receptor interaction, TGF-beta signaling pathway, ErbB signaling pathway, PI3K-Akt signaling pathway and MAPK signaling pathway[6]. In the present study, we were focusing on the role of miR-103/107 family for two reasons. First, the miR-103/107 expression has been shown to be greatly changed in bladder cancer tissues; second, miR-103/107 has been profoundly linked to multiple cellular processes and pathways especially the ones that regulate cell survival, such as autophagy, necrosis and ER stress in multiple contexts[11,16-18]. In colorectal cancer cells, miR-103/107 has been reported to promote metastasis by targeting DAPK and KLF4[12]. However, in gastric cancer, miR-103/107 is downregulated in drug resistant cancer cells, and overexpression of miR-103/107 diminished the chemoresistant phenotype[19]. Currently, no study has experimentally tested the consequence of miR-103/107 overexpression or inhibition in bladder cancer cells. To gain an accurate and novel insight into the role of miR-103/107 in bladder cancer, we studied its expression and function both *in vivo* and *in vitro*. We clearly showed that miR-103/107 is overexpressed in bladder cancers and functions as a promoting factor for cell proliferation and cell cycle progression. These findings are consistent with the previous ones showing the tumorigenicity of miR-103/107 family[12,20-22]. Our work has proposed a PI3K/AKT signaling pathway dependent mechanism, which is controlled by miR-103/107 in bladder cancer. PTEN has been identified as a novel target of this microRNA family. PTEN is a well-characterized tumor suppressor and basically serves as a phosphatase, which negatively regulates PI3K/AKT signaling. A considerable level of PTEN is required for normal cells since it is involved in cell cycle regulation and prevents cells from growing too rapidly[23]. Thus, suppression of PTEN might be a decent approach to treat cancers[24]. It has been shown that reduced expression of PTEN correlates with increased aggressiveness in bladder cancer[25]. The causal link for dysregulation of PTEN and carcinogenesis of urothelium has also been confirmed in animal model[26]. Previous investigations[27,28] have provided evidence of the regulation of PTEN by miR-103 in endometrial cancer and colorectal cancer. We reinforced these studies and demonstrated that inhibition of the PTEN suppressive factor miR-103/107 may exert favorable actions on bladder cancer.

Conclusions

We uncovered an oncomiR, miR-103/107, which is overexpressed in bladder cancer and targets the tumor suppressor PTEN to contribute to the tumorigenicity in bladder cancer cells. Inhibiting miR-103/107 may be a novel therapeutic approach for bladder cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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