

## RESEARCH ARTICLE

# MicroRNA-16 Inhibits Bladder Cancer Proliferation by Targeting Cyclin D1

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### Abstract

MicroRNA-16 (miR-16) has been demonstrated to regulate proliferation and apoptosis in many types of cancers, but its biological function in bladder cancer remains unknown. Here, we found expression of miR-16 to be downregulated in bladder cancer in comparison with the adjacent normal tissues. Enforced expression of miR-16 was able to inhibit cell proliferation in TCHu-1 cells, in line with results for miR-16 antisense oligonucleotides (antisense miR-16). At the molecular level, our results further revealed that cyclin D1 expression was negatively regulated by miR-16. Therefore, the data reported here demonstrate that miR-16 is an important regulator in bladder cancer, which will contribute to better understanding of important mis-regulated miRNAs.

**Keywords:** MicroRNA-16 - Cyclin D1 - bladder cancer - cell proliferation

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### Introduction

Bladder cancer (BC) is one of the most common urogenital cancers (Jacobs et al., 2010). Downregulation of certain tumor suppressor genes was confirmed to largely contribute to initiation, proliferation, invasion and metastasis of bladder cancer (Voutsinas et al., 2009). Therefore, targeted gene therapy has been documented as a reasonable strategy for bladder cancer treatment (Malmstrom et al., 2010). Moreover, many reports have confirmed its effectiveness and feasibility [4]. For example, it was shown that adenovirus delivery of human CD40 ligand gene confers direct therapeutic effects on carcinomas (Vardouli et al., 2009). Thus, identification of more genes responsible for the progression of bladder cancer will help to develop potential therapeutic targets for bladder cancer patients.

MicroRNAs (miRNAs) are small non-coding RNAs that play regulatory roles by inhibiting translation or cleaving RNA transcripts (Berezikov et al., 2011; Esteller et al., 2011; Pasquinelli et al., 2012). Numbers of studies have shown that miRNAs can function as either oncogenes or tumor suppressors, and the mis-regulation of specific miRNAs in diverse types of cancer is associated with cell proliferation, apoptosis, angiogenesis and metastasis (van Kouwenhove et al., 2011). Recent evidence suggests that miRNAs might also participate in treatment response across a range of therapies including radiation treatment and chemotherapy (O'Kelly et al., 2012).

Previous studies reported miR-16 plays important roles in several types of tumors. miR-16-down-regulated

transcripts were enriched with genes whose silencing by small interfering RNAs causes an accumulation of cells in G0/G1 phase (Linsley et al., 2007). Besides, systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes including CDK1 and CDK2 (Takeshita et al., 2010). However, whether miR-16 was involved in the development of bladder cancer remain unexplored.

### Materials and Methods

#### *Cell culture and tissue samples*

Bladder cancer cells (TCHu-1) were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Bladder tumor tissues and adjacent nontumor bladder tissues were collected from routine therapeutic surgery at our department. All samples were obtained with informed consent and approved by the hospital institutional review board.

#### *Analysis of miRNA expression using TaqMan RT-PCR*

Total RNA from tissue samples and cell lines was harvested using the miRNA Isolation Kit (Ambion, USA). Expression of mature miRNAs was assayed using Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-16. Briefly, 5 ng of total RNA were reverse transcribed to cDNA with specific stem-loop RT primers. Quantitative real-time PCR was performed by using Applied Biosystems 7300 Real-time PCR System and a TaqMan Universal PCR Master Mix. All the primers were

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obtained from the TaqMan miRNA Assays. Small nuclear U6 snRNA (Applied Biosystems) was used as an internal control.

#### Plasmid construction and transfection

For miR-16 expression plasmid, human miR-16 precursor was cloned into pSilencer 4.1 (Ambion, Austin, TX). The negative control plasmid consists of a scrambled sequence (Invitrogen). To inhibit miR-16 function, an Ambion miRNA inhibitor for miR-16 was used, along with the negative control. For transfection, a complex of Lipofectamine 2000 (Invitrogen, CA, USA) and 50 nM miRs mentioned above was prepared following the manufacturer's instructions. To get the stable cell lines, transfected cells were selected with puromycin 40 h after transfection and then diluted to perform clonal selection. Mature miRNA expression in selected clones was assessed by Real-time PCR as described above.

#### BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA)

#### Flow cytometric assays for cell cycle and apoptosis

To assess the distribution of nuclear DNA content, cells were collected, rinsed and fixed overnight in 75% cold ethanol at -20°C. Then, cells were treated with Tris-HCl buffer (pH 7.4) supplemented with 100 lg/mL RNase A and stained with 25 lg/mL propidium iodide (BD Biosciences, San Diego, CA). Cell cycle distribution was determined by flow cytometry (Becton Dickinson, San Jose, CA). Ten thousands cells were acquired and analyzed for DNA content. All data were collected, stored, and analyzed by ModFit software.

#### Western blotting

Cells or tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 20000× g for 10 min at 4 °C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-Cyclin D1 antibodies were purchased from Cell signaling (USA). Protein levels were normalized to total GAPDH, using a rabbit anti-GAPDH antibody (Santa Cruz, USA).

#### Luciferase reporter assay

Total cDNA from TCHu-1 cells was used to amplify the 3'UTR of Cyclin D1 by PCR. The Cyclin D1 3'UTR

was cloned into the Hind III and Xho I sites of pMir-Report (Ambion), yielding pMir-Report-Cyclin D1. Mutations were introduced in potential miR-16 binding sites using the QuikChange site-directed mutagenesis Kit (Stratagene). Cells were transfected with the pMir-Report vectors containing the 3'-UTR variants, and miR-16 precursor, miR-16-inhibitor or control plasmids for 36 hours. The pRL-TK vector (Promega, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

#### Statistical analysis

Data are expressed as the mean±SEM from at least three separate experiments. Differences between groups were analyzed using Student's t-test. A value of  $p < 0.05$  was considered statistically significant.

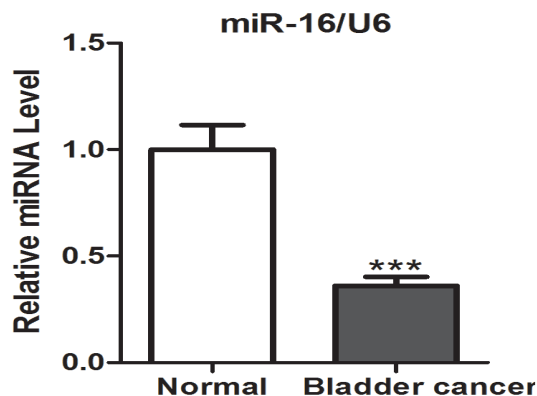
## Results

### miR-16 expression levels were down-regulated in patients with bladder cancer

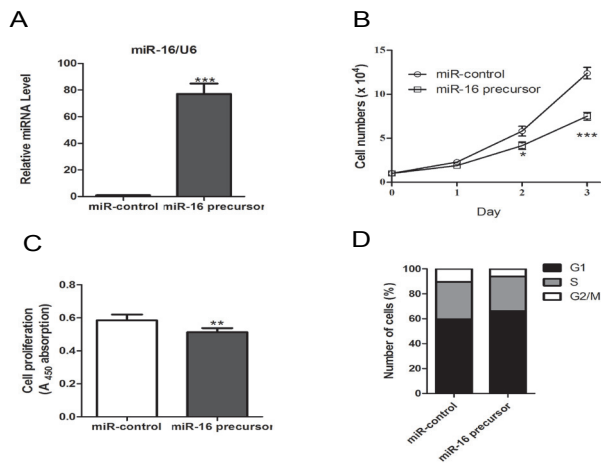
Firstly, to examine whether the miR-16 is differentially expressed in human primary gastric cancers, its expression level was determined using TaqMan real-time PCR in 40 pairs of human bladder cancer tissues and pair-matched adjacent noncancerous bladder tissues. Our results demonstrated that the expression level of miR-16 was significantly reduced in bladder cancer tissues in comparison with the adjacent noncancerous bladder tissues (Figure 1).

### The effects of miR-16 overexpression on cell growth

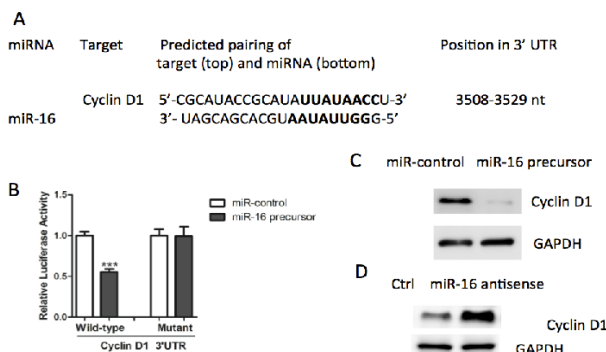
In order to assess the effects of miR-16 on bladder cancer cell growth, the miR-16 precursor was transfected into TCHu-1 cells and cell growth at various post-transfection time points was examined. miR-16 precursor was found to up-regulate miR-16 expression (Figure 2A) and significantly inhibited proliferation in cells post-transfection (Figure 2B and 2C). MiR-16-overexpressing cells had a significantly higher percentage of cells in the G1/G0 phase and decreased percentage of cells in the



**Figure 1. Expression Levels of miR-16 in Bladder Cancer Tissues.** miR-16 expression was determined by TaqMan real-time PCR in 40 pairs of human bladder cancer tissues and pair-matched adjacent noncancerous bladder tissues (Normal)



**Figure 2. Overexpression of miR-16 Inhibits Bladder Cancer Cell Proliferation.** (A) Expression of miR-16 was determined in TCHu-1 cells after miR-16 precursor transfection compared to controls. (B) The growth curve of TCHu-1 cells after miR-16 precursor transfection compared to controls. (C) The cell proliferative potential (BrdU) was determined in TCHu-1 cells transfected with miR-16 precursor or negative control (Ctrl). A450 absorption was assayed after transfection for 24 hr. (D) The cell cycle distribution was determined in TCHu-1 cells transfected with miR-16 precursor or negative control (Ctrl)



**Figure 4. miR-16 Negatively Regulates Cyclin D1 Expression in Bladder Cancer Cells.** (A) Computer prediction of miR-16 binding sites in the 3'UTRs of human Cyclin D1 genes. Potential binding site was highlighted in bold. (B) Luciferase reporter assays in TCHu-1 cells. Cells were transfected with 200 ng of wild-type 3'-UTR-reporter or mutant constructs together with 100 nM of miR-16 precursor or controls. (C) Western blotting analysis of Cyclin D1 in TCHu-1 cells transfected with miR-16 precursor or negative control (Ctrl)

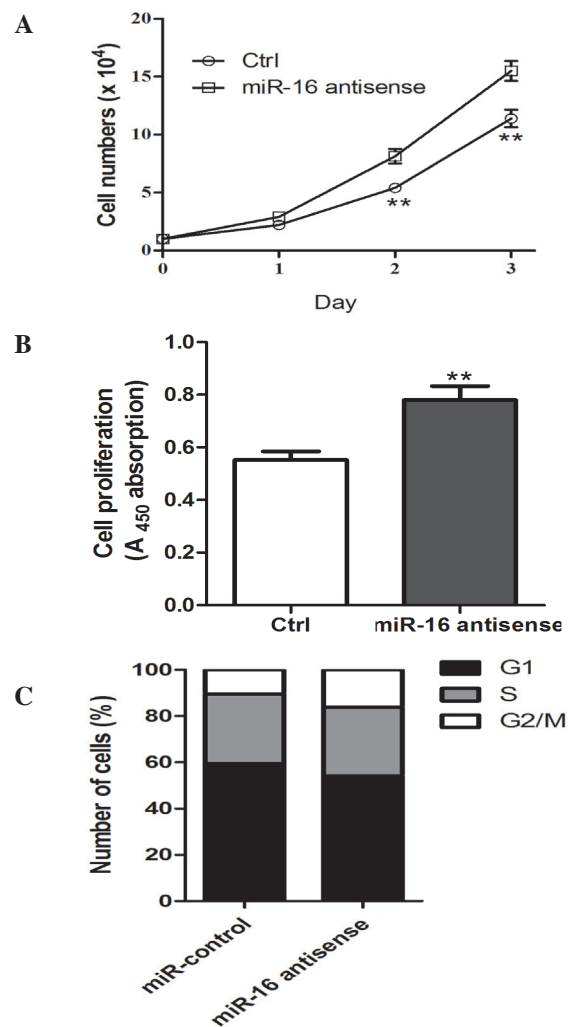
M phase, compared to negative control-transfected cells (Figure 2D).

#### *Inhibition of miR-16 promotes the proliferation of bladder cancer cells*

As described above, miR-16 plays a critical role in the proliferation of bladder cancer cells. However, it remained unknown whether inhibiting miR-16 would reduce cell proliferation. Therefore, TCHu-1 cells were transfected with miR-16 antisense. We showed that ectopic expression of the hsa-miR-16 antisense reduced the growth of TCHu-1 cancer cells, compared to NC-transfected cells (Figure 3A-C).

#### *miR-16 directly targets the Cyclin D1 in bladder cancer cells*

Using a stringent bioinformatics approach, we



**Figure 3. miR-16 Antisense Promotes the Proliferation of Bladder Cancer Cells.** (A) The growth curve of TCHu-1 cells after miR-16 antisense transfection compared to negative control (Ctrl). (B) The cell proliferative potential (BrdU) was determined in TCHu-1 cells transfected with miR-16 antisense or negative control (Ctrl). A450 absorption was assayed after transfection for 24 hr. (C) The cell cycle distribution was determined in TCHu-1 cells transfected with miR-16 antisense or negative control (Ctrl)

identified 16 putative human miR-16 target genes (data not shown), among which the gene encoding Cyclin D1 harbored a potential miR-16 binding site (Figure 4A). Overexpression of miR-16 led to a reduction of luciferase activity when the reporter construct contained the Cyclin D1 3'UTR (Figure 4B). In contrast, mutation of the conserved miR-16 binding motif abrogated the reduced luciferase expression (Figure 4B). Moreover, overexpression of miR-16 in TCHu-1 cells led to reduced Cyclin D1 protein expression (Figure 4C). Consistently, inhibition of miR-16 led to an increased expression of Cyclin D1 contents (Figure 4D), further indicating that Cyclin D1 is a target of miR-16 in bladder cancer cells.

## Discussion

In this study, we demonstrated that miR-16 expression is upregulated in gastric cancer tissues. Upregulation of miR-16 inhibited the G1/S cell cycle transition in gastric

cancer cells while inhibition of miR-16 promoted bladder cancer cell proliferation. At the molecular level, for the first time, we identified that miR-16 regulated Cyclin D1 expression through targeting its 3'UTR. Collectively, these findings suggest that downregulation of miR-16 may promote the initiation and progression of bladder cancer.

Previous studies have demonstrated that several miRNAs were mis-regulated in bladder cancer tissues (Dip et al., 2012; Majid et al., 2012; Wang et al., 2012; Noguchi et al., 2013; Shimizu et al., 2013). It was reported that a miR-21:miR-205 expression ratio that has the ability to distinguish between invasive and noninvasive bladder tumors with high sensitivity and specificity (Neely et al., 2010). Besides, miR-708 may act as an oncogene and induce the carcinogenicity of bladder cancer by down-regulating Caspase-2 level (Song et al., 2013). Moreover, expression of miR-101 is down-regulated in human bladder cancer tissue versus normal adjacent tissue (Hu et al., 2013). miR-101 was considered as a novel suppressor of bladder cancer cell migration and invasion through its negative regulation of c-Met (Hu et al., 2013). Therefore, further studies should be performed to investigate more mis-regulated miRNAs in the progression of bladder cancer.

The importance of miRNAs like miR-16 as tumor suppressors is becoming increasingly clear. The locus corresponding to miR-15a and miR-16-1 at 13q14 is deleted in more than half of B-cell chronic lymphocytic leukemias (Zhu et al., 2011). The undelleted miR-15a-miR-16-1 allele in chronic lymphocytic leukemia patients may also contain inactivating point mutations (Hanlon et al., 2009). These observations have led to the proposal that miR-15a and miR-16-1 may behave as tumor suppressors. Moreover, it was reported that the induction of microRNA-16 in colon cancer cells by protein arginine deiminase inhibition causes a p53-dependent cell cycle arrest (Zhang et al., 2010). MicroRNA-16 represses colorectal cancer cell growth in vitro by regulating the p53/survivin signaling pathway (Ma et al., 2013). In addition, miR-16 gain resulted in reduced angiogenesis and tumor growth in vivo (Sun et al., 2013). At the molecular level, miR-16 directly interacts with VEGF mRNA at the 3'-untranslated region and that the regulation of VEGF by miR-16 occurs at the translational level [24].

In summary, the key finding of the current study is that miR-16 can inhibit the proliferation of bladder cancer cell lines by targeting Cyclin D1. This data indicates that miR-16 plays an essential role in the regulation of bladder cancer cell proliferation and may function as a tumor suppressor. Understanding the precise role played by miR-16 progression will not only advance our knowledge of bladder cancer biology, but also will help determine if miR-16 has potential as a novel therapeutic target for the treatment of bladder cancer.

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