

Original Article

C-myc-regulated miR-106b promotes proliferation of human bladder cancer cells by targeting DAPK2

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Abstract: Bladder cancer is a common cancer in the worldwide, identifying biomarkers that could improve diagnostic and/or prognostic predictions is of great importance. In the present study, we aimed to investigate the relationship and the roles of c-myc and miR-106b in the development of human bladder cancer cells. ShRNA was transfected into bladder cancer cells line to interfere the expression of c-myc. Real-time polymerase chain reaction (RT-PCR) and Western blot were performed to determine the expression level of miR-106b and DAPK2 at mRNA and protein level. Then MTT and flow cytometry were respectively used to explore the proliferation and apoptosis in bladder cancer cells line in vitro. We found that c-myc influenced the expression of miR-106b and DAPK2. Additionally, DAPK2 was confirmed the direct target gene of miR-106b. Finally, the apoptosis and proliferation results illustrated the inhibition of miR-106b inhibits cell proliferation and induces cell apoptosis via down-regulating or up-regulating the DAPK2 expression. All these findings suggest that c-myc may be involved in progression of bladder cancer and could be a new therapeutic target for this disease.

Keywords: Bladder cancer, c-myc, DAPK2, miR-106b, cell proliferation

Introduction

Bladder cancer is one of the most common malignancies in developed countries, ranking as the sixth most frequent neoplasm [1, 2]. Also, with high mortality, bladder cancer is regarded as the second most frequent malignancy of the urinary tract after prostate cancer [3].

MicroRNAs (miRNAs) are single-stranded RNAs of 19 to 25 nt length, which mostly bind to the 3' untranslated regions (UTR) of classical protein coding genes and inhibit the target gene expression either by degrading the respective mRNA or by inhibiting its translation [4-7]. It was reported regulates protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner, and plays an important role in regulating various cellular activities [8, 9].

There has been accumulative evidence that miRNAs play essential roles in the tumorigenesis and progression of bladder cancer [10]. Xu X

et al, investigated the roles of miR-124-3p in human bladder cancer and proved that miR-124-3p can repress the migration and invasion of bladder cancer cells, indicating that miR-124-3p could be a tumor suppressor and may be a potential diagnostics or predictive biomarker in bladder cancer [11]. Moreover, it has been reported that miR-409-3p is down-regulated in human bladder cancer cell lines and the overexpression of miR-409-3p significantly reduced the migration and invasion of bladder cancer cells [12].

C-myc gene is frequently amplified in a number of human cancers, including breast, lung, prostate, and gastric cancer, as well as urinary bladder cancer [13-15]. Additionally, miR-106b expression is upregulated in human bladder cancer [16]. However, the molecular mechanism by which c-myc and miR-106b collaborate to contribute to the development of bladder cancer is still unknown.

Death-associated protein kinase 2 (DAPK2) is a calcium/calmodulin-regulated proapoptotic

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serine/threonine kinase that acts as a tumor suppressor, which can mediate a wide range of cellular processes, including apoptosis and autophagy [17, 18].

In this study we aimed to investigate the relationship and the roles of c-myc and miR-106b in the development of human bladder cancer cells. We sought to explore the expression of miR-106 after the silence of c-myc by sh-RNA, and then western blotting assay was used to detect the expression of DAPK2 levels. Moreover, to confirm the relationship between miR-106b and DAPK2, we silenced the miR-106 by miRNA interference, and monitoring the expression of DAPK2 by mRNA and protein levels. Finally, MTT assay and flow cytometric analysis were separately performed to determine the cell proliferation and apoptosis. The objective of our study was to evaluate the correlation of c-myc and miR-106b expression with bladder cancer cell lines, as well as its underlying mechanism. All of our efforts will provide theoretical basis and new insights into the treatment of bladder cancer.

Materials and methods

MiRNA precursors

Hsa-miR-precursors were purchased from ThermoFisher.

Cell culture

Human bladder cancer T24 cells were grown in RPMI-1640 supplemented with 10% FBS and penicillin at 37°C, 5% CO₂ in a humidified incubator [19].

MTT assay

100 µL of cells was taken from each of the culture conditions and placed in a 96-well plate. Fifty micrograms of MTT was added to each well and this mix was incubated for 4 hours at 37°C. 100 µL of 0.04 N HCl in 2-propanol was mixed thoroughly into each well before the incubation was ended. The results of each plates were read on a Molecular Devices microplate reader (Sunnyvale, CA) at a wavelength of 570 nm, with a background reading at 650 nm subtracted.

MicroRNA transfection

Synthetic miR-106b mimics and scrambled negative control RNA (control mimic and control

inhibitor) were purchased from Genepharma (Shanghai, China). Cells were seeded in 6-well plates and were transfected with Lipofectamine 2000 (Invitrogen) on the following day when the cells were approximately 70% confluent. 100 pmol of miR-106b mimic, the scrambled negative control RNAs were used in each well [20].

Stable expression of DAPK2

Full-length c-myc was cloned into the retroviral pBABE vector backbone. Retroviruses were generated from the 293T after transfection with pBABE. T24 cells were infected with virus containing BNIP3. 48 hours after infection, the cells were selected using puromycin (2 µg/ml) for 10 days and then used for the experiments as described [21].

Lentivirus-mediated stable c-myc or DAPK2 knockdown

The third-generation lentiviral packaging system was used for stable genetic alteration. The lentiviral plasmid carrying the shRNAs against c-myc or DAPK2 was publicly available (Santa Cruz Biotechnology, Santa Cruz, CA), as was for the control pLKO.1. sh. scramble plasmid (No. 1864; Addgene, Cambridge, MA).

HEK293T cells were cultured to 80% confluence in 10-cm Petri dishes. The cells were transiently transfected with 16 µg of plasmid DNA using Lipofectamine 2000 (Life Technologies). The virus was collected 48, 72, and 96 h after transfection and concentrated via ultracentrifugation using ultraspeed centrifuge (Beckman Coulter, Brea, CA). Each virus production was concentrated in 100 µL PBS and stored at -80°C [22].

Western blot analysis

The cells were washed twice with PBS and then lysed with 1× SDS-loading buffer as the whole-cell sample. The protein samples were subjected to SDS-PAGE gel electrophoresis. Immunoblottings were carried out with primary antibodies and the proteins were detected by enhanced chemiluminescence (ECL-plus, Amersham Pharmacia Biotech).

Apoptosis assay by Annexin V-FITC/PI-staining

Cells were stained using the FITC Annexin V/Dead Cell Apoptosis Kit (V13242, Invitrogen)

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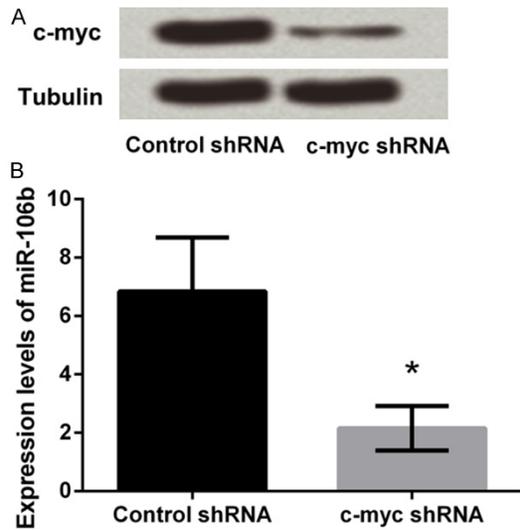


Figure 1. Correlation of c-myc and miR-106b expression. A. C-myc-shRNA down-regulates c-myc expression by Western blot assay. B. C-myc influence expression of miR-106b. * $P < 0.05$.

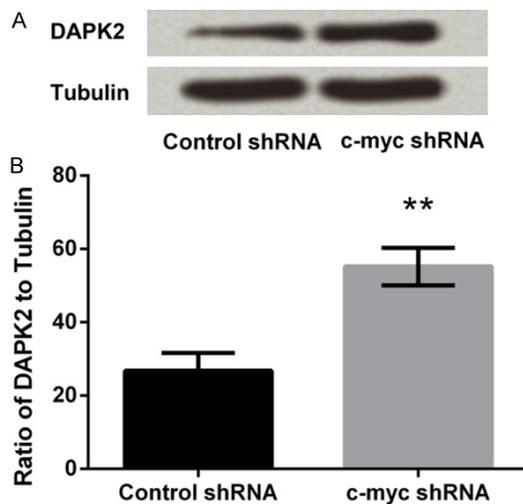


Figure 2. Correlation of c-myc and DAPK2 expression. A. C-myc-shRNA up-regulates DAPK2 expression by Western blot assay. B. C-myc influences ratio of DAPK2 to Tubulin. ** $P < 0.01$.

according to the manufacturer's instructions. Stained cells were diluted in Annexin V-binding buffer (Invitrogen). Suspended cells were used to perform flow cytometry. Annexin V-FITC/PI-stained cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Data was analyzed using FlowJo 10.0.7 software (Treestar Inc, Ashland, US) [23].

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, NY). RNA (500 ng) was polyadenylated and reverse transcribed to cDNA using an NCode miRNA First-Strand cDNA synthesis kit (Invitrogen). cDNA was used as the template for real-time PCR FastStart Universal SYBR green Master (Roche) with the universal reverse primer provided in the kit. Real-time PCR was performed on Applied Biosystems real-time detection system (Applied Biosystems), and the thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 s followed by 60°C for 30 s. Each sample was run in triplicate and was normalized to U6 snRNA levels. (U6 primers 5'-CTTCGGCAGCACATATACT-3' and 5'-AAAATATGGAA-CGCTTCACG-3') [24].

Results

C-myc regulates the expression of miR-106b in human bladder cancer T24 cells

ShRNA-mediated silencing of c-myc was performed as described in Materials and Methods. The expression of c-myc levels was determined by Western blotting (Figure 1A). Real-time PCR was conducted to examine the expression levels of miR-106b in control and c-myc knockdown cells (Figure 1B). We can read from the results shown in Figure 1 that silence of c-myc inhibits the expression of miR-106. Thus, c-myc regulates the expression of miR-106.

C-myc knockdown upregulates the expression of DAPK2 in human bladder cancer T24 cells

The expression of DAPK2 levels was determined by Western blotting in control and c-myc knockdown cells (Figure 2A). Quantitative data from triplicate samples were plotted at the same time (Figure 2B). The results showed that the knockdown of c-myc promotes the expression of DAPK2 in human bladder cancer cells.

DAPK2 is a direct target of miR-106b in human bladder cancer T24 cells

A diagram showed miR-106b that forms base-pair with the 3'UTR of DAPK2 (Figure 3A). Then miR-106b and 3'UTR of DAPK2 were co-transfected into T24 cells and luciferase activities

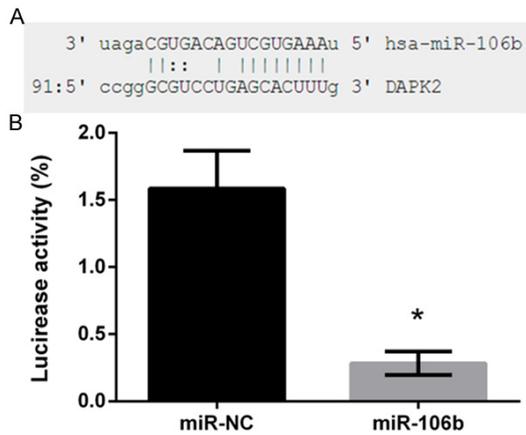


Figure 3. Correlation of miR-106b and DAPK2. A. Base sequence of miR-106b and DAPK2. B. Luciferase activities decreased in the miR-106b group then in miR-NC group. * $P < 0.05$.

were then measured as described in the materials and methods (Figure 3B). We can conclude that the activity of miR-106b directly influence DAPK2, thus DAPK2 is a direct target gene of miR-106b.

MiR-106b modulates the expression of DAPK2 in human bladder cancer T24 cells

To investigate the correlation of miR-106b and DAPK2, miR-106b mimics or inhibitors were transfected into T24 cells (Figure 4A). 72 h after the transfection, the cell were harvested to determine the expression levels of DAPK2 (Figure 4B and 4C). In line with our forecast, transfection of miR-106b mimics promotes expression of miR-106b, while transfection of inhibitors downregulates the miR-106b expression. As the results shown in Figure 4B and 4C, we can confirm that miR-106b negatively regulates the expression of DAPK2.

Overexpression of miR-106b inhibitor induces apoptosis of human bladder cancer T24 cells via upregulating the expression of DAPK2

MiR-106b mimics or inhibitors were transfected into T24 cells. 72 h after the transfection, MTT assay was performed to determine the cell proliferation (Figure 5A). The stable expression of DAPK2 or shRNA-mediated silencing of DAPK2 was performed as described in the Materials and Methods. MiR-106b mimics or inhibitors were then transfected into the cells (Figure 5B). 72 h after the transfection, flow

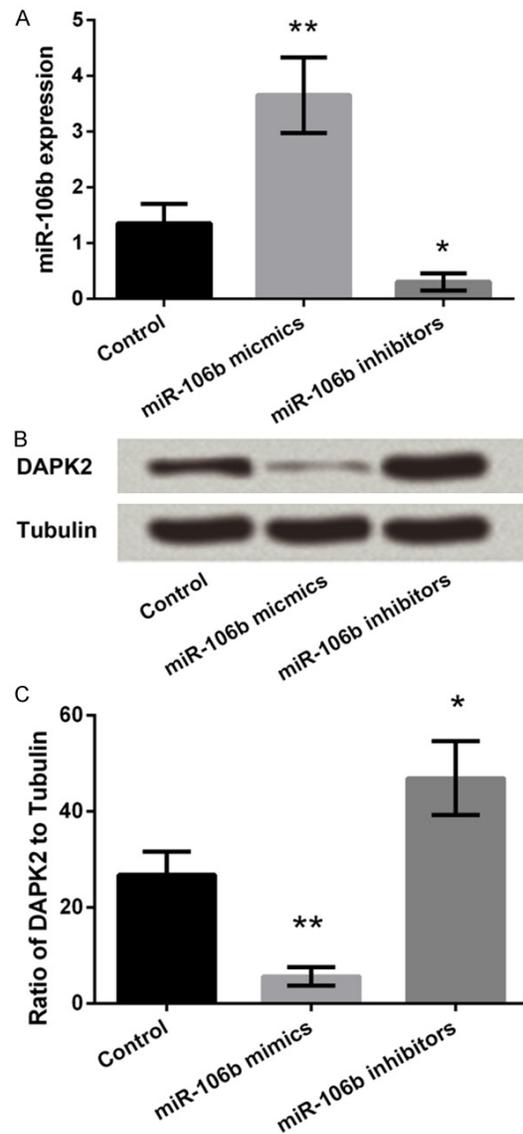


Figure 4. Effect of miR-106b on DAPK2. A. The expression level of miR-106b in mRNA level. B. The expression level of miR-106b analyzed by Western blot. C. MiR-106b influences ratio of DAPK2 to Tubulin. * $P < 0.05$, ** $P < 0.01$.

cytometric analysis was performed to measure apoptosis (Figure 5C and 5D). We can read from Figure 5 that miR-106b promotes cell proliferation and inhibits cell apoptosis via down-regulating or up-regulating the DAPK2 expression.

Discussion

Increasing evidence has suggested that miRNAs can modulate the expression of specific genes and participate in the initiation and pro-

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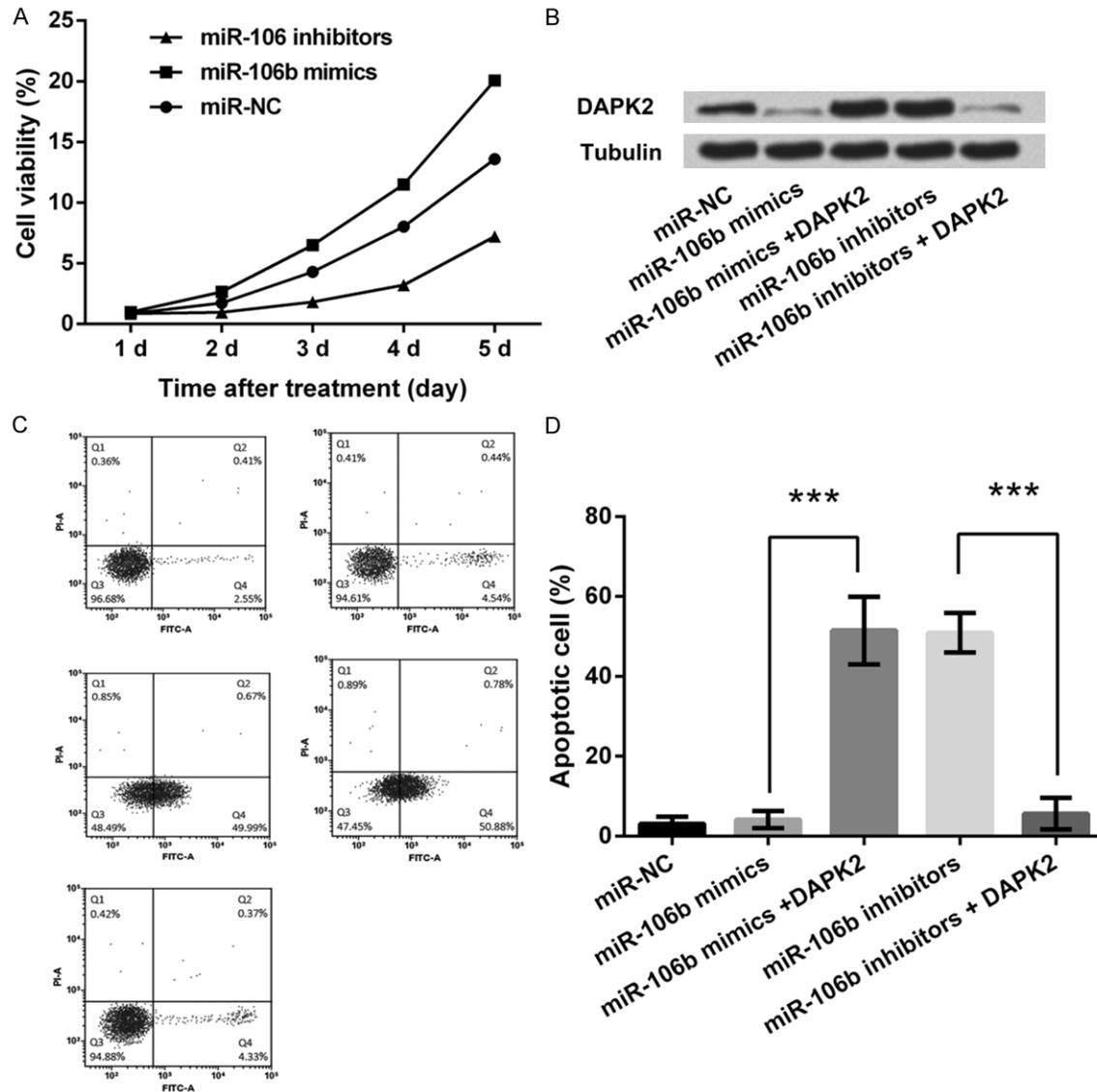


Figure 5. Effect of miR-106b on cell proliferation and apoptosis. A. The proliferation of cancer cells was analyzed by MTT. B. DAPK2 expression levels were analyzed by Western blot. C, D. The apoptosis rates of bladder cancer cells analyzed by flow cytometry. *** $P < 0.001$.

gression of multiple diseases and dysregulation of certain miRNAs may contribute to human disease including carcinogenesis and tumor metastasis in human [25, 26].

Bladder cancer is one of the leading cancers of the urinary tract, so far, no specific and effective early detection biomarkers are available [27]. MiRNAs expression profile of bladder cancer patient has been regarded as a promising tool that can serve as biomarker [28]. In the study of Xiao et al, miR-126 was confirmed negatively regulated the target gene PIK3R2 and

further inhibited the PI3K/Akt signaling pathway, thereby inhibiting proliferation, migration, and invasion and promoting apoptosis in bladder cancer cells [29]. Additionally, studies by Jin et al has told that miR-106b participates in the bladder cancer pathway by targeting E2F transcription factor 1 [30].

Moreover, researches have revealed c-myc closely involved in bladder cancer progression [31, 32]. The study of Li Y et al, has confirmed that the expression of c-myc RNA and protein was significantly higher in bladder cancer sam-

ples in comparison to normal bladder tissue and the Lin28/let-7a/c-myc pathway plays an important role in non-muscle invasive bladder cancer [33]. However, the correlation of c-myc and miR-106b and the underlying mechanism has not been clearly understood. So in this study, shRNA was transfected into bladder cancer cells line to interfere the expression of c-myc. And the results shown that silence of c-myc inhibits the expression of miR-106b. Our data suggested that c-myc functions as a transcription factor to regulate the expression of miR-106b in human urinary cancer T24 cells.

DAPK2 belongs to a family of five related serine/threonine kinases which mediate a range of cellular processes, including membrane blebbing, apoptosis, and autophagy, and possess tumor suppressive functions [34]. It has been widely studied in many kinds of biological progress, including tumors [35]. In this study, we aimed to make clear of the role of DAPK2 in bladder cancer and find miR-106b directly target DAPK2 and negatively regulates the expression of the DAPK2 in bladder cancer. And the cell proliferation and apoptosis data proved that miR-106b promotes cell proliferation and inhibits cell apoptosis via down-regulating or up-regulating the DAPK2 expression.

Taken together, our study found that c-myc could regulate the expression of miR-106b and the suppression of miR-106b expression could inhibit cell proliferation, and promote apoptosis in bladder cancer cells via targeting DAPK2. All these findings suggest that miR-106b may be involved in progression of bladder cancer and could be a new therapeutic target for this disease.

Disclosure of conflict of interest

None.

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