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MiR-302b regulates cell functions and acts as a potential biomarker to predict recurrence in bladder cancer

Zuwei Li[a,b,c,1], Liang Zhou[a,c,d,1], Canbin Lin[a,b,c], Xiang Pan[a,c], Jiansen Xie[b], Liwen Zhao[a,c], Jing Quan[a,c], Jinling Xu[a], Xin Guan[a], Weijie Xu[a], Hang Li[a], Yun Chen[e,**], Yongqing Lai[a,c,*]

[a] Department of Urology, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036, PR China
[b] Department of Urology, Shantou University Medical College, Shantou, Guangdong 515041, PR China
[c] The Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Peking University Shenzhen Hospital, Institute of Urology of Shenzhen PKU-HKUST Medical Center, Shenzhen, Guangdong 518036, PR China
[d] Department of Urology, Guangzhou Medical University, Guangzhou, Guangdong 511436, PR China
[e] Department of Ultrasound, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036, PR China

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ABSTRACT

Background: Bladder cancer is the most common urogenital tumor with substantial morbidity, high recurrence rate and mortality. miRNAs, a class of endogenous noncoding RNA, were found to involve in the genesis, maintenance and metastasis of cancer. Genomic profiling revealed that miR-302b is down-regulated in bladder cancer while its functions in bladder cancer remain to be ascertained.

Methods: Cell functional assays including wound healing assay, CCK-8 assay, Transwell assay and flow cytometry assay were performed to clarify the functions of miR-302b expression in cell proliferation, migration, invasion and apoptosis in BC. Furthermore, RT-qPCR was performed to study the expression of miR-302b in bladder cancer tissues and the prognostic value of altered miR-302b expression with 48 formalin-fixed paraffin-embedded bladder urothelial carcinoma samples.

Results: The results of RT-qPCR demonstrated that expression level of miR-302b was significantly reduced in bladder cancer tissues and cell lines. The cells after transfected with miR-302b mimic showed lower mobility, lower proliferation and increased apoptosis, while opposite results were obtained after inhibiting the expression of miR-302b. The prognosis analysis demonstrated that the patients with low expression of miR-302b experienced high risks of recurrence.

Conclusions: The results of our study demonstrate that miR-302b regulates cell functions and acts as a potential biomarker to predict recurrence in bladder cancer.

1. Introduction

Bladder cancer (BC), ranking the first in urogenital malignant tumor incidence and the ninth most common malignancy worldwide, is characterized by high morbidity and mortality rates with estimated 429,800 new cases and 165,100 deaths in 2012 worldwide [1–3]. Established risk factors for the genesis of BC include genetic and molecular abnormalities, chronic stimulation and chemical or environmental exposures [4]. Roughly 75% of BC patients are non-muscle-invasive BC and 60%–70% of non-muscle-invasive BC patients will experience recurrence [1, 5]. Therefore, it is significant to explore a novel molecular biomarker for early diagnosis, treatment and recurrence prediction of BC.

MicroRNAs(miRNAs), short and endogenous noncoding RNA with roughly 19–24 nucleotides in length, regulate the gene expression by base-pairing with the 3′ untranslated region (UTR) of target mRNAs, resulting in repression of the target Genes or inhibition of protein synthesis [6,7]. More than a thousand miRNAs were found in the human genome and one miRNA is probably involved in regulating hundreds of mRNAs [7–10]. In recent years, there has been a growing emphasis on the role of miRNAs in the early diagnosis, treatment and recurrence prediction of cancer. MiRNAs play a significant role in regulating molecular pathways associated with angiogenesis, the epithelial–mesenchymal transition, cancer-stem-cell biology, metastasis

* Correspondence to: Y. Lai, Department of Urology, Peking University Shenzhen Hospital, 1120 Lianhua Road, Shenzhen, Guangdong 518036, PR China.
** Correspondence to: Y. Chen, Department of Ultrasound, Peking University Shenzhen Hospital, 1120 Lianhua Road, Shenzhen, Guangdong 518036, PR China.
E-mail addresses: cyun126@126.com (Y. Chen), yqlord@163.com (Y. Lai).
[1] Contributed equally.

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and drug resistance in numerous type of tumors [8,11].

MiR-302b, one of the miR-302/367 cluster that is vertebrate-specific and highly conserved, is located at 4q25 chromosome region and serves as a tumor suppressor in ovarian cancer, hepatocellular carcinoma, pleural mesothelioma and gastric cancer [12–16]. MiR-302b had been reported to be down-regulated in a genomic profiling of miRNAs in BC [17]. However, the expression of miR-302b in BC has been not yet ascertained by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and the role of miR-302b in BC has not been clarified so far. In our study, RT-qPCR was performed to study the expression of miR-302b in BC and the cell functions were also evaluated by wound healing assay, CCK-8 assay, Transwell assay and flow cytometry assay. Moreover, the significance of miR-302b in the recurrence of BC was analyzed with 48 FFPE bladder urothelial carcinoma samples.

2. Materials and methods

2.1. Human tissue samples collection

A total of 39 paired urothelial carcinoma and adjacent non-carcinoma bladder tissues (3 cm far away from the BC tissue) were collected from patients who had received surgical bladder resection at Peking University Shenzhen Hospital (Shenzhen, China). None of the patients received any pre-operative chemotherapy or radiotherapy. The study was approved by the Ethics Committees of Peking University Shenzhen Hospital and Informed consents were obtained from the 39 patients. Once collected, all tissue samples were immediately submerged in RNAlater® RNA Stabilization Agent (Qiagen, Hilden, Germany) and frozen in liquid nitrogen afterwards until RNA extraction.

2.2. Cell culture and cell transfection

The normal transitional epithelial cell (SV-HUC-1) and BC cell lines (RT4, J82, UM-UC-3, 5637 and T24), purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific. Inc., Waltham, MA, USA), which is added with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 1% antibiotics (100 μl/ml penicillin and 100 mg/ml streptomycin sulfates) and 1% glutamine. All cells were maintained in 37 °C in a 5% CO_2 incubator. For transfection, 5637 and UM-UC-3 cells were sown in a 6-well plate and transfected with 100 pmol miR-302b mimic (F: 5′-ACUUUAACAUGGAAGUGCUUUC-3′; R: 5′-UAAGUGCUUCCAUGUUUUAGUAG-3′), inhibitor(5′-GAAAGCACUUCCAUGUUAAAGU-3′), negative control(NC, F:5′-UCACAACCUCCUAGAAAGAGUAGA-3′; R: 5′-UCACAACCUCCUAGAAAGAGUAGA-3′), inhibitor NC(5′-UCUACUCUUUCUAGGAGGUUGUGA-3′) when they are at 60%–70% confluence by using Lipofectamine 3000 (Invitrogen Life Technologies). Cells were transfected for 4–6 h at 37 °C. The transfection efficiency was authenticated by RT-qPCR.

2.3. RNA extraction, cDNA synthesis and RT-qPCR

The isolation and purification of total RNA of the tissues and cells depended on TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and the RNeasy Maxi kit (Qiagen GmbH) respectively according to the instructions of manufacturers. A total of 1 μg RNA was reverse transcribed into cDNA using miScript Reverse Transcription kit II (Qiagen GmbH). After that, the synthetic cDNA was subjected to RT-qPCR with primers and miScript SYBR® Green PCR kit (Qiagen GmbH) on the Roche Lightcycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). U6 acted as an internal control. RT-qPCR conditions were set as follows: 95 °C for 15 min, after that, 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. The expression level of miR-302b was calculated with $2^{-\Delta\Delta Cq}$ method [18].

2.4. Wound healing assay

Cell migration ability was assessed by wound healing assay. Succinctly, once the transfected cells reached 90%–95% confluence in 6-well plates, a straight wound was artificially scratched by a disposable 200 μl pipette tip. The detached cells were removed by phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and afterwards, the cell was maintained in humidified incubator at 37 °C containing 5% CO_2. The images of wound closure were photographed at 0 and 24 h with a digital camera system (Olympus Corporation, Tokyo, Japan). The migration distance is the furthest distance that the cells migrate to the clear area.

2.5. Transwell assay

Cell migratory and invasive ability was assessed by using Transwell chambers (BD Biosciences, New York, NJ, USA) coated with Matrigel (for invasion) or without Matrigel (for migration) following the manufacturer's protocol. At 24 h post transfection, the harvested cells were counted, suspended in 200 μl serum free DMEM and added into the top chamber. The bottom chamber was imbued with 500 μl DMEM medium containing 10% FBS. After incubation for 24 h (migration) or 36 h (invasion), the migrated or invaded cells were fixed with formaldehyde and stained with crystal violet. The cells were counted in five different fields using a microscope (Olympus Corporation, Tokyo, Japan).

2.6. Cell counting kit-8 (CCK-8) assay

Cell proliferation was assessed by CCK-8 (US Everbright Inc.) assays using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Approximately 3000 transfected cells were seeded in each well of 96-well plates and incubated for 0, 24, 48 or 72 h. According to the manufacturer's protocol, the cells of each well was added with 10ul CCK-8 (US Everbright Inc.) and cultured for at 37 °C for 1 h before the optical density (OD) was determined at 450 nm by microplate reader.

2.7. Flow cytometry assay

Apoptosis was assessed by flow cytometry assay on Beckman Coulter (EPICS XL-4; Inc., Brea, CA, USA). In this assay, a six-well plate was used and seeded with 3×10^5 cells (per well). At 48 h post transfection with 100 pmol of miR-302b mimic, inhibitor or corresponding NC, all transfection cells were stained with propidium iodide (PI; Invitrogen; Invitrogen; Thermo Fisher Scientific, Inc.) and Annexin V-fluorescein isothiocyanate (FITC; Invitrogen; Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min. Afterwards, the cells were added with binding buffer (400 μl/tube) and subjected to flow cytometry(EPICS Xl-4, Beckman Coulter, Brea, CA, USA). FlowJo software (version, X) fl (FlowJo LLC, Ashland, OR) was used to calculate the apoptosis rate.

2.8. Formalin-fixed paraffin-embedded (FFPE) bladder urothelial carcinoma tissue specimens

The significance of miR-302b in the recurrence of BC was analyzed with 48 FFPE bladder urothelial carcinoma tissue samples. Hematoxylin/eosin staining was performed to identify tumor areas and nonmalignant areas. All the cases were pathologically reviewed and diagnosed with urothelial carcinoma. The first recurrent tumor was defined as any tumor identified following a disease-free remission period, > 90 days after the date of initial primary bladder tumor diagnosis. The smoking patient was defined as the patient who smoked at least 500 cigarettes. After obtained from Department of pathology of Peking University Shenzhen Hospital, the FFPE bladder urothelial carcinoma tissues samples were treated with the miRNeasy FFPE Kit (Qiagen) for the total RNA extraction. All the bladder urothelial carcinoma patients of the FFPE samples received surgical bladder resection