Expression and role of miR-34a in bladder cancer

Wei Wang1*, Tan Li2*, Guang Han1, Ying Li1, Li-hua Shi1 and Hui Li1*

1Department of Urology, The Affiliated Hospital of Logistics University of the Chinese People’s Armed Police Force, Tianjin, 300162, China
2Department of Pathogen Biology and Immunology, Logistics University of the Chinese People’s Armed Police Force, Tianjin, 300162, China

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To investigate the biological function of microRNA-34a (miR-34a) in bladder cancer, the expression of miR-34a was determined using quantitative real-time polymerase chain (qRT-PCR) reaction in 42 cases of bladder cancer. The relationship between the expression of miR-34a and development of bladder cancer was also studied. The mature mimics of miR-34a were chemically synthesized and transiently transfected into human bladder cancer T24 cells. The effects of miR-34a on apoptosis, cell cycle and proliferation in T24 cells were evaluated by flow cytometry and MTT, respectively. The results showed that the low expression rate of miR-34a was correlated with the malignancy and tumor size of bladder cancer. The up-regulation of miR-34a in T24 cells contributed to cell growth and cell cycle arrest, but not caspase-3 pathway. These findings suggest that the relative low expression of miRNA-34a might be involved in the tumorigenesis of bladder cancer.

Keywords: microRNA-34a, Bladder cancer, Pathological variables, Proliferation, Apoptosis.

Bladder cancer is one of the most common urinary malignant tumors, and transitional cell cancer accounts for 90% of all bladder cancers which ranks ninth in the global cancer incidence, with 356,000 annual new cases and 145,000 annual deaths1,2. It presents in both superficial forms, accounting for 80% of newly diagnosed cases and invasive highly aggressive tumors. Even after complete transurethral resection, 50% to 75% of superficial tumors recur and up to 25% progress in stage or grade, even after a long tumor-free period. The development of bladder cancer is a complex multi-step process and the pathway from normal to malignant urothelium is still not clearly understood. Accurate estimation on the biological behavior of bladder cancer is important to select the appropriate treatment and predict the prognosis. Therefore, more reliable prognostic factors are urgently needed.

MicroRNAs (miRNAs) are a conserved class of non-coding small RNAs that regulate gene and protein expression by binding to mRNA, leading to mRNA degradation or inhibition of translation3,4. Either individually or as a cluster, the expression levels of miRNAs have been shown to be up-regulated or down-regulated in several cancers, including prostate cancer, breast cancer, lung cancer, medulloblastoma and bladder cancer5-6.

Recently, the miR-34 family, including miR-34a, b, c has been found to be directly correlated with p537,8. Some evidences have shown that reduced expression of miR-34a is involved in the initiation and progression of cancer and the functional activity of miR-34 indicates a potential role as a tumor suppressor9-12, whereas others have reported that the overexpression of miR-34a is related with various types of human cancers13. However, the role of miR-34a in bladder cancer has not yet been elucidated. Herein, a group of 42 Chinese patients with bladder cancer has been studied to examine the expression of miR-34a to elucidate its underlying mechanism on the occurrence and development of tumor.

*Corresponding author
Tel: +86 22 60578634
Fax: +86 22 60579305
E-mail: jinglong2000w@163.com (W Wang)
tanli20042001@yahoo.com.cn (T Li)
lihui2009wujing@163.com (H Li)
Materials and Methods

Patients
Forty-two patients, without any radiotherapy or chemotherapy, were enrolled in the study underwent a transurethral resection of bladder cancer at the Affiliated Hospital of Logistics University of the Chinese People’s Armed Police Force between July 2008 and October 2010. Of these 42 cases, 27 patients were male and 15 were female, with the mean age of 43.5 yrs (range: 35.1 to 72.6 yrs). Tumors were classified using the 2002 AJCC TNM staging system.

The clinicopathological parameters, such as gender, age, cell grade, T classification and others were reviewed. Histological classification and grade were evaluated by an independent pathologist blinded to the outcomes according to WHO 1973 guidelines. Bladder cancer and matched histologically normal mucous membrane from each subject were immediately frozen in liquid nitrogen after resection. Detailed information of patients is summarized in Table 1.

The clinical trial was performed according to the declaration of Helsinki and had been approved by the Regional Scientific Ethical Committee of Logistics University of the Chinese People’s Armed Police Force. Written informed consent and approval were obtained from all participants.

Quantitative real-time polymerase chain reaction (qRT-PCR)
qRT-PCR was performed to determine the expression of potential miR-34 target genes. Total RNA was prepared using Trizol reagent (Invitrogen Inc.) according to the manufacturer’s instruction. The first strand of cDNA was synthesized using a commercial kit (Takara Bio Inc., Japan). The transgene expression was evaluated by qRT-PCR on an ABI Prism 7500 (Applied Biosystems Inc.) with SYBR Green PCR core reagent (ABI Bioscience Inc.) according to the manufacturer’s protocol and triplicate samples were setup.

Amplification was performed using the following primers: 5’-ACCTGGCAGTGTCTTAGCTGGT-3’ and 5’-AATCCATGAGAGATCCCTACCG-3’. The level of miR-34a was normalized to RNU6B. The threshold cycle (C_T) values for each reaction were determined and averaged using TaqMan SDS analysis software (Applied Biosystems-Life Technologies). The changes in the expression of a target gene were calculated by the comparative C_T method (fold changes = 2^[ΔΔCT], ΔΔCT = ΔCTmiRNAΔCTRNU6B) as described elsewhere.

Cell line
Human bladder cancer T24 cell line was purchased from the Tianjin Institute of Urology (Tianjin, China) and maintained in RPMI 1640 medium (GIBCO, Carlsbad, CA) was supplemented with 10% fetal bovine serum (Bioind Industries, Israel), 2 mM L-glutamine and 1% penicillin and streptomycin in humidified atmosphere with 5% CO_2 at 37°C.

Transfection of miR-34 mimics
The miR-34a was purchased from Gene-Pharma Company (Shanghai, China). Sequences were as follows: hsa-miR-34a 5’-UGGCAGUGUCUUAGCUGGUUGU-3’ and miRNA scrambled (negative control, NC) 5’-UUC UCGAACGUGUCACGUTT-3’. T24 cells were transfected at the time of 70-90% confluence after being seeded in 6-well plates. The miRNA mimics (10 nmol) mixed with lipofectamine-2000 transfection reagent (Invitrogen, Carlsbad, CA) and serum-free, antibiotic-free medium according to the manufacturer’s instructions. The transfection solutions were then added to each well and replaced 6 h later. The transfection effect was evaluated using qRT-PCR as described before.

MTT assay
At the time points of 24, 48, 72 and 96 h after transfection, the cells were harvested for MTT assay. T24 cells were seeded at 5 × 10^3 cells per well of 96-well tissue culture plates with six replicate wells for each condition. Every 24 h after transfection, each well were added 20 µL MTT (5 µg/mL, Amresco Inc.) and incubated for 4 h at 37°C. Then supernatant was removed and 150 µL DMSO was added. Absorbance was determined by spectrophotometry using a wavelength of 490 nm.

Cell cycle
The cells transfected with hsa-miR-34a or miRNA scrambled were stained in propidium iodide solution (50 µg/ml propidium iodide, 0.1% Triton X-100 and 0.1% sodium citrate in PBS) for 1 h at 4°C in the dark and the fluorescence was read on a Flow Cytometer (BD Bioscience Inc.). Data from ≥10,000 cells were collected and analyzed by a FACS software (BD Bioscience Inc.).
Caspase-3 activation
After 48 h transfection, the activation of caspase-3 in transfected T24 cells was detected following the instructions of a caspase-3 activation assay kit (BioVision, Mountain View, CA). The relative alteration of fluorescence signal was calculated by dividing the normalized signal in each treated sample with that of NC mimic as 100.

Statistical analysis
Statistical analyses were performed with SPSS 15.0 software (SPSS, Chicago, IL). Chi-Square test or Fishe's exact test was used to assess the relationship between the expression of miR-34a and the clinical characteristics of bladder cancer.

Results
Expression level of miR-34a and clinical features of bladder cancer
Clinical data and histopathologic analysis were performed and the expression level of MiR-34a was categorized as low (T/S <0.5) or high (T/S ≥0.5) in relation to that of tumor surrounding tissues (Table 1, Fig. 1). The low level of MiR-34a was observed significantly higher in bladder cancer with unfavorable features, including high histological grade (p<0.05), high clinical stage (p<0.01) and recurrence (p<0.05). No significant associations were identified between MiR-34a expression and patient age, patient gender and tumor size. These data suggested that the low level of MiR-34a was unfavorable prognostic factor for bladder cancer.

Effect of miR-34a on proliferation
The in vitro growth ability of transfected T24 cells was determined by MTT assay. As shown in Fig. 2, at the time point of 24, 48, 72 and 96 h post-transfection, the inhibition rates were 17.84 ± 5.62%, 36.87 ± 7.25%, 28.20 ± 4.70%, 26.55 ± 5.53%, respectively. The maximum inhibitory rate appeared at the point of 48 h post-transfection. It implied that miR-34a could suppress cell growth of T24 cells.

miR-34a affected cell cycle distribution
T24 cells were transfected with miR-34a as described before and miRNA scrambled was set as a negative control. The overexpression of miR-34a in T24 cells 48 h after transfection was confirmed using

Table 1—Associations of miR-34a expression with clinical and pathological variables

<table>
<thead>
<tr>
<th>miR-34a</th>
<th>P value</th>
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<tbody>
<tr>
<td>T/S&lt;0.5-fold</td>
<td>T/S≥0.5-fold</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
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<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>14</td>
</tr>
<tr>
<td>&gt;60</td>
<td>12</td>
</tr>
<tr>
<td>Tumor size(cm)</td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>8</td>
</tr>
<tr>
<td>≥3</td>
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<td>Clinical T stage</td>
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</tr>
<tr>
<td>T3 + T4a</td>
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<tr>
<td>Histological grade</td>
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</tr>
<tr>
<td>I + II</td>
<td>9</td>
</tr>
<tr>
<td>III</td>
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</tr>
<tr>
<td>Recurrence in a year</td>
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<tr>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>+</td>
<td>17</td>
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Note: Expression level of MiR-34a in tumor bed was evaluated as low (T/S<0.5) or high (T/S≥0.5) in reference to that of tumor surrounding tissues. Chi-Square test or Fishe's exact test was used to assess the relationship between miR-34a expression level and clinical characteristics. P<0.05 was considered statistically significant.
Fig. 3—MiR-34a suppressed cycle phase transition from G0/G1 to S but not affect caspase-3 activation [(A): Transfection of miR-34a was evaluated using qRT-PCR assays; (B): Cell cycle analysis was produced with flow cytometry in T24 cells infected by miR-34a mimics at 48 h time point after transfection; (C): Histogram of cell percentage data revealed that miR-34a suppressed cell cycle transition from G1/G0 to S, compared to NC group; and (D): Transfection of miR-34a mimics did not affect caspase-3 activation, a key indication of the cells undergoing apoptosis]
qRT-PCR as shown in Fig. 3A and could suppress cycle phase transition from G0/G1 to S (Fig. 3B & C). However, up-regulation of miR-34a did not increase caspase-3 activation, a key indicator of the cells undergoing apoptosis (Fig. 3D). Therefore, these data suggested that the overexpression of miR-34a could inhibit the proliferation of T24 cells at least partly via cell cycle inhibition, but not caspase-3 pathway.

Discussion

Increasing evidences have revealed that the low level of miR-34a could be involved in different types of cancers. miR-34a may work as a tumor suppressor and is also associated with various biological events. Meanwhile, some other evidences suggest that miR-34a is overexpressed in various types of cancers and is associated with the proliferation cell type-dependently. Therefore, the expression of miR-34a and its effect on gene regulation may vary, depending on the cellular type. In the present study, standard clinicopathological criteria, such as tumor size, T stage, tumor grade and tumor recurrence were analyzed in the patients of bladder cancer. The low level of MiR-34a was observed significantly higher in tumors with high histological grade, high clinical stage and recurrence, which suggested that MiR-34a was a unfavourable prognostic factor for bladder cancer.

The miR-34a was transfected into T24 cells and the proliferation, caspase activation and cell cycle were further analyzed. Our data showed that the overexpression of miR-34a could inhibit the proliferation of T24 cells at least partly via inhibiting cycle phase transition from G0/G1 to S. Recent studies suggest that miR-34a could also modulate multiple cellular pathways simultaneously including Cdk6, which controls Rb phosphorylation status and E2F3. Clearly, miR-34a has the potential to play an important role in abrogating the effect mediated by the dysfunction of the p53-Rb signaling axis, that is to say, uncontrolled progression of cell cycle. The ability of miR-34a to target multiple points within this important pathway indicates that the increased expression of miR-34a could suppress cell cycle progression, regardless of which part of the p53-Rb signaling axis is dysfunctional.

It has been predicted by bioinformatics tools that p53 could combine to promoter of miR-34a and up-regulate its expression. In pancreatic cancer, the absence of miR-34a compromises p53-dependent tumor suppression. Some studies have shown that p53 is activated by oxidative stress and oncogene over activation, which consequently forms the complex of Bcl-2, CDK4 and CDK6 through miR-34a. The interaction between p53 and Drosha participates in the post-transcript regulation of miR-34a by enhancing microRNA processing from pri-miR34a to pre-miR34a, while mutant p53 retards the processing in chronic lymphocytic leukemia. In human pancreatic cancer cells, the overexpression of miR-34a could sensitize the cells to chemotherapy and radiation via Bcl-2 pathway. In other cancers, miR-34a is an independent predictor of disease progression either. Due to the complicated regulation of miR-34a, further study is needed to clarify the exact mechanism of miR34 on p53 in bladder cancer.

In conclusion, the expression level of miR-34a could be used as a surrogate biomarker for diagnosis and prognostic of bladder cancer. In the future, miR-34a might be used as a biomarker of cancers and an adjutant therapeutic way for tumor patients in clinical works.

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References


