Research on correlations of MiR-203 expression with onset and prognosis of bladder cancer

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Summary

Purpose: To investigate the correlations of the expression of micro ribonucleic acid (miR)-203 with the onset and prognosis of bladder cancer.

Methods: Human bladder cancer T24 cell line with miR-203 overexpression were constructed. Effects of miR-203 expression on cell proliferation and apoptosis were detected via methyl thiazolyl tetrazolium (MTT) assay and flow cytometry, respectively. Fifty-eight patients with bladder cancer treated in our hospital were selected, and para-carcinoma tissues were used as controls. The expression of miR-203 in bladder cancer tissues was detected via quantitative polymerase chain reaction (qPCR), and the survival time of patients with bladder cancer was recorded in detail. Moreover, correlations of miR-203 expression with onset and prognosis of patients were evaluated via multivariate Cox regression analysis.

Results: Results of qPCR revealed that after T24 cells were transfected with miR-203, the miR-203 expression level in the overexpression group was significantly higher than that in the control group (p<0.01), the cell proliferation capacity was significantly lower than that in the control group (p<0.01), and the apoptotic level was also significantly higher than that in the control group (p<0.01). The miR-203 expression level in carcinoma tissues of patients with bladder cancer was obviously lower than that in the para-carcinoma tissues (p<0.01). The miR-203 expression had close correlations with the tumor size, differentiation grade and tumor stage (p<0.01), and the 5-year survival rate of patients in low miR-203 expression group was remarkably lower than that in the high miR-203 expression group (p<0.01). Besides, the results of multivariate Cox regression analysis revealed that the tumor grade of differentiation, tumor-node-metastasis (TNM) stage and miR-203 expression were closely related to the prognosis of patients (p<0.01).

Conclusion: The high expression of miR-203 can significantly reduce the proliferation and promote the apoptosis of bladder cancer cells. The increased expression level of miR-203 can effectively increase the survival rate and improve the prognosis of patients.

Key words: miR-203, bladder cancer, onset, prognosis

Introduction

The onset of bladder cancer, one of the most common malignant tumors of the urinary system, has obvious regional characteristics. According to epidemiological surveys, the incidence rate of bladder cancer is highest in Egypt, followed by Europe and North America. The incidence rate of bladder cancer is lower in China compared to the above countries, but there are still approximately 360,000 new cases of bladder cancer every year, and the incidence rate in urban areas is generally higher than in rural areas [1,2]. According to its pathological type, bladder cancer can be divided into transitional cell carcinoma, glandular cell carcinoma and squamous cell carcinoma due to different tissue sources, the first of which is dominating. Most patients with superficial bladder cancer can...
be treated with transurethral resection of bladder tumor after diagnosis, but the high recurrence rate after operation is an important factor seriously affecting the survival of patients [3,4]. Tumor recurrence can be effectively prevented via intravesical chemotherapy after operation, but severe adverse reactions and immunosuppression often occur after chemotherapy [5,6]. Micrornucleic acids (miRs) are a kind of small non-coding RNAs existing in eukaryotes, which play an important regulatory role in cell differentiation, cell cycle and apoptosis. Studies have found that a variety of miRs are involved in the occurrence and development of bladder cancer [7]. Fujii et al. [8] found through genomic research that miR-100, miR-137 and miR-203 have close correlations with proliferation and apoptosis of a variety of tumor cells. Moreover, Shen et al. [9] found that the high expression of miR-203 can effectively increase the sensitivity of patients with bladder cancer to chemotherapeutic drugs. However, correlations of miR-203 with the onset and prognosis of patients with bladder cancer are rarely studied and this study aimed to reveal the correlations of miR-203 expression with the onset and prognosis of bladder cancer from the cellular level, so as to provide a theoretical basis for the diagnosis and treatment of this disease.

Methods

Objects of study and grouping

Patients with bladder cancer treated in our hospital from May 2011 to May 2012 were collected, and 58 cases pathologically diagnosed with single tumor were enrolled. All patients were newly diagnosed, and none of them had undergone treatment before operation. Tissues more than 3 cm away from the edge of tumor tissues served as control group. There were 49 males and 9 females aged 58-76 years. In terms of the clinical stage according to TNM criteria of the Union Internationale Contre le Cancer (UICC), there were 20 cases in stage T1, 18 cases in stage T2, 15 cases in stage T3 and 5 cases in stage T4.

Inclusion criteria

(1) patients without severe hepatic and renal dysfunction; (2) patients without a history of severe cardiovascular and cerebrovascular diseases and immune system diseases; (3) patients without chronic or acute infectious diseases; and (4) patients who agreed to be followed up.

All patients enrolled signed informed consent. The experimental scheme was ethically reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital.

Cell culture

T24 bladder cancer cell line purchased from Cell Bank of the Academy of Sciences of China was stored in liquid nitrogen for standby application. After T24 cell line was taken off the liquid nitrogen, it was immediately put in water bath box at 37°C and gently shaken to be dissolved and thawed. Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Waltham, USA) was added into a centrifuge tube, followed by centrifugation at 1,000 rpm for 5 min. Cells were inoculated into 6-well plates added with DMEM containing 10% FBS and cultured in an incubator with 5% CO₂ at 37°C. After cells grew logarithmically, basically covering the bottom of 6-well plates they were digested with trypsin, followed by passage and culture until the third generation which was used for experiments.

Cell transfection

pINDUCER21-EGFP plasmid was purchased from Nanjing Vazyme Biotech Co., Ltd., as the miR-203 vector, and amplification was performed with genomic DNA extracted from para-carcinoma tissue of bladder cancer (chr14: 104583642-1047583951). The product was 286 bp in length and the annealing temperature was 59°C. After polymerase chain reaction (PCR), the end was filled using Klenow enzyme (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China) and connected using T4 ligase (Nanjing Vazyme Biotech Co., Ltd.). The plate cloning was performed after transformation, positive clones were selected for sequencing, and plasmid construction was determined for subsequent cell transfection. T24 cells in the logarithmic growth phase were selected, inoculated into a 6-well plate at a cell density of 3×10⁵ well and cultured in the incubator with 5% CO₂ at 37°C for 24 h. Lipofectamine 2000 was diluted with DMEM, mixed evenly and incubated at 37°C for 5 min. After the 6-well plate was washed with the serum-free medium, mixed solution of Lipofectamine 2000 and serum-free DMEM were added, followed by incubation in the incubator with 5% CO₂ at 37°C for 6 h. After the transfection reagent and culture medium were discarded, the medium was replaced with DMEM containing 10% FBS, followed by incubation in the incubator with 5% CO₂ at 37°C for another 48 h. After RNA was extracted, the miR-203 expression level in each group of cells was detected via quantitative polymerase chain reaction (qPCR) to evaluate the miR-203 transfection.

Detection of cell proliferation and apoptosis

The cell proliferation was detected via methyl thiazolyl tetrazolium (MTT) assay. After successful transfection, cells in the logarithmic growth phase were paved onto 6-well plates and the cell density was adjusted to 1×10⁵/well, with the empty plasmid group as the control. Serum-free DMEM was added, followed by incubation in the incubator with 5% CO₂ at 37°C for 24 h. After 1% MTT was added, cells were incubated in the incubator for another 4 h. Finally, the cell proliferation was detected using an ultraviolet spectrophotometer.

The apoptotic level was detected via flow cytometry. After the cell density was adjusted to 5×10⁵/well,
cells were incubated in the incubator with 5% CO₂ at 37°C for 24 h. After cells were washed with phosphate buffered saline (PBS), they were digested with trypsin and collected. Finally, the apoptotic level was detected using the apoptosis kit (Thermo Fisher, Waltham, USA). Flow cytometry should be performed within 1 h, or else it would affect the experimental results.

Detection of miR-203 expression in bladder cancer tissues and cells after transfection via qPCR

After transfection, cells in each well were added with 500 μL TRIzol (TRIzol kit, Thermo Fisher, Waltham, USA), scrapped off using a cell scraper and collected into a centrifuge tube. Bladder cancer tissues and para-carcinoma tissues were taken, added with TRIzol at a ratio of 100 mg:1 mL, and smashed using an ultrasonic disrupter, followed by centrifugation at 10,000 rpm, and the supernatant was taken. Each of the above cell tubes and tissue tubes were added with 700 μL chloroform, and total RNA was extracted from cells and tissues according to instructions of the RNA extraction kit (Qiagen, Germantown, USA). The RNA integrity was detected via agarose gel assay, and the absorbance (A)_{260}/A_{280} and optical density (OD) value of RNA were measured. The reaction system was prepared strictly according to instructions of the reverse transcription kit (Invitrogen, Waltham, USA), followed by reaction at 65°C for 5 min and incubation at 37°C for 2 min. Then the reverse transcription was performed to synthesize complementary DNA (cDNA). After qPCR system was prepared, amplification was performed on a qPCR instrument, and amplification conditions are as follows: pre-denaturation at 92°C for 45 s, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, a total of 50 cycles. The primers were synthesized by Invitrogen, with U6 as an internal reference. Sequences are as follows: miR-203 forward: 5’-CGGTGAAATGTTAGG-3’, and reverse: 5’-GAGCAGGCTGGAGAA-3’. U6 forward: 5’-GCTTCGGCAGCACATA-3’, and reverse: 5’-CTTACCAGATTTCGGCGT-3’. The 2⁻ΔΔCt formula was used for calculation, and miR-203/U6 indicated the miR-203 expression level in cells and tissues.

Detection of apoptotic protein expressions in bladder cancer tissues and cells after transfection via Western blotting

After transfection, cells in each well were added with 1 mL protein lysis solution, and bladder cancer tissues and para-carcinoma tissues were added with protein lysis solution at a ratio of 100 mg·1 mL. 1% protease inhibitor was added into the above cells and tissues, followed by homogenization using an ultrasonic homogenizer until there were no tissues visible to the naked eye, and centrifugation at 12,000 rpm and 4°C for 10 min. The supernatant was taken, namely the total protein sample. The total protein concentration in each group of samples was determined using the bicinchoninic acid (BCA) protein assay kit (PIERCE). An equal concentration of protein loading buffer was prepared, and 15 μL loading buffer was added into each well after the gel plate was prepared, followed by electrophoresis and membrane transfer. Then, the protein was sealed with 5% skim milk powder for 2 h, and the target band was cut off and incubated with the primary antibody at 4°C overnight. Antibodies of B-cell lymphoma-2 (Bcl-2) (1:1000, CST, USA), Bcl-2 associated X protein (Bax) (1:1000, CST, USA), cleaved caspase-3 (1:1000, CST, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (internal reference, 1:1000, CST, USA) were incubated, respectively. The band was washed with PBST for 3 times (5 min/time), incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Shanghai YIHYSON Biotechnology Co., Ltd.) at room temperature for 1 h, and washed again with PBST for 5 times (10 min/time). Finally, enhanced chemiluminescence (ECL) solution was added and images were obtained via fluorescence imaging technique.

Survival curve plotting

The miR-203 expression in carcinoma tissues of each patient with bladder cancer was detected. All patients with bladder cancer enrolled were divided into high miR-203 expression group and low or normal miR-203 expression group, based on the miR-203 expression level in para-carcinoma tissues. Patients in both groups were followed up for 5 years, the 5-year survival rate was recorded, and the survival curves of patients in both groups were plotted. The influencing factors for the prognosis of patients in both groups were analyzed via multivariate Cox regression analysis.

Statistics

Data in this study were presented as mean ± standard deviation, and SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing. T-test was used for the intergroup comparison, and chi-square test was used for enumeration data. One-way ANOVA was performed to assess homogeneity. The Bonferroni method was adopted for pairwise comparison if the variance was homogeneous. Otherwise, the Welch method was adopted for analysis. Correlation analysis was performed via multivariate Cox regression analysis. P<0.05 suggested that the difference was statistically significant.

Results

Construction of cell lines with miR-203 overexpression

After transfection of bladder cancer T24 cell line, the miR-203 expression level in each group of cells was detected via qPCR. The miR-203 expression level in the overexpression group was significantly higher than that in the control group (p<0.01), indicating that cell lines with miR-203 overexpression were constructed successfully and could be used for subsequent experiments (Figure 1).

Effects of miR-203 on proliferation and apoptosis of bladder cancer cells

The proliferation and apoptosis of transfected cells were detected via MTT assay and flow cytometry,
respectively. The results of MTT assay revealed that the cell proliferation capacity in the overexpression group was significantly lower than that in the control group (p<0.01). The results of flow cytometry revealed that the apoptotic level in the overexpression group was significantly higher than that in the control group (p<0.01) (Figure 2).

**Figure 1.** Detection of miR-203 expression in transfected cells via qPCR. The miR-203 expression level in the overexpression group is significantly higher than that in the control group (**p<0.01).**

**Effects of miR-203 on apoptotic protein expressions in bladder cancer cells**

The expression levels of apoptotic proteins in each group of cells were detected via Western blotting. Compared with those in the control group, the expression level of cleaved caspase-3 protein in the overexpression group was obviously increased (p<0.01), while the Bcl-2/Bax ratio was obviously decreased (p<0.01) (Figure 3).

**Correlations of miR-203 expression with clinical data of patients**

Clinicopathological data of patients with bladder cancer were recorded in detail, the miR-203 expression level in bladder cancer tissues was detected, and correlations of miR-203 expression in carcinoma tissue with clinicopathological data of patients in each group were analyzed. The results showed that miR-203 expression had no correlations with gender and age of patients with bladder cancer (p>0.05), but had close correlations with the tumor size, differentiation grade and tumor stage of bladder cancer (p<0.01) (Table 1).

**MiR-203 expression in bladder cancer tissues and para-carcinoma tissues**

The miR-203 expression level was detected via qPCR in bladder cancer tissues and para-carcinoma tissues in all patients and the results demonstrated

**Figure 2.** Effects of miR-203 on cell proliferation and apoptotic level. The cell proliferation capacity in the overexpression group is significantly lower than that in the control group (A), and the apoptotic level in the overexpression group is significantly higher than that in the control group (B) (**p<0.01).**
that the miR-203 expression level in bladder cancer tissues was remarkably lower than that in para-carcinoma tissues (p<0.01) (Figure 4).

Correlations of miR-203 with survival rate of patients with bladder cancer

All patients enrolled were followed up for 5 years, the survival rate was recorded, and the survival curve was plotted. The results showed that the 5-year survival rate of patients in the high miR-203 expression group was obviously higher than that of patients in the low or normal miR-203 expression group (p<0.01) (Figure 5).

Expression levels of apoptotic proteins in patients with bladder cancer

The expression levels of apoptosis-associated proteins in bladder cancer tissues of patients in the high miR-203 expression group and the low or normal miR-203 expression group were detected via Western blotting. The results showed that the expression level of cleaved caspase-3 in carcinoma tissue of patients in the high miR-203 expression group was remarkably higher than that of patients in low or normal miR-203 expression group (p<0.01), while the Bcl-2/Bax ratio was remarkably lower than that of patients in the low or normal

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**Figure 3.** Detection of apoptotic protein expressions in bladder cancer cells. **A**: Western blot protein bands and **B**: statistical graph of protein bands of cleaved caspase-3. **C**: Statistical graph of protein bands of Bcl-2/Bax. The expression level of cleaved caspase-3 protein in the overexpressing group is obviously higher than that in the control group, while the Bcl-2/Bax ratio is obviously lower than that in the control group (**p<0.01).
miR-203 expression group (p<0.01).

Cox regression analysis of prognostic factors for bladder cancer

The influencing factors for the prognosis of patients with bladder cancer were analyzed via multivariate Cox regression analysis. The results showed that the tumor differentiation grade, TNM stage and vascular endothelial growth factor (VEGF) expression had close correlations with the prognosis of patients (p<0.05, p<0.01) (Table 2).

Discussion

Although superficial bladder cancer dominates, infiltration and metastasis of bladder cancer will occur when bladder cancer cells metastasize to the bladder subepithelial connective tissues, seriously affecting the survival and quality of life of patients [10,11]. A lot of research evidence proves that a variety of tumor-stimulating factors in vivo

Table 1. Correlations of miR-203 expression in bladder cancer tissues with clinicopathological data

<table>
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<tr>
<th>Clinicopathological data</th>
<th>n</th>
<th>Relative expression level of miR-203 (mean±SD)</th>
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<tr>
<td>Male</td>
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<td>&lt;58</td>
<td>19</td>
<td>1.96±0.55</td>
<td>&gt;0.05</td>
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<td>≥58</td>
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<td>1.95±0.38</td>
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<tr>
<td>Diameter (cm)</td>
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<td></td>
<td>&lt;0.01</td>
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<td>≥1.0</td>
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<td>Differentiation grade</td>
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<td>Low and no differentiation</td>
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<tr>
<td>High and moderate differentiation</td>
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<td>1.76±0.19</td>
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<tr>
<td>Tumor stage</td>
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<td></td>
<td>&lt;0.01</td>
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<tr>
<td>T1-T2</td>
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<td>2.18±0.16</td>
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<td>T3-T4</td>
<td>20</td>
<td>1.70±0.22</td>
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Table 2. Multivariate Cox regression analysis of prognostic factors for bladder cancer

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<th>Factors</th>
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<th>Wald</th>
<th>df</th>
<th>Sig.</th>
<th>Exp(B)</th>
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<td>0.359</td>
<td>1</td>
<td>0.683</td>
<td>1.552</td>
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<td>Age</td>
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<td>1</td>
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<td>Differentiation grade</td>
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<td>12.565</td>
<td>1</td>
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<td>TNM stage</td>
<td>1.779</td>
<td>0.813</td>
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<td>0.048</td>
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<td>miR-203</td>
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<td>0.298</td>
<td>6.987</td>
<td>1</td>
<td>0.041</td>
<td>3.267</td>
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**Figure 5.** 5-year survival curves of patients with bladder cancer. The 5-year survival rate of patients in the high miR-203 expression group is obviously higher than that of patients in the low or normal miR-203 expression group (p<0.01).

**Figure 6.** Detection of apoptotic protein expressions in bladder cancer tissues via Western blotting. **A:** Protein bands; **B:** statistical graph of protein bands of cleaved caspase-3; **C:** statistical graph of protein bands of Bcl-2/Bax. Compared with those in the low or normal miR-203 expression group, the expression level of cleaved caspase-3 protein in the carcinoma tissues of patients in the high miR-203 expression group is obviously increased, while the Bcl-2/Bax ratio is obviously decreased (**p<0.01).
and in vitro are important, leading to the occurrence and development of bladder cancer, and the increased expression level of vascular endothelial factors can further promote neovascularization and growth of tumor tissues [12]. MiRs can be involved in regulating the translation and degradation of target messenger RNA (mRNA), and proliferation and apoptosis of malignant tumors, which are considered as carcinogenic factors for a variety of tumors [13]. Research evidence shows that miR-203 is expressed abnormally in colon cancer, breast cancer and gastric cancer [14]. Gottardo et al. [15] studied and found that miR-203 is lowly expressed in carcinoma tissues of patients with lung cancer. Dusílková et al. [16] studied and found that up-regulation of miR-203 can effectively increase the sensitivity of non-small cell lung cancer cell lines to chemotherapeutic drugs. At the same time, researchers found that the low expression of miR-203 will obviously improve the tolerance of various tumor cells to chemotherapy drugs such as cisplatin. Moreover, Takano et al. [17] found that the miR-203 expression level in carcinoma tissues of drug-resistant patients with bladder cancer was obviously decreased. The above research evidence indicates that miR-203 may mediate the occurrence and development of bladder cancer.

In this study it was found that the miR-203 overexpression in bladder cancer T24 cell line can effectively reduce the proliferation capacity and significantly increase the apoptotic level of T24 cells. In addition, the expression levels of apoptosis-associated proteins were increased significantly in cell lines with miR-203 overexpression. The above results indicate that miR-203 may affect the occurrence and development of bladder cancer through affecting the expressions of apoptotic proteins. Benati et al. [18] studied and found that miR-203 can promote the expression of dickkopf 1 (DKK1) protein through the Wnt signal transduction pathway, thereby reducing the expressions of apoptotic proteins in tumor cells. The results of this study also manifested that the miR-203 expression in bladder cancer tissues had close correlations with the tumor size, tumor differentiation grade and TNM stage of bladder cancer. At the same time, the 5-year survival rate of patients in the high miR-203 expression group was also significantly increased. The above results indicate that miR-203 plays an important role in regulating the prognosis of bladder cancer. Research evidence proves that miR-203 can act on and reduce the expression of its target protein, VEGF, thus reducing vascularization in tumor tissue, so that tumor apoptosis is promoted and prognosis of cancer patients is improved [19,20]. The high expression of miR-203 in patients with bladder cancer can effectively increase the expressions of apoptotic proteins in carcinoma tissues, thereby leading to apoptosis of tumor cells and improving prognosis of patients with bladder cancer.

In conclusion, in vitro and in vivo experiments strongly indicate that miR-203 has close correlations with the occurrence, development and prognosis of bladder cancer. High expression of miR-203 can significantly reduce the proliferation and promote the apoptosis of bladder cancer cells. The increased expression level of miR-203 can effectively increase the survival rate and promote prognosis of patients. MiR-203 can serve as a screening tool for bladder cancer in the clinic and can also be used to evaluate the therapeutic effect and prognosis of bladder cancer.

Conflict of interests

The authors declare no conflict of interests.

References

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