Original Article
Expression of miR-182 and Foxo3a in patients with bladder cancer correlate with prognosis

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Abstract: Objective: FoxO3a is a specific tumor suppressor gene in the forkhead transcription factor O subfamily (FoxO). Studies show that its expression plays a role in bladder cancer. The abnormal expression of miR-182 in bladder cancer suggests that miR-182 may be an oncogene in bladder cancer. Bioinformatic analysis showed that there is a target complementary binding site between miR-182 and Foxo3a. In this study, the expression of miR-182 and Foxo3a in cancer tissues of patients with bladder cancer was detected. The expression of miR-182 and Foxo3a in bladder cancer tissues and their relationship with the prognosis of the patients were analyzed, and the role of miR-182 in regulating the expression of Foxo3a and the biologic process of cell proliferation and apoptosis in bladder cancer cells was explored. Methods: Tumor tissues of patients with bladder cancer were collected and the normal bladder mucosa was used as a control. The expression of Foxo3a was detected by western blot. The expression of miR-182 and Foxo3a mRNA was detected by qRT-PCR. The relationship between miR-182, Foxo3a mRNA and the clinical features of patients was analyzed. The median expression of miR-182 and Foxo3a mRNA was bounded, and Log Rank test was used to compare the survival rate of low and high expression of miR-182 and Foxo3a mRNA. The double luciferase reporter gene assay was used to confirm a target regulatory effect between miR-182 and Foxo3a. In vitro, RT112 and T24 cells were divided into 2 groups: group miR-NC, and group miR-182 inhibitor. qRT-PCR and western blot were used to detect the expression of Foxo3a, flow cytometry was used to detect cell apoptosis, and EdU staining was used to detect cell proliferation. Results: Compared with normal bladder tissue, the expression of miR-182 in bladder cancer tissue was significantly increased, and it was related to tumor size, TNM stage, and lymph node metastasis (P < 0.05). The expression of Foxo3a mRNA was significantly decreased, and was related to tumor size, TNM stage, histopathologic classification, and lymph node metastasis (P < 0.05). There was a significant negative correlation between the expression of miR-182 and Foxo3a mRNA in bladder cancer (r = -0.602, P < 0.05). The prognosis of patients with high expression of miR-182 was significantly worse than that of those with low miR-182 expression. The prognosis of patients with low expression of Foxo3a was significantly better than those with high Foxo3a. Double luciferase reporter gene experiments confirmed that there was a target regulatory relationship between miR-182 and Foxo3a. Transfection of miR-182 inhibitor significantly increased the expression of Foxo3a in RT112 and T24 cells, significantly reducing cell proliferation, and significantly increasing apoptosis. Conclusion: The expression of miR-182 was increased and the expression of Foxo3a was decreased in bladder cancer, which is related to prognosis. Downregulation of the expression of miR-182 can increase the expression of Foxo3a, inhibiting the proliferation of bladder cancer cells and inducing apoptosis.

Keywords: miR-182, Foxo3a, bladder cancer, proliferation, apoptosis, prognosis

Introduction

Bladder cancer (BC) is one of the most common malignant tumors in the genitourinary system. It is one of the ten major malignant tumors in the world. The incidence of bladder cancer is the ninth [1] among cancers. The incidence of bladder cancer is more obscure. There are no obvious clinical symptoms in the early stages, but the disease develops rapidly and easily invades and metastasizes. Therefore, BC is difficult to treat [2-4].

MicroRNA is an endogenous noncoding small molecule single strand RNA of 22~25 nucleotides in eukaryotes. The expression of a target gene is regulated by the combination of complementary pairing and 3'-UTR of target gene.
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mRNA to degrade or inhibit translation. MicroRNA expression and dysfunction play a vital role in the pathogenesis of tumors [5-7]. Studies have shown [8, 9], that the expression of miR-182 in bladder cancer is increased, so miR-182 may play a role in promoting pathogenesis of bladder cancer. FoxO3a is a well-defined tumor suppressor gene in the forkhead transcription factor O subfamily (FoxO), which can regulate the expression of a variety of cell proliferation, cycle, and apoptosis related genes, thus affecting the various biologic processes of tumor cells [10].

The study shows that a decrease of the expression of Foxo3a is related to the malignant biologic characteristics of bladder cancer cells [11], while the increased expression of Foxo3a has the effect of inhibiting bladder cancer. Bioinformatic analysis showed that there was a target binding site between miR-182 and the 3'-UTR of FoxO3a mRNA, suggesting a possible regulatory relationship between them. In this study, the expression of miR-182 and Foxo3a in cancer tissues of patients with bladder cancer was detected. The expression of miR-182 and Foxo3a in bladder cancer tissues and their relationships with the prognosis were analyzed, and the role of miR-182 in the regulation of Foxo3a expression and in the biologic processes of cell proliferation and apoptosis in bladder cancer cells was explored.

Materials and methods

Reagents and materials

Human bladder cancer cell line RT112, T24, and human normal bladder epithelial cell SV-HUC-1 were purchased in Guangzhou Ginny Biotechnology Co., Ltd.; DMEM medium and FBS were purchased from American Gibco; Lip 2000 was purchased from American Invitrogen; ReverTra Ace qPCR RT Kit and SYBR dyes were purchased from JapanToyobo; miR-NC, miR-182 mimic and EdU test kit were purchased from Ruibo Biology, Guangzhou; the Rabbit anti human Foxo3a, beta-actin antibody and HRP marker were purchased from American Abcam; RIPA protein extract and Annexin V/PI apoptosis detection reagent were purchased from Biyun Tian Biota, Jiangsu; Dual-Luciferase Reporter Assay System and pGL3-promoter plasmids were purchased from Promega, United States.

Clinical data

60 patients with urothelial bladder cancer were operated on in our hospital from March 2015 to April 2016, including 40 males and 20 females, aged 36-79 years, with an average age of 62.9 years. All patients had not received any antitumor treatment before operation. Among them, histopathologic grade was G1 grade: 12, grade G2: 12, G3 grade: 12, Ta-T2 stage in TNM: 37, T3-T4 stage: 23. Another 20 cases of normal bladder mucosa specimens were taken as controls. All samples were informed and agreed by the hospital ethics committee.

Cell culture

SV-HUC-1, RT112 and T24 cells were cultured in DMEM medium containing 10% FBS and 1% streptomycin in a cell culture incubator containing 5% CO₂ at 37°C. After the cells were confluent, the cells were cultured in proportion by 1:4, and the cells with a good logarithmic growth state were tested.

Double luciferase gene reporter

The HEK293T cell genome mRNA was used as a template to amplify the fragment of the target binding site in the 3'-UTR region of the FoxO3a gene or its mutant fragment, and to cut the glue to recover the PCR product. After the enzyme was cut into the pGL3 vector, the DH5 alpha receptive cells were transformed and the positive clones of the colony PCR were screened and the correct plasmids were selected and named as pGL3-Foxo3a-WT, pGL3-Foxo3a-MUT.

100 ng pGL3-Foxo3a-WT (or pGL3-Foxo3a-MUT), 50 miR-182 mimic (or miR-NC) and 50 ng pRL-TK were cotransfected to HEK293 cells with Lip 2000, and after 48 h, the culture medium was discarded. Then the cells were washed with PBS 2 times, lysed 15 min with L Passive Lysis Buffer, and centrifuged 5 min at 1000 rpm. We determined the luciferase activity immediately after the reaction between 50 L lysate and 50 L luciferase substrate.

Cell transfection and grouping

In vitro, RT112 and T24 cells were divided into 2 groups: miR-NC transfection group and miR-182 mimic transfection group. The steps of transfection: 10 μL Lip 2000, 50 nmol miR-NC,
50 nmoL miR-NC and 50 nmoL miR-182 mimic were diluted with 100 μL Opti-MEM free of serum, respectively. After 5 min, Lip 2000 was mixed gently with miR-NC and miR-182 mimic, respectively. After incubation 20 min at room temperature, the transfection was added to the cell culture medium. After mixing gently, the cells were incubated for 72 hours.

Detection of cell proliferation with flow cytometry

The cells of the above-transfected group were collected after trypsin digestion, cultured in DMEM containing 10% FBS, incubated at 10 μM EdU 37°C for 2 h, after 48 h, washed with PBS 2 times, and collected with trypsin digestion. After digestion, the cells were transferred to a centrifuge tube. After centrifugation, the above medium was thrown away, the cells were washed with PBS 1 time, fixed with paraformaldehyde, neutralized with glycine neutralization, permeabilized with 0.1% TritonX-100, added into 500 μL the Apollo staining solution, incubated 10 min at room temperature, and washed with PBS after centrifugation. We detected the proliferation of cells with Beckman Kurt FC500 MCL flow cytometry.

Detection of cell apoptosis with flow cytometry

After the cells were washed with PBS 2 times, 100 μL Binding Buffer was added to the cell precipitate. After mixing, 5 μL Annexin V-FITC and 5 μL PI were added to the above mixture. 400 μL Binding Buffer was added to the cells after Light Reaction for 20 min. The apoptosis of cells was detected with flow cytometry.

Detection of gene expression by qRT-PCR

RNA was extracted with the Trizol method. ReverTra Ace qPCR RT Kit was used to reverse RNA to cDNA. The reaction system: 2 μg RNA, 1 μL dNTP, 4 μL RT Buffer, 1 μL RT primer, 2 μL RT Enzyme, 1 μL RNase inhibitor and lastly ddH2O to 20 μL. The PCR amplification conditions: 95°C 15 s, 60°C 30 s, 74°C 30 s, and the data were collected after 40 cycles on a Bio-Rad CFX96 quantitative PCR instrument.

Western blot

Every 5 mg or 1,000,000 cells was added into 100 μL protein extract, and after fully lysing, centrifuged 15 min at 10,000 g. Then the above solution was moved to a new EP tube, and we detected the mass concentration with BCA method. Later, 50 μg was separated 3 h (10% gel, 4% concentrate) in SDS-PAGE, transferred to PVDF membrane (250 mA, 100 min), cultured in skim milk powder at room temperature 60 min, and incubated with the primary antibody for 12 h at 4°C (Foxo3a, beta-actin dilution ratio is 1:1000, 1:5000). The next day, the film was washed with PBST 3 times, then added to the second antibody with HRP marker (1:10000 dilution) incubating 1 h, washed with PBST 3 times and added into ECL luminescence reaction. After reacting 2~3 min in darkroom, the western blot films were exposed, developed and fixed. Finally, the films were scanned and the data was saved.

Statistical analysis

The statistical analysis was carried out with SPSS 18, and the measurement data were expressed as mean ± standard deviation. The comparison between the two groups was compared by the t test. The comparison of the data among multiple groups was compared first with the single factor analysis of variance and then by Bonferroni. The expression of miR-182 and Foxo3a mRNA in the brain tissue of the two groups were compared by Mann-Whitney U test. The correlation analysis of the expression of miR-182 and Foxo3a mRNA in gastric cancer tissue was carried out by Spearman method. The survival curve of the patients was determined by Kaplan-Meier method. The comparison of survival rates was carried out with Log-rank test. P < 0.05 was considered significant.

Results

Abnormal expression of miR-182 and Foxo3a in bladder cancer tissues

The results of qRT-PCR detection showed that the expression of Foxo3a mRNA in Ta-T2 bladder cancer tissue decreased significantly compared with normal bladder mucosa, and the expression of Foxo3a mRNA in the T3-T4 stages of bladder cancer was significantly lower than that of Ta-T2 stage bladder cancer tissue (Figure 1A). The results of qRT-PCR showed that the expression of miR-182 mRNA in Ta-T2 bladder cancer tissue was significantly higher than that of normal bladder mucosa, and the expression of miR-182 in T3-T4 stages of bladder cancer was significantly higher than that of.
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The expression of miR-182 was related to the size of tumor tissue, TNM stage, and lymph node metastasis ($P < 0.05$), but there was no significant relationship between the age of the patients and the pathologic grade of tumor ($P > 0.05$), Table 1. The expression of Foxo3a was related to the size of the tumor, the TNM stage, the histopathologic classification, and lymph node metastasis ($P < 0.05$). There was no significant relationship with patient age ($P > 0.05$), Table 2.

Figure 1. Abnormal expression of miR-182 and Foxo3a in bladder cancer tissues. A. Expression of miR-182 in bladder cancer by qRT-PCR. B. Expression of Foxo3a mRNA in bladder cancer by qRT-PCR. C. Analysis of the correlation between the expression of miR-182 and Foxo3a mRNA in bladder cancer tissues by Spearman. D. Expression of Foxo3a protein in bladder cancer by western blot. *represents two groups compared, $P < 0.05$.

Relationship between the expression of miR-182 and Foxo3a and clinical characteristics in bladder cancer tissues

Using the median of the expression of miR-182 and Foxo3a mRNA, patients with bladder cancer were divided into miR-182 group, high expression of Foxo3a group, and low expression of Foxo3a group, and the relationship between the patients and the clinical features was analyzed. The results showed that the level of the expression of miR-182 was related to the size of tumor tissue, TNM stage, and lymph node metastasis ($P < 0.05$), but there was no significant relationship between the age of the patients and the pathologic grade of tumor ($P > 0.05$), Table 1. The expression of Foxo3a was related to the size of the tumor, the TNM stage, the histopathologic classification, and lymph node metastasis ($P < 0.05$). There was no significant relationship with patient age ($P > 0.05$), Table 2.

The expression of miR-182 and Foxo3a is related to the prognosis of patients with bladder cancer

Using the median of the expression of miR-182 and Foxo3a mRNA, bladder cancer patients were divided into miR-182 group, high expression of Foxo3a group, and low expression of Foxo3a group, and the relationship between the expression of miR-182 and Foxo3a and the survival and prognosis of the patients was analyzed. The survival curve analysis showed that the survival and prognosis in high expression of
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Table 1. Relationship between expression of miR-182 and clinicopathologic features of bladder cancer

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Table 2. Relationship between expression of Foxo3a mRNA and clinicopathologic features of bladder cancer

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There is a target regulatory relationship between miR-182 and Foxo3a

The online prediction results of microRNA.org website showed that there was a target complementary binding site between miR-182 and Foxo3a mRNA’s 3’-UTR (Figure 3A). The results of the double luciferase gene report showed that the transfection of miR-182 mimic could significantly reduce the relative luciferase activity of pGL3-Foxo3a-WT transfected HEK293T cells, but there was no significant effect on the relative luciferase activity in HEK293T cells transfected by pGL3-Foxo3a-MUT (Figure 3B), indicating a targeting regulation between miR-182 and Foxo3a mRNA.

Expression of miR-182 was increased and the expression of Foxo3a was decreased in bladder cancer cells

The results of qRT-PCR showed that the expression of miR-182 in RT112 and T24 cells of bladder cancer was significantly higher than that of human normal bladder epithelial cells SV-HUC-1 (Figure 4A), while the expression of Foxo3a mRNA decreased significantly (Figure 4B). Western blot showed that compared with SV-HUC-1 cells, the expression of Foxo3a protein in RT112 and T24 cells of bladder cancer decreased significantly (Figure 4C).

miR-182 group were significantly lower than that in low expression of miR-182 group (Log-rank test \(Z = 4.325, P = 0.038\)) (Figure 2A). The survival and prognosis in low expression of Foxo3a mRNA group were better than those in high expression of Foxo3a mRNA group (Log-rank Chi square 3.991, \(P = 0.046\)) (Figure 2B).

Inhibition of miR-182 can increase the expression of Foxo3a, inhibit cell proliferation, and promote apoptosis

The results of qRT-PCR detection showed that, compared with the miR-NC group, the transfection of miR-182 inhibitor could obviously
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decrease the expression of miR-182 in RT112 and T24 cells (Figure 5A), and increased the expression of Foxo3a mRNA (Figure 5B). Western blot showed that compared with the miR-NC group, the expression of Foxo3a protein in RT112 and T24 cells of miR-182 inhibitor transfection group increased significantly (Figure 5C). The results of flow cytometry showed that transfection of miR-182 inhibitor increased the apoptosis of RT112 and T24 cells (Figure 5D), and significantly increased cell proliferation (Figure 5E).

Discussion

Invasion and metastasis are important features of bladder cancer. Once pelvic lymph node metastasis or distant metastasis occurs in bladder cancer, the 5 year survival rate is usually less than 20% [12]. Also, frequent recurrence is also an important feature of bladder cancer. The recurrence rate is about 70% after operation, which poses a serious threat to the health and quality of life of the patients [13]. Therefore, the study of the pathogenesis of bladder cancer and the finding of abnormal changes in the process of bladder cancer are of great significance for diagnosis, therapy, and prognosis.

The FoxO transcription factor family contains 4 members: FoxO1, FoxO3a, FoxO4 and FoxO6. FoxO3a is currently the most studied transcription factor [14, 15]. FoxO3a is a well-defined tumor suppressor gene, which controls the
miR-182 and FXO3a in bladder cancer

Figure 4. The expression of miR-182 is increased, and the expression of Foxo3a is decreased in bladder cancer cells. A. Expression of miR-182 in bladder cancer cells by qRT-PCR. B. Expression of Foxo3a mRNA in bladder cancer cells by qRT-PCR. C. Expression of Foxo3a protein in bladder cancer cells with western blot. * means compared with SV-HUC-1 cells, P < 0.05.

expression of a variety of genes related to cell proliferation, cell cycle, and apoptosis such as BIM, p27Kip1, and cyclin D1. As a key node, FoxO3 controls multiple signal pathways and multiple biologic processes of the tumor cells [10,16,17]. The decreased expression and activity of FoxO3a is related to the occurrence, progression, and drug resistance of breast cancer [18], pancreatic cancer [19], and liver cancer [20]. The study shows that the decreased expression of Foxo3a is related to the malignant biologic characteristics of bladder cancer cells [11], and increased Foxo3a expression can inhibit bladder cancer.

miR-182 is a frequently studied microRNA. A number of studies have shown that the abnormal increase of miR-182 expression is related to the occurrence, progression, metastasis and poor prognosis of lung cancer [21], gastric cancer [22], breast cancer [23], and prostate cancer [24]. Studies have shown [8,9] that the expression of miR-182 is increased in bladder cancer tissue, so that miR-182 may play a role in promoting the pathogenesis of bladder cancer. Bioinformatic analysis showed that there was a target binding site between miR-182 and 3’-UTR of Foxo3a mRNA, suggesting a possible regulatory relationship between them. This study analyzed the expression of miR-182 and Foxo3a in the tumor tissues of patients with gastric cancer and their relationship with prognosis, and the role of miR-182 in the regulation of the expression of Foxo3a and the apoptosis of bladder cancer cells.

The results of this study showed that compared with normal bladder tissue, the expression of miR-182 in the tumor tissues of patients with bladder cancer increased significantly, while the expression of Foxo3a decreased significantly, and was related to the TNM stage, tumor size, histopathologic classification, and lymph node metastasis. The survival curve analysis showed that the increased expression of miR-182 was related to a poor prognosis, while the low expression of Foxo3a was associated with poor prognosis.

Chen et al. [8] showed that the expression of miR-182 in the tumor tissues of the patients with bladder cancer was significantly higher...
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Figure 5. Inhibition of miR-182 can increase the expression of Foxo3a, inhibit cell proliferation, and promote apoptosis. A. Expression of miR-182 by qRT-PCR. B. Expression of Foxo3a mRNA by qRT-PCR. C. Expression of Foxo3a protein by Western blot. D. Flow cytometry to detect cell apoptosis. E. Detection of cell proliferation by flow cytometry. * means compared with the miR-NC group, P < 0.05.

than that of normal bladder mucosa, and the area under the curve of the expression of miR-182 for the diagnostic value of gastric cancer (area under curve) was 0.913. Hirata et al. [9]
showed that the expression of miR-182 in the tumor tissues of patients with bladder cancer was significantly higher than that of the normal bladder mucosa, and the expression of miR-182 was related to the TNM stage and depth of the tumor. The survival rate of those with higher expression of miR-182 was significantly lower than that of those with higher miR-182 expression \((P = 0.0349)\). Han et al. [25] showed that compared with normal bladder epithelial tissue, the expression of miR-182 in bladder cancer tissues increased significantly. Wei et al. [26] showed that the level of expression of miR-182 in bladder cancer tissues was significantly increased. Pignot et al. [27] showed that the expression of miR-182 in the tumor tissues of patients with bladder cancer was significantly higher than that of normal bladder tissue by 68.6 times. In the higher multiples of non-muscle-invasive bladder cancer, NMIBC had an increase of 64.3, and that in muscle-invasive bladder cancer (MIBC) it increased by 79.6. These findings suggest that elevated expression of miR-182 may play a role in promoting bladder cancer and affect prognosis, similar to findings in this study.

The results of the double luciferase gene report showed that the transfection of miR-182 mimic could significantly reduce the relative luciferase activity of pGL3-Foxo3a-WT transfected HEK-293T cells, indicating that there was a target regulation relationship between miR-182 and Foxo3a mRNA, and this was confirmed by the negative correlation between miR-182 and the amount of Foxo3a in bladder cancer tissues. In vitro, the expression of miR-182 in bladder cancer cells was increased and the expression of Foxo3a was abnormally decreased, suggesting that abnormal miR-182 and Foxo3a were related to the malignant biologic characteristics of bladder cancer cells. Our study aimed to explore whether miR-182 and Foxo3a affect the biologic effects of bladder cancer cells. The results showed that transfection of miR-182 inhibitor into RT112 and T24 cells could increase the expression of Foxo3a, promote cell apoptosis, and inhibit cell proliferation, which indicates that miR-182 plays a role of promoting cancer by targeting Foxo3a.

Hirata et al. [9] showed that overexpression of miR-182 in T24 and UM-UC-3 cells of bladder cancer could inhibit the expression of RECK and Smad4 of the target gene, promote cell proliferation and migration, and reduce cell apoptosis. Jiang et al. [28] showed that Isorhapontigenin, ISO, could inhibit the expression of miR-182 and increase the expression of the target gene p27 of miR-182, inhibit the proliferation of T24 cells in bladder cancer and induce cell cycle arrest; but the overexpression of miR-182 antagonized the proliferation inhibition and apoptosis induction of ISO to T24 cells. The down-regulation of miR-182 can attenuate the malignant biologic characteristics of bladder cancer cells.

**Conclusion**

At present, although there are many studies on the relationship between miR-182 and bladder cancer, the studies targeted other genes. Our study combining miR-182 with Foxo3a revealed that the increased expression of miR-182 plays a role in reducing the expression of Foxo3a and promoting the pathogenesis of bladder cancer, which has not been reported previously. However, it is not clear which downstream gene of Foxo3a was affected by miR-182, so further research is still needed.

In conclusion, the expression of miR-182 increased and the expression of Foxo3a decreased in bladder cancer, which are related to prognosis. Downregulation of miR-182 can increase the expression of Foxo3a, inhibit the proliferation of bladder cancer cells, and induce apoptosis.

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**Disclosure of conflict of interest**

None.

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