Original Article

miR-202 promoted cell proliferation of prostate cancer by targeting SOX7

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Abstract: Objective: To investigate whether miR-202 regulate the proliferation of prostate cancer (PC) via SOX7 gene. Methods: Real-time quantitative PCR analysis was performed to observe the expression level of miR-202 in three PC cell lines (DU145, PC3, and LNCaP) and issues. Then, miR-202 mimic and the negative control were transfected to DU145, PC3 cell lines to explore the relationship between miR-202 and the proliferation of the PC cells in vitro. Meanwhile, bioinformatical prediction was performed to discover the target gene of the miR-202 (SOX7) which was confirmed by luciferase reporter assay. Moreover, the DU145 and PC3 cell lines were transfected with siRNA-SOX7 or the negative control to confirm the influence of SOX7 on the proliferation of PC. Additionally, the proliferation assay of PC3 cells adopted MTT method. Results: miR-202 expression was up-regulated in PC cell lines and tissues. The MTT assay revealed that the over-expression of miR-202 significantly promoted the proliferation of the PC cells in vitro. The SOX7 was the direct target of the miR-202 and miR-202 down-regulated the expression of SOX7 which was confirmed by the luciferase reporter assay. The knockdown of SOX7 remarkably promoted the proliferation of PC cells in vitro that verified by the MTT assay. Conclusion: The miR-202 could promote the proliferation of PC by targeting the SOX7, indicating a new potential therapeutic target and treatment for the PC.

Keywords: Luciferase reporter assay, miR-202, SOX7, prostate cancer

Introduction

Prostate cancer (PC) is one of leading causes of disease and death among men in the world. It was reported that about 29% of men who were 30 to 40 years old and 64% of men who were 60 to 70 years old were suffered from small prostatic carcinoma [1]. There were almost 180, 890 new PC cases and 26, 120 deaths in the United states of 2016; in men, PCs accounted for 20% new diagnoses [2]. Lifestyle-related factors, dietary factors and androgens have been recognized as important causes for PC [3]. Currently, with the development of medicine and examination technology, the treatment trend to be personalized and molecular therapy for PC is a hot spot [4]. Accordingly, researchers are keeping focusing on identification of new biomarkers or pathways of PC, which is of great importance and benefit for the treatment of the PC.

The microRNAs (miRNAs), small non-coding RNAs with 20 to 24 nucleotides, could regulate the gene expression post-transcriptionally via binding to the 3'-UTR of the target mRNA and have the potential to regulate approximately one-third of human genome [5]. Most miRNA genes are solo, and exhibit their functions under the modulation of their own promoters and regulatory sequences [6]. The miR-202 plays an important role in proliferation and suppression in many cancers. For example, Yu Jiajia et al reported that expression level of serum miR-202 in multiple myeloma patients was dramatically higher than in normal controls [7]; in human hepatocellular carcinoma, over-expression of miR-202 inhibited cell migration and proliferation [8]; miR-202 inhibited cell proliferation and induced apoptosis in osteosarcoma [9].

The Sry-related high-mobility-group box (SOX) genes are transcription factors which have specific DNA binding sites, 5'-(A/T) (A/T) CAA (A/T) G-3', and Sex Determining Region Y-Box 7 (SOX7) is grouped into the SOX F subfamily, which also includes the Sex Determining Region...
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Y-Box 17 (SOX17) and Sex Determining Region Y-Box 18 (SOX18) [10, 11]. It is reported that SOX7 plays an important role in various developmental processes, such as cardiogenesis, endoderm differentiation and hematopoiesis; for instance, the expression of SOX7 was remarkably down-regulated in the breast cancer tissues compared with the nontumorous tissues [12]. In the hepatocellular carcinoma (HCC), the SOX7 was the direct target of the miR-425, and the over-expression of the SOX7 could significantly suppress migration and invasion of HCC cells [13]. However, the role of SOX7 in PC proliferation has not been identified. Therefore, we investigated the potential functions of miR-202 and SOX7 on PC cell proliferation.

Materials and methods

Experimental design

Firstly, qRT-PCR and western blot were used to detect miR-202 expression level in PC cell lines and SOX7 mutated PC cell lines. The U6 snRNA and GAPDH were adopted as the miR-202 and SOX7 normalization, respectively. According to the results, we hypothesized that miR-202 promoted PC cell proliferation by targeting SOX7.

Secondly, we explored the relationship between miR-202 and SOX7. According to the data research by TargetScan online tool, SOX7 was hypothesized as a downstream target of miR-202 which was confirmed by luciferase reporter assay. The relative luciferase activity was detected to identify whether SOX7 was the target gene of miR-202. Finally, we investigate the effect of miR-202 and SOX7 on cell proliferation. MTT assay was used to perform these experiments.

Patient samples

The PC tissues and the corresponding nontumorous counterparts were obtained from patients of our hospital. The tissues samples were frozen in liquid nitrogen and stored at -80°C immediately after resection from patients who were undergoing the surgical treatment. Written consent of tissue donation for research purposes was provided by patients and the research has been approved by the ethics Committee of our hospital.

Cell culture

PC cell line DU145, PC3 and LNCaP were purchased from American Type Culture Collection.

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All the cell lines were cultured in RPMI 1640 with 10% fetal bovine serum (Gibco, USA).

RNA extraction and real-time quantitative PCR

The total RNAs from the PC tissues and corresponding nontumorous tissues were extracted and purified with Trizol reagent (Invitrogen, California, USA) following the manufacturer’s instruction. The concentration and purity of extracted RNA were determined using the NanoDrop 2000 instrument (Thermo Fisher Scientific, Massachusetts, USA). cDNAs were synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The real-time quantitative PCR was carried out with the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China) on the LightCycler 480 Instrument II (Roche, Basel, Switzerland). The GAPDH was used as the control for the normalization of expression levels of genes. The snRNA U6 was used as the internal control for miRNA; 2−ΔΔCt method was used to analyze the results. Specific primers were used in RT-PCR as follows: GAPDH (F: 5'-TGTGTCATCAATGACCC-3'; R: 5’-CTCCCGTTCTCAGCCTTG-3'); U6 snRNA (F: 5'-CTCGCTTCGGCGACACA-3'; R: 5'-AACGCTTCACGAATTCTGG-3'); SOX7 (F: 5'-TTTTATGTATATTTTGGTGTAGAT-3'; R: 5'-CTCACTCATCACAACCTTTAAC-3'); miR-202 (F: 5'-GCGAGAGGCATAGGGCATG-3; R: 5'-CAGTGCAAGGTCCCGAGGT-3').

Plasmid construction and siRNA interference assay

The miR-202 mimic was constructed for the over-expression of the miR-202. The siRNA-SOX-07 (5’-CCAUUGAGGAGUACUGCCCAAT-3’) was synthesized and purchased from Takara, which was used for the knockdown of the SOX7. The constructed plasmids were transfected into the DU145 cell line by lipofectamine 2000 reagent (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer’s instruction.

Western blotting

Total protein was extracted from cells using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein concentration was determined using the BCA method. The proteins were separated by the SDS-PAGE with Mini-Protein Tetra instrument (Bio-Rad, California, USA), and transferred onto PVDF membranes (Bio-Rad, California, USA). The membrane was probed with the anti SOX7 (Sigma, 1:1000) or anti GAPDH (Sigma, 1:1000,
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used for internal control) antibody. The IOD was measured using the GelDoc 2000 instrument (Bio-Rad, California, USA) and the relative expression quantity was counted.

Luciferase reporter assay

A sequence of 3’-UTR of SOX7 containing the predicted miR-202 binding site was synthesized and inserted into the p-MIR-reporter plasmid (p-MIR-SOX7-3’-UTR-WT) (Ambion, California, USA). Simultaneously, a sequence that contains seven mutant nucleotides of the 3’-UTR of SOX7 was inserted into the p-MIR-reporter plasmid (p-MIR-SOX7-3’-UTR-MUT). For the luciferase assay, the DU145 cells were transfected with equivalent luciferase reporter plasmids, miR-202 mimic, or the negative control by Lipofectamine 2000. The p-miR-Report β-galactosidase vector was used as the control. The luciferase activity was measured using the One-Glo luciferase assay instrument (Promega, Wisconsin, USA).

Cell proliferation assays

In this study, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) as the indicator for the cell proliferation. After transfection, the cells were cultured for 1-5 days and detected with MTT per day. The cells were incubated for 4 h at 37°C after the adding MTT. Remove the supernatants, dissolve the formazan crystals using the DMSO (150 μl/well), observe the absorbance at 490 nm for each sample with multilabel plate reader (PerkinElmer, Waltham, MA, USA).

Statistical analysis

SPSS17.0 software was used in this study. Measurement data was expressed as mean ± SD and comparison between two groups were performed with student’s t test; One-way ANOVA was adopted for comparison within a group. All the experiments were performed in three independent replicates, and the differences were considered statistically significant at P<0.05 (using the two-tailed paired t test).

Results

miR-202 expression was up-regulated in PC cell lines and tissues

Real-time quantitative PCR analysis showed that the miR-202 expression was significantly increased in the PC cell lines (DU145, PC3, and LNCaP) compared with the noncancerous prostate tissues (all P<0.01, Figure 1A). Real-time quantitative PCR analysis also identified that the miR-202 expression was remarkably up-
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regulated in 70 cases of PC tissues compared with the corresponding adjacent nontumorous tissues (P<0.01, Figure 1B). Generally, all of these finding showed that the miR-202 expression was significantly increased in the PC cell lines and tissues.

Over-expression of miR-202 promoted the proliferation of the PC cells in vitro

To better understand the function of miR-202 in the PC tissues, we transfected the miR-202 mimic or the negative control to the PC cell lines (DU145, PC3). The successful over-expression of the miR-202 was confirmed by the real-time quantitative PCR (P<0.01, Figure 2A). The influence of miR-202 on the proliferation of the DU145, PC3 cell lines was investigated using the MTT assay. The MTT results showed that the cell growth of the PC cell lines was significantly increased by the over-expression of miR-202 (P<0.05, Figure 2B).

The SOX7 was the direct target of the miR-202 which down-regulated the expression of SOX7

In order to discover the target gene of the miR-202, bioinformatical prediction was performed using the Target Scan website (http://www.targetscan.org/mamm_31/). The SOX7 was selected as the promising target from the predicted results. As shown in Figure 3A, the SOX7 has a binding site at the 36-42 bp of the 3'-UTR for miR-202.

To verify this prediction, the DU145 cell line was transfected with the p-miR-SOX7-3'-UTR-WT or p-miR-SOX7-3'-UTR-MUT luciferase reporter, and miR-202 mimic. The relative luciferase activity was significantly reduced in cell lines co-transfected with p-miR-SOX7-3'-UTR-WT luciferase reporter and miR-202 mimic than the negative control cells (P<0.05). On the contrary, the relative luciferase activity was reduced slightly in cell lines co-transfected with p-MIR-SOX7-3'-UTR-MUT luciferase reporter and miR-202 mimic than the negative control cells (Figure 3B).

The knockdown of SOX7 promoted the proliferation of PC cells in vitro

To confirm the influence of SOX7 on the proliferation of PC, the DU145 and PC3 cell lines were transfected with siRNA-SOX7 or the negative control. Compared with the negative control, the reduction of SOX7 expression in protein level was verified using the western blotting assay (Figure 4A). Moreover, the proliferation of these two SOX7-knockdown PC cell lines were significantly increased, which was confirmed by the MTT assay (both P<0.05, Figure 4B), suggesting that the knockdown of SOX7 could promote the proliferation of PC cells in vitro.

Discussion

It is meaningful to study the PC related gene expression and miRNA alteration. Dysregulation of miRNAs is a distinct feature of cancers, which derive from dysregulated gene expression [14, 15]. It is reported that miRNA alteration could induce carcinogenesis and miR-202 plays an important role in many cancers, such as NSCLC, esophageal squamous cell carcinoma, gastric cancer, and hepatocellular carcinoma [16-20]. In our study, we found that the miR-202 significantly up-regulated in the PC tissues compared with the adjacent nontumorous tissues and promoted the proliferation of PC cells. In contrast to our research, the miR-202 down-regulated in human hepatocellular carcinoma tumor tissues compared with their normal
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Using the Target Scan, we predicted and confirmed that the miR-202 bound to the 3'-UTR of SOX7. In the meanwhile, the luciferase reporter assay confirmed that the luciferase activity was reduced slightly in cell lines co-transfected with p-MIR-SOX7-3'-UTR-MUT luciferase reporter and miR-202 mimic than the negative control cells. In addition, we wonder that whether there are other sites which can regulate the 3'-UTR binding relation between the miR-202 and SOX7. It remains to be researched.

Recently, SOX7 has been found to involve in regulation of many cancers and functioned as a suppressor. In the human breast cancer cells, the miR-492 promotes the cell cycle and proliferation through inhibiting SOX7 [22]. Liu et al. found that the SOX7 expression was remarkably reduced in the ovarian cancer tissues compared with the noncancerous tissues which was regulated through Wnt/β-catenin signaling pathway [23]. Moreover, the miR-935 increased the tumorigenesis and cell proliferation by targeting the SOX7 in the gastric cancer [24]. In addition, the SOX7 also plays a role in acute myeloid leukemia, and functions as a tumor suppressor [25]. The SOX7 contains HMG box, which plays an essential role in DNA unwinding and binding, protein transportation and interaction [26]. These important domains may explain why the SOX7 could function as tumor suppressor in a number of cancers. In our study, the results showed that the knockdown of the SOX7 promoted the proliferation of PC cell lines and the miR-202 could suppress the expression of SOX7 in the luciferase reporter assays, which was consist with our hypotheses and the trend in the previous studies.
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However, there were still some drawbacks in our study. For example, the number of samples was small. On the other hand, the results lacked the clinical experiments. In our further study, further researches are needed to verify our findings with large sample size in clinic.

In conclusion, our results demonstrated that miR-202 significantly up-regulated in the PC tissues compared with the non-tumorous tissues, and it can also promote the PC cell proliferation. Furthermore, the miR-202 could bind to the 3’-UTR of SOX7 and suppress the SOX7 expression, suggesting that the miR-202/SOX7 axis may be a promising therapeutic target for the PC treatment.

Disclosure of conflict of interest

None.

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