miR-564 inhibited metastasis and proliferation of prostate cancer by targeting MLLT3

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Abstract. – OBJECTIVE: MiR-564 has been discovered to be abnormally expressed in human malignancy. Two recent studies suggested that miR-564 plays a role in tumor inhibition in both lung and breast cancer. However, no evidence reported the mechanism and function of miR-564 in prostate cancer (PCa).

PATIENTS AND METHODS: The PCa tissues and their adjacent normal tissues were collected from 50 PCa patients. Expressions of miR-564 in tissues and cells were evaluated with RT-qPCR. The MTT [3-(4,5-dimethylthiazol-2-yl)2,5-di-phenyl tetrazolium bromide] assay, flow cytometry and Western-blot analysis, were applied to detect the proliferation, cell cycle progression and the protein expression of PCa cell lines (PC-3 and DU-145). Migration and invasion of PCa cells were analyzed by Transwell assays. Furthermore, the correlation between miR-564 and MLLT3 was assessed by luciferase reporter assay. Also, the PCa cells were transfected with miR-564 mimics control and inhibitor.

RESULTS: In our present research, miR-564 was found dysregulated in PCa cells and to act as a suppressor in PCa cell proliferation, progression of cell cycle, cell invasion and migration. MLLT3 (also known as Af9) is a proto-oncogene, which has first reported in leukemia, and the regulation of its expression remains incompletely elucidated. Also, it is first reported in our study, suggesting that MLLT3 is a direct target of miR-564. The results also showed a significant negative correlation with miR-564 in PCa cells. Furthermore, up-regulation of MLLT3 attenuates the effects of miR-564 on the ability of PCa cells.

CONCLUSIONS: Our research demonstrated the suppressor function of miR-564 in PCa, revealing restoration of miR-564 as a potential therapeutic strategy for the treatment of PCa.

Key Words: miR-564, MLLT3/Af9, Prostate cancer.

Introduction

Prostate cancer (PCa) is an important public health problem around the world. Its incidence rate ranks 2nd in the male tumors, and its fatality rate ranks 6th in all male tumors in the world. Besides, its incidence rate is 28.5/0.1 million people currently, and the incidence rate of PCa in China also shows an increasing trend year by year. The clinical symptoms of PCa lack specificity. It has no obvious symptoms in the early stage, but hematuria, lower urinary tract obstruction or irritation symptoms may occur in the progressive stage. The main complications of advanced PCa include the refractory ostealgia, pathological fractures, anemia and spinal cord compression, etc. It is reported that the incidence rate of bone metastases is about 65-75%, and the 5-year survival rate of patients is 25% with poor quality of life. Prostate Specific Antigen (PSA) combined with digital rectal examination is always recognized as the best screening method for early detection of PCa. However, PSA detection still cannot completely distinguish benign from malignant cancer, and PCa cannot be completely ruled out from patients with less than 4 ng/mL antigen, either. PLCO (Prostate, Lung, Colorectal, and Ovarian) study results showed that PSA screening may lead to over-examination and treatment, increased adverse reactions and complications, and higher medical cost, but it cannot reduce the mortality rate of males aged below 55 years old. Therefore, searching for a new screening method of PCa has become one of the research hotspots in recent years. More and more studies have focused on the diagnosis of PCa by detecting the changes in microRNA (miRNA) levels in human blood and
miR-564 inhibited prostate cancer

urine samples. For example, Chen et al. identified five miRNAs that were abnormally expressed in the serum of PCa patient for the differential diagnosis of PCa and benign prostatic hyperplasia. Brace et al. found that miR-141 and miR-375 are important indexes of prognosis estimation of PCa through the screening of plasma in patients with non-PCa metastasis and PCa metastasis.

MiR-564 has firstly been described as potential blood-based biomarkers in schizophrenia patients together with six other miRNAs. The later reports suggested miR-564 has an inhibitory effect on lung cancer and breast cancer. However, the roles of miR-564 in PCa tumorigenesis and underlying mechanisms remain unknown.

MLLT3 (also known as Af9), a proto-oncogene, was first reported in leukemia and involved in many different cellular processes, such as cell differentiation, cell fate decision and nervous system development. MLLT3 gene has been reported to play a pathological role in neurodevelopmental diseases. A previous study showed that disruption of MLLT3 was associated with mental retardation, epilepsy and ataxia in human. A later study demonstrated that MLLT3 has promotion function in lymphocytoma.

In our study, we analyzed the biological function of miR-564 in PCa cells. For the first time, MLLT3 was identified to be a direct and functional target through which miR-564 acts as tumor suppressor in PCa cells. These findings indicated that the miR-564 has a tumor suppressive function in the PCa progress and may become a potential treatment for the PCa.

**Patients and Methods**

**PCa Cases and Cells**

This study included 50 PCa patients who received treatment with radical prostatectomy at Weifang People’s Hospital and underwent pathological diagnoses to be confirmed as PCa. Preoperative chemotherapy or radiotherapy treatments were forbidden. The liquid nitrogen was used to freeze PCa tissues and corresponding adjacent normal tissues before being kept in -70°C refrigerator. The adjacent normal tissues have to be concerned by biological biopsy to be sure that they do not include cancer cells. This study was approved by the Ethics Committee of Weifang People’s Hospital. Signed written informed consents were obtained from all participants before the study.

The human PCa lines (PC-3 and DU-145) together with the adult human prostatic epithelial cell line (RWPE-1) were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) complemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 IU/mL penicillin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5% CO₂ cell culture incubator.

**qRT-PCR Analysis**

Total RNA was procured by TRizol reagent in accordance with the manufacturer’s protocol. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to measure the level of miR-564 expression normalized to miRNA U6. SYBR green qPCR assay (Life Science, San Diego, CA, USA) was used to measure the level of MLLT3 expression and endogenous controlled by GAPDH (Life Science, San Diego, CA, USA).

**Western Blot Analysis**

Protein concentration was measured by using bicinchoninic acid (BCA) reagent kit (Merek, NJ, USA). Cell lysates were separated on polyacrylamide gels and electro blotted onto nitrocellulose membranes. Then, they were blocked with Tris-buffered saline (TBS) with 0.05% Tween 20, pH 7.6 with 5% skimmed milk. After that, the blot was washed and incubated with anti-MLLT3 antibody (Abcam, Cambridge, MA, USA, Dilution 1:1000) or anti-GAPDH (Abcam, Cambridge, MA, USA, Dilution: 1:1000) antibody, which was blotted to show equal protein loading at 4°C overnight.

**Transfection**

MiR-564 mimics control and inhibitor were synthesized and transfected to PCa cell lines to analyze biological function of miR-564. Then, three groups were established to study the potential relevance between miR-564 and PCa. MiR-564 mimics (PCa cell transfected by miR-564 mimics), mimics +MLLT3 (PCa cell transfected by miR-564 mimics and siMLLT3) and NC group (negative control). All the stuff was purchased from Guangzhou RiboBio (Guangzhou, China), and were transfected by using lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions.
Cell Proliferation and Cell Cycle Assay

PCa cells were harvested and inoculated into 96-well plates at a density of $2 \times 10^3$ cells for 48 hours, MTT solution (5 mg/mL, MultiSciences, Hangzhou, China) was appended to each well after 4-hour incubation. Then, 150 μL of dimethyl sulfoxide (DMSO) was added to each well for solubilizing the formazan formed. After 30 min, the absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) set at 490 nm.

For cell cycle analysis, PCa cells were obtained 3 days after transfection. The number of PCa cells in different cell phase was measured with the cell cycle staining kit (Multi Sciences Biotech Co., Ltd., Hangzhou, China) by flow cytometry. The rate of cells in G0/G1 or S phases is presented in the results.

Luciferase Reporter Assays

PCa cells were co-transfected with pMIR-30UTR-MLLT3 or pMIR-30UTR-Mut MLLT3 and miR-564 mimic or negative control (NC), and the pMIR-Renilla plasmid (Promega, Madison, WI, USA) followed being seeded into a 24-well plate. The cells were then lysed post-transfection. The luciferase activity was assessed using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA), and results were normalized to Renilla luciferase activity.

Cell Invasion and Migration Assays

Cell invasion and migration assays were performed using Transwell plates (Corning Incorporated, Corning, NY, USA) with 8-μm-pore size membranes with Matrigel (for invasion assay) or without Matrigel (for migration assay). Briefly, $2 \times 10^4$ cells were planted into the upper chambers with serum free medium. On the other hand, the lower chamber was offered with medium containing 10% fetal bovine serum (FBS) as a chemotactrant. After 2 days incubating, the cells on the top of membrane were wiped by a brush. Subsequently, the membrane was stained by 0.2% crystal violet followed drenched by 95% ethanol. The cells of migrating or invading were noted by an inverted microscope.

Statistical Analysis

Statistical analysis was performed with a Student's t-test or F-test. All $p$-values were two-sided and $p<0.05$ were considered significantly and analyzed by Prism 6 software (Version X; La Jolla, CA, USA).

Results

MiR-564 Expression Found Reduced both Tissues and Cells of PCa

To examine the role of miR-564 in PCa, we used qRT-PCR to detect the expression level of miR-564 in PCa tissues and the adjacent normal tissues. The results showed that the expression of miR-564 is pretty lower in PCa tissues compared with the adjacent normal tissues ($p<0.05$) (Figure 1). Furthermore, we found the same results in PCa cells compared to the RWPE-1 cell ($p<0.05$) (Figure 2). Together, we thought miR-564 might have the regulatory effects in PCa progression.

Figure 1. The expressions of miR-564 in prostate tissue samples. Difference in the expression of miR-199a-3p between prostate cancer tissues and corresponding adjacent normal prostate tissues. **** $p<0.0001$ compared with RWPE-1.

Figure 2. The expressions of miR-564 in prostate cells. The expression of miR-199a-3p in prostate cancer cell lines (PC-3 and DU145) and normal prostate epithelial cells (RWPE-1). ***$p<0.001$ compared with RWPE-1.
**MiR-564 Suppressed Proliferation of PCa cells**

To examine the function of miR-564 on proliferation of PCa cells, we performed MTT assay to detect the cell proliferation rates. The MTT results showed that up-regulation of miR-564 significantly decreased cell proliferation rates in both PC-3 and DU-145 cells. In contrast, down-regulation of miR-564 promoted cell growth of PCa cells ($p<0.05$) (Figure 3A, B). In order to understand the underlying mechanism, we make a further experiment to explore whether the miR-564 influences on cell cycle progression. We found that overexpression of miR-564 significantly increased the percentage of cells at G0/G1 phase. However, inhibition of miR-564 exhibited an opposite effect ($p<0.05$) (Figure 3C, D). As a result, the present study suggested that miR-564 inhibited PCa cell growth via inducing cell-cycle arrest at G0/G1 phase.

**MiR-564 Inhibited Migration and Invasion of PCa Cells**

Migration and invasion are two most key factors in cancer cell proliferation. In the Transwell experiments, results showed that migration and invasion in both PC-3 and DU-145 cells were restricted after up-regulation of miR-564 with mimics. However, inhibition of miR-564 resulted in promotion of migration and invasion in PCa cells ($p<0.05$) (Figure 3G-H).

**MLLT3 is a Direct Target of miR-564 in PCa Cells**

In order to explore potential target of miR-564, we made a prediction via searching three publicly available algorithms (Target Scan, miRDB and microRNA) to elucidate the putative and possible targets of miR-564. Finally we found the MLLT3 was a supposed target of miR-564 (Figure 4A) (564c). A recent study has demonstrated that the MLLT3 has the function of promotion in lymphocytoma15. Thus, MLLT3 has caught our attention. To confirm whether miR-564 has regulation effect on MLLT3, Firstly, we established luciferase reporter vectors containing the wild or mutant type miR-564 seed sequences of the MLLT3 30UTR. Increase the expression of miR-564 with mimics result in the decrease of the luciferase activity of the wild type MLLT3 30UTR reporter gene, but it has no effect on mutant-type (Figure 4C). Furthermore, we found that up-regulation of miR-564 decreases the expression level of protein of MLLT3 in both PC-3 and DU-145 cells in Western blot experiment (Figure 3E-F). Secondly, we explored the correlation between MLLT3 and miR-564 on PCa cells. We set up three groups to conduct the similar experiments (miR-NC group, miR-564 mimics group, and the mimics + MLLT3 group) in PC-3 cells. As we expected, restoration of MLLT3 has the reverse force on the depressing effect induced by miR-564 on the proliferation of PCa cells (Figure 4B), cell cycle progression (Figure 4D), protein expression (Figure 4E), migration, and invasion (Figure 4F). Taken it all, the results indicated that the up-regulation of miR-564 has a negative relationship with MLLT3 in PCa cell, miR-564 suppressed the metastasis and proliferation of PCa cells, and MLLT3 restoration partially weaken the inhibition of miR-564.

**Discussion**

PCa is a major public health problem in developed countries1,16. Traditional treatments of PCa rely on hormone therapy, but the cancer cells gradually become insensitive and resistant to hormone treatment as the disease progress17. Thus, it is important to explore the pathologic process of disease development. Loss of cycle control is an important pathological factor in the occurrence of tumors including PCa. Cell cycle disorder is an important cause for unstable genetic information and malignant cell transformation18. Studies have showed a large number of cycle regulatory proteins that are abnormally expressed in PCa. Many cycle regulation-associated proteins will mutate in PCa, which may lead to the activation of oncogenes and inactivation of tumor-suppressor genes19. The pharmacological basis of a lot of therapeutic drugs of PCa, including the existing chemotherapy drugs, is the cell cycle regulation20. Therefore, the study on the new cycle regulatory genes will undoubtedly bring breakthroughs to the development of new targeted drugs of PCa. At present, studies have demonstrated that miRNAs play an important role in the transformation of stem cells from G1 phase to S phase21, but the G1 phase of tumor cells is short and G1/S detection sites are lacked. At this point, tumor cells and embryonic stem cells are similar. There are many miRNAs that inhibit the tumor proliferation by regulating cell cycle. For example, miR-765 arrests the cell cycle in G2 phase via inhibiting HMGA 122. MiR-107 and miR-449a can induce the G1 phase arrest by targeting CDK4/CDK623. This phenomenon suggests that miRNA may be closely related to
the tumor cell cycle regulation. All these cycle-related tumor suppressor microRNAs are expected to be new therapeutic targets for PCa. Here in our study, we analyzed samples from 50 PCa patients. These results indicated that compared with corresponding adjacent normal tissues, miR-564 expression was significantly down-regulated in PCa. We also found that expressions of miR-564 in PC-3 and DU-145 cell lines were significantly decreased compared with RWPE-1 cells, suggesting that miR-564 has a significant effect on PCa development. Besides, over-expressed miR-564 inhibited the proliferation of PCa cells and had the function of inducing cell-cycle dispute at G0/G1 phase. The invasion and metastasis of prostate cancer is a multi-step and multi-factor complex process, involving a variety of adhesion-related molecules, proteolytic enzymes, many cytokines.
miR-564 inhibited prostate cancer

and regulatory factors. More and more evidence suggests that miRNA is also closely related to the metastasis of PCa. For example, the suppressor gene of tumor, p-63, can inhibit the tumor metastasis via regulating the expression of miR-205, and patients with p-63/miR-205 deletion are often accompanied with high Gleason score and easy tumor metastasis. Our findings suggested that the ability of migratory and invasive of PCa cells were restricted by up-regulation of miR-564, suggesting that miR-564 has a certain inhibitory effect on tumor proliferation.

Subsequently, we searched the potential targets through which miR-564 exerted its effect on PCa cells. MLLT3 aroused our interest after it was analyzed in three online bioinformatics databases, which have been suggested to have the function of promotion in malignancy. As we expected, we found that the expression of miR-564 negatively correlated with the expression of MLLT3 utilizing in luciferase assay and Western blot. Moreover, MLLT3 overexpression seriously impacts the inhibitive function of miR-564 on PCa cells in cell proliferation, cell cycle, the invasion and metastasis of cell, indicating that miR-564/MLLT3 axis may be an important mechanism in the tumorigenesis and development of PCa.

Conclusions

We explored the tumor inhibitory function of miR-564 in PCa cells. MLLT3, as the direct and function target of miR-564, attenuated the suppressor effect in metastasis and proliferation of PCa cell. Thus, restoration of miR-564 could be a potential therapeutic strategy for the treatment of PCa.
Conflict of interest
The authors declare no conflicts of interest.

References


