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Original Article

miR-377-3p suppresses cell proliferation and promotes apoptosis of prostate cancer cells

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Abstract: Objective: The aim of the current study was to explore the effects of miR-377-3p overexpression and miR-21 downregulation on proliferation and apoptosis rates of prostate cancer cells. Methods: Quantitative reverse transcription polymerase chain reaction was employed to detect expression of miR-21 and miR-377-3p in prostate cancer and para-cancerous normal tissues. Moreover, miR-21 inhibitors and miR-377-3p overexpression vectors were transfected into cell line PC-3. Cells were divided into four groups, including experimental group A (transfected with miR-377-3p), experimental group B (transfected with miR-21 inhibitor), negative control group, and blank group. CCK-8 testing and flow cytometry were used to detect the proliferation capacity and apoptosis of prostate cancer cells, respectively. Possible target genes of miR-21 and miR-377-3p, such as PAK2 and PDCD4, were detected by RT-PCR and Western blotting, respectively. Results: Relative expression of miR-21 in prostate cancer tissues was significantly higher than that in para-cancerous tissues ($P<0.05$). Relative expression of miR-377-3p in prostate cancer tissues was significantly lower than that in para-cancerous tissues ($P<0.05$). After transfection with miR-21 inhibitors, expression of miR-21 in PC-3 cells was significantly decreased, while that of miR-377-3p in PC-3 cells was significantly increased. Cell viability in experimental group A and experimental group B was not significantly different between the two groups ($P>0.05$), but significantly lower than that in the negative control group and blank groups ($P<0.05$). Apoptosis rates in experimental group A and experimental group B were not significantly different ($P>0.05$). However, these rates were significantly higher than those in negative control and blank groups ($P<0.05$). Expression of PAK2 protein in the experimental group A was significantly lower than that in the blank group and negative control group. Levels of PDCD4 mRNA and PDCD4 protein in the experimental group B were significantly higher than those in the blank group and control group ($P<0.05$) Conclusion: miR-21 is highly expressed in PC-3 cells, while miR-377-3p is downregulated. Inhibition of miR-21 expression and overexpression of miR-377-3p may inhibit proliferation of PC-3 cells and promote apoptosis. Moreover, miR-21 and miR-377-3p may be considered molecules of significance for targeted therapy of prostate cancer.

Keywords: miR-21, miR-377-3p, prostate cancer, cell proliferation and apoptosis

Introduction

Prostate cancer is a common malignancy in males, with approximately 1.1 million cases reported worldwide in 2012. Incidence of prostate cancer has increased since 2012, making this disease a serious health threat and one of the main causes of cancer-related deaths in men [1, 2]. Patients with early-stage prostate cancer rarely present with significant symptoms. Many patients visit the doctor only when they feel obvious discomfort. Unfortunately, this often leads to diagnosis of advanced prostate cancer [3]. Surgery and radiotherapy are the principle treatments for prostate cancer. However, traditional therapy has not been very effective [4]. In recent years, molecular targeted therapy has interested many clinicians, making it a hot spot in cancer treatment [5]. MicroRNAs (miRNAs) are non-coding RNAs involved in several biological processes. They bind to specific target mRNAs and degrade or inhibit the translation of mRNAs, thereby regulating gene expression. They also participate in various cellular processes, such as cell proliferation and apoptosis, including those of tumor cells [6, 7]. Previous studies [8] have shown that miRNAs are abnormally expressed in many

malignant tumors. This is explained by the inhibition or promotion of tumor cell growth by miRNAs.

Other studies [9] have shown that miR-21 is abnormally elevated in many malignant tumors, such as breast cancer. It has been hypothesized that miR-21 promotes the proliferation of various tumor cells. Previous studies [10] have clearly indicated that miR-21 plays a role in promoting proliferation and reducing apoptosis in a variety of tumor cells. However, the mechanisms have not yet been clarified. Additionally, other studies [11, 12] have reported that the sensitivity of various tumor cells to anti-tumor drugs is affected by changing the expression of miR-21. This has an impact on the therapeutic effects of drugs used. miR-377-3p inhibits tumor growth. Its expression has been discovered in non-small cell lung cancer [13], pancreatic cancer [14], and other cancers. Moreover, miR-377-3p has been reported to be downregulated in prostate cancer tissue [15]. However, its effects on proliferation and apoptosis of prostate cancer cells have not been fully explored.

Both miR-21 and miR-377-3p are popular targets for prostate cancer. However, recent research concerning these two miRNAs in prostate cancer has been relatively simple. Therefore, the current study examined expression of miR-21 and miR-377-3p in prostate cancer tissues and para-cancerous tissues, investigating the effects of miR-377-3p inhibition on the proliferation and apoptosis of prostate cancer cells. Present data provides further evidence concerning the roles of miR-377-3p in prostate cancer and may contribute to the development of targeted therapy for prostate cancer.

Materials and methods

Tissue specimen collection

Ninety-eight patients undergoing prostate cancer resections were prospectively enrolled. The average age of these patients was 62.1±5.9 years. Of these 98 patients, 23, 34, 21, and 20 had stage I, II, III, and IV prostate cancer, respectively. During resection surgery, 98 prostate cancer tissues and 76 para-cancerous tissues were collected. Informed consent was obtained from all patients before surgery. All patients were pathologically diagnosed with prostate cancer. Patients with other tumors or serious organ failure, patients that did not consent to participation in the study, and patients with communication disorders and mental illnesses were excluded. All specimens were cryopreserved immediately after excision. Specimen collection was approved by the Ethics Committee of Yanbian University Affiliated Hospital.

Materials and instruments

Human prostate cancer PC-3 cells were purchased from the cell bank of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. SpectraMax M5 microplate reader was purchased from Shanghai Molecule Device. CytoFLEX LX flow cytometer was purchased from Beckman, USA. Real-time quantitative PCR instrumentation was purchased from BioRad, USA. TRIzol Reagent was purchased from Thermo Fisher Scientific, USA, while the qRT-PCR kit and minScript reverse transcription kit were purchased from Dalian TaKaRa Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco, USA. Fetal bovine serum (FBS) and trypsin were purchased from Hyclone, USA. CCK-8 kit was purchased from Beyotime Institute of Biotechnology. Annexin V-FITC/PI double staining kit was purchased from Becton Dickinson and company, USA. Finally, miR 37~3p mimetics and miR21 inhibitors were purchased from Shanghai Gene Pharma Co., Ltd., by which the primers were designed.

Experimental methods

Expression of miR-377-3p and miR-21 in prostate cancer tissues and para-cancerous tissues: Prostate cancer tissues and para-cancerous tissues were removed from the cryopreservation system. Total RNA was then extracted with TRIzol Reagent. Next, purity and concentration levels of RNA were detected using an ultraviolet spectrophotometer. According to manufacturer instructions, 1 μg of total RNA was reverse transcribed to cDNA. Reaction parameters were 16°C for 15 minutes, 42°C for 42 minutes, and 85°C for 5 minutes. Moreover, cDNA was used for qPCR amplification, according to manufacturer instructions, with U6 as the internal reference material. Primer sequence is shown in **Table 1**. qPCR reaction conditions were: pre-denaturation at 95°C for 5

Table 1. Related RNA primer sequence listing

	Forward primer	Reverse primer
miR-377-3p	5'-GGGAGGCAGTGTATTGTTA-3'	5'-CAGTGCGTGTCGTGGAGT-3'
miR-21	5'-GCGGTAGCTTATCAGACTGA-3'	5'-TGCGTGTCGTGGAGTC-3'
U6	5'-GCTTCGGCAGCACATATACTAAAAT-3'	5'-CGCTTCACGAATTTGCGTGTCAT-3'

minutes, followed by 40 cycles (95°C, 10 seconds; 60°C, 30 seconds) of amplification, with a final extension at 72°C for 20 seconds. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and all experiments were repeated 3 times.

Cell cultivation and transfection: Human prostate cancer PC-3 cells were placed in DMEM containing 10% PBS and cultivated at 37°C under 5% CO_2 conditions. Pancreatin (25%) was added for digestion when adherent cell growth was observed to reach 85%. Cells were placed in the medium to continue cultivation after digestion, completing the sub-cultivation. Cells in the log phase were transfected after grouping. Non-transfected cells were included in the blank group. Cells transfected with miR-377-3p and miR-21 inhibitors were included in experimental groups A and B, respectively. Cells transfected with miRNA negative control (miR-NC) were included in the negative control group. Lipofectamine 2000 and DNA were diluted and mixed, according to manufacturer instructions, incubated at room temperature for 5 minutes, and finally mixed with the cells. Transfection was carried out at 37°C in 5% CO_2.

CCK8 tests for detection of cell proliferation: After 48 hours of transfection, cells from each group were deposited onto 96-well plates, diluted to 2×10^3 cells/mL with 100 μl in each well, and cultured at 37°C in 5% CO_2. On days 1, 2, and 3 of cell growth, 10 μl of CCK8 solution was added to each well. The cells were then cultivated for 4 hours. Cell proliferation was calculated by measuring the absorbance at 450 nm using a microplate reader. The experiment was repeated 3 times.

Flow cytometry detection of apoptosis in each group: Apoptosis was detected using Annexin V-FITC/PI double staining flow cytometry. PC-3 cells transfected with miR-377-3p, miR-21 inhibitors, and miRNA NC were deposited onto a 6-well plate at a density of 3×10^5 cells/well. After incubation for 48 hours, the cells were flushed two times with pre-cooled PBS. They were then re-suspended and incubated for 20 minutes at room temperature in the dark after adding 5 μl Annexin V-FITC and 10 μl of PI. Finally, apoptosis was detected using a flow cytometer. The experiment was repeated three times.

Detection of target genes of miR-377-3p and miR-21: Possible target genes of miR-377-3p and miR-21 were analyzed using bioinformatics prediction, possibly including PAK2 and PDCD4 genes. PAK2 mRNA, PDCD4 mRNA, and PAK2 and PDCD4 proteins were detected using RT-PCR and Western blotting, respectively. The upstream sequence of PAK2 mRNA was: 5'-TGAGCACACCATCCATGTTGG-3', the downstream sequence was: 5'-AGGTCTGTAGTAATCGAGCCC-3', the upstream primer sequence of DCD4 mRNA was: 5'-AAA GGA AGGGGACTACCAAAGAAA G-3', and the downstream primer sequence was: 5'-CACCTCCTCCACATCATACACCTG-3'. With GAPDH as an internal reference, the upstream primer sequence was: 5'-GGAGCGAGATCCCTCCAAAAT-3' and the downstream primer sequence was: 5'-GGCTGTTGTCATACTTCTCATGG-3'. RT-PCR was conducted according to the description above.

Western blotting was used as follows. After 48 hours of cell transfection, cells were lysed and total proteins of PAK2 and PDCD4 of each group were collected. Proteins were then separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk at room temperature for 1 hour. PAK2 PDCD4 primary antibody was then added and kept at 4°C overnight. Next, the secondary antibody was added for incubation Finally, color development was carried out.

Statistical analyses

Experimental data were analyzed using SPSS 20.0 software (Beijing Net Data Times Technology Co., Ltd.). Data are expressed as mean ± standard deviation. Two groups were compared using t-tests, while differences among multiple

Table 2. Expression of miR-377-3p and miR-21 in prostate cancer tissues

	Prostate cancer tissue n=98	Para-cancerous tissue n=76	t	P
miR-377-3p	0.367±0.104	1.257±0.216	35.81	<0.001
miR-21	3.137±0.793	0.911±0.169	24.04	<0.001

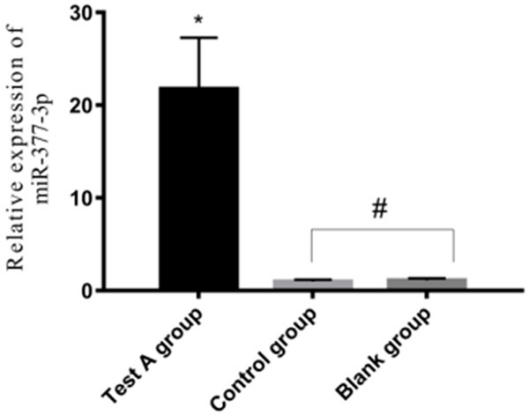

Figure 1. Relative expression levels of miR-377-3p in each group. Expression of miR-377-3p in experimental group A was significantly higher than that in the negative control and blank group (P<0.05). There was no significant difference in expression of miR-377-3p between the negative control and blank group (P>0.05). Note: *P<0.05 compared with the experimental group and control group; #indicates P>0.05.

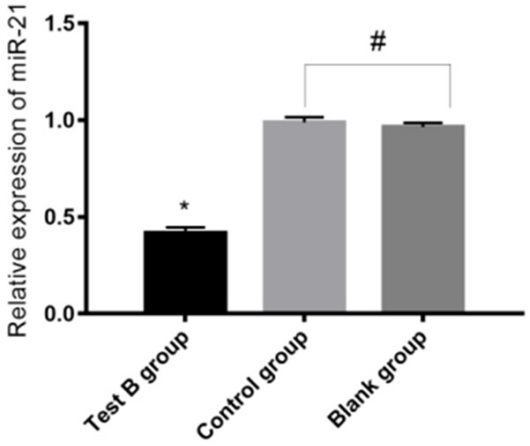

Figure 2. Expression of miR-21 in experimental group B was significantly higher than that in the negative control and blank group (P<0.05). There was no significant difference in expression of miR-21 between the negative control and blank group (P>0.05). Note: *P<0.05 compared with the experimental group and control group; #indicates P>0.05.

groups were compared using one-way ANOVA. P<0.05 indicates statistical significance.

Results

Expression of miR-377-3p and miR-21 in prostate cancer tissues

The relative expression level of miR-21 in prostate cancer tissues was 3.137±0.793, significantly higher than that in para-cancerous tissues (0.911±0.169). The difference was statistically significant (P<0.05). The relative expression level of miR-377-3p in prostate cancer tissues was 0.367±0.104, significantly lower than that of miR-377-3p in para-cancerous tissues (1.257±0.216). The difference was statistically significant (P<0.05) (**Table 2**).

Comparison of miR-377-3p and miR-21 expression levels in transfected PC-3 cells in the four groups

Expression levels of miR-377-3p in experimental group A, negative control group, and blank group were 21.763±5.519, 0.986±0.193, and 1.102±0.235, respectively. Expression of miR-377-3p in experimental group A was significantly higher than that of the negative control group and blank group (P<0.05). There were no significant differences in expression of miR-377-3p between the negative control group and blank group (P>0.05). Expression levels of miR-21 in experimental group B, negative control group, and blank group were 0.417±0.029, 0.988±0.027, and 0.964±0.021, respectively. Expression of miR-21 in experimental group B was significantly higher than that in the negative control group and blank group (P<0.05). There were no significant differences in expression of miR-21 between the negative control group and blank group (P>0.05) (**Figures 1** and **2**).

Comparison of the proliferation of transfected PC-3 cells in the four groups

Cell viability of experimental group A, experimental group B, negative control group, and blank group was 71.29±4.64%, 69.91±3.17%, 96.14±2.09%, and 97.02±2.29%, respectively. There were no significant differences in cell viability between experimental group A and experimental group B (P>0.05). Cell viability in experimental group A and experimental group B was significantly lower than that of the negative control group and blank group (P<0.05). There were no significant differences in cell viability

Table 3. Survival rates of PC-3 cells in each group after transfection (%)

Factor	Test A group	Test B group	Negative control group	Blank group
Survival rate	71.29±4.64*	69.91±3.17*	96.14±2.09	97.02±2.29

Note: *Compared with the control group and blank group, P<0.05.

between the negative control group and blank group (P>0.05) (**Table 3**).

Comparison of apoptosis rates of transfected PC-3 cells in the four groups

Apoptosis rates of experimental group A, experimental group B, negative control group, and blank group were 19.15±1.43%, 18.79±1.48%, 2.07±0.52%, and 2.12±0.61%, respectively. The apoptosis rate in group A was not significantly higher than that in group B (P>0.05). However, the apoptosis rate in group B was significantly higher than those in the negative control group and blank group (P<0.05). There were no significant differences in apoptosis rates between the negative control and blank group (>0.05) (**Figures 3** and **4**).

Expression of PAK2 mRNA, PDCD4 mRNA, and PAK2 and PDCD4 protein

After transfection of miR-377-3p and miR-21 inhibitors, expression of PAK2 mRNA and PAK2 protein in experimental group A was significantly lower than that in the blank group and negative control group (P<0.05). Expression of PDCD4 protein in experimental group B was significantly higher than that in the blank group and negative control group (P<0.05) (**Figures 5** and **6**).

Discussion

A common malignant tumor of the reproductive system, prostate cancer has high incidence and mortality rates, worldwide. Therefore, it is a serious threat to the health of males [16]. A previous study [17] showed that prostate cancer, if diagnosed and treated in the early stages, can be effectively controlled and survival rates of patients can be greatly improved. Although prostate cancer has always been treated with traditional methods, such as surgery and chemotherapy, molecular targeted therapy has become a research hotspot in the treatment of prostate cancer [18]. Understanding the roles of miRNAs in cancer is important in the development of molecular targeted therapy. Some studies [19] have shown that miRNAs play diverse roles in tumor development by participating in target gene regulation. Therefore, miRNAs may provide a new breakthrough concerning the diagnosis and treatment of cancer. miR-21 and miR-377-3p are current miRNAs of great interest in tumor molecular targeted therapy [20]. Some studies [21, 22] have reported that miR-21 affects various biological processes, such as proliferation and apoptosis of tumor cells, by inhibiting genes, such as tumor programmed apoptosis factor 4 and tropomyosin. Additional studies [23, 24] have found that overexpression of miR-21 in tumor tissues results in poor prognosis and poor sensitivity to chemotherapeutic drugs in some patients with cancer. However, the detailed molecular mechanisms underlying this phenomenon remain unclear and have rarely been reported. Unlike expression of miR-21 in tumor cells, some studies [25] have reported that miR-377-3p inhibits tumor growth. For example, [26] it reduced the proliferation and migration of melanocytes in melano-blastoma by regulating E2F signaling pathways. Another study [27] found that miR-377-3p overexpression inhibited proliferation and promoted apoptosis of non-small cell carcinoma. However, there are few reports regarding the roles of miR-377-3p in prostate cancer cells. Therefore, the current study investigated expression levels of miR-21 and miR-377-3p in prostate cancer cells, examining their effects on proliferation and apoptosis of prostate cancer cells.

This study observed and compared expression levels of miR-21 and miR-377-3p in prostate cancer tissues and para-cancerous tissues, finding that relative expression levels of miR-21 in prostate cancer tissues were significantly higher than those of miR-21 in para-cancerous tissues (P<0.05). Relative expression levels of miR-377-3p in prostate cancer tissues were significantly lower than those of miR-377-3p in para-cancerous tissues (P<0.05), indicating that miR-21 is highly expressed and miR-377-3p is lowly expressed in patients with prostate cancer. This finding is consistent with the results of previous studies. Furthermore, the

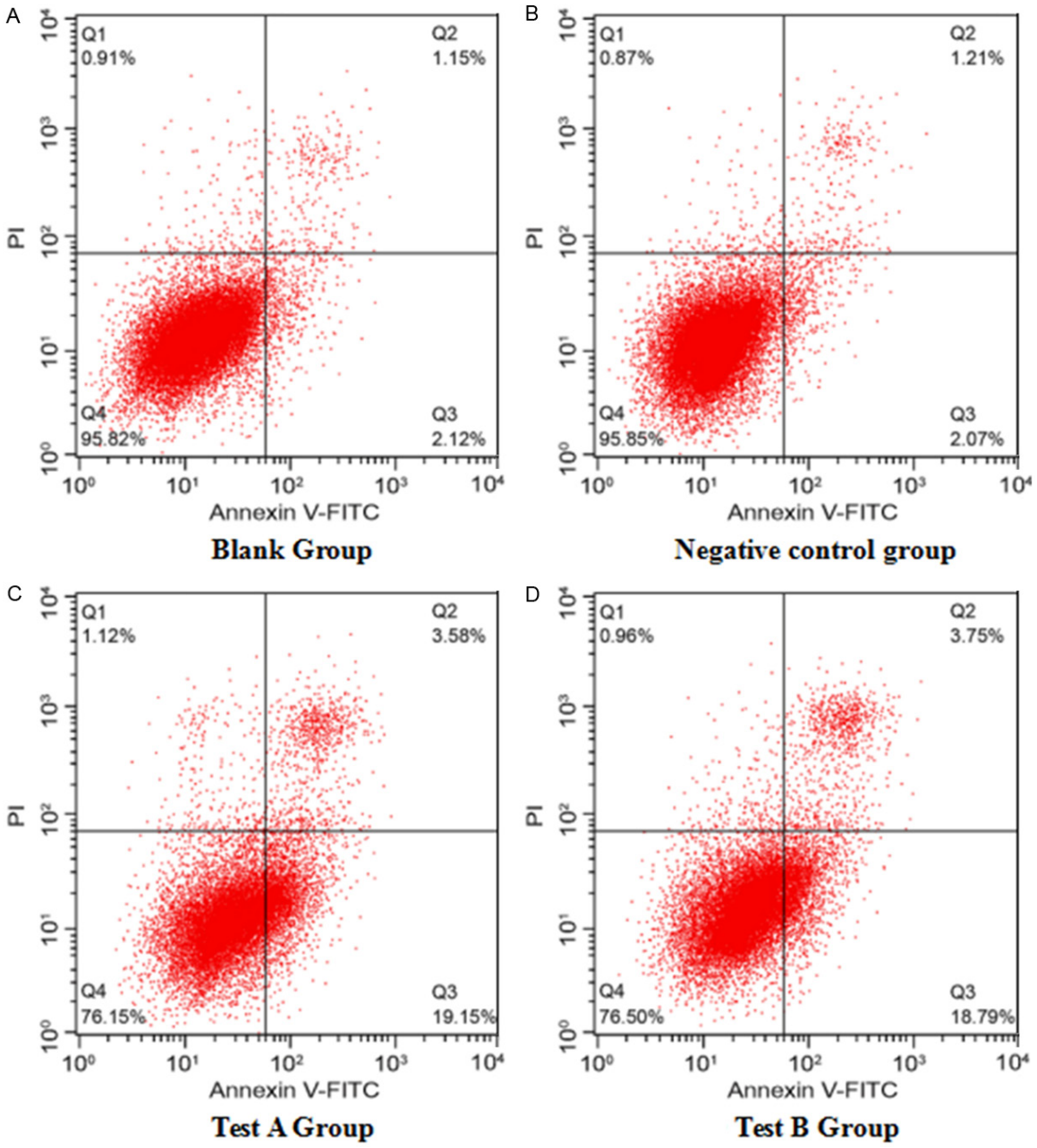

Figure 3. Flow cytometry results of early apoptosis transfected with miR-377-3p and miR-21 inhibitors.

current study investigated the effects of miR-21 and miR-377-3p on proliferation and apoptosis of prostate cancer PC-3 cells. Results showed that, when miR-377-3p was overexpressed and miR-21 was inhibited, cell viabilities of experimental group A and experimental group B were significantly lower than those of the negative control group and blank group, respectively. However, apoptosis rates of experimental group A and experimental group B were significantly higher than those of the negative control group and blank group ($P<0.05$). Results indicate that inhibition of miR-21 expression and miR-377-3p overexpression inhibits cell proliferation and promotes apoptosis. One previous study [28] examined the roles of miR-21 in esophageal cancer, finding that inhibition of miR-21 expression also inhibited proliferation and promoted apoptosis of esophageal cancer cells, in accord with present results. Using bioinformatics software, the current study predicted that the target gene of miR-21 in prostate

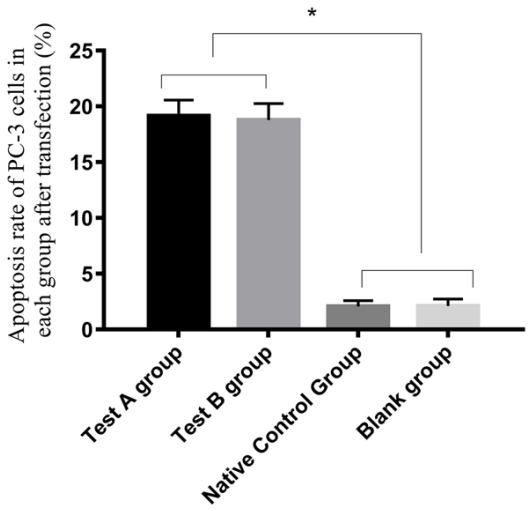

Figure 4. Early apoptosis rates of cells transfected with miR-377-3p and miR-21 inhibitors were significantly higher than those of the negative control group and blank group (P<0.05), but there were no significant differences between the negative control group and blank group (P>0.05).

cancer may be PDCD4. PDCD4 mRNA and PDCD4 proteins transfected with miR-21 inhibitor cells were detected. Results showed that levels of PDCD4 mRNA and PDCD4 protein in experimental group B were significantly higher those in the blank group and control group (P<0.05). It was hypothesized that miR-21 may regulate the proliferation and apoptosis of prostate cancer cells by targeting PDCD4.

Another study [29] investigated the relationship between PAK2 and prostate cancer, finding that PAK2 expression in prostate cancer tissues was higher than that in para-cancerous tissues, with PAK2 predicted to be a miR-377-3p target gene. Although the current study did not investigate the mechanisms of action of miR-377-3p on prostate cancer PC-3 cells, results suggest that miR-377-3p may affect the proliferation and apoptosis of prostate cancer PC-3 cells by inhibiting expression of PAK2. A previous study investigating the roles of miR-377-3p in other cells discovered that [30] miR-377-3p can induce the senescence of human skin fibroblasts, indicating that miR-377-3p can induce apoptosis.

In conclusion, miR-21 is highly expressed in prostate cancer PC-3 cells, while miR-377-3p is downregulated. Furthermore, inhibition of miR-21 expression and miR-377 overexpression

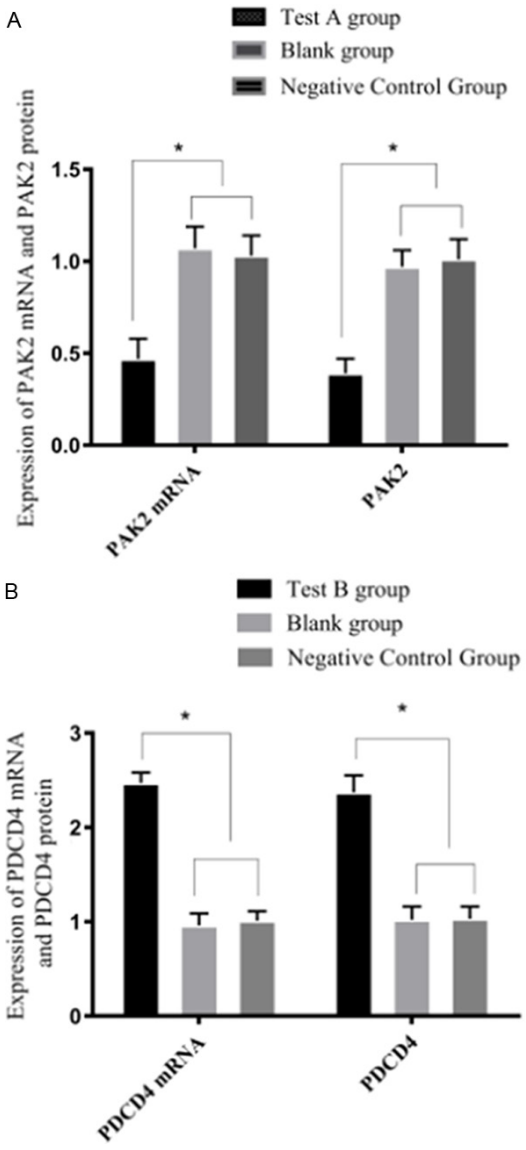

Figure 5. A. Expression of PAK2 mRNA and PAK2 protein. B. Expression of PDCD4 mRNA and PDCD4 protein. *P<0.05.

inhibited proliferation of PC-3 cells and promoted the apoptosis of PC-3 cells. Present data suggests that miR-21 and miR-377-3p are involved in various biological processes of prostate cancer cells. They can be used as a diagnostic markers and therapeutic targets for prostate cancer. However, the mechanisms of action of miR-21 and miR-377-3p in PC-3 cells remain unexplored, an interesting field for further research. Although miR-21 and miR-377-3p are related to prostate cancer, further exploration is necessary to determine whether miR-377-3p and miR-21 are related.

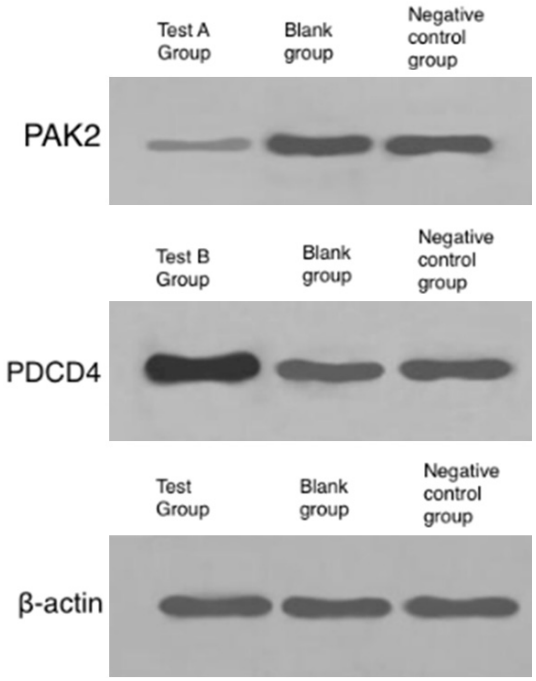

Figure 6. Expression of PAK2 protein in experimental group A was significantly lower than that in the blank group and negative control group. The difference was statistically significant ($P<0.05$). Expression of PDCD4 protein in experimental group B was significantly higher than that in the blank group and negative control group. The difference was statistically significant ($P<0.05$).

Disclosure of conflict of interest

None.

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