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Original Article

Enhanced expression of miR-375 increases chemotherapy sensitivity of prostate cancer to cisplatin

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Abstract: Objectives: Prostate cancer (PCa) is one of the most common malignancies in men. This study is to determine if human miR-375 affects the effect of cisplatin on PCa. Methods: Prostate cancer tissue samples were obtained during urological surgery from 10 patients. The adjacent normal tissues were collected during surgeries and served as the control sample groups. Real-time PCR, MTT assay, flow cytometry, and transwell chamber invasion assay were performed to detect the effects of transfected miR-375 analogs on the cells in combination with treatment of cisplatin. Results: The miR-375 levels were significantly decreased in PCa tissues when compared with the adjacent normal tissues. miR-375 analog nucleotide and cisplatin treatment significantly decreased cell survival rates ($p < 0.05$), suggesting that miR-375 increases the chemotherapy sensitivity of PCa cells to cisplatin. Additionally, miR-375 analog nucleotide and cisplatin significantly increased apoptosis rate and decreased cell invasion ability of PCa cells ($p < 0.05$). Conclusion: miR-375 analog nucleotide can increase the chemotherapy sensitivity of PCa cells to cisplatin, resulting in a synergistic effect by inducing apoptosis and reducing the cancer cell invasion ability.

Keywords: Prostate cancer, miR-375, cisplatin, apoptosis

Introduction

Prostate cancer (PCa) is one of the most common malignancies in men. It is reported that PCa ranked first (29%) in the new cancers in men in the United States. In China, the incidence of PCa increased significantly in the recent years [1-3]. Radical prostate resection and radiotherapy are used in patients with early and localized PCa, but there is more than 40% of recurrence or metastasis [4]. The efficacy of Taxol-based combined chemotherapy in the improvement of patients' quality of life and survival rate is limited [5]. Many signal transduction pathways are imbalanced in PCa, which participate in tumor genesis, including cell proliferation, apoptosis and angiogenesis. These pathways may provide potential targets for tumor treatment [6].

MicroRNA (miRNA) is a kind of small non-coding RNA that is commonly found in cells, with a length of 21 to 23 nucleotides. miRNA genes exist in many forms, including single copy, multiple copies and gene clusters, and they are non-randomly located on the chromosome, with separate promoter and regulatory sequences [7]. Human miR-375 gene is located on the CpG island of chromosome 2q35 with 22 nucleotides. Recently it is found that miR-375 expression is abnormal in many malignant tumors, including the squamous cell carcinoma in head and neck, gastric cancer, and liver cancer [8-10]. By analyzing miRNA expression profile of laryngeal squamous cell carcinoma (LSCC) and adjacent normal tissue, it is found that in LSCC, expression of miR-375 is low and is not relevant with tumor stages [11]. Komatsu S et al. [12] found that high levels of miR-21 and low levels of miR-375 in plasma were independent and reliable prognostic factors for esophageal squamous cell carcinoma. In a study of 123 patients with primary head and neck squamous cell carcinoma, it is found by multivariate

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequences
miR-375 forward	5'-TTGTTCGTTCGGCTCGCG-3'
miR-375 reverse	5'-TTTGGCACTAGCACATT-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
miR-375 analog	5'-UUUGUUCGUUCGGCUCGCGUGA-3'
miR-375 negative control	5'-UUGUACUACACAAAAGUACUGC-3'

Cox proportional hazard ratio that the survival rates of patients and the incidences of distant metastasis are significantly correlated with the low expression of miR-375 [13]. It is shown that miR-375 is down-regulated in cells and tissues of hepatocellular carcinoma. miR-375 independently regulates autophagy related gene ATG7 to inhibit the autophagy of mitochondrial in liver cancer cells, reduce the elimination of damaged mitochondria by hypoxia, and increase the release of mitochondrial apoptotic proteins [14].

By gene chip detection of the differences in miRNA expression in the plasma of PCa patients, it showed that miR-375 is highly expressed in PCa patients [15, 16]. But miR-375 is lowly expressed in most tumors, and it is found that miR-375 can inhibit tumor cell proliferation by down-regulating the cellular MTDH gene expression in breast cancer and liver cancer [17-19]. Therefore, further studies on expression of miR-375 in PCa patients and its influence on PCa cells are needed. In this study, it is shown that miR-375 was closely related with PCa cell growth, apoptosis, migration, and the increased sensitivity of PCa cells to chemotherapy drugs. These findings may provide evidence for anti-tumor therapy of PCa patients.

Materials and methods

Patients

Prostate cancer tissue samples were obtained during urological surgery from 10 patients (age, 55-70 years; mean age, 66.5 years). According to the clinical TNM staging criteria, 7 cases were T1-T2 stages and 3 cases were T3a stages. According to the pathological grading, 6 cases were G1-G2 grades and 4 cases were G3 grade. The adjacent normal tissues were collected during surgeries and served as the control sample groups. The postoperative pathologic examinations indicated that no tumor tissues were detected in these adjacent normal tissues. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Central South University, China.

Cell lines, reagents, and transfection

PCa cells (LNCap cell line) were purchased from Chinese Academy of Sciences, Beijing, China. Cells were cultured in RPMI-1640 medium containing 10% FBS at 37°C, 5% CO_2. Cell transfections were performed using Lipofectamine™ 2000 (Invitrogen, CA, USA). Two days later, total RNAs were harvested and subjected to real-time PCR analyses. The miR-375 analog nucleotide and the control nucleotide were synthesized by Shanghai GenePharma Ltd. (Shanghai, China). The miR-375 analog nucleotide and the control nucleotide were given in (**Table 1**). Cisplatin was purchased from Biyuntian Company, (Shanghai, China).

PCa cell treatment

PCa cells were transfected with miR-375 analog nucleotides or the negative control nucleotides, with or without treatment of cisplatin (1 μg/ml) for 24 hours.

Real-time PCR

Total RNAs were harvested from cells or tissues using the Trizol RNA extraction kit (Qiagen, USA). RNA (1 μl) was reverse transcribed into cDNA using random primers in a Reverse Transcription system according to the manufacturer's instructions (RT-PCR reaction kit, GeneCopoeia, USA). Expression of miR-375 was quantified by quantitative PCR on an ABI Prism Sequence Detection System (Applied Biosystems, USA). Template-negative and RT-negative conditions were used as controls. Amplification of miR-375 and the endogenous U6 were monitored by levels in FAM and VIC fluorescent intensities, respectively, using the ABI 7900 software. The relative amounts of transcript miR-375 were normalized to the amount of U6 mRNA in the same sample. The Ct values relative to the control group were calculated. The RT-PCR experiments were repeated at least 3 times. The primers used in this study were given in (**Table 1**).

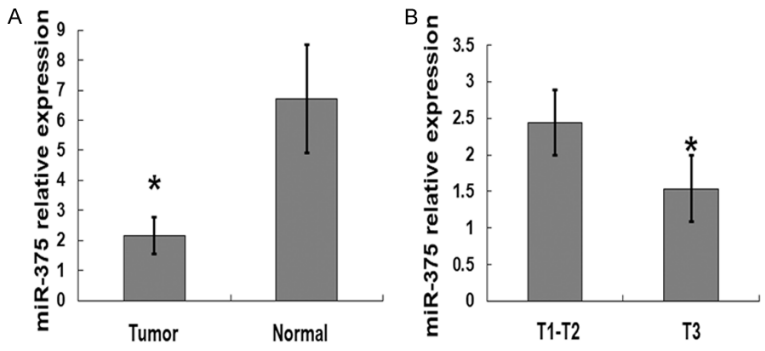

Figure 1. Real-time PCR detection of miR-375 levels in the PCa tissues and the adjacent normal tissues. The total RNAs were extracted from tissues of 10 patients. The primers were given in (**Table 1**). A. Levels of miR-375 expression in PCa tissues and the adjacent normal tissues. Compared with the adjacent normal tissues, *, $p < 0.01$. B. Levels of miR-375 expression in PCa tissues of patients at different clinical PCa stages. Compared with T1-T2 stage, *, $p < 0.05$.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay

PCa cells were treated as above described. Then, 50 μl of MTT was added for incubation for 4 h. The medium was removed, and 150 μl of DMSO was added and incubated at 37°C for 10 min. The absorbance was determined at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, USA). The above procedure was repeated at least for 3 times.

Cell apoptosis

Cell apoptosis was detected via an Annexin V-FITC kit purchased from Nanjing Kaiji Biotech Company (Nanjing, China) according to the manufacturer's instructions. Samples were analyzed with a FACSAria™ flow cytometer (Becton Dickinson, USA) after incubation in the dark at room temperature for 15 min.

Transwell chamber invasion assay

Matrigel, TGF-β, entactin and fibroblast growth factor was diluted with FBS-free RPMI-1640 with a ratio of 1:8. A volume of 60 μl of the diluted Matrigel was added to the Transwell bottom chamber. A volume of 60 μl of RPMI-1640 was also added to the chamber. After 30 min, LNCap cells at exponential growth stage were treated with 0.25% trypsin and then RPMI-1640 was added to prepare 1×10^6/ml single-cell suspension. A Transwell chamber was put into a 24-well plate and 600 μl of RPMI-1640 containing 10% FBS was added. The prepared single-cell suspension (200 μl) was also added into the Transwell chamber. The cells were cultured at 37°C with 5% CO_2 for 24 h. All of the liquid was removed from the Transwell chamber and the bottom chamber. The cells on the upper surface of the Transwell membrane were also removed. The membrane was washed with PBS for three times, immersed in methanol and incubated for 20 min at room temperature, followed by Hematoxylin staining for 10 min. The cells that invaded through the pores to the lower surface of the membrane were counted under microscope (×400).

Statistical analyses

SPSS16.0 statistical software was used. Measurement data was expressed as mean ± standard deviation. The t-test was used to compare the differences between two groups. Comparisons among groups were performed with one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

The expression level of miR-375 is decreased in PCa tissues

To determine the expression levels of miR-375 in PCa patients, the cancer tissues and the adjacent normal tissues were collected from 10 cases. The total RNAs were isolated and subjected to real-time PCR analyses. As shown in **Figure 1A**, the relative expression of miR-375 in carcinoma tissue and adjacent normal tissues were 2.16 ± 0.61 and 6.72 ± 1.81, respectively, suggesting that the miR-375 levels were significantly decreased in PCa tissues ($p < 0.01$).

Among these 10 PCa cases, 7 cases were in T1 or T2 stages and the other 3 cases were in T3a stage. Further PCR analyses (**Figure 1B**) showed that the relative expression of miR-375 was even lower in T3 stage cases than those in

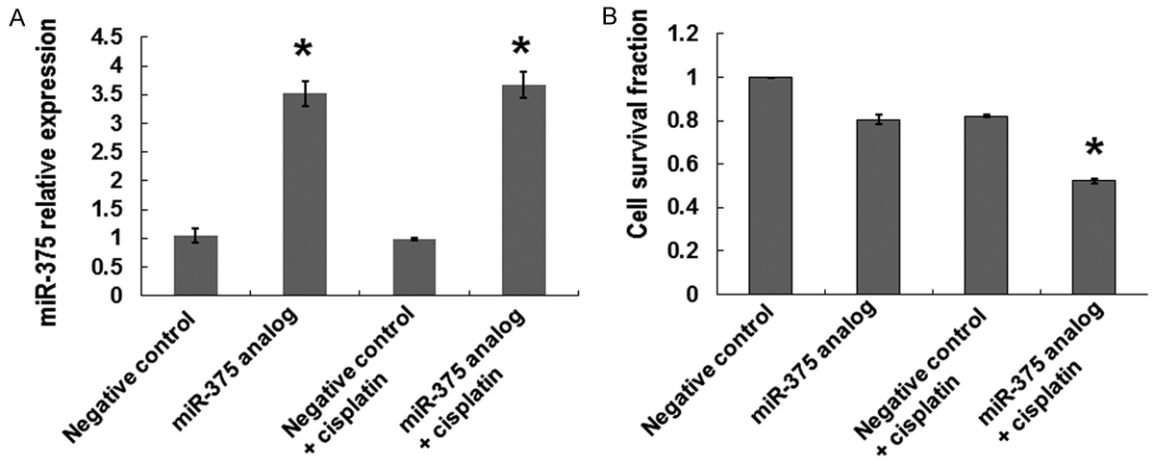

Figure 2. Effects of miR-375 analog nucleotides on PCa cells. A. PCa cells were transfected with miR-375 analog nucleotides or the negative control nucleotides, with or without treatment of cisplatin (1 μg/ml) for 24 hours. The real-time PCR were performed to detect expression levels of miR-375 in cells. Compared with negative control or negative control+cisplatin, *$p < 0.05$. B. The MTT experiments were performed to determine effects of miR-375 analog nucleotides or the negative control nucleotides, with or without treatment of cisplatin (1 μg/ml) for 24 hours. The experiments were independently repeated for at least 3 times. Compared with negative control, miR-375 analog, or negative control + cisplatin, *$p < 0.05$.

the T1 or T2 stages ($p < 0.05$). These results suggest that the miR-375 levels were significantly down-regulated in PCa tissues.

miR-375 analog nucleotide increases sensitivity of PCa cells to cisplatin

Since the miR-375 levels were altered in PCa tissues in comparison with the adjacent normal tissues, regulation of miR-375 levels may be an alternative method to treat cancers alone or together with some clinical drugs, such as cisplatin. Therefore, PCa cells were transfected with miR-375 analog nucleotides or the negative control nucleotides. As shown in **Figure 2A**, the real-time PCR results indicated that transfection of miR-375 analog nucleotides resulted in efficient expression of miR-375 in cells.

The MTT experiments were performed to determine effects of transiently expressed miR-375 and cisplatin treatment on cell survival. As shown in **Figure 2B**, compared with the control group, miR-375 analog nucleotide or cisplatin treatment decreased the cell survival rates, but without significant differences. And, the combination of miR-375 analog nucleotide transfection and cisplatin treatment significantly decreased the cell survival rates ($p < 0.05$). These results suggest that miR-375 analog nucleotide transfection can increase the chemotherapy sensitivity of PCa cells to cisplatin, resulting in a synergistic effect.

miR-375 analog nucleotide and cisplatin synergistically increase apoptosis rate and decrease cell invasion ability of PCa cells

To further study the effects of miR-375 analog nucleotide transfection and cisplatin, the PCa cells were transfected with miR-375 analog nucleotides or/and treated with cisplatin. As shown in **Figure 3**, cell apoptosis rates were detected with flow cytomery. Transfection with miR-375 analog nucleotides or treatment with cisplatin increased apoptosis rates. Transfection with miR-375 analog nucleotides and treatment with cisplatin synergistically increased apoptosis rates ($p < 0.05$).

Transwell chamber invasion experiments were also performed to further analyze the effects of miR-375 analog nucleotides and cisplatin. As shown in **Figure 4**, the cell invasion was decreased obviously in the cells transfected with miR-375 analog nucleotides and treated with cisplatin when compared with the control cells which were not transfected and not treated with cisplatin either ($p < 0.05$). The results indicated that transfection with miR-375 analog nucleotides and treatment with cisplatin synergistically decreased the invasion ability of

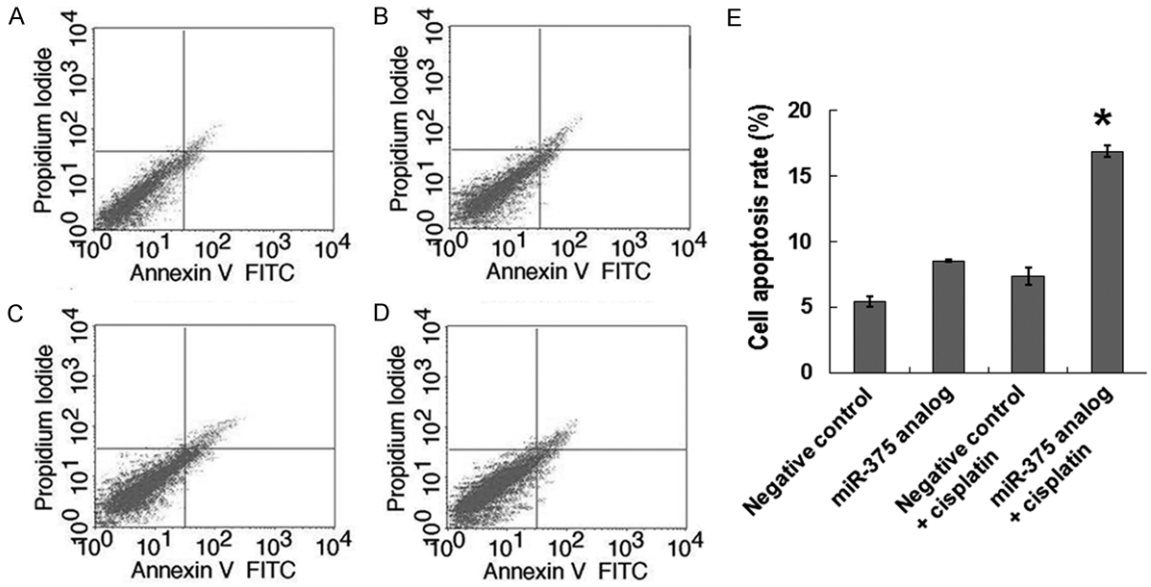

Figure 3. Cell apoptosis rates were detected with Annexin V-PE kit. PCa cells were transfected with miR-375 analog nucleotides or the negative control nucleotides, with or without treatment of cisplatin (1 μg/ml) for 24 hours. A. Cells were transfected with the negative control nucleotides. B. Cells were transfected with the miR-375 analog. C. Cells were treated with cisplatin (1 μg/ml) for 24 h. D. Cells were transfected with miR-375 analog and treated with cisplatin (1 μg/ml) for 24 h. E. Apoptosis rate in each group were compared with that of samples transfected with the negative control nucleotides. Compared with negative control, miR-375 analog, or negative control + cisplatin, *, $p < 0.01$.

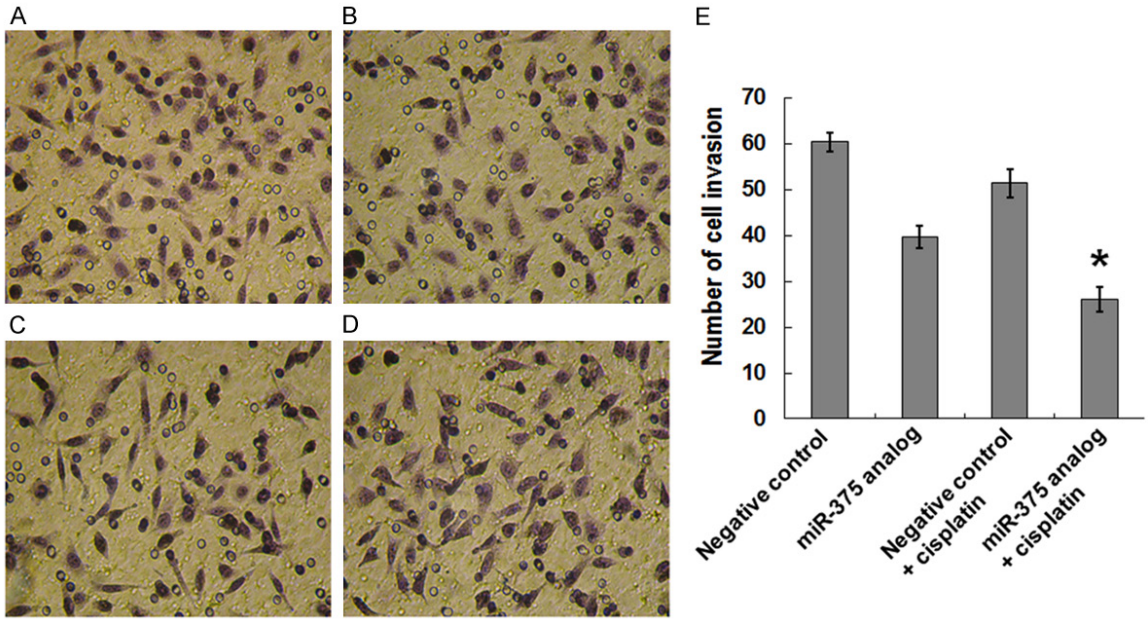

Figure 4. Transwell chamber invasion experiments were performed to further analyze the effects of transfection with miR-375 analog nucleotides and treatment with cisplatin. PCa cells were transfected with miR-375 analog nucleotides or the negative control nucleotides, with or without treatment of cisplatin (1 μg/ml) for 24 hours. A. Cells were transfected with the negative control nucleotides. B. Cells were transfected with the miR-375 analog. C. Cells were treated with cisplatin (1 μg/ml) for 24 h. D. Cells were transfected with miR-375 analog and treated with cisplatin (1 μg/ml) for 24 h. E. Numbers of cell invasions in each group were compared with that of samples transfected with the negative control nucleotides. Compared with negative control, miR-375 analog, or negative control + cisplatin, *, $p < 0.01$.

PCa cells. These results suggest that miR-375 analog nucleotide increases the chemotherapy sensitivity of PCa cells to cisplatin via a synergistic effect on apoptosis rates and the invasion ability of cancer cells.

Discussion

In this study, expression levels of miR-375 in prostate cancer tissues and the adjacent normal tissues were determined in 10 cases of prostate cancer. It was found that miR-375 levels were significantly down-regulated in 10 cases of prostate cancer tissues. Yoshiyuki [20] found that in gastric cancer tissues, down-regulation of miR-375 increased tumor cell invasion. We also found that transfection of miR-375 analog nucleotide decreased cell survival and increased the chemotherapy sensitivity of PCa cells to cisplatin. Such an effect is mediated by a synergistic effect of miR-375 analog nucleotide and cisplatin on inducing apoptosis and reducing the invasion ability of cancer cells.

In recent years, abnormal expression of miR-375 was found in many malignant tumors, including head and neck squamous cell carcinoma, gastric cancer, liver cancer. For example, Komatsu found that [12] high levels of plasma miR-21 and low levels of miR-375 were independent prognostic factors of esophageal squamous cell carcinoma. Yoshiyuki [20] found in gastric carcinoma that down-regulation of miR-375 increased tumor cell invasiveness by regulating PDK1 and 14-3-3 expression.

Zhao et al. found that miR-375 expression levels were significantly increased in small cell lung cancer NCI-H209 cells [21]. In squamous cervical cancers, miR-375 levels are decreased and affect tumors via a mechanism related to transcription factor SP1 [22]. In this study, we found that miR-375 enhanced the chemotherapy sensitivity of prostate cancer cells to cisplatin. However, the downstream target of miR-375 was not investigated.

In conclusion, our findings demonstrate that miR-375 expression is decreased in prostate cancer tissues. Additionally, miR-375 analog nucleotide transfection decreases cell survival and increases the chemotherapy sensitivity of PCa cells to cisplatin. These results suggest that miR-375 may act as a tumor suppressor in prostate cancer. The downstream target of miR-375 in prostate cancer should be further investigated.

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Disclosure of conflict of interest

None.

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