MicroRNA-122 regulates docetaxel resistance of prostate cancer cells by regulating PKM2

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Abstract. Prostate cancer (PCa), an epithelial malignancy that occurs in the prostate, is the second leading cause of cancer death worldwide. MicroRNAs (miRs/miRNAs) are reported to have important applications in the field of cancer diagnosis and treatment. The present study aimed to investigate the function of miRNA-122 in the chemoresistance of PCa cells and the underlying mechanism. Significantly decreased miR-122 and increased pyruvate kinase (PKM2) levels were observed in docetaxel-resistant PCa cells, and PKM2 was negatively correlated with miR-122. MiR-122 mimic transfection in docetaxel-resistant LNCaP cells significantly inhibited cell proliferation, promoted apoptosis and decreased glucose uptake and lactate production, which was counteracted by PKM2 overexpression. Inhibition of miR-122 in LNCaP cells had an opposite effect to miR-122 mimic transfection. In addition, miR-122 mimic transfection significantly increased the sensitivity of docetaxel-resistant LNCaP cells to docetaxel, while inhibition of miR-122 significantly decreased the sensitivity of LNCaP cells to docetaxel. Luciferase reporter assays showed that miR-122 regulated PKM2 expression by binding to the 3'-untranslated region of PKM2. The results suggest that upregulation of miR-122 could enhance docetaxel sensitivity, inhibit cell proliferation and promote apoptosis in PCa cells, possibly through the downregulation of its target protein PKM2.

Introduction

Prostate cancer (PCa) is an epithelial malignancy that occurs in the prostate (1). PCa mainly occurs in men over the age of 50 and is the second leading cause of cancer death worldwide (2,3). Currently, endocrine therapy, including surgical or drug castration and antiandrogen (bicalutamide or flutamide) therapy, is the main treatment for hormone-sensitive advanced PCa patients (4). However, the vast majority of patients are eventually treated with androgen-deprivation therapy and progress to metastatic castration-resistant PCa (CRPC), which is the leading cause of PCa-related mortality (5-7). Novel treatments, such as docetaxel and abiraterone, were shown to improve the survival of patients with metastatic CRPC; however, most patients develop drug resistance (8,9).

While healthy cells rely on carbohydrate molecules oxidized in mitochondria to acquire energy, most tumor cells obtain their energy supply through relatively low-yield glycolysis, which does not involve oxygen or mitochondria (10). Malignant, rapidly growing tumor cells typically have a 200-fold higher rate of glycolysis compared with normal tissues, even under oxygen-sufficient conditions (11). Therefore, it is speculated that this change in metabolism is the root cause of cancer (12). It was reported that glycolysis in cancer cells is characterized by high glucose consumption and lactate production (13). Cancer cells often take up high amounts of glucose and rely on glycolysis for ATP generation, more efficiently converting glucose into macromolecules that are needed for a variety of cellular processes (14-16). Pyruvate kinase (PKM2), a key rate-limiting enzyme that catalyzes the final step in glycolysis, was reported to be highly expressed in multiple cancers (17,18) and can promote glucose metabolism and cell growth (19). A number of studies showed that upregulation of PKM2 can promote malignancy and downregulation of PKM2 can inhibit cell growth, migration and invasion in various types of cancer (20-24).

MicroRNAs (miRs/miRNAs) are small noncoding, single-stranded RNAs which regulate gene expression by regulating the stability or translation of target miRNAs by binding to their 3'-untranslated regions (UTRs) (25,26). Studies showed that miRNAs have important applications in the field of cancer diagnosis and treatment (27-32). For example, exo-anti-miR-214 can reverse the resistance of gastric cancer cells to cisplatin (33). MiR-122, an abundant liver-specific...
miRNA, was shown to reverse doxorubicin resistance in liver cancer cells by inhibiting glycolysis in tumors via PKM2 inhibition (34,35). In colon cancer, overexpression of miR-122 can increase the sensitivity of fluorouracil (5-FU)-resistant colon cancer cells to 5-FU by PKM2 downregulation (36). Previous findings showed that miR-34a and miR-21 play a role in the chemoresistance of PCa cells (37-40). The present study aimed to investigate the function of miRNA-122 in the chemoresistance of PCa cells and the underlying mechanism.

Materials and methods

**Cell culture.** Prostate cancer docetaxel-resistant (LNCaP/Doctetaxel) and docetaxel-sensitive (LNCaP) LNCaP cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured with DMEM (cat. no. SH30243.01; HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (100X; cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) in a 37°C incubator (Forma 3111; Thermo Fisher Scientific, Inc.) with 5% CO₂.

**Isolation of primary PCa cells.** PCa cells were isolated from 20 patients with PCa who were treated in Shaoxing People's Hospital, Shaoxing, China from February 2018 to February 2019 (age range, 60-80 years; mean age, 70.12±8.43 years). The inclusion criteria were as follows: i) patients did not receive any treatment and ii) clinical data of patients were complete. All cases were confirmed by a review by the Shaoxing People's Hospital Pathology Center. The exclusion criteria were cases without complete clinical data. Fresh prostate cancer tissues were washed three times with D-Hank's balanced salt solution containing 500 IU/ml penicillin and streptomycin. Once surrounding inactivated tissues (cloudy appearance, dull and loss of normal tissue elasticity) were removed with ophthalmic scissors, the tissues (normal color and elasticity) were cut into pieces (~300 times) in a sterile 5 ml syringe and incubated with 5 ml trypsin-EDTA (0.125% trypsin and 0.53 mol/l EDTA) for 5 min at 37°C. A total of 10 ml RPMI-1640 medium (cat. no. 88365; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to terminate digestion in a 15 ml centrifuge tube. After centrifugation at 4°C and 800 x g for 5 min, the pellet was resuspended in 5 ml RPMI 1640 containing 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 0.5 µg/ml bovine pituitary extract (cat. no. 13028014; Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS. A total of 5 ml of the suspension was seeded in the T25 cell flasks for incubation in a 37°C and 5% CO₂ incubator. Fibroblasts can adherent growth in the T25 cell flasks after 4-6 days. Medium was replaced every two days, incubator. Fibroblasts can adherent growth in the T25 cell flasks after 4-6 days. Medium was replaced every two days, incubator.

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**Cell transfection.** LNCaP and LNCaP/Doctetaxel cells in the logarithmic growth phase were suspended to 1x10⁶ cells/ml after trypsinization. Subsequently, 2 ml cell suspension was inoculated into six-well plates for overnight culture at 37°C with 5% CO₂. Once the cells grew to 60-70% confluence, LNCaP and LNCaP/Doctetaxel cells were transfected with 5 µl negative control (NC)-miRNA (100 pmol; 5'-CAGUAC UUUUGUGUAGUACAA-3'), 5 µl hsa-miR-122-5p inhibitor (100 pmol; 5'-CAACACACATGTCACACATCA-3') and 5 µl of hsa-miR-122-5p mimic (100 pmol; 5'-UGGAGUG ACAUGUGGUUUUG-3') using Lipofectamine™ 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h of transfection, serum-free transfer solution was replaced with complete medium to culture for a further 48 h.

**Docetaxel treatment.** LNCaP and LNCaP/Doctetaxel cells were treated with gradient concentrations of docetaxel (0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/ml; cat. no. 114977-28-5; Shanghai Aladdin Biochemical Technology Co., Ltd.), followed by the detection of cell inhibition rate. After treatment with docetaxel, the apoptosis levels of primary PCa cells, treated LNCaP or treated LNCaP/Docetaxel cells were detected.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated from PCa cells (LNCaP, LNCaP/Docetaxel or primary PCa cells) with or without miR-122 inhibitor or mimic, and/or combined with PKM2 overexpression (oePKM2) lentivirus using TRIzol® reagent (cat. no. 15996026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following RNA quantification and integrity confirmation, ~1 µg of RNA was reversed transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) using a miRNA RT-PCR Detection Kit (cat. no. AOMD-Q020; GeneCoepia, Inc.) or a Maxima SYBR Green/ROX qPCR master mix (cat. no. K0223; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 45 sec and a final extension step at 95°C for 15 sec. miR-122 expression was normalized to U6 levels and PKM2 expression was normalized to GAPDH levels using the 2⁻ΔCq method (41). The primer pairs used for the qPCR are listed in Table 1.

**Western blot analysis.** Total protein was isolated from PCa cells (LNCaP, LNCaP/Docetaxel or primary PCa cells) with
or without treatment of miR-122 inhibitor or mimic, and/or combined with oePKM2 lentivirus using RIPA buffer supplemented with protease and phosphatase inhibitors (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). Following protein quantification using a BCA protein quantification kit (Thermo Fisher Scientific, Inc.), 25 µg of protein/lane was separated via 10% SDS PAGE followed by semi-dry transfer onto PVDF membranes (cat. no. HATF00010; EMD Millipore). Protein bands were visualized using an Tanon 5200 chemiluminescent imaging system (Tanon Science and Technology Co., Ltd.) at 4°C with gentle agitation.Following six washes with TBS-Tween-20, membranes were incubated for 2 h at room temperature with goat anti-rabbit horseradish peroxidase (HRP)-labeled secondary antibodies (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology). Protein expression was quantified using ImageJ (version 1.47; National Institutes of Health) with GAPDH as the loading control.

**Cell proliferation assay.** PCA cells (LNCaP or LNCaP/Docetaxel) in the logarithmic growth phase were trypsinized and a 3x10^5 cells/ml suspension was prepared by counting the cells under an inverted microscope at x40 magnification (XDS-500C; Shanghai Caikon Optical Instrument Co., Ltd.). Subsequently, in a 96-well culture plate, 100 µl of the suspension was inoculated and cultured at 37°C overnight. A total of 100 µl DMEM was used as the blank control. Cell Counting Kit-8 solution (CCK-8; cat. no. ab228554; Abcam) and GAPDH (1:2,000; cat. no. 5174; Cell Signaling Technology, Inc.) at 4°C with gentle agitation.Following six washes with PBS, cells were trypsinized and centrifuged at 1,000 x g for 5 min at 4°C. After discarding the supernatant, the cells were gently resuspended in 195 µl Annexin V-FITC binding solution, followed by a 15-min incubation with 5 µl Annexin V-FITC at 4°C in the dark. Subsequently, 5 µl of propidium iodide (PI) staining solution was added and the cells were incubated for 5 min at 4°C in the dark. A tube without Annexin V-FITC and PI was used as a negative control. Flow cytometry was performed and apoptosis percentages were assessed with BD Accuri C6 software (version 1.0.264.21; BD Biosciences).

**Detection of glucose uptake and lactate production.** PCA cells (LNCaP or LNCaP/Docetaxel) were seeded in 24-well plates and cultured overnight and treated with miR-122 inhibitor or miR-122 mimic and oePKM2 lentivirus. A 2-NBDG Glucose Uptake Assay kit (cat. no. K682-50; BioVision, Inc.) was used for glucose uptake detection. Following 72 h of treatment, the cells were incubated with 100 µM 2-NBDG for 1 h. After two washes with PBS, cells were trypsinized and resuspended in DMEM containing 10% FBS, followed by incubation with 5 µg/ml PI for staining. Subsequently, flow cytometry was performed to measure the proportion of PI-negative and 2-NBDG-positive cells, and glucose uptake was calculated. Lactate production was measured using a lactate test kit (cat. no. A019-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions. The absorbance was measured at a wavelength of 530 nm using a spectrophotometer and lactate production was calculated.

**Luciferase reporter assay.** TargetScanHuman 7.2 (http://www.targetscan.org/vert_72/) was used to predict miR-122 target sites on PKM2. PCA cells (LNCaP or LNCaP/Docetaxel) in the logarithmic growth phase were trypsinized and centrifuged at 800 x g for 5 min at room temperature. After discarding the supernatant, the cells were gently resuspended in 1 ml DMEM and counted under an inverted microscope at x40 magnification (XDS-500C; Shanghai Caikon Optical Instrument Co., Ltd.). The cell suspension was inoculated into a six-well plate at a density of 5x10^5 cells/well and cultured in an incubator at 37°C. Following 24 h of culture, the cells were co-transfected with 1.5 µg luciferase plasmid (pGL3-Promoter-PKM; Promega Corporation) and miR-122-5p inhibitor or mimic

| Gene                        | Forward sequence | Reverse sequence |
|-----------------------------|------------------|------------------|
| RT primer for miR-122       |                  |                  |
| miR-122                     | 5'-CGCCATTATCACACTAATAATGCTACTG-3' | 5'-AGTGCAAGGGTCCAGATT-3' |
| PKM2                       | 5'-TCCAGGTGAACGAGAAG-3'            | 5'-CGGATGATAGGCCAAAC-3'    |
| U6                          | 5'-CTCGTTCTCGCAGCACA-3'            | 5'-AAACGCTTACAGATTTCGT-3'  |
| GAPDH                      | 5'-AATCCCATACACATCTTC-3'           | 5'-AGGCTGTGGTCATACTTC-3'   |

Table I. Primer sequences used for reverse transcription-quantitative PCR.
using Lipofectamine™ 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection, the cells were washed with PBS, and then incubated in 500 µl PLB for 15 mins with gentle agitation at room temperature. The luciferase activity of the PKM2 reporter activity was detected following addition of 100 µl LAR II and 20 µl sample lysate in 96-well plates. Renilla luciferase activity was detected following addition of 100 µl Stop & Glo reagent.

**Statistical analysis.** GraphPad Prism 7.0 (GraphPad Software, Inc.) was used for statistical analysis. Data are presented as the mean ± SD from triplicate experiments. Unpaired Student’s t-test was used to determine the significance between two groups, while multiple groups were compared by one-way ANOVA and Tukey’s post hoc test. The Pearson correlation coefficient was used to analyze the correlation between miR-122 and PKM2. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of miR-122 significantly decreased in LNCaP/Docetaxel cells, and inhibition of miR-122 in LNCaP cells significantly promotes proliferation and glycolysis and inhibits apoptosis.** Following treatment with gradient concentrations of docetaxel (0.25, 0.5, 1, 2, 4, 8, 16, and 32 µg/ml), cell proliferation was assessed to determine resistance to docetaxel in LNCaP and LNCaP/Docetaxel cells. "P<0.01. (B) Baseline proliferation levels of LNCaP and LNCaP/Docetaxel cells were detected. "**"P<0.001. (C) Expression of miR-122 in LNCaP and LNCaP/Docetaxel cells was detected. "***"P<0.001. (D) The expression of miR-122 in miR-122-treated LNCaP cells was detected. "***"P<0.001 vs. NC. (E) Cell proliferation was detected at 0, 24, 48 and 72 h. "**P<0.01 and "***"P<0.001. (F) Cell apoptosis was detected at 48 h. The ordinate of the histogram is the sum of early and late apoptosis. "***"P<0.001. (G) Glucose uptake and (H) lactate production were detected. "***"P<0.001. miR-122, microRNA-122; OD, optical density; NC, negative control; PI, propidium iodide.
(LNCaP/Docetaxel) and -sensitive (LNCaP) cell strains. The results in Fig. 1C show that compared with LNCaP cells, miR-122 levels significantly decreased in LNCaP/Docetaxel cells. Furthermore, LNCaP cells were treated with miR-122 inhibitor (Fig. 1D). Following miR-122 inhibition, the proliferation of LNCaP cells significantly increased (Fig. 1E), and apoptosis was significantly decreased (Fig. 1F), which was accompanied by significantly increased glucose uptake (Fig. 1G) and lactate production (Fig. 1H) compared with the NC group. The findings suggested that miR-122 expression may be associated with docetaxel resistance in PCa.

PKM2 may be a target gene of miR-122 in regulating PCa. Both mRNA (Fig. 2A) and protein (Fig. 2B) expression of PKM2 significantly increased in LNCaP/Docetaxel cells compared with LNCaP cells. PKM2 expression was negatively correlated with miR-122 expression in primary PCa cells isolated from tumor tissues of 20 patients with PCa (Fig. 2C). Furthermore, TargetScan was used to predict the binding site between the PKM2 3'-UTR and miR-122. Compared with the NC group, the luciferase reporter assay showed that following inhibition of miR-122, the luciferase activity of the PKM2 reporter was significantly increased (Fig. 2D), accompanied by increased expression of PKM2 (Fig. 2E and F), while miR-122 mimic transfection had the opposite effect. The results indicated that PKM2 might be a target gene of miR-122 in regulating PCa.

miR-122 possibly regulates the docetaxel resistance of PCa cells via PKM2 regulation. The human PCa docetaxel-resistant cell strain LNCaP/Docetaxel was treated with both miR-122 mimic and oePKM2 lentivirus. As shown in Fig. 3A-C, oePKM2 and miR-122 mimic treatment
in LNCaP/Docetaxel cells significantly increased PKM2 and mir-122 expression compared with the vector and NC groups, respectively. Upregulation of mir-122 following mir-122 mimic transfection resulted in significantly increased cell proliferation (Fig. 3D), glucose uptake (Fig. 3F) and lactate production (Fig. 3G) in LNCaP/Docetaxel cells, whereas cell apoptosis (Fig. 3E) increased, concurrent with a decrease in the expression of PKM2 (Fig. 3H and I). Overexpression of PKM2 counteracted the effects of mir-122 mimic transfection, and the effect of 10 µg/ml docetaxel was more significant compared with 5 µg/ml docetaxel (Fig. 4C). By contrast, inhibition of mir-122 in LNCaP cells significantly decreased docetaxel-induced apoptosis (Fig. 4D). The results demonstrated that high expression of mir-122 could promote docetaxel-induced apoptosis in PCa cells and upregulation of mir-122 could reverse the resistance of LNCaP/Docetaxel cells to docetaxel.

**Discussion**

An increasing number of studies have reported that the sensitivity of tumor cells to anticancer drugs can be altered by miRNAs (43-45). miR-34a was reported to enhance the chemosensitivity of PC3 cells to paclitaxel and camptothecin and PCa cells treated with miR-143 showed higher chemosensitivity to docetaxel (37,46). Previous research also showed that miR-122 can reverse drug resistance in several cancers (34,36). The results demonstrated that high expression of miR-122 could promote docetaxel-induced apoptosis in PCa cells and upregulation of miR-122 could reverse the resistance of LNCaP/Docetaxel cells to docetaxel.
proliferation, increased apoptosis and inhibited glycolysis, while miR-122 inhibitor transfection in LNCaP showed the opposite effect, suggesting that miR-122 expression may be associated with docetaxel resistance in PCa by regulating cell proliferation, apoptosis and glycolysis. In primary PCa cells, high expression of miR-122 could promote docetaxel-induced apoptosis in PCa cells, and miR-122 mimic transfection significantly increased docetaxel-induced apoptosis in LNCaP/Docetaxel cells, while inhibition of miR-122 showed the opposite effect. Thus, it was speculated that upregulation of miR-122 could reverse the resistance of LNCaP/Docetaxel cells to docetaxel, which may contribute to the treatment of PCa chemoresistance.

Furthermore, the underlying mechanism of miR-122 in regulating docetaxel resistance in PCa was investigated. In tumors, miRNAs primarily function via regulation of their target genes by targeting specific mRNAs for degradation or translation inhibition (47). A study reported that upregulation
of miR-328 can enhance docetaxel sensitivity, decrease cell proliferation and increase apoptosis in PCa cells by directly targeting p21-activated protein kinase 6 (48). Additionally, the ectopic expression of miR-21 can increase the resistance of PC3 cells to docetaxel by targeting the tumor suppressor programmed cell death protein 4 (40). In addition, a previous study revealed that in human lung cancer xenografts in mice, inhibition of PKM2 could enhance the efficacy of docetaxel (49). Results of the present study showed that the expression of PKM2 in PCa cells negatively correlated with miR-122 expression, and the luciferase reporter assay showed that miR-122 regulated PKM2 expression by binding to the 3′-UTR of PKM2. In LNCaP/Docetaxel PCa cells, miR-122 mimic-induced cell proliferation decreased, apoptosis increased and glycolysis inhibition was counteracted by PKM2 overexpression. Consistent with previous reports on miR-122 in cancer chemoresistance (34-36), it can be inferred that the upregulation of miR-122 expression may reverse the resistance of PCa LNCaP/Docetaxel cells to docetaxel via downregulation of its target gene PKM2. However, the present study also had limitations, such as the lack of sequencing data and validation, as well as the lack of studies on other prostate cancer cell types. Mechanisms of miR-122 involved in docetaxel resistance and the function of miR-122 in other prostate cancer cell types can be investigated in future studies to further confirm the current results.

In conclusion, the results demonstrated that high expression of miR-122 could promote docetaxel-induced apoptosis in PCa cells and that the upregulation of miR-122 could reverse the resistance of LNCaP/Docetaxel cells to docetaxel, possibly via the regulation of its target protein PKM2 by binding to the 3′-UTR. These findings may provide a link between PCa chemoresistance and miRNAs, and targeting miRNA-122 may offer a novel therapy for the chemoresistance of PCa.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ZZ and JY conceived and designed the study. ZZ, JY and GT performed the experiments. ZZ and JY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments conducted in this study were approved by the Ethics Committee of Shaoxing People’s Hospital. Written informed consent was obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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miR-122 in hepatocellular carcinoma leads to chemoresistance

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