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Research paper

Up-regulated miR-29c inhibits cell proliferation and glycolysis by inhibiting SLC2A3 expression in prostate cancer

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ABSTRACT

Prostate cancer (PCa) is the most commonly cancer in male worldwide. However, the molecular mechanisms underlying the progression of PCa remain unclear. MiR-29c was reported to be down-regulated in several kinds of tumors. Here, we for the first time demonstrated miR-29c was down-regulated in PCa samples. SLC2A3, a regulator of glycolysis, was validated as a direct target of miR-29c. Moreover, functional studies showed miR-29c could inhibit cell growth, induce apoptosis and deceased the rate of glucose metabolism. Accordingly, we identified miR-29c acted as a tumor-suppressor and was down-regulated in PCa. We thought this study will provide useful information to explore the potential candidate biomarkers for diagnosis and prognosis targets of PCa.

1. Introduction

Prostate cancer (PCa) is the second most common cancer in male worldwide, while the most in developed country (Torre et al., 2015). In recent years, the incidence of prostate cancer has also risen sharply in China, with an estimated incidence of 10 cases/100,000 individuals in 2011 compared with 5 cases/100,000 individuals in 2000 (Kirby et al., 2011). However, the molecular mechanisms that regulate the tumorigenesis, progression and metastasis of PCa remain unclear. Substantial evidences had showed noncoding RNAs played important roles in the pathogenesis of multiple types of cancers via regulating diversity of biological processes. MicroRNAs are a class of non-coding RNAs (20–22 nucleotides long) (Ying et al., 2008). MicroRNAs play an important role in regulating progression of cancer cells by inhibiting target genes expression through binding to the 3′-untranslated region (Ambros, 2004; Bartel, 2004; Ventura and Jacks, 2009). In prostate cancer, some miRNAs including miR-27a (Wan et al., 2016b), miR-135a (Wan et al., 2016a) and miR-449a (Noonan et al., 2010) had been shown to regulate cell growth, apoptosis, migration and invasion. In our early work, we also found downregulated miR-29a suppressed prostate cell proliferation by inhibiting KDM5B expression (Li et al., 2015). These findings provided a novel insight to explore the mechanisms that underlie PCa carcinogenesis.

MiR-29c was reported as a biomarker predicting cancer progression for non-small lung cancer, esophageal and gastric cancer (Zhu et al., 2014; Hezova et al., 2015; Vidal, 2016). MiR-29c has been reported as an anti-oncogenic miRNA in gastric, colorectal and pancreatic cancer (Matsuo et al., 2013; Cristóbal et al., 2015; Lu et al., 2016). MiR-29c could induce cell cycle arrest by inhibiting CCNE1 expression in esophageal squamous cell carcinoma (Ding et al., 2011) and suppress epithelial-to-mesenchymal transition (EMT) in lung cancer (Zhang et al., 2016). However, the biological processes of miR-29c in PCa still remain unknown.

Warburg effect was that cancer cells converted much more glucose into lactic acid even with ambient oxygen supply compared to normal cells (Weljie and Jirik, 2011; Liberti and Locasale, 2016). High rates of glycolysis were widely observed in cancers (Diaz-Ruiz et al., 2011). Recently studies suggested that a few miRNAs, such as miR-1 (Xu et al., 2017), miR-186 (Liu et al., 2017) and miR-199 (Guo et al., 2015), were involved in cancer cell metabolism. However, the detail mechanisms of miRNAs in PCa glycolysis are still unclear.

In this study, we speculated that miR-29c was a tumor suppressor in prostate cancer and was significantly down-regulated in prostate cancer. Thus, in this study, we analyzed levels of miR-29c in different stages of prostate cancer and picked out potential target mRNAs by bioinformatics ways, and we confirmed the biological function of miR-

Abbreviations: 3′-UTR, 3′-untranslated region; FBS, fetal bovine serum; GLUT3, glucose transporter type 3; HCC, hepatocellular carcinoma; Mut, mutant; NC, negative control; PCa, prostate cancer; PSA, prostate-specific antigen; qRT-PCR, quantitative real time polymerase chain reaction; SLC2A3, solute carrier family 2 member 3

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29c in PCa. Then, the interaction between miR-29c and mRNA was confirmed by luciferase assay. Importantly, we demonstrated miR-29c also reduced anaerobic glycolysis in PCa cells.

2. Materials and methods

2.1. Cell culture

LNCaP cells were purchased from the American Type Cult. Collection (Manassas, USA) which were confirmed by short tandem repeat (STR) analysis. 22RV1, DU145, PC-3 and WPMY-1 were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) where they were authenticated by mycoplasma detection, DNA-Fingerprinting, isozyme detection and cell vitality detection. All experiments were carried out with cell lines at passages below 30. The four prostate cancer cell lines were maintained in RPMI 1640 medium (Corning, USA) supplemented with 10% FBS (Hyclone, USA) and WPMY-1 in DMEM medium (Corning, USA) with 10% FBS, and they all cultured at 37 °C in 5% CO_2.

2.2. RNA interference and transient transfection

Synthetic miR-29c mimic (miR-29c) and its scrambled control miRNA (miR-NC) were purchased from GenePharma (Shanghai, China), and used at the concentration of 50 nM. Transfection was carried out with Lipofectamine 2000 Transfection Reagent (Life, USA) according to the manufacturer's procedure. The Opti-MEM medium and Lipofectamine 2000 were both purchased from Life Technologies.

2.3. RNA isolation and real-time qPCR

qRT-PCR for mRNAs was performed as described previously. Specific primers for mature miR-29c were from GenePharma (Shanghai, China). Primers used for qRT-PCR were listed in Supplementary Table 1. The Ct values were normalized using β-actin or RNU6 as internal control to estimate the different expression of genes. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate to ensure quantitative accuracy.

2.4. Western blotting analysis

Western blot was performed as described previously (29) with antibodies against SLC2A3 (1:1000, Sigma–Aldrich, MO, USA; 1:500, Proteintech, Chicago, USA), and β -Actin (1:4000, Sigma-Aldrich). Goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Sigma–Aldrich, USA) secondary antibodies were used to visualize bands using Amersham ECL Prime (GE Healthcare, UK). Signal intensity of Western blots was quantified by Quantity One Software (Bio-Rad, USA).

2.5. Reporter constructs and luciferase assay

700-bp nucleotide sequences corresponding to portion of the 3′-UTR of SLC2A3, including the conserved predicted binding site (seed sequence) for miR-29c, were inserted into psi-CHECK2 Dual-Luciferase miRNA Target Expression Vector (Promega, USA) within the XhoI/NotI sites. Mutagenesis was performed using Mut Express® II Fast Mutagenesis Kit V2 (Vazyme, USA). All insertions were verified by sequencing. The relative luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, USA) 48 h after transfection (Primer sequences show in Supplementary Table 1).

2.6. Cell proliferation assay

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) in octuplicate according to the manufacturer's instructions. Absorbance was measured at 450 nm with Microplate Reader ELx808 (Bio-Tek, USA). The absorbance at 630 nm was used as a reference.

2.7. Cell cycle and apoptosis assay

Cells were harvested 48 h after transfection. For cycle assay, cells were incubated with 0.03% triton X-100 and propidium iodide (PI) (50 ng/mL) for 15 min; the percentages of cells in different phases of cell cycle were measured with a FACScalibur flow cytometer (BD, USA) and analyzed with ModFit software (Verity Software House, USA). For apoptosis assay, cells were assayed with FITC Annexin V Apoptosis Detection Kit (BD, USA) and analyzed by flow cytometry.

2.8. Glucose consumption and lactate production

Cells were seeded on a 12-well plate well, and the culture medium was changed to RPMI-1640 medium without FBS 4 h after transfection. The concentrations of glucose and L-lactate were measured after another 36 h of incubation with a glucose test kit (Sigma, GAHK20) and L-lactate assay kit (Sigma, MAK064), respectively.

2.9. Statistical analysis

The numerical data were presented as mean ± standard deviation (SD) of at least three determinations. Statistical comparisons between groups of normalized data were performed using *t*-test or Mann–Whitney *U* test according to the test condition. A $P < 0.05$ was considered statistical significance with a 95% confidence level.

3. Results

3.1. MiR-29c expression is downregulated in prostate cancer

To explore the expression levels of miR-29c in PCa, we first detected the expression of miR-29c in 18 cases of normal prostate tissues and 57 cases of prostate cancer tissues. Compared to the normal prostate tissues, miR-29c expression was significantly downregulated in 57 samples of prostate cancer tissues (Fig. 1A). To further confirm that miR-29c was downregulated in PCa tissues, we investigated miR-29c expression levels in prostate tumors using publicly database GSE21036 (Taylor et al., 2010). Analysis of GSE21036 database showed that miR-29c was significantly decreased in metastatic prostate cancers compared with local prostate cancers and normal prostate tissues ($P < 0.001$, Fig. 1B).

To validate whether miR-29c was correlated with PCa progression, we analyzed TCGA database. We found miR-29c was significantly upregulated in tumors of Gleason grades 6 ($P < 0.01$), 7 ($P < 0.001$) and 8 ($P < 0.05$) compared to tumors of Gleason grades 9 (Fig. 1C). Moreover, we observed significantly lower expression of miR-29c was found in T3a ($P < 0.05$) and T3b ($P < 0.01$) patients compared to the T2 patients (Fig. 1D). These results highlight the potential role of miR-29c in PCa progression.

3.2. SLC2A3 is a direct target of miR-29c in PCa cells

To identify possible miR-29c target genes and obtain insight into the potential molecular pathways miR-29c participated, we performed bioinformatics analysis using complementary sites of miR-29c in their 3′-UTR by four publicly available algorithms including miRDB (http://www.mirdb.org) (Wong and Wang, 2015), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (Dweep et al., 2011), starBase (http://starbase.sysu.edu.cn/) (Yang et al., 2011) and miRanda (http://www.microrna.org/microrna/home.do) (Kou et al., 2015), which revealed there were 407 potential target mRNAs in common (Fig. 2A). All the potential target mRNAs were divided into different groups according to their biological functions using Kyoto