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Original Article

miR-181a-5p is downregulated and inhibits proliferation and the cell cycle in prostate cancer

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Abstract: Prostate cancer (PCa) is one of the most common cancers in men worldwide. However, the detailed molecular mechanisms underlying PCa tumorigenesis and progression remain largely unclear. MicroRNAs are key regulators of gene post-transcriptional expression in human cancer. In this study, we used public datasets, including GSE21036, GSE14857 and GSE45604 to analyze the expression of miR-181a in PCa. We also explored the potential role of miR-181a by using bioinformatics analysis and gain of function assay. miR-181a was down-regulated in PCa. Bioinformatics analysis revealed miR-181a was significantly involved in regulating cell metabolic process and gene expression. Of note, gain of function assay results showed overexpression of miR-181a could significantly inhibit cell proliferation by inducing G1 cell cycle arrest. Our results suggest miR-181a-5p may be adiagnostic and therapeutic biomarker for prostate cancer.

Keywords: Prostate cancer, miR-181a-5p, proliferation, cell cycle

Introduction

Prostate cancer (PCa) is one of the most common cancers in men worldwide [1-3]. However, the detailed molecular mechanisms underlying PCa tumorigenesis and progression remain largely unclear. Recently, emerging studies demonstrated the important roles of non-coding RNAs in the pathogenesis of multiple types of cancer. MicroRNAs (miRNAs) are the most well-known non-coding RNAs, which play pivotal roles in posttranscriptional regulation of gene expression. Previous reports showed altered expression of miRNAs in numerous human malignancies, including prostate cancer [4-7]. For example, microRNA-141 was down-regulated and suppressed prostate cancer stem cells and metastasis by targeting a cohort of pro-metastasis genes [8], and microRNA-424 was up-regulated and impaired ubiquitination to activate STAT3 and promoted prostate tumor progression [9]. These findings suggested that exploring the functions of miRNAs in PCa could provide a novel insight to search the mechanisms that underlie PCa carcinogenesis.

miR-181a, a member of the miR-181 family, was indicated as a novel biomarker for the diagnosis and prognosis of some cancers by recent studies. In recent years, more studies confirmed the implication of miR-181a in cancer biology and reported that miR-181a could serve as either an oncogene or tumor suppressor. miR-181a expression levels were down-regulated in primary squamous lung cell carcinoma and inhibited cancer cell proliferation by targeting oncogene KRAS [10]. miR-181a was also found to be downregulated in non-small-cell lung cancer and glioblastoma [11, 12]. In contrast, miR-181a was observed to be overexpressed in gastric cancer [13, 14] and thyroid cancer [15] and promote their progression. In prostate cancer, the roles of miR-181a remain largely unclear. Zhai et al. [16] reported miR-181a promoted bufalin-induced apoptosis in PC-3 by targeting BCL-2; however, Zhiping et al. [17] found miR-181a promoted epithelial to mesenchymal transition of prostate cancer cells by targeting TGIF2.

In the present study, we found miR-181a was down-regulated in PCa samples by analyzing

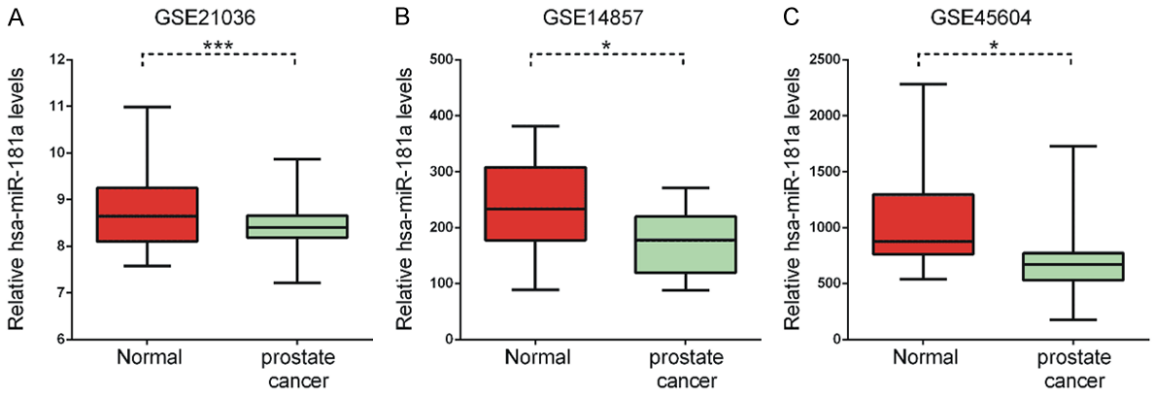

Figure 1. miR-181a-5p was downregulated in PCa tissues compared with non-tumor tissues in three TCGA databases GSE21036 (A), GSE14857 (B) and GSE45604 (C). (*, P < 0.05; ***, P < 0.001).

public datasets, including GSE21036 [18], GSE14857 [19] and GSE45604 [20]. Furthermore, we performed bioinformatic analysis to reveal the potential mechanisms of miR-181a in PCa. Finally, we constructed experiments to explore the effects of miR-181a on PCa cell cycle and proliferation.

Material and methods

miRNA and mRNA profile data collection

miRNA profiles data were downloaded from GEO database (www.ncbi.nlm.nih.gov/gds, GSE21036, GSE14857 and GSE45604). The comparison of miRNA profiles between prostate cancer samples and normal tissues was performed using raw microarray data. The cutoff of significant differentially expressed miRNA is identified with t test methods using a *P* value < 0.05.

Cell culture

LNCaP, 22RV1, WPMY-1, PC-3, and DU145 cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The four prostate cancer cell lines cultured in Ham's F12K media (Invitrogen, Beijing, China) supplemented with 10% (vol/vol) fetal bovine serum (FBS).

Cell transfection

Synthetic miR-181a-5p mimic and its scrambled control miRNA (miR-NC) were purchased from GenePharma (Shanghai, China). Prostate cancer cells were seeded at $3*10^5$ cells/wells in 6-well plates and incubated overnight. PC-3 and LNCaP cells were transfected with miRNA mimics using Lipofectamine 3000 (Life Technologies). Total RNAs were extracted at 48 h after transfection.

RNA extraction and quantitative RT-PCR

Total RNA for RT-qPCR was extracted using TRIzol (Invitrogen). Reverse transcription (RT) was performed with PrimeScript reagent kit (TAKARA, Japan) following the manufacturer's instructions. miR-181a-5p-specific RT primer was purchased from GenePharma (Shanghai, China). For analysis of microRNA expression, RT-qPCR was performed using SYBR Green Reagents (Bio-Rad, Hercules, CA, USA) on the LightCyclerR 480 (Roche, Switzerland). Expression level of miR-181a-5p was normalized to U6. The PCR primers for mature miR-181a-5p were designed and purchased from GenePharma; and U6 primers forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. Relative miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was assayed in triplicate to ensure quantitative accuracy.

Cell proliferation assay

CCK-8 assays were performed to evaluate changes in cell viability. Five thousand transfected cancer cells were seeded in 96-well plates at a final volume of 100 μl medium/well. Proliferation was assessed at 0, 24, 48, 72 and 96 hours. Cell viability was quantified by adding 10 μl CCK-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's protocol. After a 1.5 h incubation, the plates

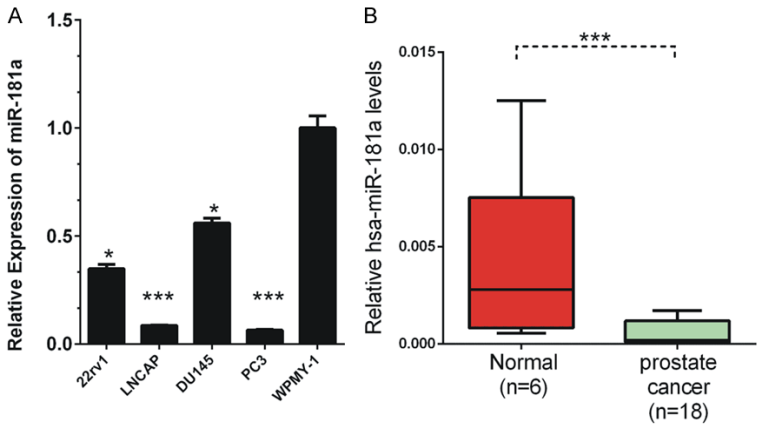

Figure 2. A. miR-181a-5p was significantly down-regulated in PCa cell lines compared to WPMY-1. B. miR-181a-5p were down-regulated in 18 prostate cancer specimens compared with 6 normal tissues. (*, $P < 0.05$; ***, $P < 0.001$).

were monitored at specific time points using a PowerWave XS Microplate reader (BioTek, Winooski, VT, USA), which measured absorbance at 450 nm. The absorbance at 630 nm was used as a reference. Each experiment was performed at least in triplicate.

Cell cycle assay

Transfected LNCaP and PC-3 cells in the log phase of growth were collected and fixed in 0.03% triton X-100 and propidium iodide (PI) (50 ng/mL) for 20 min. The transfected cells were examined with a fluorescence-activated cell sorting (FACS) flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with ModFit software (Verity Software House, ME, USA). Each test was performed in triplicate.

Statistical analysis

The numerical data are presented as mean ± standard deviation (SD) of at least three determinations. Statistical comparisons between groups of normalized data were performed using T-test. A $P < 0.05$ was considered significant with a 95% confidence level.

Results

miR-181a-5p was down-regulated in prostate cancer

To explore the clinical relevance of miR-181a-5p in PCa, we investigated its expression in publically available databases including, GSE-21036, GSE14857 and GSE-45604. We found that miR-181a-5p was downregulated in PCa tissues compared with non-tumor tissues in these databases (**Figure 1A-C**).

Furthermore, we evaluated miR-181a-5p expression levels in PCa cell lines (including LNCaP, PC-3, DU145, 22Rv1) and noncancerous prostatic cells WPMY-1. We observed that miR-181a-5p was significantly down-regulated in PCa cell lines compared to WPMY-1 by using RT-qPCR assay. In order to validate our results, we measured the expression of miR-181a-5p in PCa patients. As presented in **Figure 2B**, the expression of miR-181a-5p was lower in 18 prostate cancer specimens compared with 6 normal tissues.

Bioinformatic analysis for miR-181a-5p in prostate cancer

In the present study, we performed bioinformatics analysis for miR-181a-5p to explore its potential mechanisms in PCa by using its targets. Four miRNA target prediction websites were used, including TargetScan [21-25], miRWALK [26, 27], miRDB [28-31] and Starbase [32, 33] (**Figure 3A**). A total of 146 targets of miR-181a-5p were used to perform the Gene Ontologies (GO) categories analysis. Our data revealed that miR-181a-5p affects a series of biological processes, including regulation of metabolic process, positive regulation of gene expression, regulation of RNA metabolic process, positive regulation of macromolecule biosynthetic process, positive regulation of transcription, regulation of cellular biosynthetic process, and positive regulation of cellular biosynthetic process (**Figure 3B**).

Moreover, the protein-protein interaction network involved in miR-181a-5p targets was constructed. Interestingly, we found miR-181a-5p was significantly associated with glycolysis regulation by targeting SLC2A1, SLC2A3, HK2 and PKFFB2 (**Figure 3C**). RT-PCR results showed SLC2A1, SLC2A3, HK2 and PKFFB2 were decreased significantly after transfecting with miR-181a-5p in PC-3 and LNCaP cells (**Figure 3D**, **3E**).

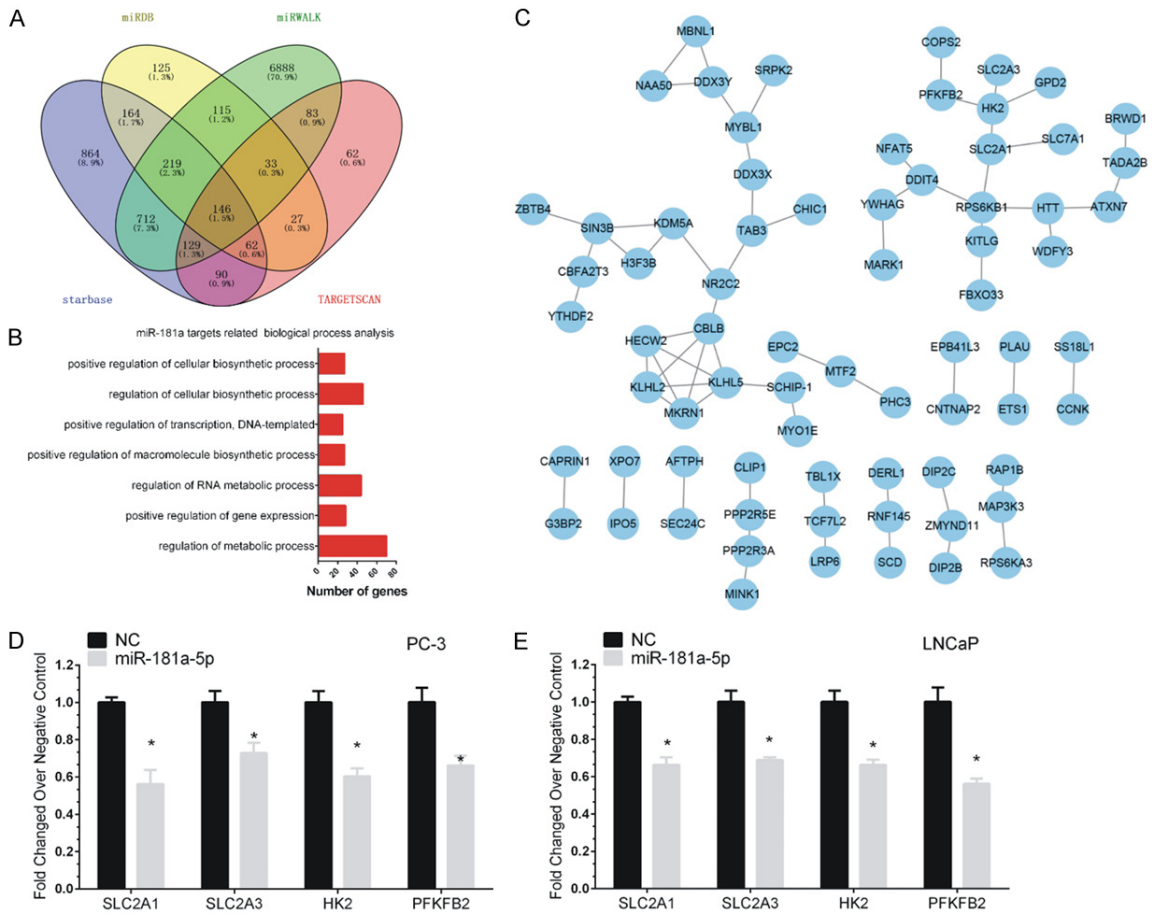

Figure 3. Bioinformatic analysis for miR-181a-5p in prostate cancer. A. The miR-181a-5p target prediction results of TargetScan, miRWALK, miRDB and starbase. B. The Gene Ontologies (GO) category analysis of miR-181a-5p. C. The protein-protein interaction network involved in miR-181a-5p targets. D, E. RT-PCR verified the downstream target genes of miR-181a-5p. (*, $P < 0.05$).

Overexpression of miR-181a-5p inhibited prostate cancer cell proliferation

To explore the potential effects of miR-181a-5p on the proliferation of PCa, we performed CCK-8 assay by transfecting LNCaP and PC-3 with NC or miR-181a-5p mimics. As shown in **Figure 4**, we found miR-181a-5p significantly inhibited the growth rate of LNCaP ($P < 0.001$) and PC-3 ($P < 0.001$) cells. The transfection efficiency is shown in **Figure 4A**, **4B**.

Overexpression of miR-181a-5p induced G1-phase cell cycle arrest in vitro

We then assessed the function of miR-181a-5p on the cell cycle profile of LNCaP (**Figure 5A**) and PC-3 (**Figure 5B**) cells. Flow cytometry analysis revealed that miR-181a-5p in prostate cancer cells underwent a significant decrease in the proportion of cells in the S-phase population and increase in the proportion of cells in the G1-phase population. Taken together, these results suggested miR-181a-5p inhibited PCa proliferation by inducing G1-phase cell cycle arrest.

Discussion

Prostate cancer (PCa) is a leading cause of cancer-related deaths in men worldwide [1-3], but the precise molecular mechanisms of the progression of PCa remain unclear. miRNAs play pivotal roles in posttranscriptional regulation of gene expression. A series of miRNAs was reported to be involved in PCa progression. For example, microRNA-141 suppressed prostate cancer stem cells and metastasis by targeting a cohort of pro-metastasis genes [8], and microRNA-424 inhibited ubiquitination to acti-

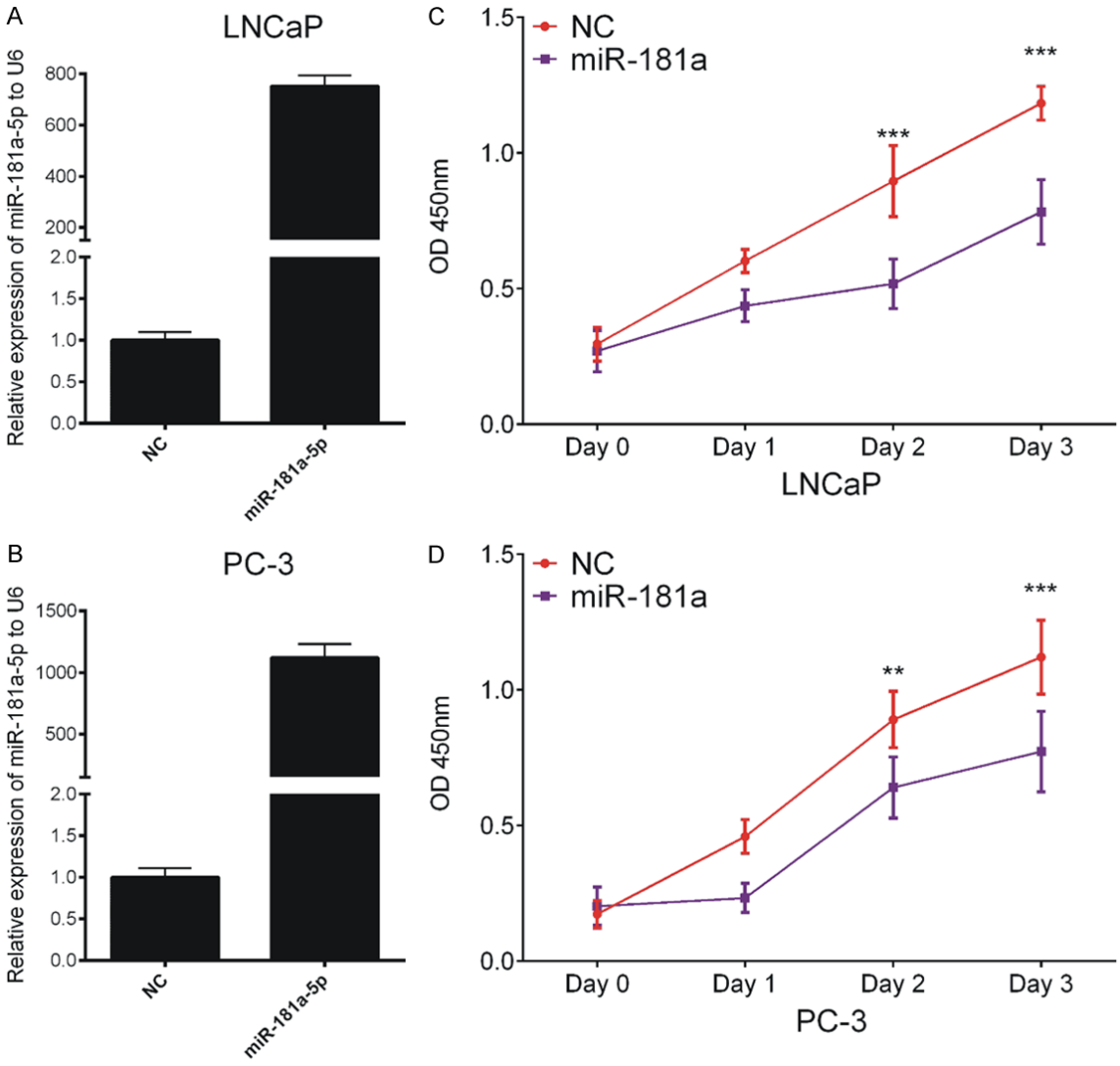

Figure 4. Overexpression of miR-181a-5p inhibited prostate cancer cell proliferation in LNCaP (C) and PC-3 (D) cell lines. (A, B) The transfection efficiency of miR-181a-5p. (**, $P < 0.01$; ***, $P < 0.001$).

vate STAT3 and promote prostate tumor progression [9]. Exploring the molecular functions and prognostic values could provide useful information for identifying biomarkers for PCa. In this study, we found miR-181a-5p was significantly down-regulated in PCa samples by analyzing public datasets. RT-PCR assay validation also showed miR-181a-5p expression was reduced in PCa cell lines and samples.

miR-181a was found to be a novel biomarker for the diagnosis and prognosis of some cancers, including lung cancer, glioma, gastric cancer, thyroid cancer, and prostate cancer. miR-181a could serve as either an oncogene or tumor suppressor. For example, miR-181a was downregulated in non-small-cell lung cancer and glioblastoma [11, 12], but was up-regulated in gastic and thyroid cancer [13-15]. In prostate cancer, miR-181a could promote bufalin-induced apoptosis and epithelial to mesenchymal transition [16]. In this study, we found overexpression of miR-181a significantly inhibited cell proliferation by inducing G1-phase cell cycle arrest. Consistent with our finding that miR-181a was down-regulated in PCa samples, our results strongly suggested miR-181a may act as a tumor suppressor in PCa.

Although several studies showed miR-181a could target KRAS, BCL2, and GATA6 in human cancers, the mechanism of miR-181a in regu-

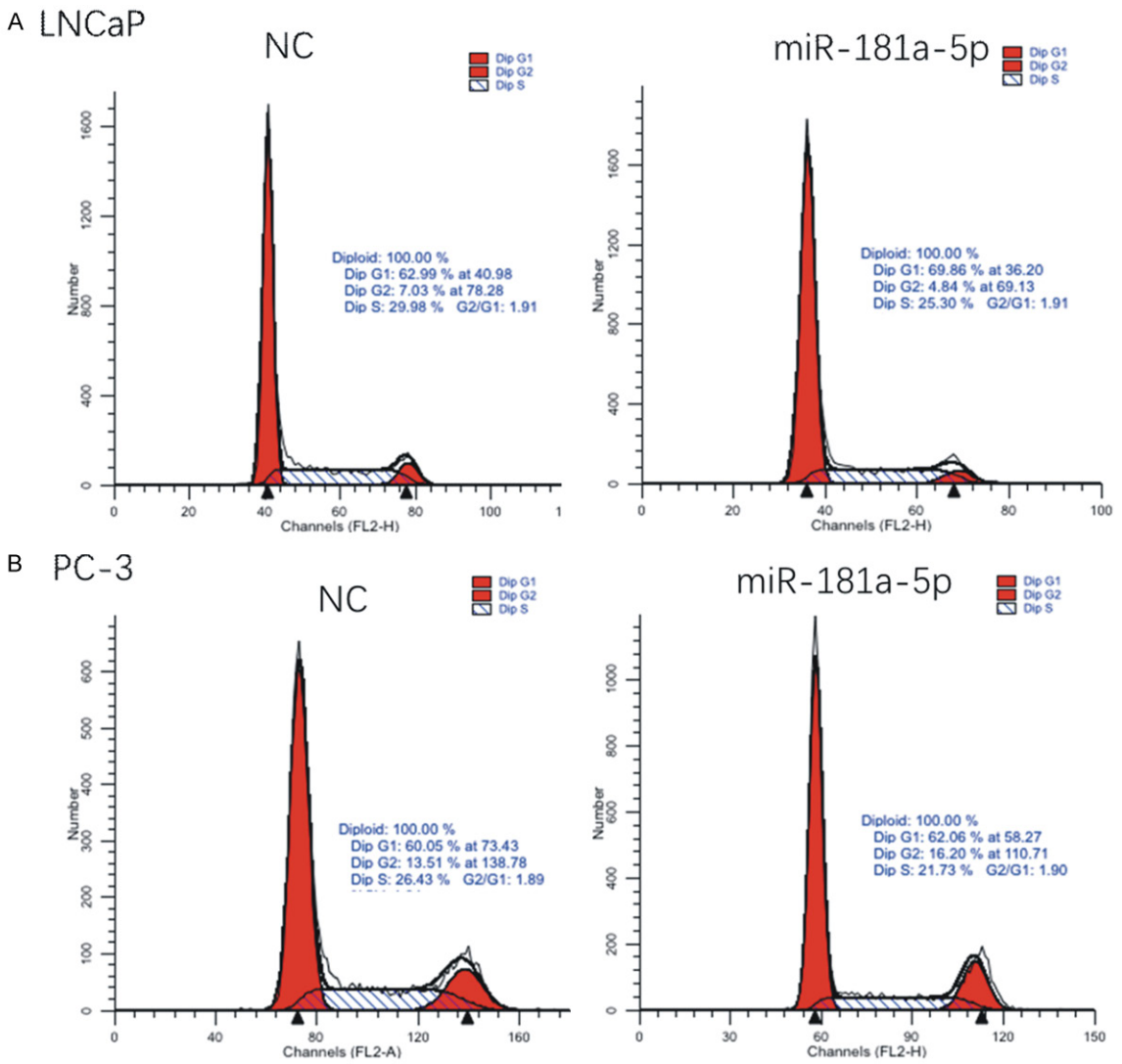

Figure 5. Overexpression of miR-181a-5p induced G1-phase cell cycle arrest in LNCaP (A) and PC-3 (B) cell lines (A, B).

lating PCa progression remained unclear. In the present study, we performed bioinformatics analysis for miR-181a-5p to explore its potential mechanisms in PCa by using its targets. A total of 146 targets of miR-181a-5p was used to perform Gene Ontologies (GO) categories analysis. Our data revealed that miR-181a-5p affects a series of biological processes, including regulation of metabolic process, positive regulation of gene expression, and regulation of RNA metabolic process. Interestingly, we found miR-181a-5p was significantly associated with glycolysis regulation by targeting SLC2A1, SLC2A3, HK2 and PKFFB2. RT-PCR results showed SLC2A1, SLC2A3, HK2 and PKFFB2 were decreased significantly after transfection with miR-181a-5p in LNCaP and PC-3 cells. Aerobic glycolysis is widely considered as an emerging hallmark of cancer and plays an important role in cancer progression by providing energy and metabolism precursor for cell proliferation. This result suggests a key role of miR-181a-5p in regulating PCa cellular energy metabolism.

Conclusions

In this study, we found miR-181a was down-regulated in PCa sample by analyzing public datasets, including GSE21036, GSE14857 and GSE45604. Furthermore, we found miR-181a was significantly involved in regulating cell met-

abolic process and gene expression. Moreover, we found overexpression of miR-181a could significantly inhibited cell proliferation and induced G1 cell cycle arrest. Our results suggest miR-181a-5p may be a diagnostic and therapeutic biomarker for prostate cancer.

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Disclosure of conflict of interest

None.

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