MicroRNA-512-3p is upregulated, and promotes proliferation and cell cycle progression, in prostate cancer cells

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Abstract. Prostate cancer (PCa) is the most commonly diagnosed cancer in males worldwide. MicroRNAs (miRNAs/miRs) are small non-coding RNAs that participate in the regulation of various biological processes by regulating post-transcriptional gene expression. However, whether dysregulation of miRNA expression may be associated with the carcinogenesis of PCa remains to be elucidated. The present study identified differentially expressed miRNAs in PCa by analyzing two publicly available gene expression datasets, GSE14857 and GSE21036. The results demonstrated that miR-512-3p was significantly upregulated in PCa. Furthermore, the present study explored the molecular functions of miR-512-3p in PCa, and demonstrated that overexpression of miR-512-3p promoted PCa cell proliferation and reduced G_1 phase cell cycle arrest in PCa. These results indicated that miR-512-3p may act as an oncogene in PCa. To the best of our knowledge, this is the first study revealed the molecular functions of miR-512-3p in PCa. To obtain valuable insights into the potential mechanisms of miR-512-3p, bioinformatics analyses were performed to identify the targets of miR-512-3p. Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology category analyses revealed that miR-512-3p may be associated with the mitogen-activated protein kinase signaling pathway and numerous biological processes, including cell adhesion, cell proliferation, cell cycle and apoptosis. These results suggested that miR-512-3p may be considered a potential diagnostic and therapeutic target of PCa.

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Introduction

Prostate cancer (PCa) is one of the most common types of cancer, and the leading cause of cancer-associated mortality in men worldwide (1,2). The molecular mechanisms that underlie the tumorigenesis, progression and metastasis of PCa remain unclear, regardless of a large number of research studies. Noncoding RNAs have been reported to serve pivotal roles in the pathogenesis of numerous types of cancer via regulating the diversity of biological processes. Therefore, identifying the noncoding RNAs associated with PCa may provide a novel insight into the mechanisms underlying PCa carcinogenesis (3-6).

MicroRNAs (miRNAs/miRs) are small post-transcriptional regulatory noncoding RNAs, 20-22 nt in length. Previous studies have reported the aberrant expression of miRNAs in numerous types of human malignancy, including breast and lung cancer, and PCa (7-11). In PCa, numerous dysregulated miRNAs, inducing miR-27a (12,13), miR-135a (14), miR-186 (15), miR-4638-5p (16), miR-124 (17-19) and miR-320 (20,21), have been reported to regulate cell growth, apoptosis, migration and invasion. These findings indicated that dysregulation of miRNA expression may be associated with carcinogenesis of PCa.

miR-512-3p has been reported to act as a tumor suppressor in hepatocellular carcinoma (22) and lung adenocarcinoma (23). In addition, miR-512-3p has also been revealed to be upregulated in non-small cell lung cancer (NSCLC) A549 cells following retinoic acid (RA) treatment, and has been demonstrated to inhibit the adhesion, migration and invasion of NSCLC cells (23). However, the role of miR-512-3p in PCa remains poorly understood and therefore requires further investigation.

The present study identified differentially expressed miRNAs in PCa, by analyzing two publicly available gene expression datasets, GSE14857 (24) and GSE21036 (25). Furthermore, the molecular functions of miR-512-3p in PCa were investigated, in order to identify their potential roles in the carcinogenesis of PCa.

Materials and methods

miRNA profile data collection. miRNA profile datasets (GSE14857 and GSE21036) were collected from the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/gds).

Comparison of the miRNA profiles between PCa samples and normal tissue samples was performed with limma package in R software using raw microarray data. Significantly differentially expressed miRNAs were identified with thresholds of |logFC|>1.0 and P<0.05.

Cell culture. LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). PC-3 and 22RV1 cells, and the noncancerous prostatic cell line WPMY-1, were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were confirmed by short tandem repeat analysis. The four cell lines were cultured in Ham's F12K media (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (vol/vol) fetal bovine serum (cat. no. 10099141M; Gibco; Thermo Fisher Scientific, Inc.), at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell transfection. The synthetic miR-512-3p mimics and a scrambled control miRNA [miR-negative control (NC)] were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences were as follows: miR-512-3p mimics, 5'-AAGUGCUGUCAUAGCUGAGGUC-3' (sense) and 5'-CCUCAGCUAUGACAGCACUUUU-3' (antisense); NC mimics, 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). PCa cells were seeded at $3x10^5$ cells/wells in 6-well plates and were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 overnight. Subsequently, transfection with the miR-512-3p mimic or miR-NC was performed using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were transfected with 300 nmol miRNA according to the manufacturer's protocol. Total RNA was extracted from the cells 48 h post-transfection and western blotting was also performed.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA, which was used for RT-qPCR analysis, was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT was performed using the PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. The sequence of the miR-512-3p-specific RT primer was 5'-GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACGACCTC-3'. To analyze miRNA expression, RT-qPCR was performed using SYBR-Green Reagents (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a LightCycler 480 system (Roche Diagnostics, Basel, Switzerland). The expression levels of miR-512-3p were normalized to U6. The PCR primers for mature miR-512-3p, Rho family GTPase 3 (RND3), MX dynamin like GTPase 1 (MXI1), mitofusin 2 (MFN2), forkhead box O1 (FOXO1), RNA binding motif protein38 (RBM38), transforming coiled-coil containing protein 1 (TACC1) and U6 were as follows: miR-512-3p forward, 5'-CGG CGGCACTCAGCCTTGAGGG-3' and reverse, 5'-GTGCAG GGTCCGAGGT-3'; RND3 forward, 5'-AAAAACTGCGCT GCTCCAT-3' and reverse, 5'-TCAAAACTGGCCGTGTAA TTC-3'; MXI1 forward, 5'-CATGGAGCGGGTGAAGAT-3' and reverse, 5'-ATGAAGAGGCGTAGCCATGT-3'; MFN2 forward, 5'-TGCCTCAGAGCCCGAGTA-3' and reverse, 5'-CTGGTACAACGCTCCATGTG-3'; FOXO1 forward, 5'-AAGGGTGACAGCAACAGCTC-3' and reverse, 5'-TTC CTTCATTCTGCACACGA-3'; RBM38 forward, 5'-TTGATC CAGCGGACTTACG-3' and reverse, 5'-AATGTAGGGCGA GGACAGC-3'; TACC1 forward, 5'-GCGAAATGGACGTGG TCT-3' and reverse, 5'-CACCTTACAGCCACTCCTGAA-3'; and U6 forward, 5'-CGCTTCGGCAGCACATATACTAA-3' and reverse 5'-TATGGAACGCTTCACGAATTTGC-3'. The results were normalized to those of β-actin or U6 as the internal control to estimate the different expression of genes. Relative mRNA and miRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method (26). Each sample was assayed in triplicate to ensure quantitative accuracy.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer (Boston Bioproducts, Inc., Ashland, MA, USA) supplemented with cOmplete™, EDTA-free Protease Inhibitors (Roche Diagnostics) and phenylmethylsulfonyl fluoride (Calbiochem; EMD Millipore, Billerica, MA, USA). The protein concentration was determined using the Pierce™ Bicinchoninic acid Protein Assay (cat. no. 23222; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions. A total of 30 μg/lane protein was loaded and separated by 12% SDS-PAGE, which was then transferred to PVDF membranes. Membranes were blocked in Tris buffered saline with 0.05% Tween-20 containing 5% non-fat dry milk at room temperature for 1 h. Immunoblots were incubated overnight at 4˚C with the following primary antibodies: Anti-p21 (1:1,000; cat. no. ab109520; Abcam, Cambridge, MA, USA) and anti-β-actin (1:3,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Subsequently, the blots were incubated at room temperature for 1 h with goat anti-mouse immunoglobulin (Ig)G-horseradish peroxidase (HRP)-conjugated and goat anti-rabbit IgG-HRP-conjugated secondary antibodies (1:4,000; cat. nos. A4416 and A6154, respectively; Sigma-Aldrich; Merck KGaA). An Electrochemiluminescence Plus kit (cat. no. RPN2132; GE Healthcare Life Sciences, Uppsala, Sweden) was used for visualization.

Cell proliferation assay. Cells were seeded into 96-well plates at 2,000 cells/well 6 h post-transfection. The Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to detect relative cell proliferation for 4 days. Briefly, 10 μl/well CCK-8 agent was added to the cells, which were incubated for 2 h at 37˚C; subsequently, absorbance was measured at 450 nm using an ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Cell cycle analysis. Transfected LNCaP, 22Rv1 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) at room temperature for 15 min, 48 h post-transfection. For cell cycle analysis, the transfected cells were examined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and were analyzed with ModFit version 4.1 software (Verity Software House, ME, USA). Each test was performed in triplicate.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The Molecule

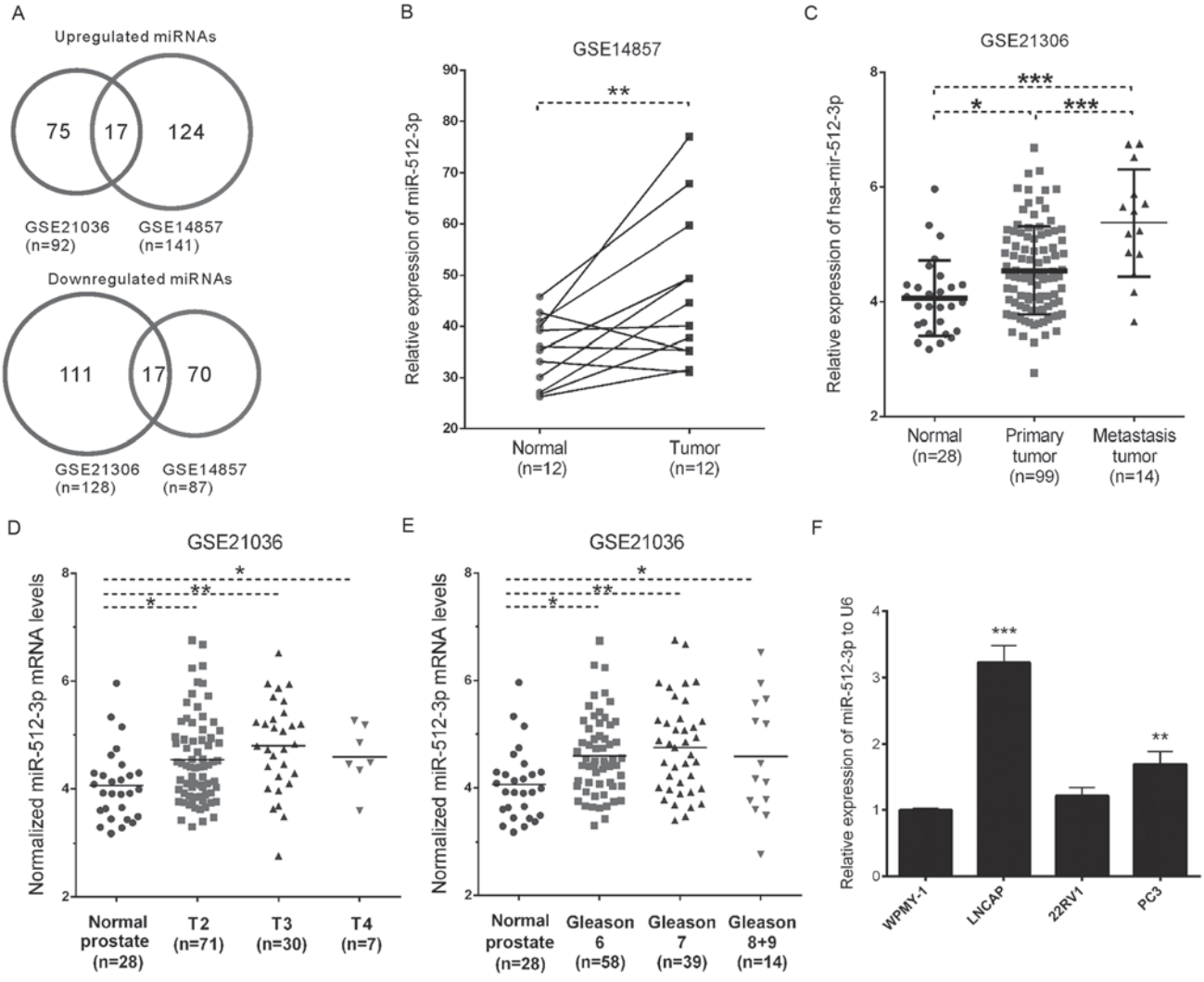

Figure 1. miR-512-3p is overexpressed in prostate cancer. (A-C) miR-512-3p was overexpressed in two publicly available gene expression datasets, GSE14857 and GSE21036. (D) miR-512-3p expression levels were overexpressed in patients with T2 (P<0.05), T3 (P<0.01) and T4 (P<0.05) prostate cancer compared with the normal controls. (E) Expression levels of miR-512-3p were upregulated in Gleason 6 (P<0.05), Gleason 7 (P<0.01) and Gleason 8 (P<0.05) prostate cancer when compared with the normal controls (all vs. NC). (F) Expression levels of miR-512-3p were upregulated in LNCaP (P<0.001) and PC-3 (P<0.01) when compared with the WPMY-1 normal prostate cell line (both vs. WPMY-1). *P<0.05, **P<0.01 and ***P<0.001, as indicated. miR, microRNA; T2, intraprostatic localized tumors; T3+T4, invasive extraprostatic tumors.

Annotation System (MAS; version 3.0), provided by CapitalBio Corporation (Beijing, China; bioinfo.capitalbio.com/mas3/) was used to determine the biological roles of differentially expressed mRNAs. Gene functions were classified in to three subgroups: Biological process, cellular component and molecular function. The enriched GO terms were presented by enrichment scores. KEGG pathway analysis was carried out to determine the involvement of differentially expressed mRNAs in different biological pathways. The recommended hypergeometric-P-value used as the cut-off was P<0.05.

Statistical analysis. Numerical data were presented as the mean ± standard deviation of at least three determinations. Statistical comparisons between groups of normalized data were performed using an unpaired Student's t-test and SPSS v13.0 software (SPSS, Inc., Chicago, IL, USA) or a Mann-Whitney U-test according to the test condition. Statistical comparisons among multiple groups of normalized data were performed using one-way analysis of variance followed by a Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference with a 95% confidence level.

Results

miR-512-3p is overexpressed in PCa. To identify the differentially expressed miRNAs in PCa, two publicly available gene expression datasets, GSE14857 and GSE21036, were analyzed (Fig. 1A). A total of 17 miRNAs were downregulated and 17 miRNAs were overexpressed in PCa in both databases. The present study primarily focused on the 17 upregulated miRNAs (miR-106b, miR-93, miR-148a, miR-25, miR-375, miR-130b, miR-512-3p, miR-18a, miR-518c*, miR-7, miR-95, miR-96, miR-32, miR-663, miR-182, miR-183 and miR-153) as putative biomarkers. The majority of these miRNAs have been reported to be involved in the carcinogenesis of PCa (27-37); however, the molecular functions of miR-512-3p and miR-518c* in PCa remained unclear.

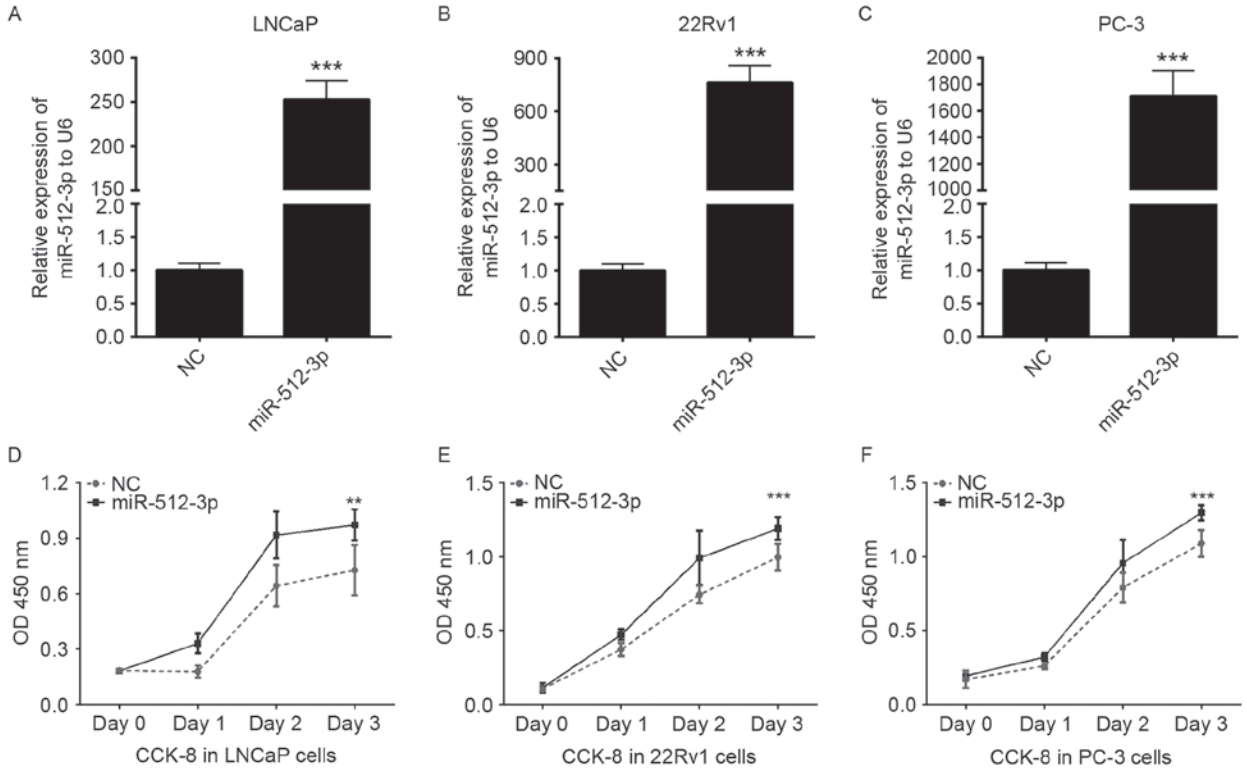

Figure 2. miR-512-3p promotes prostate cancer cell proliferation. miR-512-3p expression was significantly upregulated in (A) LNCaP, (B) 22Rv1 and (C) PC-3 prostate cancer cells following transfection with miR-512-3p mimics for 48 h using reverse transcription-quantitative polymerase chain reaction. Overexpression of miR-512-3p promoted (D) LNCaP, (E) 22Rv1 and (F) PC-3 prostate cancer cell proliferation via a CCK-8 assay. Data are presented as the mean ± standard deviation (n=8). **P<0.01 and ***P<0.001 vs. NC. CCK-8, Cell Counting kit-8; miR, microRNA; NC, negative control; OD, optical density.

Analysis of the GSE14857 and GSE21036 datasets indicated that miR-512-3p expression was significantly upregulated in tumor samples compared with in normal samples (P<0.01; Fig. 1B), and was overexpressed in metastatic samples compared with in primary tumor tissues (P<0.001; Fig. 1C). A clinical significance analysis of GSE21036 demonstrated that miR-512-3p expression levels were overexpressed in patients with T2 (P<0.05), T3 (P<0.01) and T4 (P<0.05) PCa compared with the normal controls (Fig. 1D). Subsequently, the patients in GSE21036 were categorized based on Gleason grades, and the results demonstrated that tissues from patients with Gleason grades 6, 7, 8 and 9 PCa exhibited significantly higher levels of miR-512-3p compared with the matched normal tissues (Fig. 1E).

The present study also detected miR-512-3p expression in PCa cell lines. RT-qPCR was conducted to detect the expression levels of miR-512-3p in PCa cell lines LNCaP, 22Rv1 and PC-3, and in the noncancerous prostatic cells WPMY-1 cell line. The results demonstrated that miR-512-3p was upregulated in PCa cells (including LNCaP and PC-3; Fig. 1F). However, no significant upregulation of miR-512-3p was observed in 22Rv1 cells. These results were consistent with the previous findings in PCa and normal tissues.

Overexpression of miR-512-3p promotes PCa cell proliferation. The present study aimed to explore the potential effects of miR-512-3p on the proliferation of PCa cells. Initially, the effects of a miR-512-3p mimic were determined on the expression of miR-512-3p. LNCaP, 22Rv1 and PC-3 cells were transfected with NC or miR-512-3p mimics. A total of 48 h post-transfection, the expression levels of miR-512-3p were significantly increased in the miR-512-3p mimic group compared with the NC group (P<0.001; Fig. 2A-C). Subsequently, cell proliferation was investigated using a CCK-8 assay, overexpression of miR-512-3p significantly promoted the proliferation of LNCaP, 22Rv1and PC-3 cells (P<0.01 and P<0.001; Fig. 2D-F).

Overexpression of miR-512-3p prevents G_1 phase cell cycle arrest in vitro. The present study assessed the function of miR-512-3p on cell cycle progression in LNCaP, 22Rv1 and PC-3 cells. Flow cytometric analysis revealed that overexpression of miR-512-3p in LNCaP and 22RV1 cells resulted in a significant increase in the proportion of cells in S phase and a decrease in the proportion of cells in G1 phase. However, overexpression of miR-512-3p in PC-3 cells decreased the proportion of cells in S phase and increased the proportion of cells in the G2/M phase (P<0.05; Fig. 3A-C). In addition, a decrease in the protein expression levels of cell cycle inhibitor p21 was detected in cells overexpressing miR-512-3p (Fig. 3D). Downregulation of p21 promotes cell cycle progression, thus these results suggest that the overexpression of miR-512-3p may promote cell cycle progression by inhibiting p21 (38,39).

GO category and KEGG pathway analyses. To obtain valuable insights into the potential mechanisms of miR-512-3p, a bioinformatics analysis was performed to identify the target genes of miR-512-3p using starBase (40). starBase is a database that combines data from six prediction programs TargetScan (41), PicTar (www.pictar.org/), miRanda

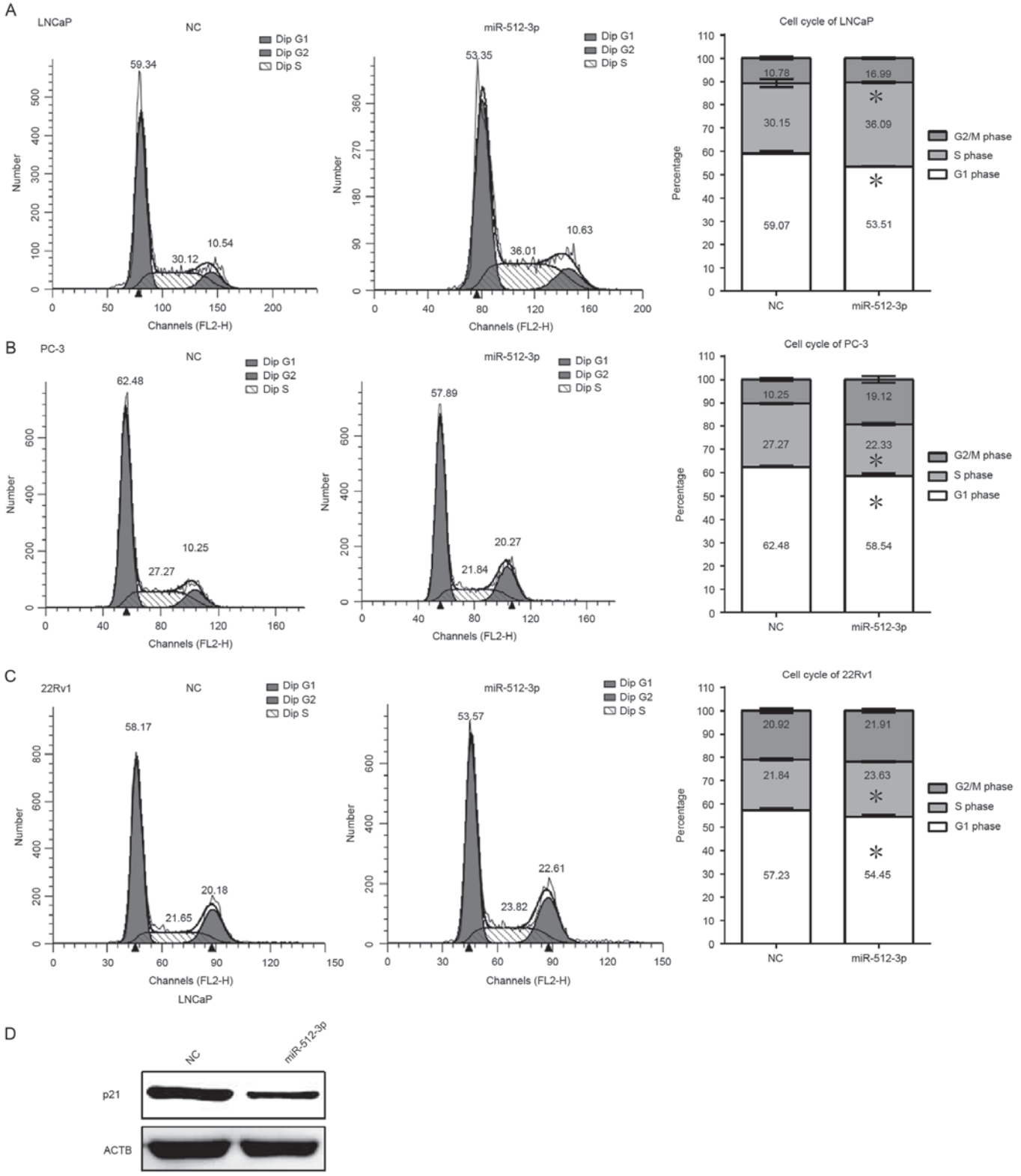

Figure 3. Cell cycle analysis was performed in LNCaP, 22RV1 and PC-3 cells. (A-C) Cells were transfected with a miR-512-3p mimic for 48 h, and were then stained with propidium iodide and evaluated using a FACSCalibur flow cytometer. (D) Protein expression levels of p21 were detected in cells overexpressing miR-512-3p. A western blot assay revealed that overexpression of miR-512-3p inhibited p21 expression. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. NC. ACTB, β-actin; miR, microRNA; NC, negative control.

(www.microrna.org/microrna/home.do), PITA (https://genie.weizmann.ac.il/), RNA22 (https://cm.jefferson.edu/rna22/) and CLIP-Seq (www.starbase.sysu.edu.cn/). A total of 663 targets of miR-512-3p were used to perform the KEGG pathway (www.genome.jp/kegg/) and GO category (www.geneontology.org/) analyses using MAS 3.0 system (http://bioinfo.capitalbio.com/mas3/). The results revealed that miR-512-3p may affect numerous biological processes, including cell adhesion, cell proliferation, cell cycle and apoptosis (Fig. 4A). Pathway enrichment analysis demonstrated that miR-512-3p

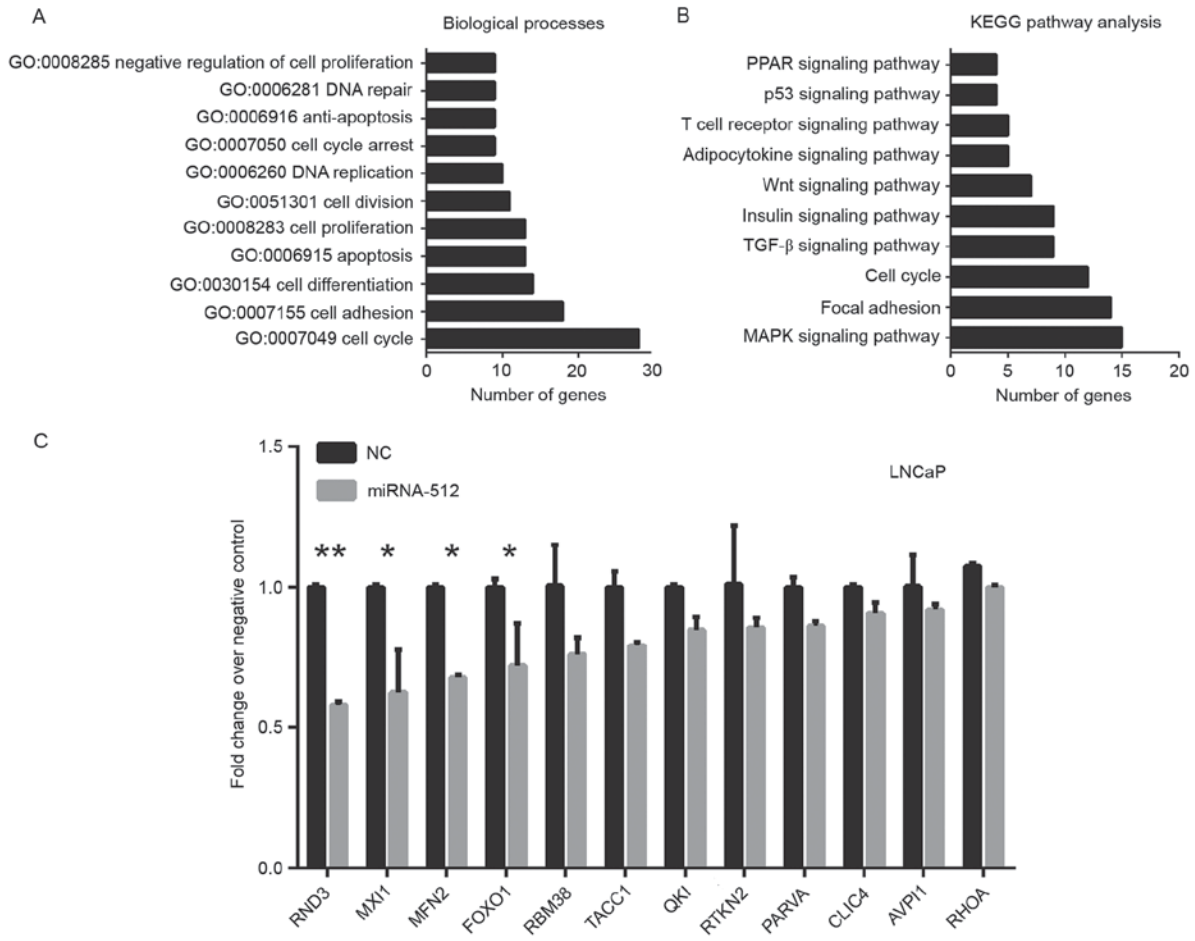

Figure 4. (A) Biological processes and (B) KEGG pathway analyses of miR-512-3p. (C) mRNA expression levels of numerous pathway-related genes following overexpression of miR-512-3p. *P<0.05 and **P<0.01 vs. NC. AVPI1, arginine vasopressin induced 1; CLIC4, chloride intracellular channel 4; FOXO1, forkhead box O1; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; miR, microRNA; MXI1, MX dynamin like GTPase 1; NC, negative control; PARVA, parvin α; PPAR, peroxisome proliferator-activated receptor; QKI, QKI, KH domain containing RNA binding; RBM38, RNA binding motif protein 38; RND3, Rho family GTPase 3; TACC1, transforming coiled-coil containing protein 1; TGF, transforming growth factor; MFN2, mitofusin 2; RHOA, Ras homolog family member A; RTKN2, rhotekin 2.

predominantly participated in the mitogen-activated protein kinase (MAPK) signaling pathway, focal adhesion, cell cycle and transforming growth factor (TGF)-β pathway (Fig. 4B). To further validate these findings the present study detected the expression of numerous pathway-associated genes using RT-qPCR. LNCaP cell lines retain characteristics associated with early androgen-dependent molecular biology and tumor cytology (42), and LNCaP is one of the most commonly used cell lines in the PCa research field (43). Thus, LNCaP cell selected for further validation. Overexpression of miR-512-3p was able to significantly reduce the expression levels of Rho family GTPase 3, MAX interactor 1, dimerization protein, MFN2 and forkhead box O1 (Fig. 4C).

Discussion

PCa is a leading cause of cancer-associated mortality in men worldwide; however, the precise molecular mechanisms underlying the progression of PCa remain unclear. Numerous studies have revealed that miRNAs regulate several biological processes in PCa, including proliferation, cell cycle progression and metastasis (44-47). miRNA expression profiles provide valuable insights into the molecular mechanisms of PCa, and may be used to identify novel biomarkers of PCa. The present study analyzed two publicly available gene expression datasets and screened differentially expressed miRNAs in PCa.

A total of 17 miRNAs were downregulated and 17 miRNAs were overexpressed in PCa samples compared with normal controls in both datasets. The present study primarily focused on the 17 upregulated miRNAs (miR-106b, miR-93, miR-148a, miR-25, miR-375, miR-130b, miR-512-3p, miR-18a, miR-518c*, miR-7, miR-95, miR-96, miR-32, miR-663, miR-182, miR-183 and miR-153) as putative biomarkers. The majority of these miRNAs have been reported to be involved in the carcinogenesis of PCa (27-37).

The present study revealed that miR-512-3p was significantly upregulated in PCa compared with in normal tissues; however, to the best of our knowledge, the molecular functions of miR-512-3p in PCa have yet to be reported in PCa. In lung adenocarcinoma, miR-512-3p has been reported to act as a tumor suppressor that inhibits cell adhesion, migration and invasion of NSCLC cells (23). However, the roles of miR-512-3p in PCa remain unclear. The present study demonstrated that overexpression of miR-512-3p promoted PCa cell proliferation and reduced G_1 phase cell cycle arrest in PCa. The results indicated that miR-512-3p may act as an

oncogene in PCa and may serve varying roles in different types of cancers.

To obtain valuable insights into the potential mechanisms of miR-512-3p, a bioinformatics analysis was conducted to identify miR-512-3p target genes using starBase (40). A total of 663 targets of miR-512-3p were used to perform KEGG pathway and GO category analyses. The results revealed that miR-512-3p may affect numerous biological processes, including cell adhesion, cell proliferation, cell cycle and apoptosis. Pathway enrichment analyses demonstrated that miR-512-3p was associated with the MAPK signaling pathway, focal adhesion, cell cycle and TGF-β pathway. Further validation revealed that overexpression of miR-512-3p significantly reduced the expression levels of RND3, MXI1, MFN2 and FOXO1. These results suggest that miR-512-3p may serve an important role in the regulation of PCa progression by regulating several genes, including RND3, MXI1, MFN2 and FOXO1.

In conclusion, the present study analyzed two publicly available gene expression datasets, and screened differentially expressed miRNAs in PCa. The results demonstrated that miR-512-3p may promote PCa cell proliferation and cell cycle progression, thus suggesting that miR-512-3p may be considered a potential diagnostic and therapeutic target of PCa.

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