

miR-20b-5p is a novel biomarker for detecting prostate cancer

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Abstract. Prostate cancer (PCa) is the second most frequently diagnosed solid tumor and the fifth leading cause of cancer mortality among men worldwide. The prostate specific antigen (PSA) test for PCa remains controversial. Therefore, the development of more effective non-invasive biomarkers for PCa is necessary. The present study evaluated the diagnostic value of microRNA (miR)-20b-5p in PCa. Tissue miR-20b-5p expression levels and their correlation with clinical parameters were assessed using The Cancer Genome Atlas (TCGA) datasets, and the diagnostic value of the miR-20b-5p expression levels in PCa tissues was assessed using receiver operating characteristic curve analysis. Reverse transcription-quantitative PCR (RT-qPCR) was used to assess the relative expression levels of miR-20b-5p in PCa tissues compared with benign prostate hyperplasia (BPH) tissues. In addition, miR-20b-5p expression levels in PCa cell lines and non-tumorigenic prostate epithelial cells were compared. In this study, exosomes were extracted from the prostatic fluid as a source of liquid biopsy for the detection of PCa. The prostatic fluid exosomal miR-20b-5p expression levels between patients with PCa and the biopsy-negative patients were compared, and the diagnostic efficiency of prostatic fluid exosomal miR-20b-5p expression levels in PCa was compared with PSA and with the European Randomized Study of Screening for Prostate Cancer (ERSPC) risk calculator. The mechanism by which miR-20b-5p may function in PCa was assessed using bioinformatic analysis and validation experiments. miR-20b-5p was expressed at a markedly higher level in PCa tissues compared with normal prostate tissues with high diagnostic efficiency (area under the curve: 0.826). The expression levels of miR-20b-5p were

also significantly higher in PCa tissues compared with BPH tissues; similarly, miR-20b-5p was more highly expressed in PCa cells compared with non-tumorigenic prostate epithelial cells. Prostatic fluid exosomal miR-20b-5p expression levels in patients with PCa were significantly higher compared with confirmed to be biopsy-negative, and the diagnostic performance of miR-20b-5p was superior to PSA and ERSPC risk calculator. The results of RT-qPCR and western blotting following transfection of DU145 cells with miR-20b-5p mimics and inhibitor showed that miR-20b-5p reduced the expression of retinoblastoma-associated protein 1 (RB1). Therefore, RB1 may be a significant target gene for miR-20b-5p. In conclusion, the present study demonstrated that miR-20b-5p was upregulated in PCa at the tissue and cellular levels, as well as in prostatic fluid exosomes. Therefore, miR-20b-5p may be a promising early diagnostic biomarker for PCa and an important tool to guide the decision-making of prostate biopsy.

Introduction

Prostate cancer (PCa) is the most common malignant tumor in the male genitourinary system (1). According to the Global Cancer Observatory 2020 database, PCa is the second most frequently diagnosed solid tumor and the fifth leading cause of cancer-related mortality among men worldwide (2). Prostate specific antigen (PSA), secreted by prostatic epithelial cells, is widely used for the screening and diagnosis of PCa. The application of PSA has greatly improved the detection rate of PCa. However, as a prostate organ-specific but not a PCa-specific biomarker, PSA levels can also be elevated in benign prostate hyperplasia (BPH), prostatic inflammation or other benign diseases (3). The low specificity of PSA has led to unnecessary prostate biopsies and the detection of clinically indolent tumors, which results in pain, bleeding, infections and other complications along with over-treatment, including surgery, radiation and additional biopsies (4). Therefore, the development of more specific biomarkers for the early diagnosis of PCa and to guide the decision-making for prostate biopsy is necessary.

MicroRNAs (miRNAs) are endogenous, short (18-25 nucleotides), single-stranded, non-coding RNAs widely found in both animals and plants (5); they bind to the 3' untranslated region (3' UTR) of target mRNAs leading to their degradation or the inhibition of mRNA translation (6). miRNAs

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have been reported to be associated with cancer progression, apoptosis, proliferation, migration, metastasis and drug resistance, which indicates that they serve a vital role in the pathogenesis of cancer and are, therefore, good choices for the diagnosis and treatment of cancer (7). miR-20b-5p belongs to the tumor-related miR-106a/363 cluster, which together with the miR-106b/25 cluster and miR-17/92 cluster forms the large miR-17 family (8). The role of miR-20b-5p in human cancers is controversial. miR-20b-5p has been reported to function as an oncomiR in non-small cell lung cancer (9), breast cancer (10), gastric cancer (11), esophageal cancer (12) and laryngeal squamous cell carcinoma (13), but as a tumor-suppressor miRNA in colon cancer (14) and papillary thyroid carcinoma (15). miR-20b-5p was found to promote the tumor aggression of PCa and can predict aggressive PCa after radical prostatectomy (16,17). In the present study, the diagnostic value and biological mechanism of miR-20b-5p in PCa was evaluated.

Liquid biopsies, including exosomes, circulating tumor cells and circulating nucleic acids, have been used as minimally invasive methods to monitor patients with PCa (18). Exosomes, a type of extracellular vesicles with a diameter of 50-150 nm, are regarded as fundamental mediators of cell-to-cell communication, and serve a critical role in multiple biological processes (19). In the process of cell-to-cell communication, cell-derived exosomes transfer genetic information, such as mRNAs and miRNAs, to neighboring cells or distant organs. Prostatic fluid, instead of other commonly used liquid biopsies, such as blood, urine or saliva, originates from prostatic epithelial cells, which can directly reflect the changes in prostate organ function, so it has a significant advantage in screening for PCa. In the present study, exosomes were extracted from the prostatic fluid as a source of liquid biopsy for the detection of PCa.

Materials and methods

TCGA database. miR-20b-5p expression data from 493 human PCa tissues and 51 adjacent normal tissues were accessed using TCGAbiolinks package (version 2.18.0) (20) in R (version 4.0.3; <https://www.r-project.org/>) and were normalized and standardized using the limma package (version 3.44.3) using the voom method with the threshold of \log_2 fold change (FC) > 1 and $P < 0.05$ (21). The clinical data of the 493 cases with complete clinical information were also downloaded and were used for secondary analysis in the present study (Table I). The miR-20b-5p expression level data and the clinical data of the 493 patients were combined and the clinical information was evaluated to assess the relationship between the miR-20b-5p expression levels and clinical variables, including age, race, Gleason score, International Society of Urological Pathology (ISUP) grade, Tumor-Node-Metastasis (TNM) stage, radiation, cancer subtype and survival status. The dataset analyzed for this study can be accessed from TCGA database (TCGA-PRAD; <https://portal.gdc.cancer.gov>).

Cell culture and transfection. DU145 and 22Rv1 human prostate carcinoma cell lines were purchased from the American Type Culture Collection and grown in RPMI-1640 medium (Procell Life Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (Biological Industries Israel Beit

Haemek, Ltd.), 100 U/ml penicillin and 100 μ g/ml streptomycin. The RWPE-1 human prostate epithelium cell line was purchased from Procell Life Science & Technology Co., Ltd. and maintained in keratinocyte serum-free medium supplemented with 50 μ g/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Procell Life Science & Technology Co., Ltd.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂. miR-20b-5p mimics (5'-CAAAGU GCUCAUAGUGCAGGUAG-3'), inhibitor (5'-CUACCUGCA CUAUGAGCACUUUG-3'), mimics negative control (NC) (5'-UUCUCCGAACGUGUCACGUTT-3') and inhibitor negative control (INC) (5'-CAGUACUUUUGUGUAGUACAA-3') were purchased from Applied Biological Materials, Inc. They were transiently transfected into DU145 cells using Lipo6000™ Transfection Reagent (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Specifically, DU145 cells were seeded into a 6-well plate and incubated at 37°C in a CO₂ incubator until cells were 70-90% confluent at the time of transfection. miR-20b-5p mimics, inhibitor, NC and INC with a concentration of 40 nM were transfected into DU145 cells using Lipo6000™ Transfection Reagent (5 μ l) at room temperature. After incubating at 37°C for 5-8 h, the transfection solution was removed. The subsequent experiments were performed 48 h later. Transfection efficiency was assessed using reverse transcription-quantitative PCR (RT-qPCR).

RNA extraction and RT-qPCR. Total RNA was extracted from tissues, cells and exosomes using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.). The total RNA (290 ng) was reverse transcribed using the Evo M-MLV RT Kit (Hunan Aikerui Biological Engineering Co., Ltd.) using the following reaction conditions: 37°C for 15 min, 85°C for 5 sec and hold at 4°C. Subsequent qPCR was performed with SYBR® Green Premix Pro Taq HS qPCR Kit (Hunan Aikerui Biological Engineering Co., Ltd.) on a Bio-Rad CFX Connect Real-Time PCR Detection System. The RT-qPCR conditions consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. miR-20b-5p expression was normalized to U6, and retinoblastoma-associated protein (RB1) expression was normalized to GAPDH. The primers for miR-20b-5p, U6, RB1 and GAPDH were synthesized by Hunan Aikerui Biological Engineering Co., Ltd., and the sequences were as follows: miR-20b-5p specific stem-loop RT5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCTACCT-3', forward, 5'-GCGCAAAGT GTCATAGTGC-3' and reverse, 5'-AGTGCAGGGTCCGAG GTATT-3'; U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; RB1 forward, 5'-CCAGACCCAGAAGCCATTGA-3' and reverse, 5'-TTCACAAAGTGTATTTAGCCGGAGA-3'; and GAPDH forward, 5'-CAGTATGACTCCACTCACGGC-3' and reverse, 5'-GAGGGGCCATCCACAGTCTTC-3'. All reactions were repeated three times, and the data were quantified using the 2^{- $\Delta\Delta$ C_q} method (22).

Specimens. PCa (n=9) and BPH (n=10) tissues used in this study were obtained from prostate biopsy specimens from patients admitted to the Urology Department of The Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) between December 2021 and May 2022. Prostatic fluid samples were

Table I. Clinical characteristics of patients with prostate cancer in The Cancer Genome Atlas database.

Clinicopathological parameters	Patients (n=493)	Percentage
Age, years		
<65	330	66.94
≥65	163	33.06
Race		
White	409	82.96
Black or African American	58	11.76
Asian	12	2.43
American Indian or Alaska native	1	0.20
NA	13	2.64
Gleason score		
<7	45	9.13
≥7	448	90.87
ISUP grading group		
1	45	9.13
2	146	29.61
3	100	20.28
4	64	12.98
5	138	27.99
T stage		
T1	176	35.70
T2	171	34.69
T3	53	10.75
T4	2	0.41
Tx	91	18.46
N stage		
N0	343	69.57
N1	78	15.82
Nx	72	14.60
M stage		
M0	454	92.09
M1	3	0.61
NA	36	7.30
Radiation		
Yes	91	18.46
No	361	73.23
NA	41	8.32
Cancer subtype		
Adenocarcinoma, NOS	481	97.57
Infiltrating duct carcinoma, NOS	9	1.83
Adenocarcinoma with mixed subtypes	3	0.61
Survival status		
Alive	481	97.57
Dead	10	2.03
NA	2	0.41

NA, not available; NOS, not otherwise stated; T, tumor; x, cannot be assessed; N, node; M, metastasis; ISUP, International Society of Urological Pathology.

obtained at the Urology Department of The Second Affiliated Hospital of Xi'an Jiaotong University from patients before they underwent a prostate biopsy between December 2021 and May 2022. A total of 10 patients with PCa and 27 patients with negative results were enrolled. The patients were ≥45 years old and were undergoing initial prostate biopsy for either moderately elevated serum PSA levels (limit range, 2.0-50.0 ng/ml) and/or a suspicious digital rectal examination (DRE). Those with a history of PCa or invasive treatment for BPH within 6 months or taking drugs that affect serum PSA levels within the past 6 months were excluded. This study was approved by the Ethics Committee of the Xi'an Jiaotong University Health Science Center (approval no. 2021-1700) and was performed in accordance with the principles of The Declaration of Helsinki. All participants included in the study provided written informed consent. The authors had access to information that could identify individual participants during or after data collection. Samples were collected by prostate massage (independently without being together with urine or semen), which was performed by systematically applying pressure to the prostate from the base to the apex and from the lateral to the median line of each lobe. Samples were collected immediately in 1.5-ml centrifuge tubes after the prostate massage, snap frozen and kept at -80°C until further processing.

Extraction of exosomes. The ExoQuick-TC™ Exosome Precipitation Solution kit (System Biosciences, LLC) was used for exosome extraction as previously described (23). Briefly, 200 µl prostatic fluid was collected and centrifuged at 3,000 x g for 15 min at 4°C to separate off the cells and cell debris. The supernatant was transferred to a clean centrifuge tube, one-fifth of its volume of ExoQuick-TC Exosome Precipitation Solution was added to it and it was then refrigerated overnight at 4°C. The ExoQuick-TC/prostatic fluid mixture was centrifuged at 1,500 x g for 30 min at 4°C. The supernatant was aspirated, the residual ExoQuick-TC solution was spun down using centrifugation at 1,500 x g for 5 min at 4°C and all traces of fluid were removed.

Transmission electron microscopy (TEM). Exosomes were suspended in 100 µl of 1X PBS, then dropped onto Formvar carbon-coated 400 mesh copper electron microscopy grids and left to sit for 5 min at room temperature. Samples were stained using 1% uranyl acetate for 30 sec at room temperature, after the grids were air-dried, micrographs were captured using a FEI TecnaiG2 spirit transmission electron microscope at 80 kV.

Nanoparticle tracking analysis. Exosome particle size and concentration were assessed using nanoparticle tracking analysis (NTA) using a ZetaView PMX 110 with ZetaView 8.04.02 software (Particle Metrix GmbH) as previously described (24). Briefly, isolated exosome samples were diluted using 1X PBS to measure the particle size and concentration. NTA measurement was recorded and analyzed at 11 positions. The ZetaView system was calibrated using 110 nm polystyrene particles. The temperature was maintained at ~23°C.

Western blotting. The protein of exosomes extracted from 200 µl of prostatic fluid and cells in 1 well of a 6-well plate was extracted using RIPA Lysis Buffer (Beyotime Institute

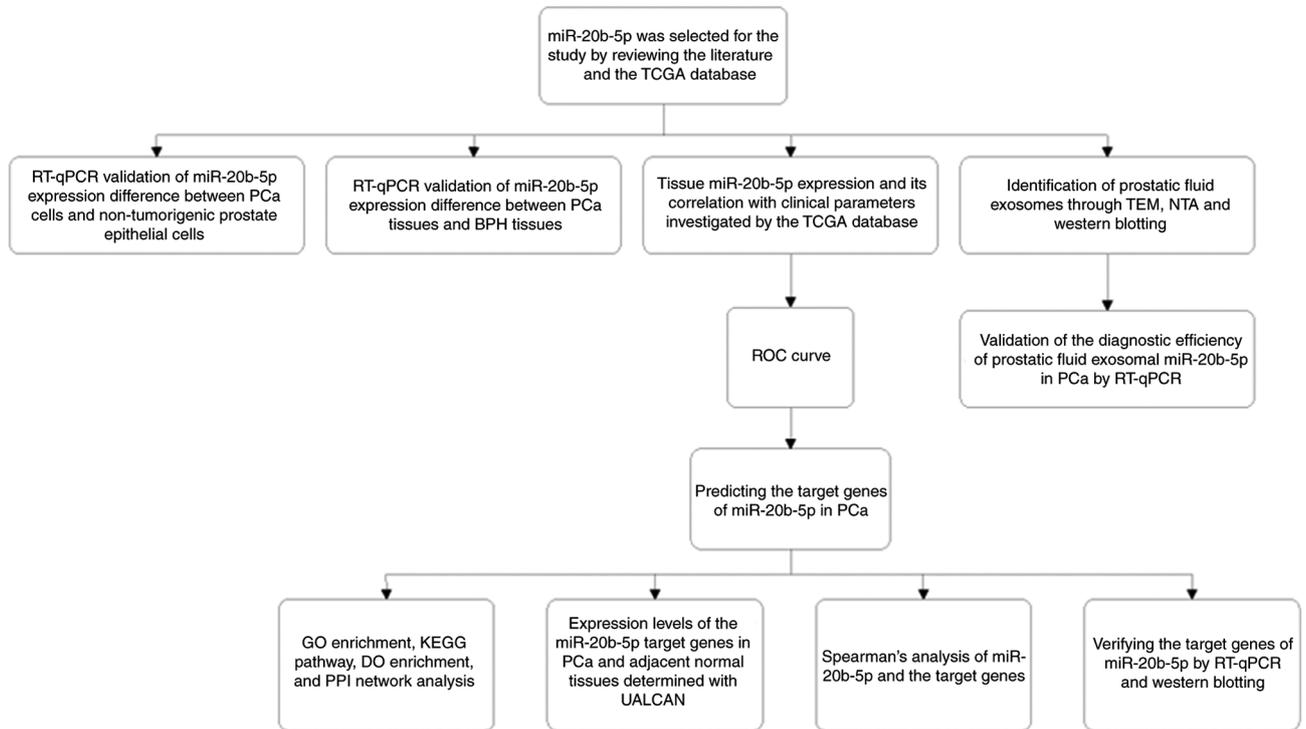


Figure 1. Overall research design and experimental sequence of the study. BPH, benign prostate hyperplasia; DO, Disease Ontology; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; miR, microRNA; NTA, nanoparticle tracking analysis; PCa, prostate cancer; PPI, protein-protein interaction; ROC, receiver operating characteristic; RT-qPCR, reverse transcription-quantitative PCR; TCGA, The Cancer Genome Atlas; TEM, transmission electron microscopy.

of Biotechnology) according to the manufacturer's protocol. Protein concentrations were determined using the BCA protein assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μ g) in each group were resolved on 8% gels using SDS-PAGE and transferred to polyvinylidene fluoride membranes. After being blocked using 5% non-fat milk for 1 h at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: Anti-CD63 (1:2,000; WL02549; Wanleibio, Co., Ltd.), anti-tumor susceptibility gene 101 (TSG101; 1:1,000; WL05130; Wanleibio Co., Ltd.), anti-RB1 (1:1,000; WL02216; Wanleibio, Co., Ltd.) and anti-GAPDH (1:1,000; WL01114; Wanleibio, Co., Ltd.). Following washing of the membranes three times with TBS-Tween-20 (0.05% Tween), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG H+L secondary antibodies (1:10,000; WLA023a; Wanleibio, Co., Ltd.) for 2 h at room temperature, and the visualized using ECL chemiluminescence reagent (Beyotime Institute of Biotechnology). The protein expression levels were semi-quantified using ImageJ software (version, 1.52a; National Institutes of Health).

Predicting the target genes of miR-20b-5p in prostate cancer. The miRDB (25) and miRWalk (26) databases were used to identify the target genes of miR-20b-5p. Gene Ontology (GO) term, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Disease Ontology (DO) enrichment analyses (27) were performed on the target genes using the ClusterProfiler package (version 3.18.0) with the threshold of $\log_2FCI > 1$ and adjusted $P < 0.05$ (28). The Search Tool for the Retrieval of

Interacting Genes (<https://string-db.org>) was then used to generate a protein-protein interaction (PPI) network of the miR-20b-5p target genes associated with the significantly enriched pathways with the threshold of interaction score ≥ 0.4 . The expression levels of the miR-20b-5p target genes in PCa and adjacent normal tissues were determined using UALCAN (29). Finally, the LinkedOmics Spearman's analysis tool (30) was used to assess the correlation between the expression levels of miR-20b-5p and the potential target genes involved in important signaling pathways with the threshold of $|\text{rhol}| \geq 0.2$ and $P < 0.05$.

Statistical analysis. Comparisons between two groups were evaluated by unpaired Student's t-test or the Mann-Whitney U test, and multiple comparisons were made using a one-way ANOVA with Tukey's post hoc test or the Kruskal-Wallis rank test with Dunn's post hoc test. A receiver operating characteristic (ROC) curve was generated to evaluate the diagnostic value of miR-20b-5p expression levels in the TCGA cohort and prostatic fluid specimens, and the area under the curve (AUC) of the ROC curve was calculated. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc.) or SPSS (version, 20.0; IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference. The overall experimental design flow chart is presented in Fig. 1.

Results

miR-20b-5p is upregulated in PCa. Data from TCGA on the miR-20b-5p expression levels in 493 PCa and 51 normal prostate tissues were compared. The results demonstrated

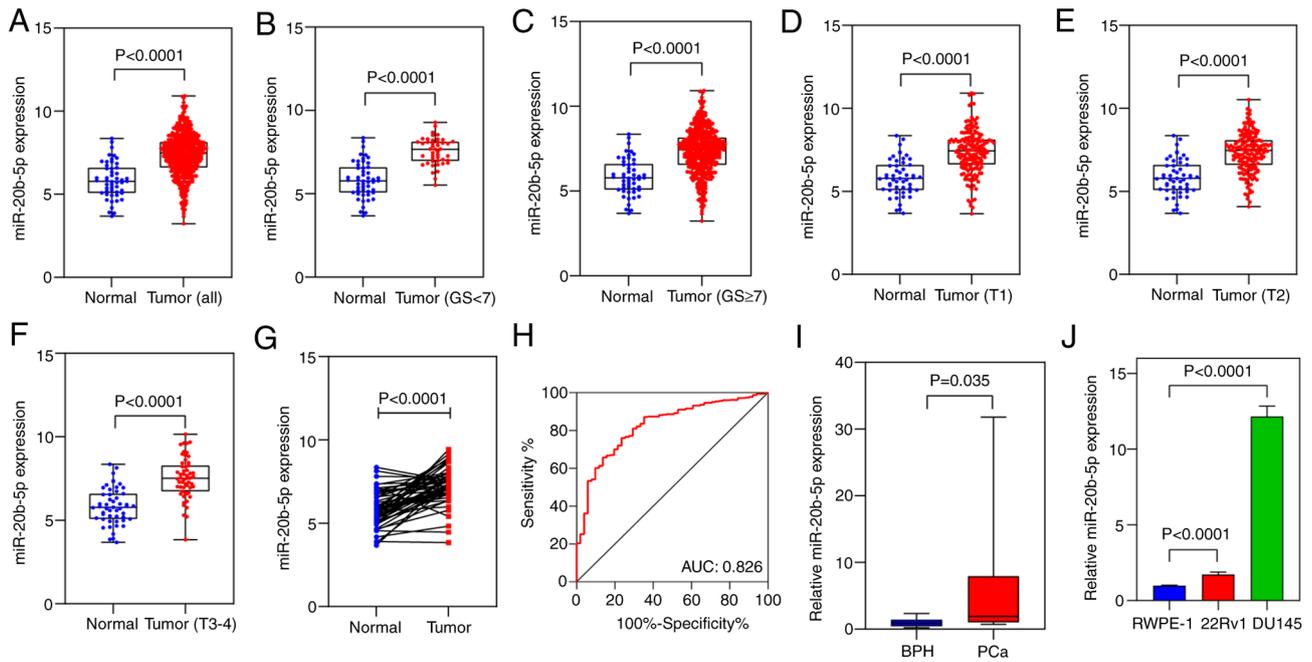


Figure 2. miR-20b-5p expression is upregulated in PCa. (A) miR-20b-5p demonstrated significantly higher expression in all PCa tissues (n=493) compared with normal tissues (n=51). miR-20b-5p expression levels in PCa tissues with (B) GS<7 (n=45) and (C) GS≥7 (n=448) compared with normal tissues. miR-20b-5p expression levels in PCa tissues at (D) T1 (n=176), (E) T2 (n=171) and (F) T3 + T4 stages (n=55) compared with normal tissues (n=51). (G) miR-20b-5p was expressed at a higher level in PCa tissues compared with 51 adjacent paired tissues. (H) Receiver operating characteristic curve for miR-20b-5p expression in PCa and normal prostate tissues. (I) Analysis of the expression levels of miR-20b-5p in PCa (n=9) and BPH (n=10) tissue samples. (J) Reverse transcription-quantitative PCR was used to demonstrate that miR-20b-5p was significantly upregulated in PCa (DU145 and 22Rv1) cells compared with non-tumorigenic prostate epithelial (RWPE-1) cells. BPH, benign prostate hyperplasia; GS, Gleason score; miR, microRNA; PCa, prostate cancer; T, tumor.

significantly higher miR-20b-5p expression levels in PCa compared with normal tissues (P<0.0001; Fig. 2A). Subgroup analysis demonstrated significantly higher miR-20b-5p expression in all different PCa groups compared with normal tissues (all P<0.0001; Fig. 2B-F). After matching the first characters of the patients' identifier, 51 pairs of PCa and matched adjacent tissues from the TCGA cohort were identified. In the paired tissues, miR-20b-5p was significantly overexpressed in PCa tissues compared with normal tissues (P<0.0001; Fig. 2G).

Furthermore, the relationship between clinicopathological parameters and tissue miR-20b-5p expression levels were assessed. miR-20b-5p expression levels were compared according to age, race, Gleason score, ISUP grade, TNM stage, radiation, cancer subtype and survival status of the patients. However, no significant associations were found between miR-20b-5p expression and the clinicopathological parameters (Fig. S1). An ROC curve was generated using the tissue expression data from 493 human PCa tissues and 51 normal adjacent tissues to evaluate the diagnostic value of miR-20b-5p. The AUC was 0.826 (95% CI, 0.771-0.881), which indicated a marked diagnostic value (Fig. 2H). RT-qPCR data demonstrated that miR-20b-5p expression levels were significantly higher in PCa tissues compared with those in BPH tissues (P<0.05; Fig. 2I) and significantly higher in PCa (DU145 and 22Rv1) cells compared with non-tumorigenic prostate epithelial (RWPE-1) cells (P<0.0001; Fig. 2J).

Identification of isolated exosomes. Exosomes isolated from the prostatic fluid of the patients were characterized using TEM, NTA and western blotting (Fig. 3A-C). TEM demonstrated that

the exosomes were round or saucer-shaped with a 50-150 nm diameter, which was consistent with the results from NTA. Furthermore, the western blotting demonstrated that exosomes were positive for both CD63 and TSG101, two typical exosome marker proteins. Therefore, these results demonstrated that the vesicles isolated from the prostatic fluid were exosomes.

Prostatic fluid exosomal miR-20b-5p as a biomarker for the detection of PCa. To assess the diagnostic efficiency of prostatic fluid exosomal miR-20b-5p in PCa, RT-qPCR was performed using samples from 37 patients who underwent prostate biopsy with moderately elevated serum PSA levels (limit range, 2.0-50.0 ng/ml), including 10 patients with PCa and 27 patients that were biopsy-negative confirmed by subsequent pathological findings. The level of exosomal miR-20b-5p expression was significantly increased in patients with PCa compared to patients with negative results (P<0.05; Fig. 3D). To evaluate the diagnostic value of miR-20b-5p for PCa, an ROC curve was generated and the diagnostic efficiency of exosomal miR-20b-5p, PSA and the diagnostic efficiency of exosomal miR-20b-5p, PSA and European Randomized Study of Screening for Prostate Cancer (ERSPC) risk calculator were compared (Fig. 3E). The data show that exosomal miR-20b-5p (AUC, 0.715; 95%CI, 0.526-0.904) was a better predictor than both PSA (AUC, 0.596; 95%CI, 0.369-0.823) and the ERSPC risk calculator (AUC, 0.650; 95%CI, 0.437-0.863).

Identification of putative target genes of miR-20b-5p in PCa. To further evaluate the potential mechanism of miR-20b-5p in PCa, GO, KEGG and DO analyses were performed. Using

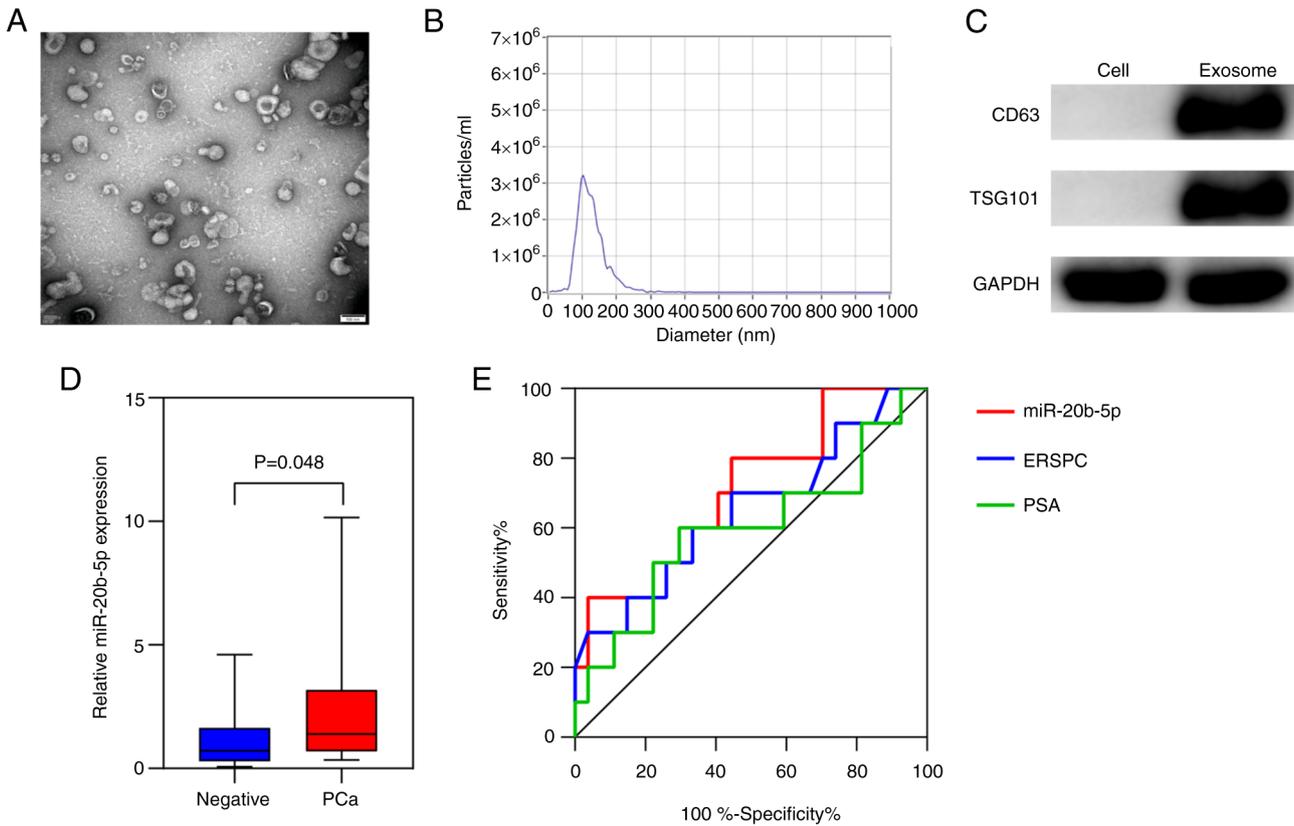


Figure 3. Prostatic fluid exosomal miR-20b-5p as a biomarker for PCa. (A) Transmission electron microscopy showing that the exosomes were round or saucer-shaped with 50-150 nm diameter (scale bar, 100 nm; high voltage, 80 kV). (B) Assessment of the size and number of exosomes using nanoparticle tracking analysis. (C) Western blotting of the exosomal markers CD63 and TSG101 as exosomal markers in prostatic fluid cells and exosomes. GAPDH was used as an endogenous control. (D) Exosomal miR-20b-5p expression levels in the prostatic fluid of patients with PCa and patients with biopsy-negative results. (E) Receiver operating characteristic curves were used to compare exosomal miR-20b-5p with PSA and the ERSPC risk calculator in distinguishing patients with PCa and patients with negative results. ERSPC, European Randomized Study of Screening for Prostate Cancer; miR, microRNA; PCa, prostate cancer; PSA, prostate specific antigen; TSG101, tumor susceptibility gene 101.

the miRDB and miRWalk databases, 1,082 overlapping target genes were retrieved (Fig. 4A). The biological process GO term enrichment analysis demonstrated that the miR-20b-5p target genes were particularly enriched in axonogenesis, dephosphorylation and regulation of GTPase activity (Fig. 4B). KEGG and DO enrichment analysis demonstrated that miR-20b-5p target genes participated in multiple cancer-related pathways, including PCa (Fig. 4C and D, respectively). The PCa-related genes included E2F1, E2F2, E2F3, RB1, SOS1, CREB1, CREB5, CCND1, CDKN1A, MAPK1, PIK3R1, PDGFRA, PDGFRB and TCF7L1, and a PPI network of these genes was generated (Fig. 4E). In the PPI network, 14 nodes and 36 lines illustrated strong interactions (average node degree, 5.14; enrichment $P=9.55 \times 10^{-15}$) between the potential key target genes.

Analysis of the miR-20b-5p target genes related to PCa. The expression levels of target genes in TCGA dataset demonstrated that 10 of the target genes related to PCa (RB1, SOS1, CREB1, CREB5, CCND1, CDKN1A, MAPK1, PIK3R1, PDGFRA and TCF7L1) were significantly downregulated in PCa compared with normal tissues and 3 of the target genes associated with PCa (E2F1, E2F2 and E2F3) were significantly upregulated in PCa compared with that in normal tissues (all $P<0.05$; Fig. 5). Spearman correlation analysis demonstrated

that the expression of RB1, SOS1, CREB1, MAPK1, PIK3R1, PDGFRA and PDGFRB was negatively correlated with miR-20b-5p expression, and the expression of E2F1 was positively correlated with miR-20b-5p expression in PCa (all $P<0.05$; Fig. 6).

miR-20b-5p affects the mRNA and protein expression levels of RB1. RT-qPCR data demonstrated that RB1 mRNA expression levels were significantly lower in PCa tissues compared with those in BPH tissues ($P<0.05$; Fig. 7A). To assess whether miR-20b-5p affected RB1 mRNA and protein expression levels in PCa, DU145 cells were cultured and transfected with miR-20b-5p mimics or miR-20b-5p inhibitor and the transfection efficiencies were assessed using RT-qPCR. Transfection of miR-20b-5p mimics significantly increased the expression of miR-20b-5p, while transfection of miR-20b-5p inhibitor significantly decreased the expression of miR-20b-5p ($P<0.05$; Fig. 7B and C). RB1 mRNA and protein expression levels were assessed using RT-qPCR and western blotting, respectively. miR-20b-5p mimics significantly reduced the mRNA and protein expression levels of RB1 compared with the negative control (Fig. 7D and F, respectively); the miR-20b-5p inhibitor significantly increased the mRNA and protein expression levels of RB1 compared with the INC (Fig. 7E and G, respectively).

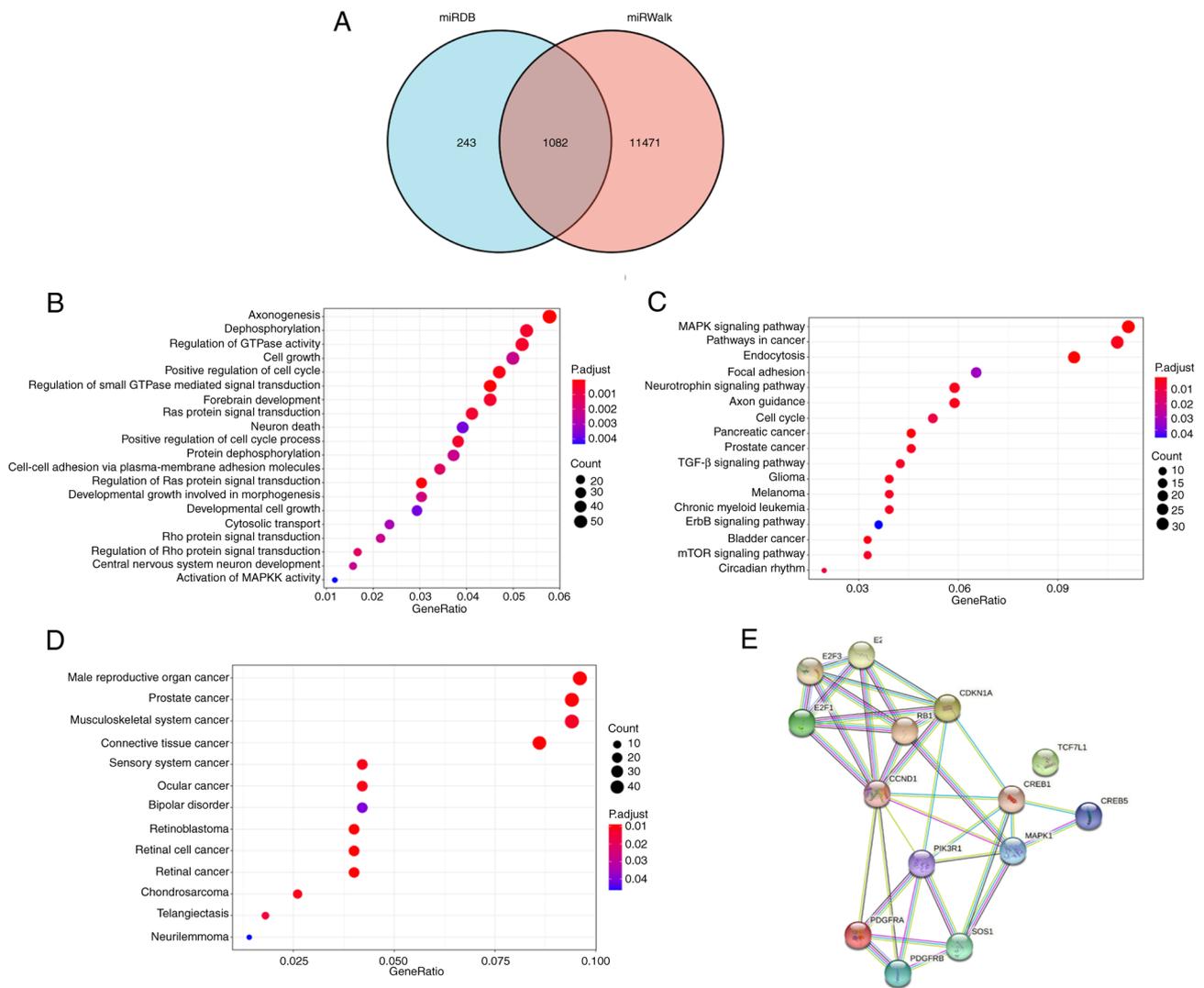


Figure 4. Putative target genes of miR-20b-5p in PCa. (A) Venn diagram of the potential target genes of miR-20b-5p from miRDB and miRWalk databases. (B) Gene Ontology, (C) Kyoto Encyclopedia of Genes and Genomes and (D) Disease Ontology analysis of miR-20b-5p target genes. (E) Protein-protein interaction network of miR-20b-5p target genes associated with PCa. The nodes represented miR-20b-5p target genes and the lines were interactions between genes. miR, microRNA; PCa, prostate cancer.

Discussion

Although the application of PSA testing has been reported to greatly improve the detection rate of PCa, its poor specificity and inability to identify high-grade PCa lead to unnecessary prostate biopsies along with over-diagnosis and over-treatment (31). Therefore, the development of more effective biomarkers for PCa is necessary. Several assays based on urinary exosomes have been used to detect PCa (32); however, a recent tracking analysis reported that urinary exosomes mainly expressed tissue-specific genes of the bladder (33). In the present study, exosomes were extracted from prostatic fluid, which is specifically secreted by the prostate, as a source of liquid biopsy for detecting PCa. In the present study, the role of miR-20b-5p in detecting PCa was assessed at the tissue and cellular levels, as well as using liquid biopsy. At the tissue level, using TCGA data and tissue samples, significantly higher expression of miR-20b-5p was demonstrated in PCa compared with that in BPH and normal prostate tissues. No correlation was demonstrated between miR-20b-5p expression levels and

clinicopathological parameters, which suggested that it may be an independent predictor for PCa. ROC curve results demonstrated the promising diagnostic value of tissue miR-20b-5p for PCa. At the cellular level, it was demonstrated that miR-20b-5p was significantly upregulated in PCa cells compared with non-tumorigenic prostate epithelial cells. Furthermore, prostatic fluid exosomal miR-20b-5p expression levels in patients with PCa were markedly higher compared with patients with biopsy-negative results. The use of prostatic fluid exosomal miR-20b-5p expression levels in predicting PCa was superior to PSA and the ERSPC risk calculator, a widely-used risk calculator which estimates the possibility of PCa on prostate biopsy by calculating a probability based on clinical, biochemical and image findings (34). The findings of the present study indicated that miR-20b-5p may serve as a clinically significant biomarker for the detection PCa and to guide the decision-making involved in prostate biopsy.

miR-20b-5p, a member of the tumor-related miR-106a/363 cluster, was reported to be frequently dysregulated in numerous human malignancies (9,15,35,36). Mechanistically,

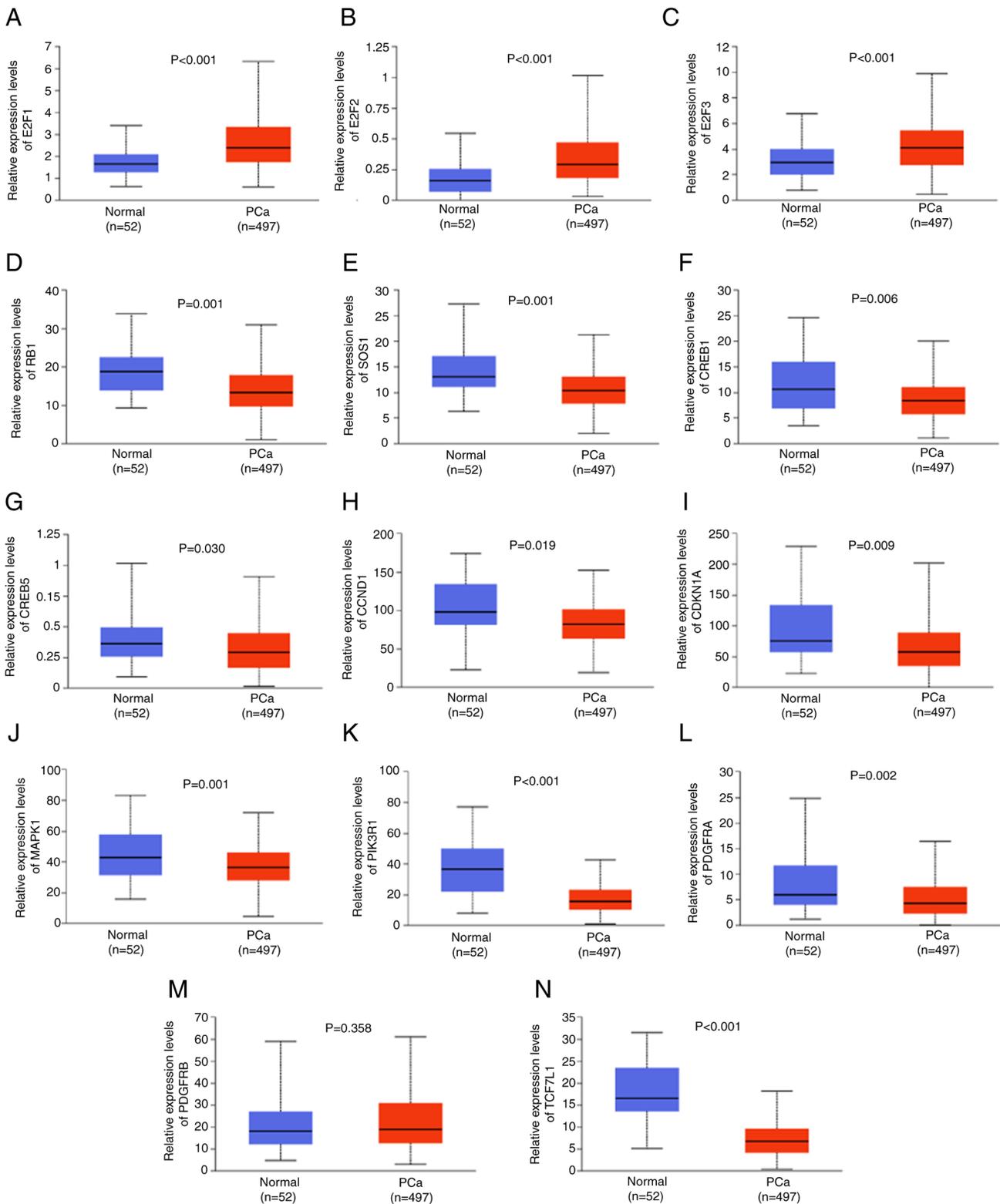


Figure 5. Expression levels of miR-20b-5p target genes related to PCa. Relative expression levels of (A) E2F1, (B) E2F2, (C) E2F3, (D) RB1, (E) SOS1, (F) CREB1, (G) CREB5, (H) CCND1, (I) CDKN1A, (J) MAPK1, (K) PIK3R1, (L) PDGFRA, (M) PDGFRB and (N) TCF7L1 in normal and PCa tissue in The Cancer Genome Atlas dataset. miR, microRNA; PCa, prostate cancer.

miR-20b-5p facilitated the proliferation and inhibited the apoptosis of breast cancer stem cells through the bidirectionally regulation of CCND1 and E2F1 (10). Conversely, miR-20b-5p was reported to inhibit migration, invasion and the cell cycle of colon cancer cells by regulating the

CCND1/CDK4/FOXM1 axis (14). miR-20b-5p was reported to be overexpressed in PCa tissue and in plasma samples, and was demonstrated to promote proliferation and migration of PCa cells (16,37). Hoey *et al* (17) reported that circulating miR-20b-5p was one of the non-invasive biomarkers that

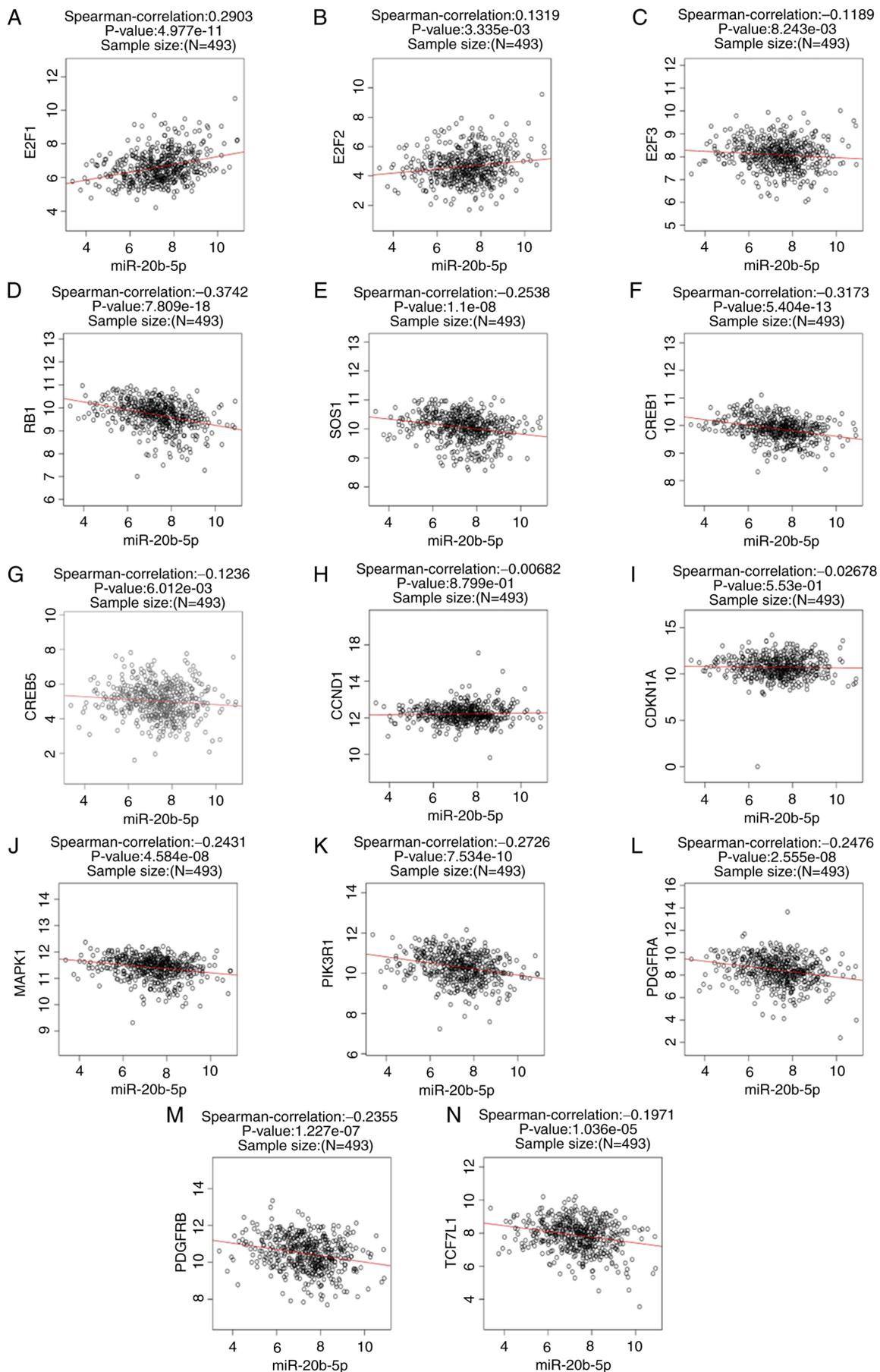


Figure 6. Spearman's correlation analysis. Correlation analyses between miR-20b-5p and (A) E2F1, (B) E2F2, (C) E2F3, (D) RB1, (E) SOS1, (F) CREB1, (G) CREB5, (H) CCND1, (I) CDKN1A, (J) MAPK1, (K) PIK3R1, (L) PDGFRA, (M) PDGFRB and (N) TCF7L1 assessed using Spearman's correlation analysis. miR, microRNA.

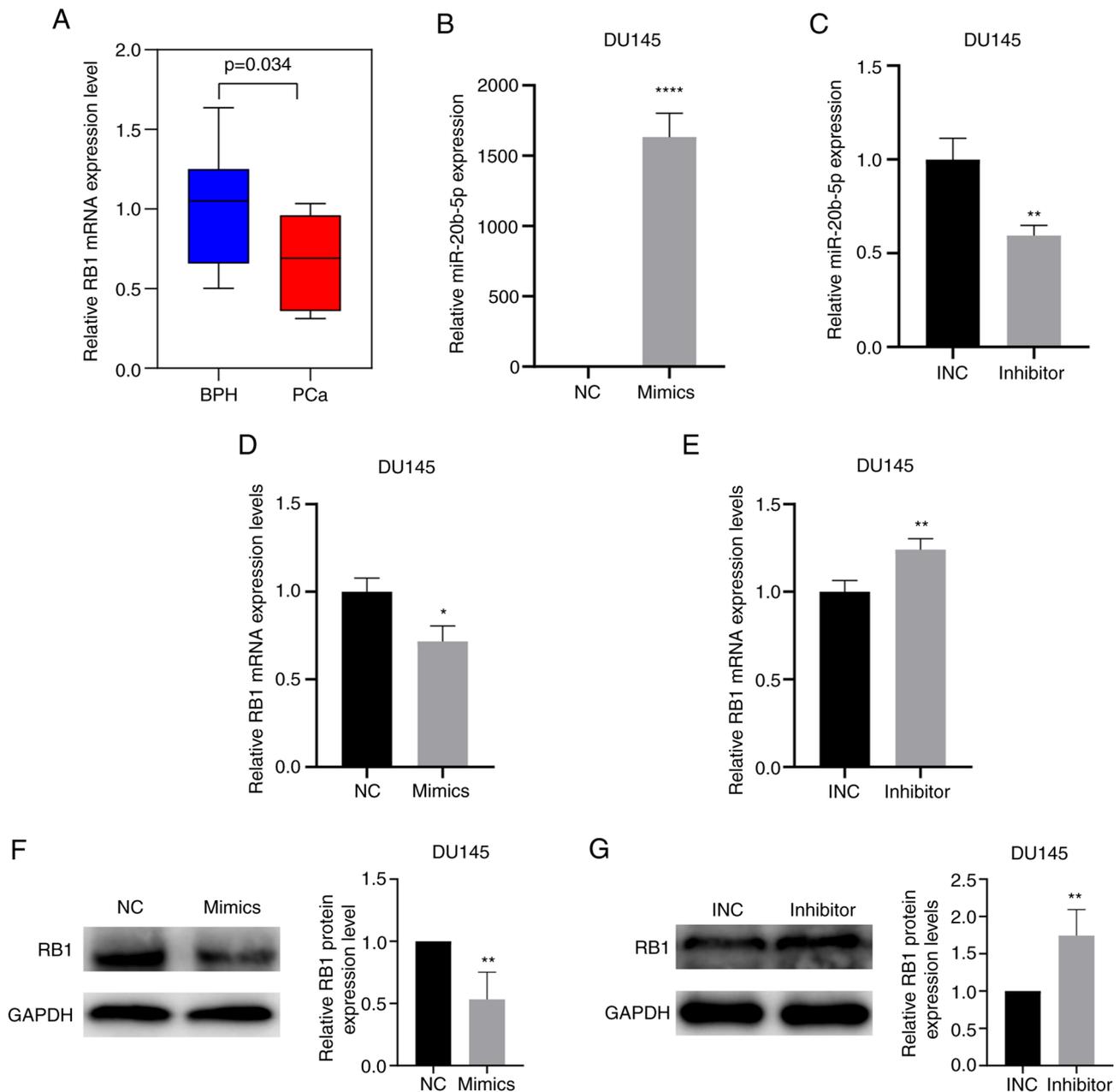


Figure 7. miR-20b-5p affects the expression of RB1. (A) mRNA expression levels of RB1 in 9 PCa tissue samples and 10 BPH tissue samples. Relative expression levels of miR-20b-5p in (B) miR-20b-5p mimics-transfected (**** $P < 0.0001$ vs. NC) and (C) miR-20b-5p inhibitor-transfected DU145 cells (** $P < 0.01$ vs. INC). RB1 mRNA expression levels were assessed using reverse transcription-quantitative PCR in DU145 cells after transfection with (D) miR-20b-5p mimics ($P < 0.05$ vs. NC) or (E) miR-20b-5p inhibitor (** $P < 0.01$ vs. INC). RB1 protein expression levels were assessed in DU145 cells after transfection with (F) miR-20b-5p mimics (** $P < 0.01$ vs. NC) or (G) miR-20b-5p inhibitor (** $P < 0.01$ vs. INC) using western blotting and semi-quantified. GAPDH was used as an endogenous control. BPH, benign prostate hyperplasia; INC, inhibitor negative control; miR, microRNA; NC, negative control; PCa, prostate cancer; RB1, retinoblastoma-associated protein.

predicted aggressive PCa after radical prostatectomy, and miR-20b-5p promoted the tumor aggression of PCa, which indicated that miR-20b-5p functioned as an oncomiR in PCa. GO analysis in the present study included GO terms such as axonogenesis, dephosphorylation and regulation of GTPase activity, indicating that miR-20b-5p may have affected the development of PCa by participation in these biological processes and molecular functions. Among the putative target genes of miR-20b-5p, RB1, SOS1, CREB1, MAPK1, PIK3R1 and PDGFRA were demonstrated to be associated with miR-20b-5p, and their expression levels were significantly lower

in PCa compared with normal tissue. Among these, RB1, a tumor suppressor that restricts the transcription of cell cycle genes by regulating the E2F transcription factor (38), has been previously reported to be commonly mutated in PCa, especially in androgen deprivation therapy-recurrent and metastatic PCa (39). A previous study also reported that miR-20b-5p promoted esophageal squamous cell carcinoma cell proliferation, migration and invasion by directly targeting RB1 (35). RT-qPCR and western blotting results from the present study demonstrated that miR-20b-5p overexpression significantly reduced the mRNA and protein expression

levels of RB1. These data suggested that miR-20b-5p may serve a vital role in the promotion of the development of PCa by reducing the expression of RB1 in PCa.

Several limitations should be noted in the present study. First, the sample size was small when verifying the diagnostic efficacy of prostatic fluid exosomal miR-20b-5p in predicting PCa, and further studies using larger cohorts are needed to validate the conclusion of the present study. Second, the role of miR-20b-5p in PCa still needs to be evaluated using *in vivo* and *in vitro* studies in the future.

In conclusion, the present study demonstrated that miR-20b-5p expression was significantly upregulated in PCa at both tissue and cellular levels. Prostatic fluid exosomal miR-20b-5p may be used as a non-invasive and effective biomarker for the diagnosis of PCa. RB1 is a potential target of miR-20b-5p, which may promote the development of PCa. Further studies are needed to confirm the findings of the present study and to explore the potential mechanism underlying the role of miR-20b-5p in PCa.

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Availability of data and materials

The dataset analyzed for this study can be found in The Cancer Genome Atlas Prostate Adenocarcinoma (TCGA-PRAD) database (<https://portal.gdc.cancer.gov>). The remaining datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TYZ, TC, LDZ, ZMW and LX conceived and designed the study, and revised the manuscript. TYZ and YBM performed the experiments, and TYZ and MD wrote the manuscript. TYZ, MD, HW and FL performed the statistical analysis. HW and FL collected the clinical samples and data of the patients, and ZMW supervised the project. TYZ and LX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants. The present study was approved by the Ethics Committee of the Xi'an Jiaotong University Health Science Center (Xi'an, China; approval no. 2021-1700) and was conducted following the principles of The Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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