

miR-505 suppresses prostate cancer progression by targeting NRCAM

XIAO-HUI LING^{1,2*}, HAO FU^{3*}, ZHI-YUN CHEN^{2*}, JIAN-MING LU⁴, YANG-JIA ZHUO⁴,
JIA-HONG CHEN², WEI-DE ZHONG^{1,4-6} and ZHENYU JIA⁷

¹Guangdong Provincial Institute of Nephrology, Southern Medical University, Guangzhou, Guangdong 510515;

²Reproductive Medicine Centre, Huizhou Central People's Hospital, Guangdong Medical University, Huizhou, Guangdong 516001; ³Department of Urology, Nanhua Affiliated Hospital, University of South China, Hengyang, Hunan 421001;

⁴Department of Urology, Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, Guangdong 510180;

⁵Department of Urology, Huadu District People's Hospital, Southern Medical University, Guangzhou, Guangdong 510800;

⁶Department of Urology, Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics, Guangzhou First People's Hospital, The Second Affiliated Hospital of South China University of Technology, Guangzhou, Guangdong 510180, P.R. China; ⁷Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA

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Abstract. Previous researchers have demonstrated that microRNA-505 (miR-505) is negatively correlated with progression in various malignancies. However, the detailed function and molecular mechanisms of miR-505 have yet to be completely elucidated in prostate cancer (PCa). The present study initially identified the potential role of miR-505 in PCa using *in vitro* experiments, and demonstrated that restoration of miR-505 inhibited proliferation, invasion and migration, yet induced cell cycle arrest and promoted apoptosis in PCa cells. The present study also demonstrated that the expression of neuron-glial-related cell adhesion molecule (NRCAM) was markedly upregulated in PCa cells when compared with benign prostate epithelium. A luciferase reporter assay demonstrated that miR-505 directly targeted NRCAM in PCa cells. In addition, NRCAM stimulation antagonized the inhibitory effects of miR-505 on the proliferation, migration, and invasion of PCa cells. Furthermore,

lower levels of miR-505 and higher levels of NRCAM may serve as a predictor of worse biochemical recurrence-free survival or disease-free survival in patients with PCa. In conclusion, the present study revealed the inhibitory effects of miR-505 on PCa tumorigenesis, which potentially occur by targeting NRCAM. The combined analysis of NRCAM and miR-505 may predict disease progression in patients with PCa following radical prostatectomy.

Introduction

Prostate cancer (PCa) is common in American males and is the second leading cause of cancer-related mortality (1,2). Prostate specific antigen (PSA) has been widely accepted for diagnosis in patients with PCa in the early stages of disease. However, the use of the PSA test is controversial and the subject of much debate due to its high false positive rate. For example, a consensus has not been reached on whether PSA serves as an effective diagnostic marker, and, more importantly, whether PSA level is associated with the risk of the disease (3). Overall, it is imperative to evaluate the biological role behind PCa tumorigenesis and progression in order to identify more effective prognostic biomarkers that can predict which patients only require active surveillance, and which patients require more aggressive treatment.

Distinct miRNA profiles have been observed in different human tumor types (4-7). The aberrations of certain miRNA signatures have been reported to be correlated with cancer progression and can predict treatment response and prognosis (8,9). Our previous studies and others have demonstrated that the dysregulation of miRNAs are associated with PCa tumorigenesis and progression (10-13). However, the precise mechanism governing how PCa-associated miRNAs affect disease progression, has yet to be completely elucidated.

Correspondence to: Dr Wei-De Zhong, Guangdong Provincial Institute of Nephrology, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China
E-mail: zhongwd2009@live.cn

Dr Zhenyu Jia, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA
E-mail: zhenyuj@ucr.edu

*Contributed equally

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In recent years, and increasing amount of research has demonstrated that miR-505 may serve as the tumor-suppressive gene in several tumor types. For example, miR-505 may inhibit cell proliferation and induce cell apoptosis in breast cancer (14). Whereas, another study indicated that miR-505 depletion enhanced tumorigenesis and epithelial-mesenchymal transition (EMT) in hepatoma cells (15). A study by Lu *et al* reported that decreased levels of miR-505 were observed in endometrial carcinoma and its restoration inhibited the activities of cell proliferation, invasion and migration, yet increased apoptosis (16). However, the tumor-suppressing function of miR-505 and its mediatory mechanism in PCa require further investigation.

Neuronal cell adhesion molecule is a member of the immunoglobulin superfamily (17). Neuron-glial-related cell adhesion molecule (NRCAM) was initially identified and has been extensively explored in the peripheral and central nervous system (18). Furthermore, the overexpression of NRCAM has been frequently identified in several malignancies including melanoma, papillary thyroid cancer, colorectal cancer and PCa (19-22). In melanoma cell lines, increased NRCAM expression was associated with increased tumorigenicity (19). In addition, the aberration of NRCAM significantly promoted cell viability and invasiveness in thyroid cancer (20). Nevertheless, the oncogenic role of NRCAM in PCa has not been adequately studied. In summary, the aim of the present study was to demonstrate the tumor-suppressing function of miR-505 and highlight its effects on NRCAM in PCa via *in vitro* studies.

Materials and methods

Ethical approval. The approval of this research by the Ethical Committee of Guangzhou First People's Hospital (Guangzhou Medical University, China) was provided prior to the commencement of the project. All patients recruited in the present study provided written informed consent.

Patients and tissues samples. The present study collected 20 pairs of PCa tissues and adjacent normal tissues following radical prostatectomy at Guangzhou First People's Hospital. The tumor tissue sections were frozen in liquid nitrogen. The tissue microarrays (TMAs; PR807c; Alenabio) contained 50 primary PCa tissues, 10 normal prostate tissues and 20 benign prostatic hyperplasia (BPH) tissues along with detailed follow-up data for further immunohistochemical staining. No previous treatment had been performed on the patients with PCa. Additional investigations were conducted using The Cancer Genome Atlas (TCGA) database, which contains 499 human primary PCa tumors with clinicopathological information, to complement our TMA results. The TCGA dataset was downloaded from the cBioPortal for Cancer Genomics. Biochemical recurrence (BCR) survival and disease-free survival was calculated (11-13). BCR was defined as post-operative serum prostate-specific antigen (PSA) >0.2 ng/ml. Disease-free survival was defined as the time to the first evidence of loco-regional or distant clinical recurrence after the initial surgery.

Cell culture, cell lines construction and transfection. PC-3, DU145 and LNCaP cell lines (PCa) and RWPE-1 (normal pros-

Table I. Oligonucleotide sequence for all the primers used in the present study.

Name	Oligonucleotide sequence (5'-3')
miR-505-F	ATGGGATGAAGTGATGATGCAAA
miR-505-R	ACGCAAATATTGTGAAACACTGGTA
SOX6-F	GCAGCAACAGATCCAGGTTCA
SOX6-R	CAGAGTCCGCTGGTCATGTG
IRF6-F	GGACGTCATGGACAGAGGAC
IRF6-R	GGTGGGCAATGAGATCGCTA
NRCAM-F	GAGCGAAGGGAAAGCTGAGA
NRCAM-R	ACAATGGTGATCTGGATGGGC
NOV-F	AGCAGCCAACAGATAAGAAAGGA
NOV-R	TATTGTGGGGAGTGCAGCAG
LPL-F	CACCTCATTCCCGGAGTAGC
LPL-R	TCCTGTTACCGTCCAGCCAT
GREM1-qF	GCTTGTGCGTAGTTCGTGTG
GREM1-qR	CCCGCCCCCTTTAGATGTGAG
AMOT-F	GCAATCCAGACAAAACAGATGGG
AMOT-R	TCTGCAGCTCTTGATTGGC
CACNA2D3-F	CAGTTGGTGGCACTCCGATA
CACNA2D3-R	GCTGGATGACAAAGGACTTGGA
18S rRNA-F	GTAACCCGTTGAACCCCAT
18S rRNA-R	CCATCCAATCGGTAGTAGCG
RNU6B-F	CTCGCTTCGGCAGCACA
RNU6B-R	AACGCTTCACGAATTTGCGT

F, forward; R, reverse; NRCAM, neuron-glial-related cell adhesion molecule.

tate) were purchased from American Type Culture Collection (ATCC). Cells were cultured in a humidified incubator at 37°C with 5% CO₂ according to the protocol outlined in a previous study (11-13). Human premicroRNA Expression Construct Lenti-miR-505 [PMIRH505PA-1; System Biosciences (SBI), LLC] was used as the pMIRNA1 lentivectors to express miR-505 precursor (pre-505). Scramble control hairpin in pCDH-CMV-MCS-EF1α-copGFP (CD511B-1) was designed as a negative control (pre-NC; cat. no. PMIRH000PA-1; SBI). With pPACKH1 Packaging Plasmid Mix (cat. no. LV500A-1; SBI), pre-505/pre-NC were transfected into 293TN cells (SBI) in order to package the construct. By using the LentiConcentin Virus Precipitation Solution (cat. no. LV810A-1; SBI), the virus particles were collected according to the manufacturer's protocol. Following transfection, PC-3 and LNCaP cells were isolated and then seeded.

NRCAM expression plasmid (pCMV-NRCAM) was obtained from Biogot Technology Co., Ltd. The cells transfected with pCMV-NRCAM or pCMV (empty vector) were used as a corresponding control. The cells were isolated for functional analyses at 48 h following transfection.

Gene expression profiling. Gene expression profiles of miR-505-overexpressing LNCaP cells and its corresponding control cells were conducted and normalized. The experi-

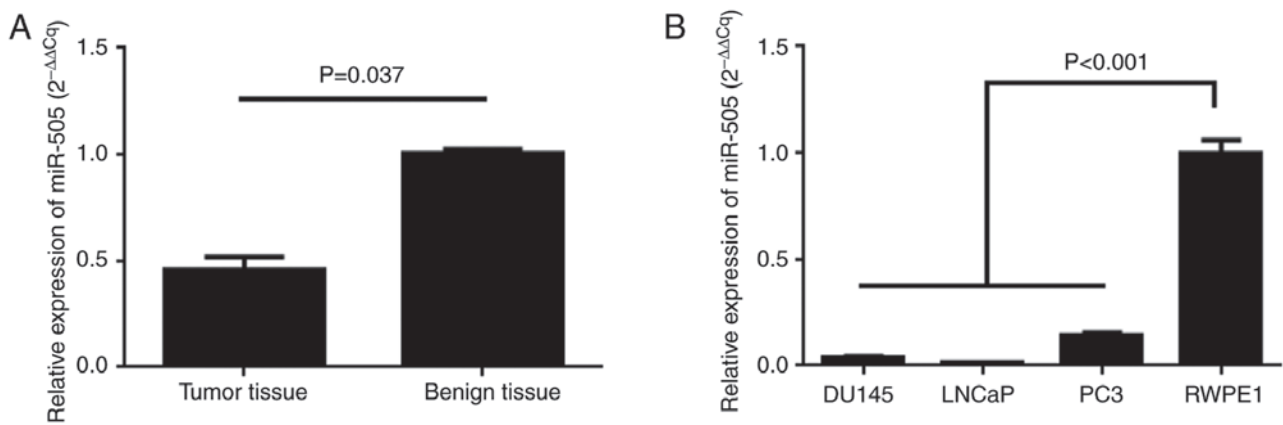


Figure 1. The decreased expression of miR-505 is observed in PCa cells and tissues. (A) Using 20 pairs of PCa tissues and normal tissues, RT-qPCR was performed to evaluate the expression of miR-505. (B) PCa cell lines (PC-3/DU145/LNCaP) and a normal prostate cell line (RWPE-1) were used to detect the expression level of miR-505 by RT-qPCR. PCa, prostate cancer.

ments were performed according to the manufacturer's protocol as outlined in a previous study (12). Briefly, total RNA from the transfected cells was amplified, labeled and purified. Then array hybridization, wash and scan were determined. Gene Spring Software (Agilent Technologies, Inc.) was used to analyze the data, and genes with a fold change greater than two folds ($P < 0.05$) were selected.

RT-qPCR. As previously described (11-13), total miRNA of PCa cells and tissues was isolated with the miRNA Isolation Kit (BioTek China), and total RNA was extracted with the RNeasy mini kit (Qiagen GmbH) when detecting the miRNA and mRNA expression, respectively. The cDNA was synthesized using an RT-qPCR Detection kit (GeneCopoeia). The primer sequences were obtained from Thermo Fisher Scientific, Inc. and are presented in Table I. RT-qPCR analysis was performed and the relative changes of miR-505 and the predictive targets were normalized to the levels of RNU6B RNA or 18S rRNA. The $2^{-\Delta\Delta Cq}$ values were calculated to the relative expression (23).

Immunohistochemical staining. Immunohistochemistry was performed on the tissue sections of TMAs of the patients with PCa to gauge the expression levels of NRCAM. Following deparaffinization and dehydration, sections were processed to reveal antigens using a microwave oven. Briefly, the sections were blocked and then incubated with primary antibody against NRCAM (1:100, cat. no. ab87427; Abcam). The slides were subsequently incubated with anti-rabbit secondary antibody and visualized by DakoCytomation Liquid DAB plus Substrate Chromogen System (DakoCytomation).

Two pathologists, who were blind to patient data independently evaluated the TMA samples. The immunohistochemistry score was calculated by the sum of the staining intensity and the fraction of positive tumor cells as previously described (11-13).

Target gene prediction programs. The potential targets of miR-505 were determined by three different predicting programs, including TargetScan (24), miRanda (25), and miRWalk (26). The miRanda-miRSVR scores, TargetScan

context score or miRWalk scores were defined to aggregate per gene and miR-505. The intersection of miRanda (score < -0.5) and TargetScan (contextscore < -0.2) and miRWalk (score > 0.80) were used to predict the targets of miR-505.

In vitro luciferase assay. As previously described (11-13), luciferase vectors were designed to express the wild-type (WT) or mutant (MUT) 3'-UTR NRCAM sequences. The PCa cells were co-transfected with WT luciferase vector and miR-505 mimic and the corresponding controls. At 24 h post-transfection, the fluorescence reader (Promega Corporation) was employed to calculate the luciferase signals.

Cell proliferation assay. As previously described (11-13), a CCK-8 assay was performed to monitor the proliferative ability of PCa cells following transfection. Briefly, cells were incubated with CCK-8 (Beyotime Institute of Biotechnology) at 37°C containing 5% CO₂ for 4 h. The proliferative ability of PC-3/LNCaP cells was determined at 4, 24, 48 and 72 h.

Wound healing analysis. This assay was performed according to the protocol outlined in previous studies (11-13). When LNCaP and PC-3 cells reached confluence, a linear scratch wound was induced in monolayers with a sterile pipette tip. After washing, the migrated cells through the scratch wound were observed and calculated at 0 and 48 h.

Cell invasion analysis. As previously described (11-13), the CytoSelect Cell Invasion Kit supplied by Cell Biolabs was used to detect invasive abilities of PCa cells according to the manufacturer's protocol. Briefly, PC-3/LNCaP cells were collected, and suspended in serum-free DMEM. These cells were located on the Transwell inserts coated with Matrigel (BD Biosciences), while the normal medium was used as an attractant. Following a 48-h incubation, 4% paraformaldehyde was used to fix the membranes at room temperature for 15 min, which were then stained with 0.1% crystal violet at room temperature for 10 min. Finally, the number of cells that had invaded through the membranes were counted in 16 randomly-selected fields of view under an optical microscope (Olympus Corporation) using x100 and x400 magnifications.

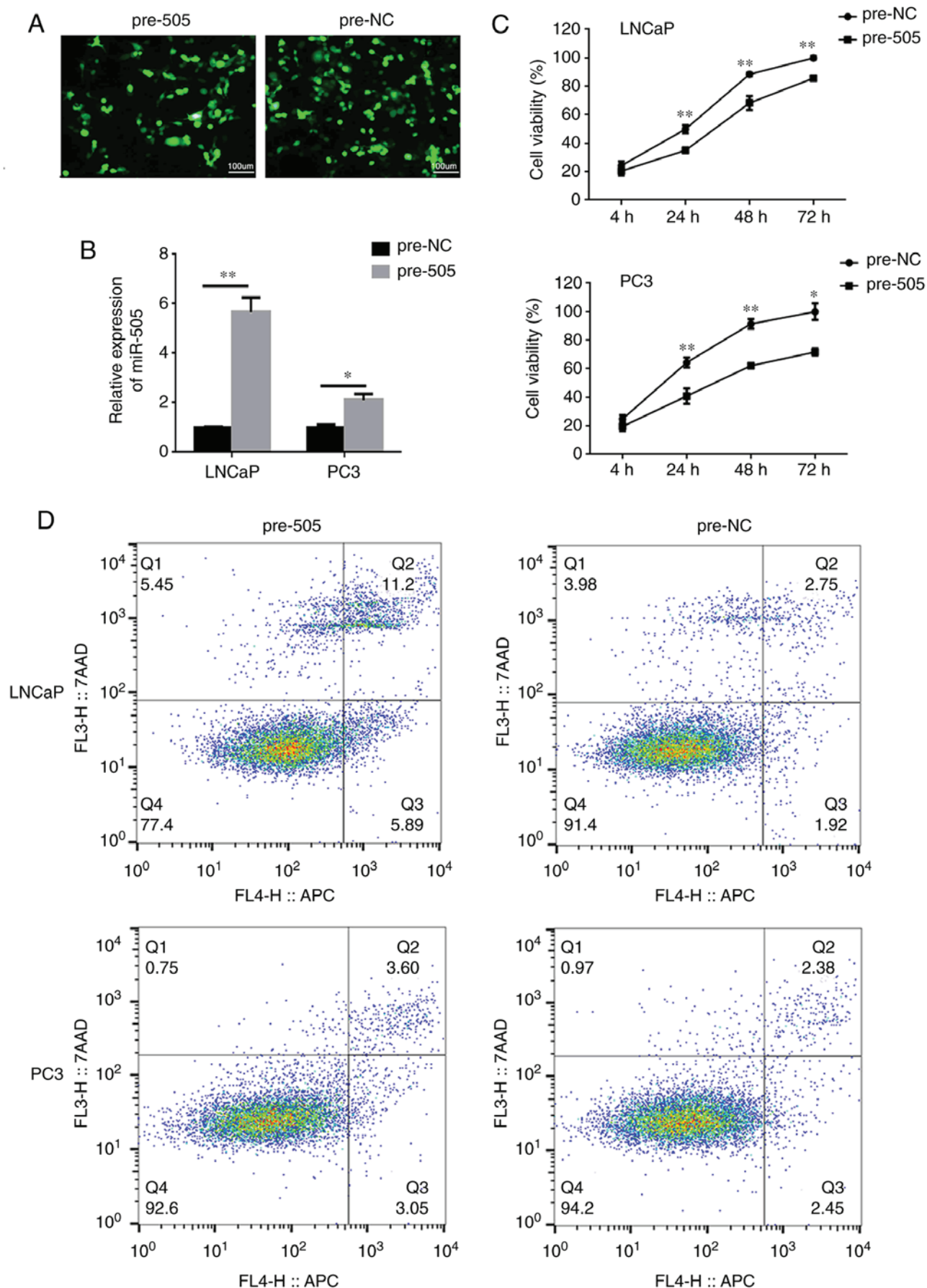


Figure 2. miR-505 inhibits proliferation, but induces cell cycle arrest and promotes apoptosis of PCa cells *in vitro*. (A and B) The stable cell lines LNCaP and PC-3 expressing miR-505 (pre-505) following lentivector transduction were constructed and further confirmed by RT-qPCR. The control group was designated as pre-NC. Scale bar, 100 μ m. (C) The cells post miR-505 transfection exhibited the lower growth features in comparison with control cells. (D) miR-505 transfection significantly promoted apoptosis in comparison with the control cells. The assays were performed in triplicate. * $P < 0.05$; ** $P < 0.01$.

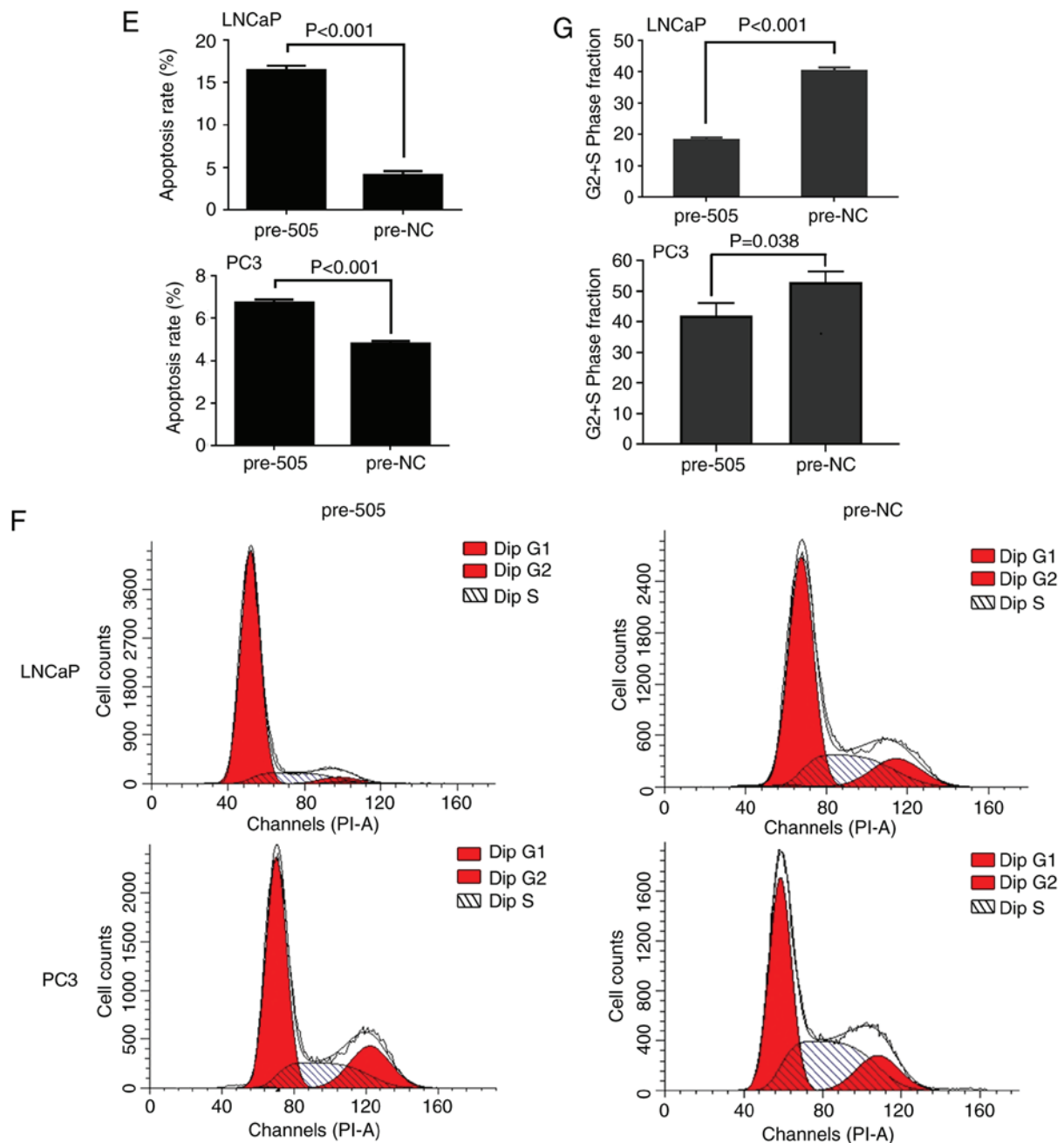


Figure 2. Continued. (E) miR-505 transfection significantly promoted apoptosis in comparison with the control cells. (F and G) Cytometry flow demonstrated that miR-505 transfection resulted in a significant decrease in G2+S phase of the cell cycle for PCa cells. The assays were performed in triplicate. *P<0.05; **P<0.01. PCa, prostate cancer.

Cell cycle analysis. As previously described (11-13), the present study trypsinized PC-3/LNCaP cells following transfection according to the manufacturer's protocol. Briefly, the cells were washed with phosphate-buffered saline (PBS), stained with propidium iodide (Sigma-Aldrich; Merck KGaA) and incubated for 30 min at room temperature. Finally, flow cytometry (BD Biosciences) was conducted to obtain propidium iodide signals.

Apoptotic assay. The experiment was performed according to the manufacturer's protocol (11-13). Briefly, cells were collected, washed, stained with Annexin V and propidium iodide, and then subjected to the FACSscan flow cytometer (BD

Biosciences). BD FACSsuite™ software (BD Biosciences) was conducted for further calculation.

Statistical analysis. The results are presented as the mean \pm SD and were analyzed using SPSS version 17.0 for Windows (SPSS, Inc.). The unpaired Student's t-test or Mann-Whitney U test were conducted to compare the two groups. One-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni's post hoc test were also used when comparing more than two groups. To determine the associations between NRCAM and clinicopathological features, Fisher's exact test or Pearson χ^2 test were performed. Kaplan-Meier method and Cox proportional hazards regression model were conducted

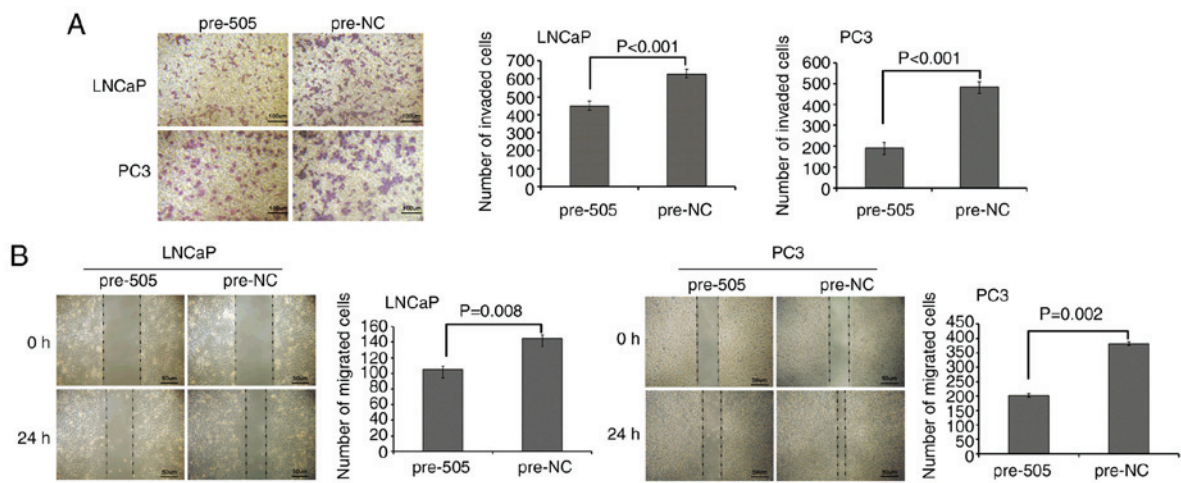


Figure 3. miR-505 inhibits invasion and migration of PCa cells. Transwell assays demonstrated that miR-505 markedly inhibited the (A) invasive (scale bar, 100 μ m) and (B) migratory (scale bar, 50 μ m) activities compared with the control cells. PCa, prostate cancer.

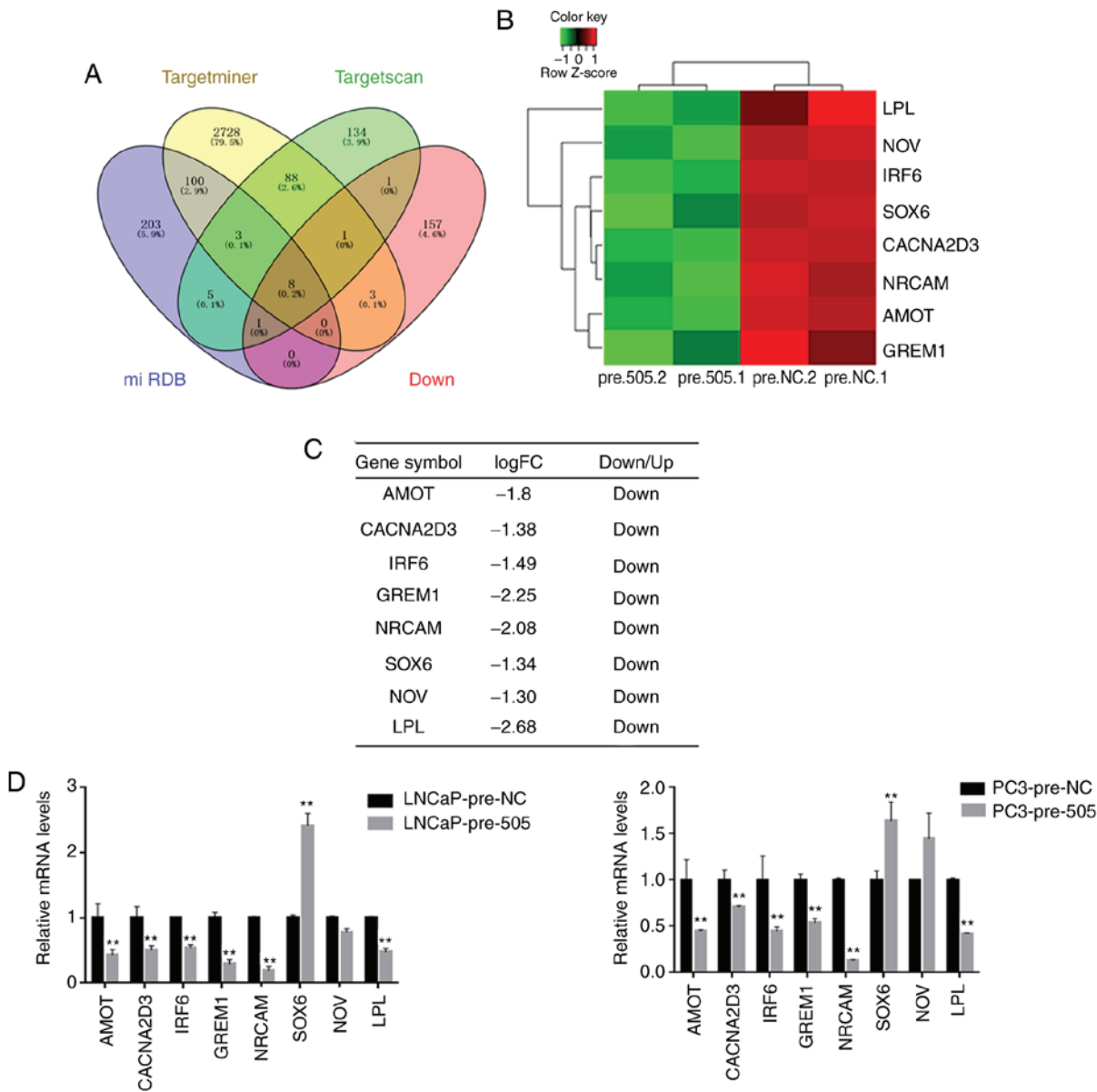


Figure 4. miR-505 may modulate several potential targets in PCa. (A) Intersections among three different target gene predicting software systems and our microarray data of miR-505-overexpressing PCa cells. (B) Cluster analysis for the potential targets. (C and D) The relative expression of the eight potential targets from our microarray data, and further confirmation by RT-qPCR. **P<0.01 vs. pre-NC. PCa, prostate cancer.

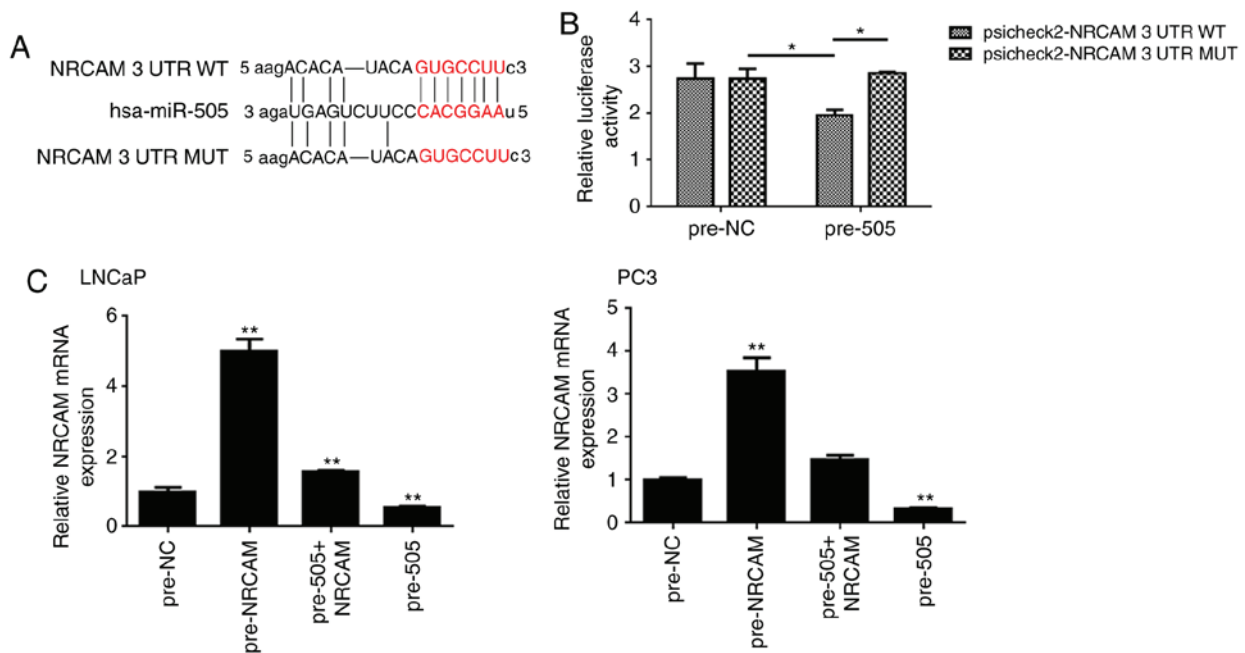


Figure 5. miR-505 directly targets NRCAM in PCa cells. (A and B) A Dual luciferase reporter assay was performed to evaluate the association whether miR-505 can bind to the 3'UTR of NRCAM. (C) RT-qPCR revealed that the inhibitory effect of miR-505 on NRCAM levels in PCa cells could be rescued by NRCAM overexpression. PCa, prostate cancer; NRCAM, neuron-glia-related cell adhesion molecule. * $P < 0.05$ vs. pre-NC; ** $P < 0.01$ vs. pre-NC.

for survival estimation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Decreased expression of miR-505 is observed in PCa. The present study, aimed to validate the expression of miR-505 with RT-qPCR in PCa cells and tissues, and the RT-qPCR results demonstrated that miR-505 expression was significantly decreased in PCa tissues when compared with normal tissues ($P = 0.037$; Fig. 1A). The data also revealed that the expression levels of miR-505 were also significantly reduced in PCa cells ($P < 0.001$; Fig. 1B).

miR-505 suppresses tumorigenic potential of PCa cells in vitro. The present study then constructed stable LNCaP and PC-3 cell lines expressing miR-505 following lentiviral transduction, and the success of this was confirmed via RT-qPCR ($P < 0.05$; Fig. 2A and B). CCK-8 assays confirmed that the proliferative activities of miR-505-overexpressing LNCaP and PC-3 cells was significantly inhibited when compared with control cells at 24, 48 and 72 h following transfection (all groups $P < 0.05$; Fig. 2C). In addition, flow cytometric analysis indicated that miR-505 could induce cell apoptosis ($P < 0.01$; Fig. 2D and E), which resulted in significant decrease in the number of PCa cells in the G2+S phase ($P < 0.05$; Fig. 2F and G). Transwell assays revealed that miR-505 suppressed the invasion of PCa cells ($P < 0.001$; Fig. 3A), and wound healing assays revealed that miR-505 also markedly inhibited the migration of PCa cells ($P < 0.01$; Fig. 3B).

miR-505 directly targets NRCAM in PCa cells. Gene expression profiles of miR-505-overexpressing LNCaP cells and its corresponding control cells were compared. From the microarray

data, 406 differentially expressed genes (235 genes upregulated and 171 genes downregulated) were identified. It is well established that miRNAs negatively modulate mRNA stability and translation by directly binding to the 3'UTR of specific target genes (4). Therefore, the 171 downregulated genes detected were further analyzed using bioinformatics tools.

The potential targets of miR-505 were determined by three different types of predicting software. In combination with our microarray data of miR-505-overexpressing PCa cells, eight candidate targets were selected, including LPL, NOV, IRF6, SOX6, CACNA2D3, NRCAM, AMOT, and GREM1, which were downregulated in LNCaP cells of miR-505 overexpression (Fig. 4A-C, $P < 0.05$ vs. pre-NC; $P < 0.01$ vs. pre-NC). RT-qPCR confirmed that AMOT, CACNA2D3, NRCAM, IRF6, GREM1 and LPL were significantly decreased in PCa cells (LNCaP and PC-3) with miR-505 overexpression (Fig. 4D, $P < 0.01$ vs. pre-NC). As the oncogenic role of NRCAM had been observed in several malignancies (18-21), NRCAM was selected for subsequent investigation.

In addition, the luciferase reporter assay demonstrated that miR-505 interacts directly with the 3'UTR of NRCAM (Fig. 5A and B, $P < 0.05$). Furthermore, RT-qPCR indicated that restoration of miR-505 attenuated the NRCAM protein expression in PC-3 and LNCaP cells (Fig. 5C, $P < 0.01$ vs. pre-NC).

miR-505 suppresses tumorigenicity by targeting NRCAM in PCa cells. To reveal whether the inhibitory effect of miR-505 in PCa functioned by mediating NRCAM, pCDNA3.1(+)-Vectors expressing NRCAM were constructed. RT-qPCR revealed that the tumor-suppressive role of miR-505 on NRCAM protein levels in PCa cells could be rescued by NRCAM overexpression (Fig. 5C, $P < 0.01$ vs. pre-NC). In addition, NRCAM stimulation antagonized the inhibitory role of proliferation

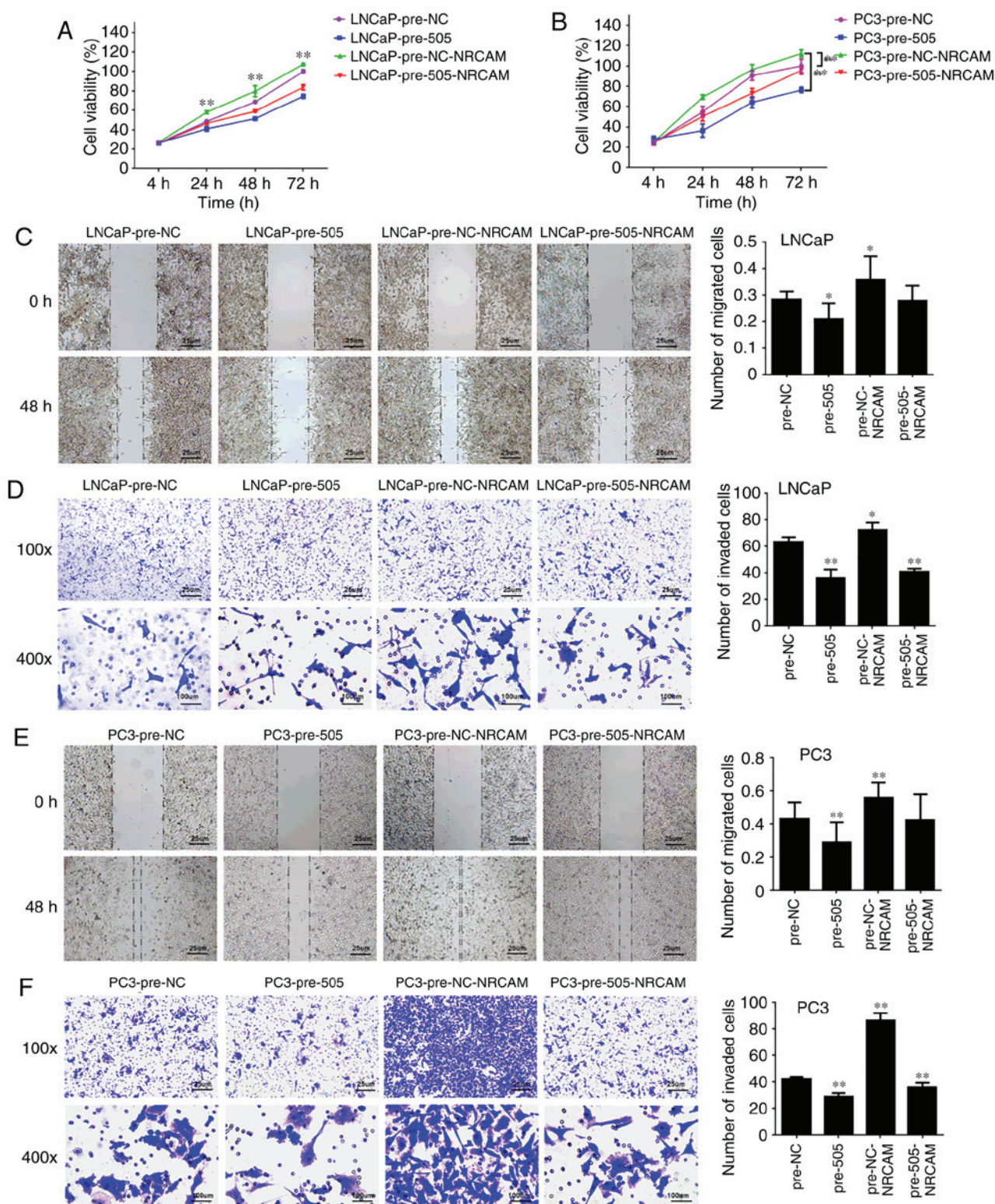


Figure 6. miR-505 suppresses tumorigenicity via NRCAM activation in PCa cells. NRCAM overexpression antagonized the inhibitory effects of miR-505 on the abilities of (A and B) proliferation, (C and E) migration, and (D and F) invasion of PCa cells; x100, magnification; scale bar, 50 μ m; x400, magnification; scale bar, 200 μ m. * P <0.05; ** P <0.01. PCa, prostate cancer; NRCAM, neuron-glia-related cell adhesion molecule.

(Fig. 6A and B), migration (Fig. 6C and E), and invasion (Fig. 6D and F) of LNCaP and PC-3 cells, which were induced by miR-505 upregulation.

NRCAM is upregulated in PCa tissues. To analyze the protein expression of NRCAM in PCa, a TMA was used consisting of 50

PCa tissues, 20 benign hyperplasia tissues, and 10 normal tissues. The immunohistochemical staining revealed that NRCAM was primarily expressed in the cytoplasm of the PCa cells (Fig. 7A, C-F). However, faint NRCAM staining was also identified in the majority of BPH and normal tissues (Fig. 7A, G and H). The present study demonstrated that NRCAM expression was

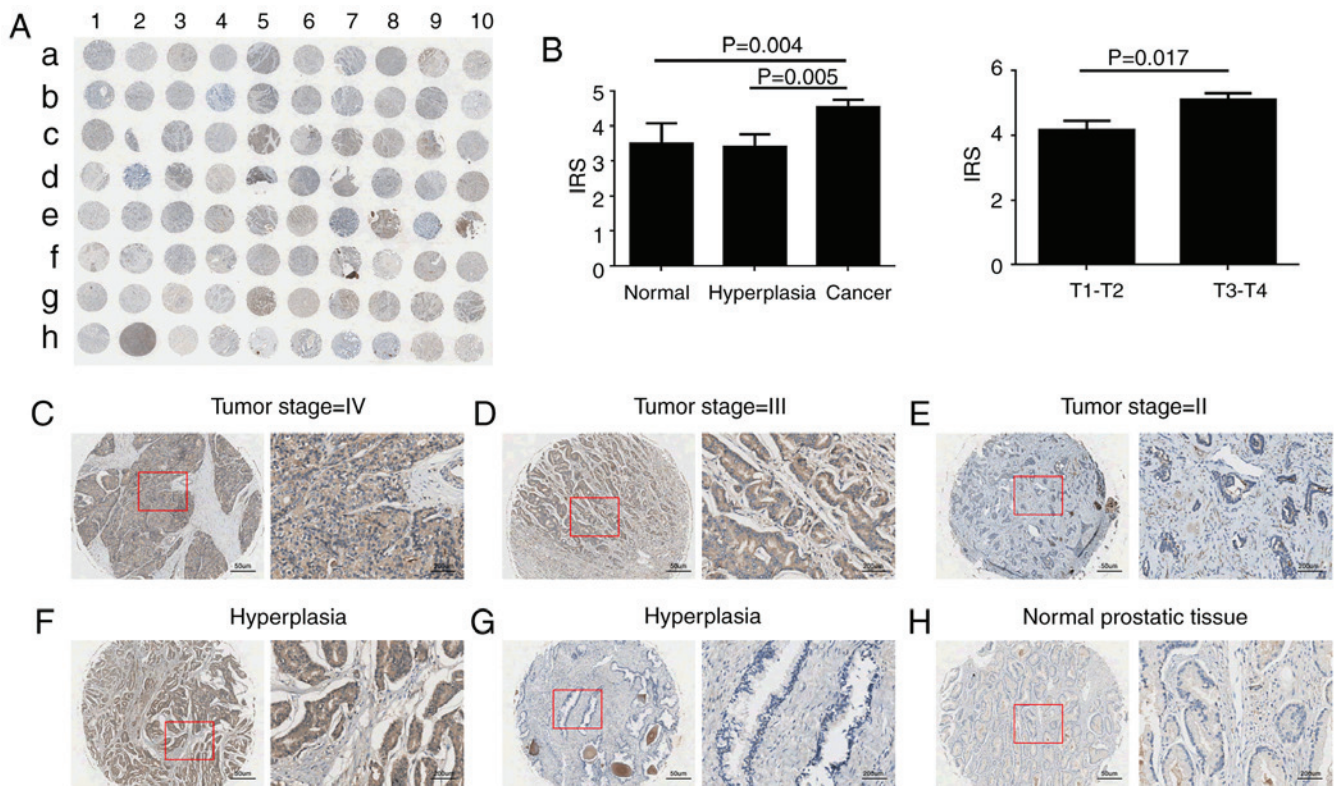


Figure 7. NRCAM is upregulated in PCa tissues. (A) A TMA was conducted to detect the NRCAM levels in PCa tissues, consisting of 50 PCa tissues, 20 benign hyperplasia tissues, and 10 normal tissues. (B) Immunohistochemical staining revealed increased levels of NRCAM expression in PCa cells in comparison with benign prostate epithelium. The higher stage tumor exhibited markedly increased NRCAM expression than that in lower stage tumor. (C-H) The representative image of NRCAM staining in different stages of PCa are presented; x100, magnification; scale bar, 50 μ m; x400, magnification; scale bar, 200 μ m. PCa, prostate cancer; NRCAM, neuron-glial-related cell adhesion molecule.

significantly upregulated in PCa cells when compared with benign prostate epithelium (Fig. 7B, $P<0.05$). In addition, the PCa patients with advanced stage exhibited an increased NRCAM expression when compared with lower stage tumors (Fig. 7B, $P=0.017$).

Combined expression of NRCAM and miR-505 may predict PCa progression. The present study also performed investigations using The Cancer Genome Atlas (TCGA), which contains data from 499 primary human tumors with PCa-specific mortality and other clinicopathological information. As presented in Table II, the TMA datasets indicated that high NRCAM expression was correlated with a higher Gleason score ($P=0.032$) and advanced pathological stage ($P=0.010$), data from TCGA also revealed NRCAM was strongly associated with lymph node metastasis ($P<0.001$) and Gleason score ($P=0.013$).

Patients with a low miR-505 expression had a significantly worse BCR after radical prostatectomy as analyzed by the Kaplan-Meier method ($P=0.009$; Fig. 8A); however, a significant trend for disease-free survival was not observed ($P=0.081$; Fig. 8B). In addition, high levels of NRCAM were significantly correlated with unfavorable BCR-free survival ($P=0.001$; Fig. 8C) and disease-free survival ($P=0.011$; Fig. 8D), respectively. The combined use of these two biomarkers in prognosis is worthy of further investigation. Collectively, these results indicated that patients with PCa with a low expression of miR-505, and a high expression of NRCAM exhibited poor BCR-free survival ($P<0.001$;

Fig. 8E) and disease-free survival ($P<0.01$; Fig. 8F) when compared with contrasting groups of patients. The multivariate model revealed that a high expression of NRCAM (Hazard Ratio 3.63; $P=0.016$) may serve as an independent prognostic factor for unfavorable disease-free survival (Table III).

Discussion

The present study provided novel evidence revealing decreased levels of miR-505 in PCa. Additionally, miR-505 may exert a tumor-suppressive role by inhibiting PCa cell viability, invasion and migration, and inducing cell cycle arrest and increasing apoptotic activity. The results also indicated that miR-505 could directly bind to the 3'UTR of NRCAM in PCa cells, and the inhibitory effect of miR-505 potentially functions by mediating NRCAM. Based on the TMA and TCGA data, the low expression of miR-505 and the high expression of NRCAM were associated with the progression and poor prognosis of PCa following radical prostatectomy.

Other than in the regulation of physiological processes in normal tissues, miR-505 has also been revealed to be implicated in the development of malignant tissues (14-16,27-32). For example, Verduci *et al* observed the suppressive role of miR-505 in a mouse embryonic fibroblast (27). Previous studies indicated that miR-505 was underexpressed and it served as a candidate tumor suppressor in different malignancies, such as cervical (28), colorectal (29,30), breast (14)

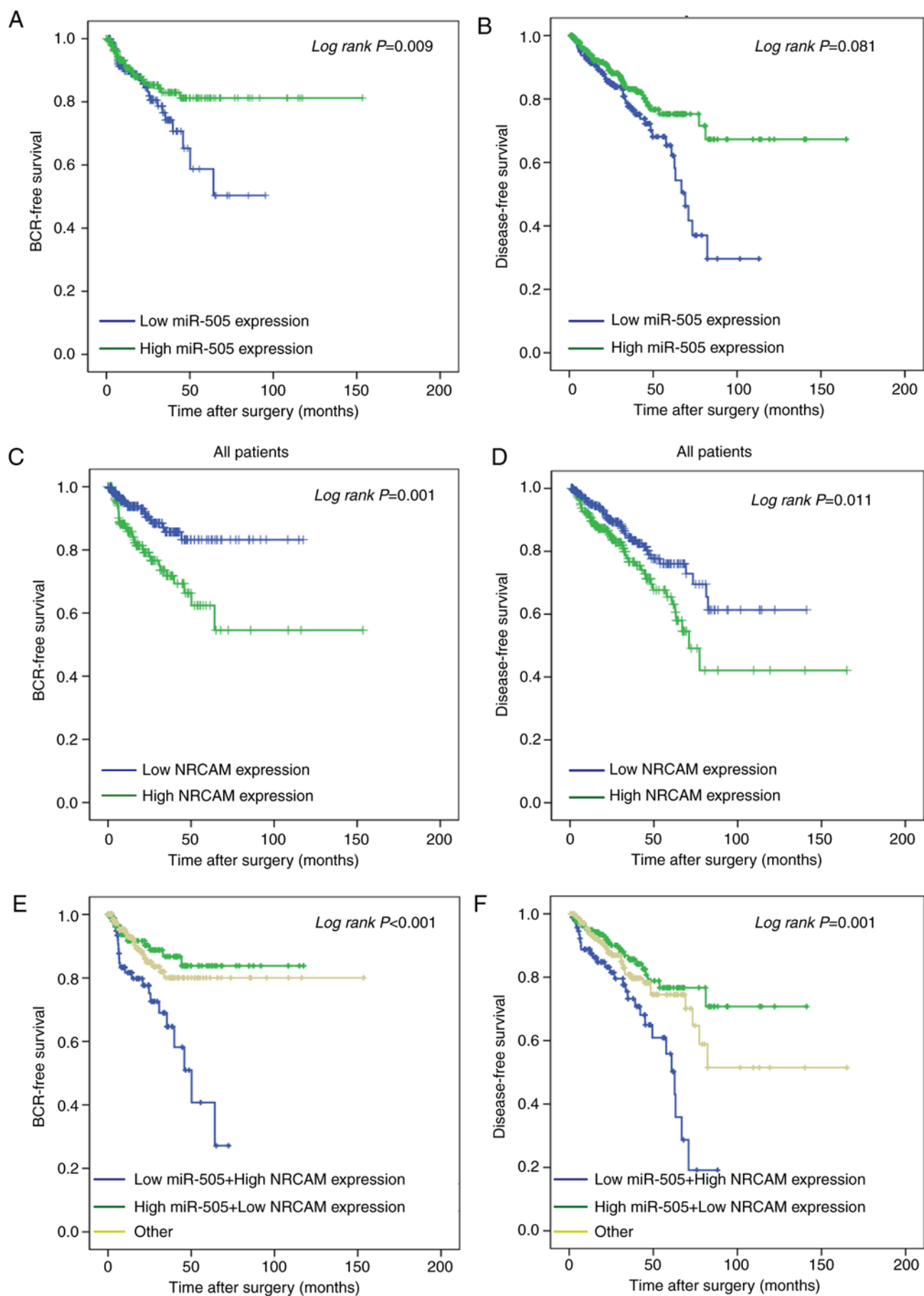


Figure 8. Combined expression of NRCAM and miR-505 may predict PCa progression. (A-D) Kaplan-Meier analysis of BCR-free and disease-free survival for PCa patients with differential expression of miR-505 or NRCAM. (E and F) Combined status of NRCAM and miR-505. PCa, prostate cancer; NRCAM, neuron-glia-related cell adhesion molecule; BCR, biochemical recurrence.

Table II. Correlation of NRCAM expression with clinicopathologic characteristics in patients with prostate cancer.

Clinical features	Case	TMA		P-value	Case	TCGA		P-value
		NRCAM expression				NRCAM expression		
		Low, n (%)	High, n (%)			Low, n (%)	High, n (%)	
Tissue								
Cancer	50	17 (34.0)	33 (66.0)	0.432	499	250 (50.1)	249 (49.9)	-
Non-cancer	23	9 (39.1)	14 (60.9)					
Age								
≤60	12	3 (25.0)	9 (75.0)	0.331	222	117 (52.7)	105 (47.3)	0.132
>60	68	25 (36.8)	43 (63.2)		273	129 (47.3)	144 (52.7)	
Sex								
Male	80	28 (35.0)	52 (65.0)	-	499	250 (50.1)	249 (49.9)	-
Serum PSA levels (ng/ml)								
<4					411	198 (48.2)	213 (51.8)	0.293
≥4					27	15 (55.6)	12 (44.4)	
Gleason score								
≤7	31	14 (45.2)	17 (54.8)	0.032 ^a	291	159 (54.6)	132 (45.4)	0.013 ^a
>7	19	3 (15.8)	16 (84.2)		204	90 (44.1)	114 (55.9)	
Pathological grade								
≤2	4	4 (100.0)	0 (0.0)	0.010 ^a				
>2	46	13 (28.3)	33 (71.7)					
Tumor stage								
T1	29	11 (37.9)	18 (62.1)	0.351	176	88 (50.0)	88 (50.0)	0.470
T2-T4	21	6 (28.6)	15 (71.4)		228	116 (50.9)	112 (49.1)	
Lymph node metastasis								
N0	43	15 (34.9)	28 (65.1)	0.554	342	190 (55.6)	152 (44.4)	<0.001 ^b
N1	7	2 (28.6)	5 (71.4)		80	25 (31.2)	55 (68.8)	
Distant metastasis								
M0	44	13 (29.5)	31 (70.5)	0.093	453	223 (49.2)	230 (50.8)	0.513
M1	6	4 (66.7)	2 (33.3)		3	1 (33.3)	2 (66.7)	

^aP<0.05 and ^bP<0.01. NRCAM, neuron-glial-related cell adhesion molecule.

Table III. Prognostic value of NRCAM expression for disease-free survival by Cox proportional hazards model.

Variables	Disease-free survival	
	HR (95% CI)	P-value
Univariate analysis		
Gleason score (≤7 vs. >7)	3.930 (2.187-7.063)	<0.001 ^b
Tumor stage (T1 vs. T2-T4)	3.416 (1.714-6.810)	<0.001 ^b
Distant metastasis (M0 vs. M1)	3.536 (0.488-25.641)	0.212
PSA (<4 vs. ≥4)	10.426 (5.309-20.474)	<0.001 ^b
NRCAM expression (low vs. high)	0.517 (0.303-0.880)	0.015 ^a
Multivariate analysis		
Tumor stage (T1 vs. T2-T4)	2.443 (1.037-5.754)	0.041 ^a
NRCAM expression (low vs. high)	3.74 (1.68-8.30)	0.016 ^a

^aP<0.05 and ^bP<0.01. NRCAM, neuron-glial-related cell adhesion molecule.

and liver cancer (15) as well as endometrial carcinoma (16), which were corroborated by the results of the present study. However, other studies have reported contradictory findings in the serum of certain tumors in which the level of circulating miR-505 was higher than that in control patients (31,32). Therefore, additional experiments should be performed in order to investigate the role of miR-505 in PCa serum.

miRNAs can target 20-30% of mRNA transcripts (33). Additionally, recent studies have identified several known miR-505 targets in various malignancies. For example, enforced expression of miR-505 decreased tumorigenic activities by targeting transforming growth factor- α (TGF- α) from *in vitro* and *in vivo* assays in endometrial carcinoma (15). In addition, cell proliferation and invasion were inversely modulated by miR-505 by targeting HMGB1 in hepatoma cells (16). Previous studies have also provided evidence that miR-505 could directly target FZD4 and S100A4 in cervical and colorectal cancer, respectively (28,30).

The present study combined microarray data of miR-505-overexpressing cells and three miRNA target prediction algorithms for a bioinformatics analysis, yielding eight potential target genes including AMOT, CACNA2D3, NRCAM, IRF6, GREM1 and LPL. Since the oncogenic role of NRCAM had been observed in several malignancies (19-22), NRCAM was selected for additional investigation. NRCAM is a member of the immunoglobulin superfamily, and is an adhesion molecule which is associated with axonal guidance and growth (17,18). Notably, NRCAM has been frequently reported to have an increased expression in human cancers, and can stimulate cell motility, and promote cell transformation in thyroid and melanoma cancer by activating the PI3K/AKT and ERK/MAPK signaling via interactions with $\alpha 4\beta 1$ integrins and EGFR (19,20). Although a previous study revealed that the increased expression of NRCAM was evident in PCa (22), its oncogenic role has yet to be completely elucidated. Cai *et al* reported that NRCAM may be targeted by miR-203 in esophageal cancer as indicated by the results of their bioinformatics analysis (34). However, to the best of our knowledge, no studies have stated that NRCAM expression is mediated by miR-505 in the investigated malignancies. In the present study, NRCAM was significantly upregulated in PCa tissues as demonstrated by a TMA assay, and the results verified that NRCAM was targeted by miR-505 through dual luciferase reporter assays. In addition, miR-505 restoration reduced NRCAM protein expression from our *in vitro* study. To the best of our knowledge, the present study is the first to establish the association between miR-505 and NRCAM in PCa.

The roles of the other potential targets (AMOT, GREM1, CACNA2D3, IRF6, and LPL) with PCa progression were elusive. AMOT serves a role in PCa proliferation via the Hippo/YAP pathway (35). miR-205 regulated the proliferation and the invasion of breast cancer cells by suppressing the expression of AMOT (36). In addition, another study indicated that miR-497 could directly target the AMOT gene in human osteosarcoma cells to inhibit cell proliferation and invasion (37). GREM1 can serve as the bone morphogenetic protein antagonist during human cancer progression (38). miR-128-3p suppressed the proliferation and metastatic

potential of glioma cells by targeting GREM1 (39). CACNA2D3 may serve as a pivotal gene in ERG-positive prostate cancer (40). The developmental transcription factor IRF6 may be associated with cell proliferation, cancer stem cell properties and chemotherapeutic sensitivity in nasopharyngeal carcinoma (41). The LPL gene is commonly methylated in PCa and may be involved in tumor progression (42). However, the role between these potential gene targets and miR-505 should be further revealed.

The biological significance of miR-505 and NRCAM in the carcinogenesis of PCa is substantiated by our TMA and TCGA data, in which the expression of miR-505 and NRCAM were closely associated with PCa recurrence and disease progression. This is consistent with the finding in other human tumor types. For example, patients with a low expression of miR-505 had advanced pathological stage or a poor predicted survival in colon adenocarcinoma, endometrial cancer and cervical cancer (16,28,29). Previous studies have suggested that unfavorable tumor phenotype and disease prognosis are associated with NRCAM overexpression in several other tumor types, such as colorectal cancer and PCa (21,22). However, this controversial data has been also reported by Tsourlakis *et al* for NRCAM in PCa tissues, in which the high expression of NRCAM was associated with a favorable clinical disease course (22). We can infer that this conflicting role may be due to the tissue heterogeneity and complex interactions with other molecules.

In conclusion, the present study demonstrated the inhibitory effects of miR-505 on PCa tumorigenesis, potentially by targeting NRCAM. The combined analysis of NRCAM and miR-505 may be associated with an unfavorable progression and prognosis in PCa.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

WDZ, XHL, HF, ZYC participated in the study design and coordination, analysis and interpretation of data, material support for obtained funding, and supervised the study. XHL

performed most of the experiments and statistical analysis and drafted the manuscript. JML, YJZ, JHC and ZJ carried out the experiments and sample collection. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The approval of this research by the Ethics Committee of Guangzhou First People's Hospital (Guangzhou Medical University, China) was provided prior to the commencement of the project. All patients recruited in the present study provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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