MiRNA-215-5p alleviates the metastasis of prostate cancer by targeting PGK1

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Abstract. – OBJECTIVE: MicroRNAs (miR-NAs) are endogenous, non-coding RNAs, which exert crucial functions in regulating biological progressions. Previous studies have demonstrated the anti-tumor effect of miRNA-215-5p. However, its specific role in influencing the progression of prostate cancer (PCa) remains unclear. This study aims to uncover the regulatory effect of miRNA-215-5p on the metastasis and prognosis of PCa.

PATIENTS AND METHODS: MiRNA-215-5p levels in collected PCa tissues (n=52) and paracancerous tissues (n=52) were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miRNA-215-5p level and pathological indexes, as well as overall survival of PCa patients, was analyzed. Regulatory effects of miRNA-215-5p on proliferative and metastatic capacities of LN-CaP and DU-145 cells were evaluated through cell counting kit-8 (CCK-8) and transwell assay, respectively. Bioinformatics prediction was performed to search for the target genes of miRNA-215-5p and PGK1 was selected. The biological role of PGK1 in the progression of PCa was finally clarified by a series of rescue experiments.

RESULTS: MiRNA-215-5p was lowly expressed in PCa tissues and cell lines. Low level of miRNA-215-5p predicted poor prognosis in PCa patients. The silence of miRNA-215-5p enhanced viability, migratory, and invasive capacities of LNCaP cells, while the overexpression of miRNA-215-5p yielded the opposite trends in DU-145 cells. PGK1 was predicted to be the target of miRNA-215-5p. PGK1 was upregulated in PCa tissues and cell lines and its high level predicted poor prognosis of PCa. Moreover, PGK1 level was negatively correlated to that of miRNA-215-5p in PCa tissues. PGK1 was able to reverse the regulatory effects of miRNA-215-5p on metastatic potentials of PCa cells.

CONCLUSIONS: Downregulated miRNA-215-5p in PCa is closely related to distant metastasis and poor prognosis of affected patients. MiRNA-215-5p alleviates the malignant progression of PCa by targeting and downregulating PGK1.

Introduction

Prostate cancer (PCa) is one of the most common malignancies throughout the world, which is the leading cause of cancer-related deaths in the United States and Europe. In recent years, the incidence of PCa in China has rapidly increased year by year[1-3]. Detective rate of PCa achieved a peak in the early 1990s after the introduction of prostate-specific antigen (PSA) screening[4,5]. So far, approximately 85% of newly diagnosed PCa cases are limited to early-stage cancer[5]. Although PSA screening greatly improves the diagnostic rate of early-stage PCa, its potential effect on decreasing the mortality of PCa remains controversial[6]. Since PCa is characterized as tumor heterogeneity, developing diagnostic, therapeutic, and prognostic hallmarks is the priority[7,8]. Currently, target therapy based on tumor-related miRNAs presents a promising application[9-11].

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs, exerting negative translation regulation on expressions of protein-coding genes[12,13]. They exert the biological function by degrading or inhibiting translation of mRNAs[14]. It is reported[15] that miRNAs are extensively involved in affecting cellular behaviors and disease progression. Increasing evidence[16,17] has demonstrated the effects of abnormally expressed miRNAs on the occurrence and progression of tumors. These certain miRNAs may be utilized for developing anti-tumor drugs or biological hallmarks[18]. About 30% human genome could be regulated by miRNAs. Most of the human miRNAs (52%) locate on tumor-associated genomic regions or gene

fragile sites[19,20]. Dysregulation of these certain miRNAs results in tumorigenesis. Bioinformatics has been widely applied to analyze the genome to further uncover the gene expression pattern[21]. By analyzing miRNA profiling microarray of PCa, miRNA-215-5p was selected[22]. Its potential targets and the biological function in PCa were further analyzed[23]. Our findings provide a new idea for the clinical treatment of PCa.

Patients and Methods

Patients and Tissue Samples

A total of 52 PCa patients undergoing rectal surgery in the Second Affiliated Hospital of Fujian Medical University were enrolled in this experiment. Tumor tissues and matched adjacent normal tissues (5 cm away from tumor edge) were surgically resected from PCa patients and preserved within 5 min *ex vivo*. Pathological indexes and follow-up data of PCa patients were collected for further analyses. Patients and their families have been fully informed. This research was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University.

Cell Culture

Prostate epithelial cells (RWPE-1) and PCa cells (LNCaP, 22RV1, PC3, and DU-145) were provided by Cell Bank (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). The medium was replaced every 2-3 days. The cell passage was conducted at 90% confluence.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells seeded in the 6-well plates were cultured until 70% confluence and transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 48 h, cells were harvested for verification of transfection efficacy and subsequent experiments.

Cell Counting Kit-8 (CCK-8)

Cells were seeded in the 96-well plate with 2×10^3 cells per well. At 6, 24, 48 and 72 h, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Assay

Cells were adjusted to a dose of 2.0×10^5/mL. For determining cell invasion, diluted Matrigel was pre-coated on the bottom of each transwell chamber. 200 µL of suspension was applied in the upper side chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. In the bottom side, 700 µL of medium containing 10% FBS was applied. After 48 h of incubation, cells penetrated to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. The numbers of migratory and invasive cells were counted in 5 randomly selected fields per sample.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. QRT-PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. Each sample was performed in triplicate and the relative level was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer 5.0 was used for designing qRT-PCR primers. Primer sequences used in this study were as follows: PGK1, F: 5'-CATG-GAGGAACCATTAGTC-3', R: 5'-GCTCCGAT-GTCCTCCAGAACGGA-3'; microRNA-215-5p, F: 5'-GATGTAGAACAGTCCTGCGACTG-3', R: 5'-GATAAGATGTCATGGAGCTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTA-AAAT-3', R: 5'-CGCTTCAGAATTTGC-GTGTCAT-3'; GAPDH: F: 5'-CGCTCTCT-GCTCCTCCTGTTC-3', R: 5'-ATCCGTT-GACTCCGACCTTCAC-3'.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk for 2 h and reacted with primary and secondary antibodies. Bands

were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism 6 V6.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier curves were introduced for survival analysis. Chi-square test was performed to evaluate the correlation between miRNA-215-5p levels with pathological indexes of PCa patients. $p < 0.05$ was considered as statistically significant.

Results

MiRNA-215-5p Was Downregulated in PCa

Expression pattern of miRNA-215-5p in PCa was first determined by qRT-PCR. Compared with adjacent normal tissues, miRNA-215-5p was downregulated in PCa tissues (Figure 1A). Similarly, miRNA-215-5p was downregulated in PCa cell lines than that of the prostate epithelial cell line (Figure 1B).

MiRNA-215-5p Expression Was Correlated with Distant Metastasis and Overall Survival in PCa Patients

Pathological indexes and follow-up data of enrolled PCa patients were collected. According to the median level of miRNA-215-5p, PCa patients were assigned to high-level and low-level miRNA-215-5p groups. MiRNA-215-5p level was related to distant metastasis, but not related to age, gender, TNM staging, and lymphatic metastasis of PCa patients (Table I). Moreover, Kaplan-Meier curves revealed worse prognosis in PCa patients of low-level group than that of high-level group (Figure 1C).

MiRNA-215-5p Influenced Proliferative and Metastatic Capacities of PCa

MiRNA-215-5p overexpression and knockdown models were constructed in DU-145 and LNCaP cells, respectively (Figure 2A). Transfection of miRNA-215-5p inhibitor enhanced viability, numbers of migratory and invasive LNCaP cells (Figure 2B, 2C). On the contrary, the viability, migratory, and invasive capacities were attenuated in DU-145 cells overexpressing miRNA-215-5p (Figure 2B, 2C).
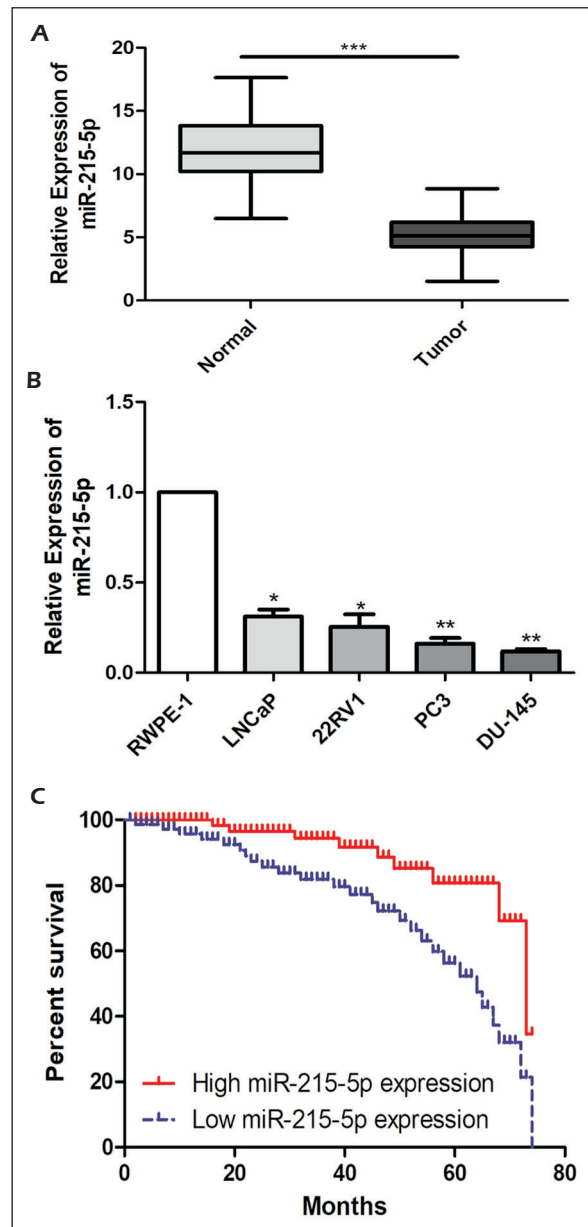

Figure 1. MiR-215-5p was downregulated in PCa. **A,** MiR-215-5p level in adjacent normal tissues and PCa tissues. **B,** MiR-215-5p level in prostate epithelial cells (RWPE-1) and PCa cells (LNCaP, 22RV1, PC3, and DU-145). **C,** Overall survival in PCa patients with high and low expression of miR-215-5p.

Interaction Between MiRNA-215-5p and PGK1

Potential target genes of miRNA-215-5p were predicted in the miRDB, TargetScan, and StarBase (Figure 3A). At last, the intersection contained 6 potential targets (PGK1, NIPAL1, PRKD3, RPAP2, ZEB2, and RAB2A). Among them, PGK1 was the most differentially expressed one after transfection of miRNA-215-5p

Table I. Association of miR-215-5p expression with clinicopathologic characteristics of prostate cancer.

Parameters	No. of cases	MiR-215-5p expression		p-value
		High (%)	Low (%)	
Age (years)				0.857
< 60	20	12	8	
≥ 60	32	20	12	
T stage				0.254
T1-T2	36	24	12	
T3-T4	16	8	8	
Lymph node metastasis				0.316
No	33	22	11	
Yes	19	10	9	
Distance metastasis				0.023
No	31	23	8	
Yes	21	9	12	

inhibitor in LNCaP cells (Figure 3B). In addition, PGK1 level was markedly downregulated in DU-145 cells overexpressing miRNA-215-5p (Figure 3C). To further uncover the role of PGK1 in PCa, we constructed pcDNA-PGK1 and si-PGK1. MiRNA-215-5p level was found to be remarkably downregulated in LNCaP cells transfected with pcDNA-PGK1, which was upregulated in DU-145 cells transfected with si-PGK1 (Figure 3D).

PGK1 Was Upregulated in PCa

In PCa tissues and cell lines, PGK1 was remarkably upregulated relative to controls (Figure 4A, 4B). By detecting expression levels of PGK1 and miRNA-215-5p in PCa tissues, a negative correlation between them was identified (Figure 4C). Furthermore, the prognostic potential of PGK1 was confirmed. PCa patients with a high level of PGK1 suffered worse prognosis relative to those with low level (Figure 4D).

MiRNA-215-5p Regulated Cell Behaviors of PCa by Targeting PGK1

Rescue experiments were performed to uncover the role of miRNA-215-5p/PGK1 axis in influencing the progression of PCa. In LNCaP cells transfected with miRNA-215-5p inhibitor, the upregulated PGK1 was reversed after co-transfection of si-PGK1. Moreover, downregulated PGK1 in DU-145 cells overexpressing miRNA-215-5p was reversed by overexpression of PGK1 (Figure 5A, 5B). Notably, the silence of miRNA-215-5p accelerated migratory and invasive capacities in

LNCaP cells, which was reversed by co-transfection of si-PGK1. Similarly, attenuated metastatic capacities of DU-145 cells overexpressing miRNA-215-5p were reversed by overexpression of PGK1 (Figure 5C).

Discussion

MiRNAs not only influence normal physical activities, but also regulate tumor progression at transcriptional and post-transcriptional levels[10,11]. Whole-genome gene expression analysis and DNA copy number analysis have demonstrated inactivated tumor-suppressor genes (PTEN, ATBF, KLF5, and KLF6) and activated oncogenes (c-MYC, AR and TEMPRESS/ERG) in PCa cells[8,9]. Microarray-based Comparative Genomic Hybridization has identified several chromosomal changes in PCa cell lines and primary tissues[5-7]. In these chromosomal deletion or amplification regions, the presence of cancer-related genes or miRNAs may be existed[10]. Oncogenes or tumor-suppressor genes were used to develop as drug targets for tumor treatment. Nowadays, tumor-related miRNAs have been well concerned, exerting more crucial application in early-stage diagnosis, target therapy, and effective prognosis of tumors[11].

MiRNA-215-5p is a recently discovered miRNA[23]. In this paper, miRNA-215-5p was downregulated in PCa tissues and cell lines. Low level of miRNA-215-5p was correlated with distant metastasis and poor prognosis of PCa patients, suggesting its anti-tumor effect. The strong metastatic ability of PCa cells is the leading cause of
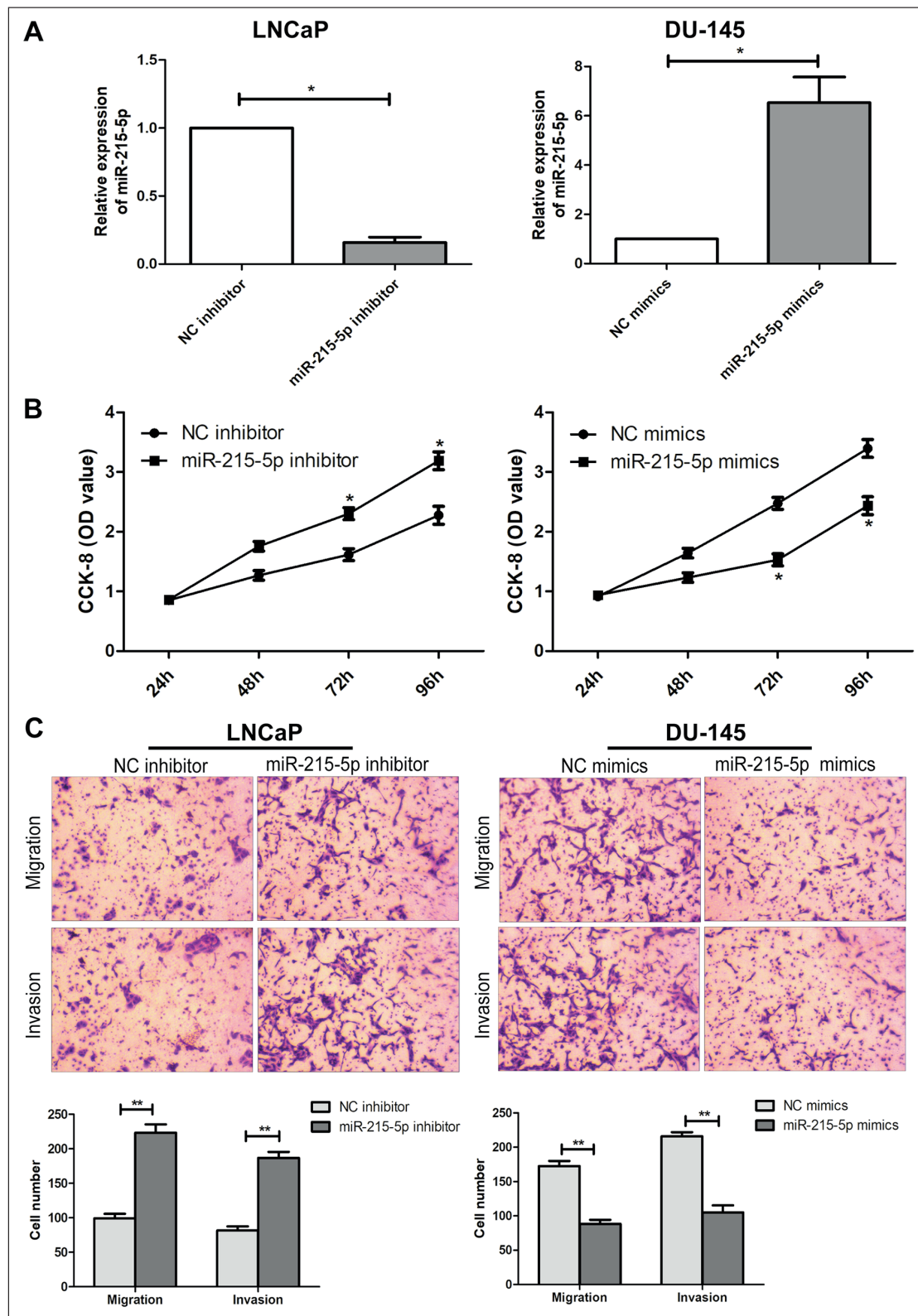
Figure 2. MiR-215-5p influences proliferative and metastatic capacities of PCa. **A,** Transfection efficacy of miR-215-5p inhibitor in LNCaP cells, and miR-215-5p mimics in DU-145 cells. **B,** Viability in LNCaP cells with miR-215-5p knockdown and DU-145 cells overexpressing miR-215-5p. **C,** Migration and invasion in LNCaP cells with miR-215-5p knockdown and DU-145 cells overexpressing miR-215-5p (magnification: 200×).
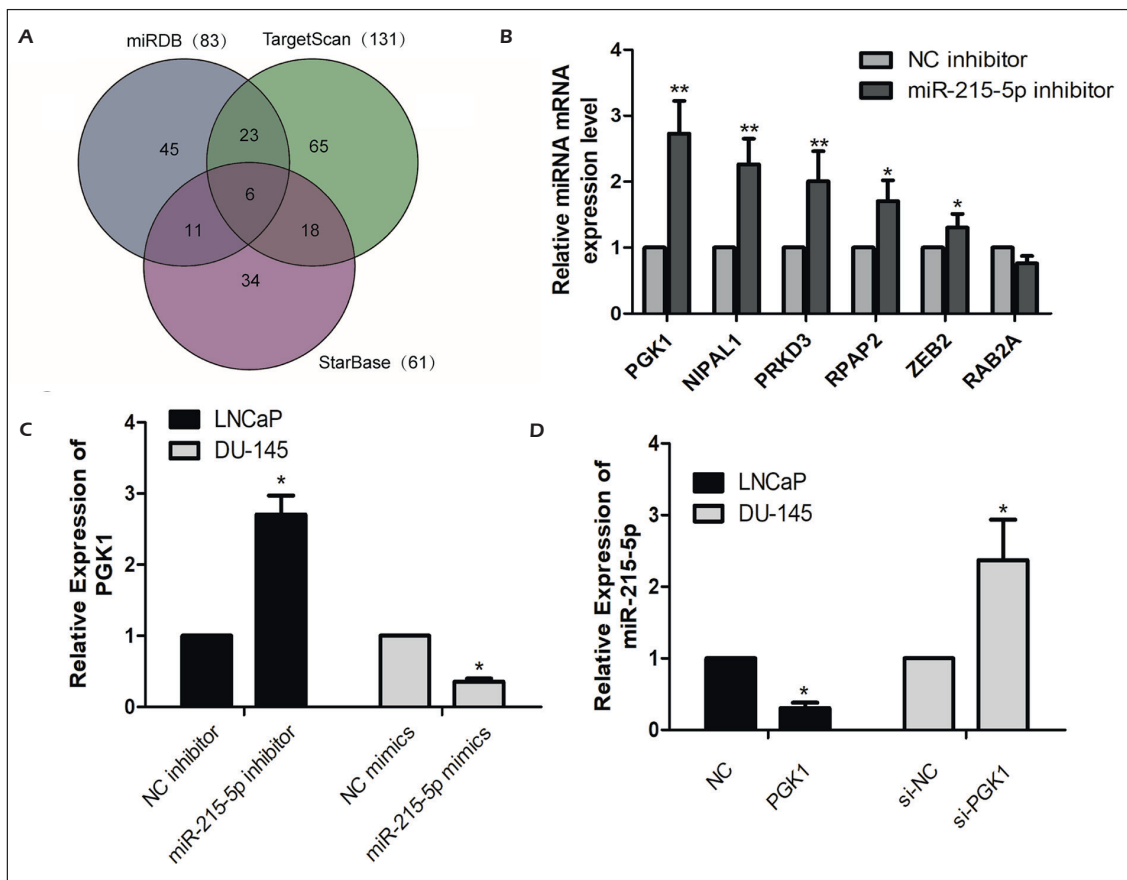
Figure 3. Interaction of miR-215-5p and PGK1. **A,** Potential target genes of miR-215-5p predicted in the miRDB, TargetScan and StarBase. **B,** Expression levels of six potential targets (PGK1, NIPAL1, PRKD3, RPAP2, ZEB2, and RAB2A) of miR-215-5p in LNCaP cells transfected with NC inhibitor or miR-215-5p inhibitor. **C,** PGK1 level in LNCaP cells with miR-215-5p knockdown and DU-145 cells overexpressing miR-215-5p. **D,** Mir-215-5p level in LNCaP cells overexpressing PGK1 and DU-145 cells with PGK1 knockdown.

poor prognosis in PCa patients. Here, our results revealed that silence of miRNA-215-5p enhanced viability, migratory, and invasive capacities of PCa. Conversely, overexpression of miRNA-215-5p attenuated the proliferative and metastatic capacities.

Regulatory mechanism of miRNAs depends on the expressions and functions of their target genes[15,16]. A miRNA degrades target mRNA or suppresses its translation by base pairing with 3'UTR of the mRNA[17]. The degree of base pairing decides the degradation or translation suppression of miRNAs, that is, complete base pairing leads to mRNA degradation. Otherwise, translation inhibition is achieved[18]. MiRNAs only account for only 1% of the whole human genome, but they are able to regulate more than 30% protein-encoding genes. Multiple miRNAs could precisely regulate a single target gene[12-15].

Our results showed that PGK1 was the target gene of miRNA-215-5p through prediction in miRDB, TargetScan, and StarBase. PGK1 level was upregulated in PCa and negatively regulated by miRNA-215-5p. Of note, PGK1 could reverse the regulatory effects of miRNA-215-5p on metastatic potentials of PCa cells. Hence, it is concluded that miRNA-215-5p alleviated the malignant progression of PCa *via* negatively regulating PGK1.

Conclusions

We first showed that downregulated miRNA-215-5p is closely related to distant metastasis and poor prognosis of PCa patients. It alleviates the malignant progression of PCa by targeting and downregulating PGK1.
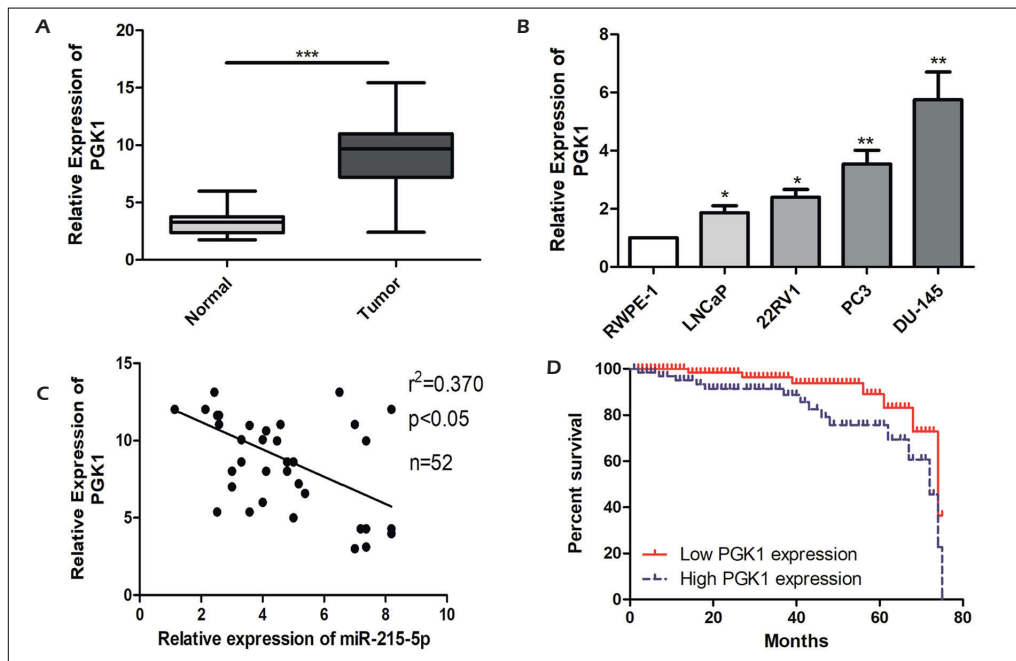
Figure 4. PGK1 was upregulated in PCa. **A,** PGK1 level in adjacent normal tissues and PCa tissues. **B,** PGK1 level in prostate epithelial cells (RWPE-1) and PCa cells (LNCaP, 22RV1, PC3, and DU-145). **C,** A negative correlation between expressions of miR-215-5p and PGK1 in PCa tissues. **D,** Overall survival in PCa patients with high and low expression of PGK1.
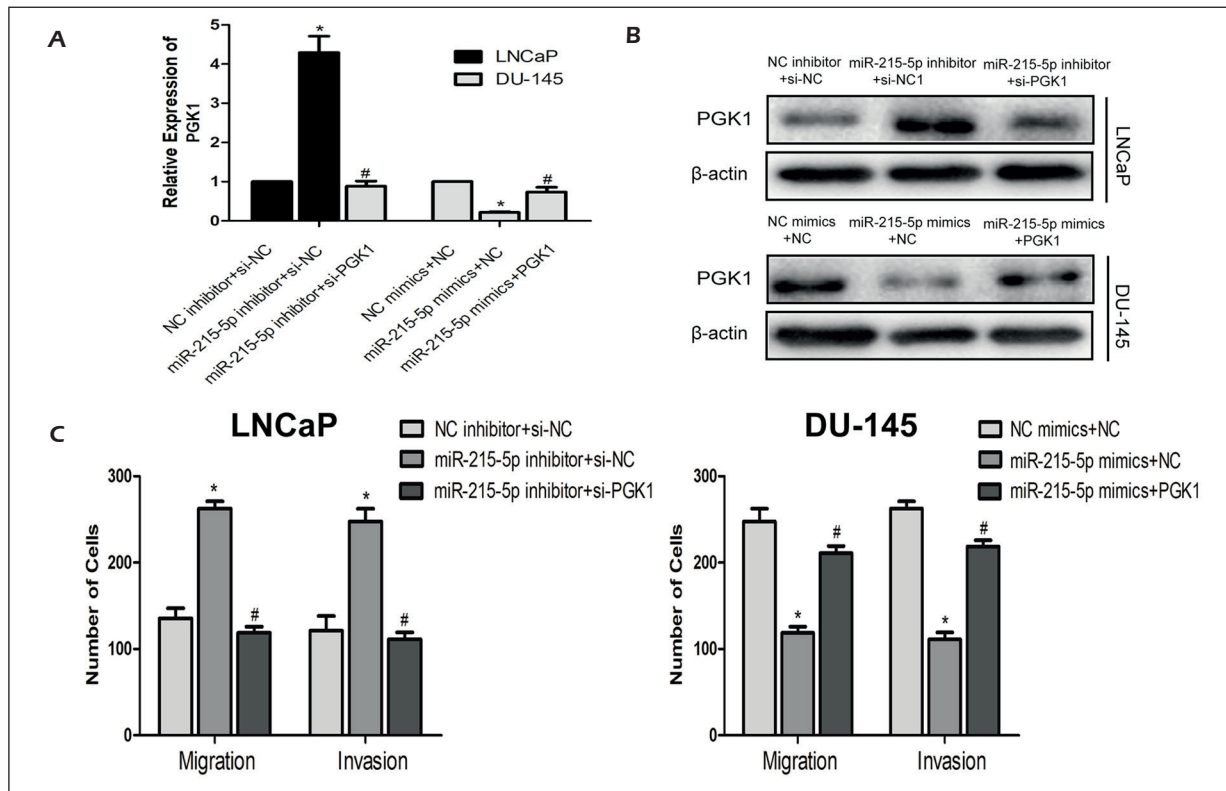

Figure 5. MiR-215-5p regulated PCa cell behaviors by targeting PGK1. LNCaP cells were transfected with NC inhibitor + si-NC, miR-215-5p inhibitor + si-NC or miR-215-5p inhibitor + si-PGK1. DU-145 cells were transfected with NC mimics + NC, miR-215-5p mimics + NC or miR-215-5p mimics + pcDNA-PGK1. **A,** mRNA level of PGK1; **B,** Protein level of PGK1; **C,** Migration and invasion abilities.

References

1) Barry MJ, Simmons LH. Prevention of prostate cancer morbidity and mortality: primary prevention and early detection. Med Clin North Am 2017; 101: 787-806.

2) Zhang JY, Su XP, Li YN, Guo YH. MicroRNA-425-5p promotes the development of prostate cancer via targeting forkhead box J3. Eur Rev Med Pharmacol Sci 2019; 23: 547-554.

3) Grozescu T, Popa F. Prostate cancer between prognosis and adequate/proper therapy. J Med Life 2017; 10: 5-12.

4) Pezaro C, Woo HH, Davis ID. Prostate cancer: measuring PSA. Intern Med J 2014; 44: 433-440.

5) Tabayoyong W, Abouassaly R. Prostate cancer screening and the associated controversy. Surg Clin North Am 2015; 95: 1023-1039.

6) Yilmaz U, Komek H, Can C, Altindag S. The role of (68Ga) PSMA I&T in biochemical recurrence after radical prostatectomy: detection rate and the correlation between the level of PSA, Gleason score, and the SUVmax. Ann Nucl Med 2019 May 8. doi: 10.1007/s12149-019-01360-x. [Epub ahead of print]

7) Garcia-Cruz E, Ramon-Barcelo C, Carrion-Puig R, Alcaraz A. Multiparametric magnetic resonance imaging in prostate cancer detection. Arch Esp Urol 2019; 72: 374-380.

8) Topaktas R, Urkmez A, Kutluhan MA, Caliskan S, Erel O. Does plasma thiol and disulphide be a new marker for prostate cancer in prostate-specific antigen level between 10 and 20 ng/ml? Aging Male 2019: 1-5.

9) Kanwal R, Plaga AR, Liu X, Shukla GC, Gupta S. MicroRNAs in prostate cancer: functional role as biomarkers. Cancer Lett 2017; 407: 9-20.

10) Malla B, Zaugg K, Vassella E, Aebersold DM, Dal Pra A. Exosomes and exosomal microRNAs in prostate cancer radiation therapy. Int J Radiat Oncol Biol Phys 2017; 98: 982-995.

11) Pashaei E, Pashaei E, Ahmady M, Ozen M, Aydin N. Meta-analysis of miRNA expression profiles for prostate cancer recurrence following radical prostatectomy. PLoS One 2017; 12: e0179543.

12) Tutar Y. MiRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.

13) Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.

14) Andres-Leon E, Cases I, Alonso S, Rojas AM. Novel miRNA-mRNA interactions conserved in essential cancer pathways. Sci Rep 2017; 7: 46101.

15) Yin H, Xiong G, Guo S, Xu C, Xu R, Guo P, Shu D. Delivery of Anti-miRNA for triple-negative breast cancer therapy using RNA nanoparticles targeting stem cell marker CD133. Mol Ther 2019; 27: 1252-1261.

16) Wang X, Shi Z, Liu X, Su Y, Li W, Dong H, Zhao L, Li M, Wang Y, Jin X, Huo Z. Upregulation of miR-191 promotes cell growth and invasion via targeting TIMP3 in prostate cancer. J BUON 2018; 23: 444-452.

17) Braicu C, Zimta AA, Harangus A, Iurca I, Irimie A, Coza O, Berindan-Neagoe I. The function of non-coding RNAs in lung cancer tumorigenesis. Cancers (Basel) 2019; 11. pii: E605.

18) Uhr K, Prager-van der Smissen WJC, Heine AAJ, Ozturk B, van Jaarsveld MTM, Boersma AWM, Jager A, Wiemer EAC, Smid M, Foekens JA, Martens JWM. MicroRNAs as possible indicators of drug sensitivity in breast cancer cell lines. PLoS One 2019; 14: e0216400.

19) Chen SN, Chang R, Lin LT, Chern CU, Tsai HW, Wen ZH, Li YH, Li CJ, Tsui KH. MicroRNA in ovarian cancer: biology, pathogenesis, and therapeutic opportunities. Int J Environ Res Public Health 2019; 16. pii: E1510.

20) Filipow S, Laczmanski L. Blood circulating miRNAs as cancer biomarkers for diagnosis and surgical treatment response. Front Genet 2019; 10: 169.

21) Khawar MB, Mehmood R, Roohi N. MicroRNAs: recent insights towards their role in male infertility and reproductive cancers. Bosn J Basic Med Sci 2019; 19: 31-42.

22) Aghdam SG, Ebrazeh M, Hemmatzadeh M, Seyfizadeh N, Shabgah AG, Azizi G, Ebrahimi N, Babaie F, Mohammadi H. The role of microRNAs in prostate cancer migration, invasion, and metastasis. J Cell Physiol 2019; 234: 9927-9942.

23) Vychytilova-Faltejskova P, Merhautova J, Machackova T, Gutierrez-Garcia I, Garcia-Solano J, Radova L, Brchnelova D, Slaba K, Svoboda M, Halamkova J, Demlova R, Kiss I, Vyzula R, Conesa-Zamora P, Slaby O. MiR-215-5p is a tumor suppressor in colorectal cancer targeting EGFR ligand epiregulin and its transcriptional inducer HOXB9. Oncogenesis 2017; 6: 399.