Original Research Article

MicroRNA-300 inhibits the metastasis of prostate cancer through the regulation of TRIM63

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Abstract

Purpose: *To investigate the role of microRNA-300 in the tumorigenesis of prostate cancer (PCa), and the relationship between microRNA-300 level and clinical data of PCa patients.*
Methods: *MicroRNA-300 levels in 63 matched PCa and adjacent tissues were determined via quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between microRNA-300 level and the clinical profile of PCa patients was assessed. PCa cell phenotypes influenced by microRNA-300 were evaluated by a series of functional experiments, including CCK-8, colony formation, Transwell and wound healing assays. The role of microRNA-300/TRIM63 axis in the development of PCa was also examined.*
Results: *MicroRNA-300 was more lowly expressed in PCa tissues than in adjacent normal tissues. PCa patients expressing lower levels of microRNA-300 had a higher Gleason score, higher rates of lymphatic metastasis and distant metastasis, and lower survival. Overexpression of microRNA-300 suppressed its proliferative and metastatic potential. Dual-luciferase reporter assay data confirmed that microRNA-300 specifically binds Tripartite Motif-containing Protein 63 (TRIM63). TRIM63 level was downregulated in PCa cells overexpressing microRNA-300. Moreover, overexpression of TRIM63 abolished the role of microRNA-300 in influencing PCa cell phenotypes.*
Conclusion: *MicroRNA-300 is downregulated in PCa. Its level is related to Gleason score, lymphatic metastasis, distant metastasis and poor prognosis of PCa patients. MicroRNA-300 stimulates proliferative and metastatic abilities in PCa cells by targeting TRIM63. This study may provide new targets for the development of new therapeutics for of PCa as well as its diagnosis.*

Keywords: *MicroRNA-300, TRIM63, Prostate cancer, Gleason score, Lymphatic metastasis, Malignant progression*

INTRODUCTION

Prostate cancer (PCa) is the second among male cancers with the highest mortality rate in European and American countries, second to lung cancer [1,2]. In the past decade, there were 200,000 newly onset cases of PCa in the United States per year, and the mortality rate was about 20 % [3,4]. With an ageing population, changes in dietary and improvement on disease surveillance, the incidence of PCa in China is on the increase [5,6]. Currently, diagnosis and treatment of PCa have greatly improved owing to the extensive detection of serum prostate

specific antigen (PSA) [7]. Radical prostatectomy is preferred for PCa patients. However, about 25 – 50 % PCa patients develop postoperative increase in PSA, that is, biochemical recurrence [8,9]. Since PCa is a heterogeneous disease, patients with the same PSA level, Gleason score and pathological grade suffer different clinical outcomes [10,11]. It is therefore necessary to identify novel tumor markers to reduce recurrent and metastatic rates of PCa [12,13].

MicroRNAs (miRNAs) are conservative non-coding RNAs with 22 nucleotide in length. They are extensively expressed in viruses, plants and mammals [14]. Through post-transcriptional regulation, miRNAs negatively regulate downstream genes by inhibiting their translation or directly degrading them. In mammals, some miRNAs are abnormally expressed in tumors, which target downstream genes by inhibiting their translation, thereby serving as oncogenes or tumor-suppressor genes. MicroRNA-300 is involved in multiple aspects of cell behaviors, which are of significance in regulating several vital pathways [15].

In this study, the prognostic potential of microRNA-300 as a serum biomarker was explored in PCa. We further clarified the role of microRNA-300/TRIM63 axis in the development of PCa, thus providing a new direction in the clinical treatment of PCa.

EXPERIMENTAL

Patients and PCa samples

A total of 63 matched PCa and adjacent tissues were collected from PCa patients undergoing radical prostatectomy or transurethral resection of the prostate, which were stored at -80 °C. The average age of enrolled PCa patients was 66.4 years (range: 56.6 - 79.3 years). All cases were independently diagnosed by two experienced pathologists. This study was approved by the Ethics Committee of Hangzhou Hospital of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study.

Cell transfection

PCa cell lines (PC-3, DU-145, 22RV1 and Lncap) and immortalized human prostate stromal cell line (WPMY-1) were inoculated in 6-well plates and cultured to 50 - 70 % confluence. Cell transfection was conducted via Lipofectamine 2000. Rapamycin (1: 3000) was added in cells transfected with microRNA-300 mimics.

Cell proliferation assay

Cells were inoculated in a 96-well plate at density of 2×10^3 cells per well. CCK-8 assay was performed at the appointed time points. After cell culture for 2 h, optical density at 490 nm per sample was measured via a microplate reader, and the cell activity curve was plotted.

Colony formation assay

Cells were harvested 48 h after transfection. Cells (200 cells per well) were seeded in 6-well plates. After culturing in complete medium for 2 weeks for colonies to form, the medium was aspirated, cells were lightly washed twice with PBS, and 2 ml of methanol was used to fix the cells for 20 minutes. After methanol was aspirated, cells were washed with PBS, and 0.1% crystal violet staining solution was added to boil cells for 20 minutes. Cells were washed 3 times with PBS, and photographed and counted in a well-lit environment.

Transwell cell migration and invasion assay

After 48 hours of transfection, the cells were trypsinized and resuspended in serum-free medium. After the cells were counted, the diluted cell density was adjusted to 2.5×10^5 cells/mL, and the Transwell chambers with and without Matrigel were placed in a 24-well plate. Cell suspension (200 μl) was added to the upper chamber, and 500 μl of medium containing 10% FBS was added to the lower chamber in a 37ºC incubator. After 48 hours, the chamber was taken out, fixed with 4% paraformaldehyde for 30 minutes, stained with crystal violet for 15 minutes and then washed with PBS. The transmembrane cells stained in the outer layer of the basement membrane of the chamber were observed under a microscope. Five visual fields were randomly selected for counting. Invasion assay was conducted in Transwell chambers pre-coated with Matrigel.

Wound healing assay

Cell suspension was prepared at 5.0×10^5/mL. After overnight culture to 90 % confluence, a pipette tip was used to establish the model of the artificial wound, which was observed at 0 and 24 h, respectively.

Quantitative real-time PCR (qRT-PCR)

RNA in cells or tissues was extracted via TRIzol reagent and then were purified using DNase I treatment. After being reversely transcribed into complementary deoxyribose nucleic acid (cDNA),

the resultant cDNA was subjected to qRT-PCR. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. Each sample was performed in triplicate, and relative level was calculated using $2^{-\Delta\Delta Ct}$. The primer sequences used were shown in Table 1.

Western blot

Total protein was extracted from cells or tissues. The obtained protein was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then blocked in 5 % defatted milk for 1 h. Membranes were incubated with the specific primary antibody at 4 °C overnight. Thereafter, secondary antibodies were added for further incubation for 2 h followed by bands being exposed via ECL kit.

Statistical analysis

Statistical Product and Service Solutions 18.0 (IBM, Armonk, NY, USA) was used for data analysis. One-way ANOVA test followed by Least Significant Difference was employed for the comparisons between multiple groups were analyzed using. Chi-square test was conducted to analyze the relationship between microRNA-300 level and clinical data of PCa patients. Kaplan-Meier curves were depicted for survival analyses. *P* < 0.05 was considered as statistically significant.

RESULTS

MicroRNA-300 was lowly expressed in PCa

Differential expressions of microRNA-300 in PCa and adjacent tissues were determined. It is shown that microRNA-300 was lowly expressed in PCa tissues (Figure 1 A). Similarly, microRNA-300 was downregulated in PCa cell lines, especially PC-3 and DU-145 cell lines (Figure 1 B). It is suggested that microRNA-300 may be a tumor-suppressor gene in PCa.
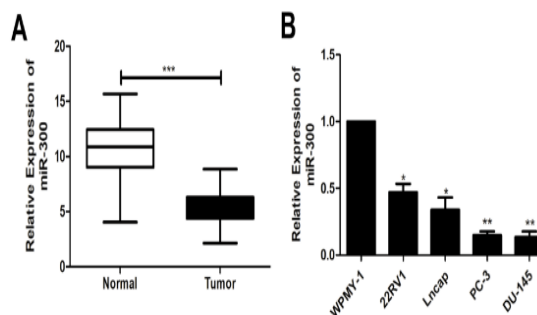
Figure 1: MicroRNA-300 was lowly expressed in PCa. (A) MicroRNA-300 levels in PCa tissues and adjacent ones (compared with Normal group). (B) MicroRNA-300 level in PCa cell lines (compared with WPMY-1). Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001

MicroRNA-300 expression correlated with clinical stage and overall survival in PCa patients

Chi-square test was conducted to analyze the relationship between the microRNA-300 level and the clinical data of PCa patients. It indicated that microRNA-300 level was negatively correlated to Gleason score, rates of lymphatic metastasis and distant metastasis of PCa patients (Table 2). Moreover, Kaplan-Meier curves were depicted based on the follow-up data of enrolled PCa patients. It showed that PCa patients expressing low level of microRNA-300 had worse prognosis.

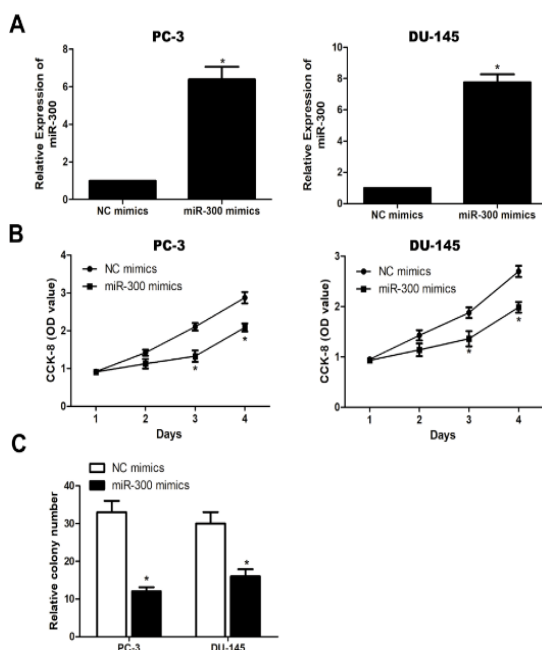## Overexpression of microRNA-300 inhibited cell proliferation in PCa

Transfection efficacy of microRNA-300 mimics was tested in PC-3 and DU-145 cells (Figure 2 A). Overexpression of microRNA-300 reduced viability and colony number in PCa cells (Figure 2 B and C). It is demonstrated that overexpression of microRNA-300 inhibited proliferative ability in PCa.

Table 1: Primer sequences used for PCR

Gene		Primer sequence
microRNA-300	Forward	5'-TATACAAGGGCAGACTCTCTCT-3'
	Reverse	5'-GTGCAGGTTCCGAGGT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
TRIM63	Forward	5'-CTTCCAGGCTGCAAATCCCTA-3'
	Reverse	5'-ACACTCCGTGACGATCCATGA-3'
GAPDH	Forward	5'-AATGGGCAGCCGTTAGGAAA-3'
	Reverse	5'-TGAAGGGGTCATTGATGGCA-3'

Table 2: Association of miR-300 expression with clinicopathologic characteristics of prostate cancer

| Parameter	Number of cases	miR-300 expression		*P*-value
		High (n=35)	*Low (n=28)*	
Age (years)				0.651
<60	29	17	12	
≥60	34	18	16	
Gleason score				0.015
≤7	31	22	9	
>7	32	13	19	
Lymph node metastasis				0.040
No	36	24	12	
Yes	27	11	16	
Distance metastasis				0.011
No	38	26	12	
Yes	25	9	16	

Figure 2: Overexpression of microRNA-300 inhibited cell proliferation in PCa. (A) Transfection efficacy of microRNA-300 mimics in PC-3 and DU-145 cells. (B) Viability of PC-3 and DU-145 cells transfected with NC or microRNA-300 mimics. (C) Colony formation in PC-3 and DU-145 cells transfected with NC or microRNA-300 mimics (×10). Data are expressed as mean ± SD. *P < 0.05

Overexpression of microRNA-300 inhibited metastatic potential in PCa

Transwell assay revealed that overexpression of microRNA-300 reduced migratory and invasive cell numbers in PC-3 and DU-145 cells (Figure 3 A). Identically, decreased percentage of wound closure in PCa cells overexpressing microRNA-300 also proved the inhibitory effect of microRNA-300 on metastasis of PCa (Figure 3 B).
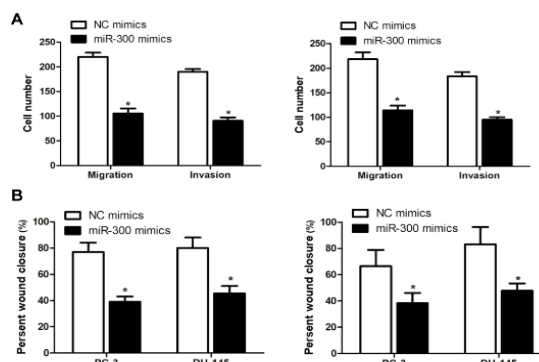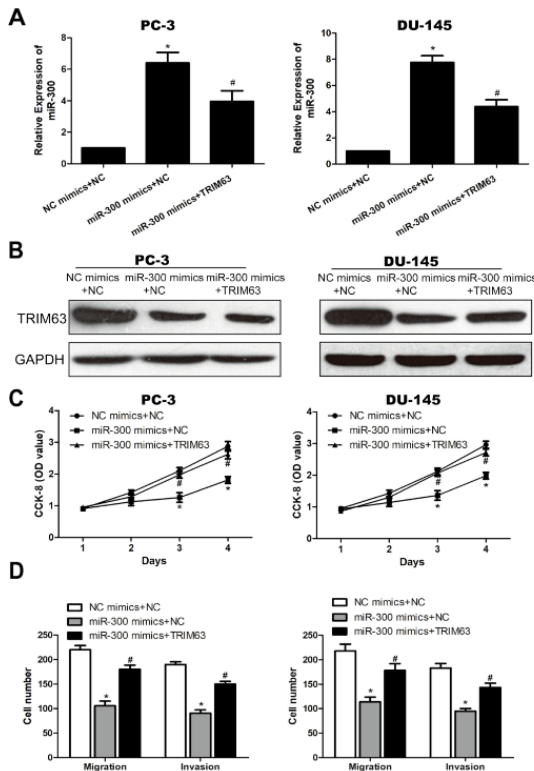

Figure 3: Overexpression of microRNA-300 inhibited metastatic abilities in PCa. (A) Migration and invasion in PC-3 and DU-145 cells transfected with NC or microRNA-300 mimics (×40). (B) Percentage wound healing in PC-3 and DU-145 cells transfected with NC or microRNA-300 mimics (×10). Data are expressed as mean ± SD. *p < 0.05
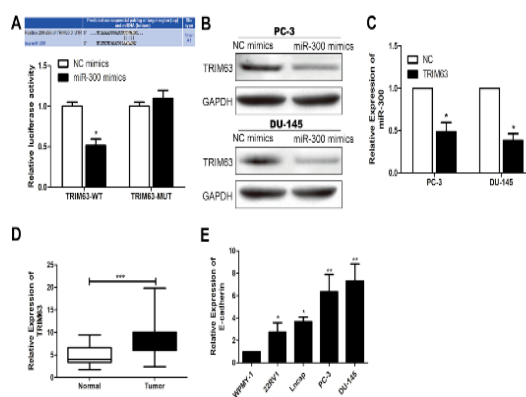
Figure 4: MicroRNA-300 was bound to TRIM63. (A) Luciferase activity after co-transfection of NC/microRNA-300 mimics and TRIM63-WT/TRIM63-MUT. (B, C) Protein (B) and mRNA (C) levels of TRIM63 in PC-3 and DU-145 cells transfected with NC or microRNA-300 mimics. (D) TRIM63 levels in PCa tissues and adjacent tissues. (E) TRIM63 level in PCa cell lines. Data are expressed as mean ± SD. *P < 0.05

MicroRNA-300 was bound to TRIM63

Bioinformatics analysis predicted a potential interaction between microRNA-300 and TRIM63. Furthermore, overexpression of microRNA-300 decreased luciferase activity in TRIM63-WT vector, confirming that TRIM63 was the target gene binding microRNA-300 (Figure 4 A). Relative level of TRIM63 was downregulated in PCa cells overexpressing microRNA-300 (Figure 4 B and C). Its level was lowly expressed in both PCa tissues and cell lines (Figures\ 4 D and E). In addition, there was a negative correlation between expression levels of microRNA-300 and TRIM63 in PCa tissues.

Figure 5: Overexpression of TRIM63 reversed regulatory roles of microRNA-300 in PCa. (A, B) The mRNA (A) and protein (B) levels of TRIM63 in PC-3 and DU-145 cells transfected with NC, microRNA-300 mimics + NC or microRNA-300 mimics + pcDNA-TRIM63. (C) Viabilities of PC-3 and DU-145 cells transfected with NC, microRNA-300 mimics + NC or microRNA-300 mimics + pcDNA-TRIM63. (D) Migration and invasion of PC-3 and DU-145 cells transfected with NC, microRNA-300 mimics + NC or microRNA-300 mimics + pcDNA - TRIM63 (×40). Data are expressed as mean ± SD. *#P < 0.05

Overexpression of TRIM63 reversed regulatory roles of microRNA-300 in PCa

Interestingly, overexpression of TRIM63 reduced upregulated level of microRNA-300 in PCa cells overexpressing microRNA-300 (Figure 5 A and B). We have already proven that overexpression of microRNA-300 inhibited viability and metastatic abilities in PCa cells. However, the above inhibitory trends were partially alleviated by co-overexpression of TRIM63 (Figure 5 C and D). Hence, TRIM63 was responsible for PCa cell phenotypes regulated by microRNA-300.

DISCUSSION

Prostate cancer (PCa) is a malignant tumor of the urinary system. Therapeutic efficacy in advanced, metastatic PCa is extremely low [1,4,5]. Its development varies in affected people because of differences in Gleason scores and pathological grades [6-8]. Besides, clinical features vary among PCa patients according to their different subtypes [8,9]. Detection of serum PSA is currently applied for diagnosing and monitoring PCa conditions [7]. In recent years, the potential of miRNAs as tumor markers have been highlighted [12,13]. MiRNAs are of significance in tumor diagnosis and prognosis as gene targets [15].

Dysfunctional miRNAs are closely linked to tumor development. MicroRNA-300 is identified to be differentially expressed in PCa [16]. In this study, microRNA-300 was downregulated in PCa tissues and cell lines, suggesting a potential anti-tumor role. *In vitro* experiments confirmed the inhibitory effects of microRNA-300 on viability and metastatic abilities in PCa cells.

TRIM63 was ascertained to be the target gene binding microRNA-300. TRIM63 protein has a typical domain unique to the TRIM family, that is, a RING domain, a B-box domain, a transmembrane domain and two coaled-coil domains. TRIM63 is localized on the endoplasmic reticulum membrane and mainly composed of multiple amino acid residues [17]. Here, both mRNA and protein levels of TRIM63 were negatively regulated by microRNA-300 in PCa cells. Also, overexpression of TRIM63 partially abolished regulatory effects of microRNA-300 on PCa cell phenotypes. Findings in this study showed that microRNA-300/TRIM63 axis may be potential targets for clinical treatment of PCa.

CONCLUSION

MicroRNA-300 is down-regulated in PCa. Its level is related to lymphatic metastasis, Gleason score, distant metastasis and poor prognosis of patients with PCa. MicroRNA-300 stimulates proliferative and metastatic activities in PCa cells by targeting TRIM63. The findings of this

research may provide new targets for the diagnosis and treatment of PCa.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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