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RESEARCH

MicroRNA-335 Acts as a Candidate Tumor Suppressor in Prostate Cancer

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Abstract MicroRNA-335 (miR-335) acts as a tumor suppressor or a tumor promoter in different human malignancies. However, the involvement of miR-335 in prostate cancer (PCa) is still unclear. The purpose of this study was to investigate the functional and clinical significance of miR-335 in PCa. miR-335 expression in 3 PCa cell lines (LNCaP/DU145/PC3) and in 20 clinical PCa tissues were detected by real-time quantitative reverse transcriptase-PCR compared with corresponding controls. The function of miR-335 was investigated for cell proliferation, invasion and migration in PCa cells transfected with agents containing EGFP-miR-335 expression vector. Additionally, miR-335 expression in 104 clinical PCa tissues was detected by in situ hybridization. Its assocaitions with clinicopathological features and prognosis in patients with PCa were also determined. miR-335 was significantly down-regulated in PCa cell lines than in the normal prostate cell line ($P<0.01$). With the similar results in vitro, the reduced expression of miR-335 was also found in human PCa tissues comparing with paired adjacent benign prostate tissues ($P<0.05$). Moreover, the increased expression of miR-335 suppressed cell proliferation, invasion and migration of PCa cell lines in vitro. Turning to its clinical significance, the low expression of miR-335 was significantly associated with high Gleason Score ($P=0.04$), advanced clinical stage ($P=0.04$), and positive metastasis ($P=0.02$), but not with prognosis in PCa patients. Our data demonstrated for the first time the inhibitory effect of miR-335 on cell proliferation and invasion for PCa cells. The loss of this microRNA might be associated with clinical progression of PCa patients.

Keywords Prostate cancer · microRNA-335 · Tumor suppressor · Clinicopathological feature · Prognosis

Introduction

Our research group is devoted to the study of molecular mechanisms of prostate cancer (PCa), which represents one of the most prevalent malignancies in men and the second leading cause of male cancer-related death in European and Western countries [1]. PCa is so highly variable that current diagnostic and prognostic indicators have led to failure to diagnose it correctly and determine its outcome. For example, Gleason score is frequently underestimated and serum prostate specific antigen (PSA) levels can be increased in other diseases, such as benign prostatic hyperplasia (BPH) and prostatitis [2, 3]. Carcinogenesis and the mechanisms influencing the progression and prognosis of PCa is a multistep process, involving a number of genetic changes and a series of molecular events. Understanding the carcinogenic process and its corresponding molecular basis for PCa may provide a useful insight that aid in the enhancement of diagnostic efficiency, the establishment of effective therapeutic strategies, and the improvement of patients' survival.

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MicroRNAs (miRNAs) are small, evolutionary conserved, noncoding RNAs that regulate a variety of gene expression post-transcriptionally by silencing with specific target genes [4]. MiRNAs have been found to function as regulators of diverse cellular processes, such as cell proliferation, differentiation, cell cycle regulation, and apoptosis. It has been found the dysregulation of numerous miRNAs in the initiation and progression of various cancers, which allows miRNAs to be potentially used for cancer diagnosis and as therapeutic targets. MiRNAs expression has the specificity of the time and space, that is, miRNAs has different profiles in cells of different stages or different cells of same stages [5]. Meantime, miRNAs reflect directly of signal regulations not only in its kinds but also its abundance. Recent studies have demonstrated that miRNAs function as tumor suppressors or oncogenes in several tumors. Similar with other malignancies, PCa owns specific miRNA profile. In 2007, Porkka KP et al. [6] firstly demonstrated that 37 miRNAs were down-regulated and 14 up-regulated in PCa compared to BPH tissues detected using an oligonucleotide array hybridization method. In 2011, our research group detected miRNA profile in PCa tissues by miRNA microarray analysis, and data are available at the Gene Expression Omnibus (GEO) repository database [http://www.ncbi.nlm.nih.gov/geo/, accession number GSE34932]. As the results of miRNA differential expression analysis, we found 11 up-regulated and 17 down-regulated miRNAs in PCa. Among these, miR-335 is one of the down-regulation miRNAs in PCa (Table 1).

Table 1 Down-regulated miRNA list in prostate cancer tissues relative to pair-matched adjacent benign prostate tissues by miRNA microarray analysis

MiRNAs	Fold_change	*p*-value
hsa-miR-193a-5p	4.31	<0.001
hsa-miR-205	58.96	0.01
hsa-miR-224	3.32	0.01
hsa-miR-374b	2.11	0.01
hsa-miR-455-3p	4.39	0.01
hsa-miR-200b	3.44	0.01
hsa-miR-335	3.36	0.01
hsa-miR-26b	1.93	0.02
hsa-miR-30c	2.09	0.02
hsa-miR-155	4.83	0.02
hsa-miR-181d	1.70	0.03
hsa-miR-23b	2.70	0.03
hsa-miR-221	5.15	0.04
hsa-miR-222	3.94	0.04
hsa-miR-505	2.72	0.04
hsa-miR-374a	1.61	0.04
hsa-miR-221*	3.50	0.05

MiR-335 is transcribed from the genomic region chromosome 7q32.2 [7] and plays a role in various fields. For example, the up-regulation of miR-335 in liver and white adipose tissue might contribute to the pathophysiology of obesity [8]; Tavazoie et al. [9] found that the overexpression of miR-335 was associated with an inhibition of metastatic cell invasion in breast cancer cell lines; In clinical breast cancer tissues, Wang et al. [10] detected the decreased expression of miR-335, which was closely associated with clinicopathologic features of breast cancer, such as histological tumor grades and sex hormone receptor expression; Sorrentino et al. [11] found that miR-335 was downregulated in drug-resistant ovarian cancer cell lines, suggesting its involvement in chemoresistance; but in astrocytoma cells, Shu et al. [12] found that miR-335 targeted a potential tumor suppressor Daam1 and promoted several malignant features such as growth and invasion, whereas miR-335 inhibition could potently induce growth arrest, apoptosis and invasion repression both in vitro and in vivo. These findings suggest miR-335 acts as an oncogenic or a tumor suppressor depending on the specific cancer types. The aim of this study was to investigate the functional and clinical significance of miR-335 in PCa, which is still missing and perplexing.

Materials and Methods

Cell Culture

Human prostate carcinoma cell lines, LNCaP, DU145 and PC-3 and a non-malignant epithelial prostate cell line, RWPE-1 were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI 1640 medium (*#SH30027.01, Hyclone, Logan,USA*) supplemented with 10 % fetal bovine serum (*#10438-026, GIBCO, NY, USA*), 2 mML-glutamine and antibiotics. All cell lines were maintained at 37 °C in a humidified chamber supplemented with 5 % CO_2.

Patients and Tissue Samples

The study was approved by the Research Ethics Committee of Guangzhou First Municipal People's Hospital Affiliated Guangzhou Medical College, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

For quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis, frozen samples of prostate primary tumor tissues and adjacent benign prostate tissues were collected from the tissue bank at Guangzhou Medical College. Before RNA extraction, the frozen sections are

confirmed by staining with hematoxylin and eosin for pathological analysis. If the tissue on slice is full of tumor, then the rest uncut section was choose to direct RNA extraction. If the tissue on slice is benign then the rest uncut section was used for laser capture microdissection (LCM) [13].

For miRNA in situ hybridization detection, frozen tissues were got from company (Jieqing, China), including with the detail clinical infromation. These data is from Caucasian, and African-American race patients. Frozen tissues include 104 PCa tissues (aging 45~86 years, mean ± SD=58.55±7.51 years, TNM staging from I to III) and 20 paired adjecent benign prostate tissues. The detail information on the clinical features of all patients in this study was shown in Table 2.

All of the tissues for RNA extraction were obtained immediately during the operation of transurethral resection prostate and suprapubic prostatectomy. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. The pathological diagnosis was performed preoperatively and confirmed postoperatively. All patients were reviewed and all specimens were re-examined in December, 2010.

The patients involved in situ hybridization detection were given a follow-up ranging from 3 to 12 years. For the analysis of survival and follow-up, the date of prostatectomy was used to represent the beginning of the follow-up period. The primary analysis endpoint was biochemical recurrence-free survival (BRS). Others analysis endpoints were overall survival (OS) and metastasis-free survival. All the patients who died from diseases other than PCa or from unexpected events were excluded from the case collection.

Table 2 Clinical features of all patients

Sample type & clinical features	Experiment type	
	QRT-PCR	In situ hybridization
Prostate cancer (Cases)	20	104
Mean age (range,years)	68.75±4.99	58.35±7.54 (42–86)
≤60	2	62
>60	18	42
Serum PSA levels (ng/ml)		
<4	3	21
≥4	17	83
Gleason score		
<8	10	86
≥8	10	18
Metastasis	1	15
Adjacent benign tissue of prostate cancer (paired)	20	20

QRT-PCR

MiRNA was extracted from 20 pairs of prostate primary tumor tissues and adjacent benign prostate tissues, and three human prostate carcinoma cell lines (LNCaP, DU145 and PC-3) and a non-malignant epithelial prostate cell line (RWPE-1) using miRNA extraction kit (*#RP5301, Bioteke, Beijing, China*). For miRNA detection, qRT-PCR was processing as the protocol of All-in-One™ miRNA qRT-PCR Detection Kit (*#AOMD-Q020, GeneCopoeia, Guangzhou, China*) described. MiRNA expression in each sample was normalized with the housekeeping gene (RNU6B and hsa-miR-130b) expression. The specificity of amplification was confirmed by melting curve analysis and also by running PCR products on agarose gels (3 %). Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method [14]. Mean normalized gene expression ± SE was calculated from independent experiments.

miRNA in Situ Hybridization in Frozen Section

Locked nucleic acid-modified probes biotinylated at the 5′ end (*#38160-15, Exiqon, Vedbaek, Denmark*) were used to detect the in situ hybridization signal for miR-335 on frozen section of prostate tissues. In situ hybridization was performed as described in protocol of Enhanced sensitivity of in situ hybridization detection kit I (peroxidase) (*#MK1030, Boster, Wuhan, China*) [15].

In situ hybridization result was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells in ten representative microscopic fields was counted and the percentage of positive cells was calculated. Given the homogenicity of the staining of the target gene, tumor specimens were scored in a semi-quantitative manner. The percentage scoring of chemoreactive tumor cells was as follows: 0 (0 %), 1 (1–10 %), 2 (11–50 %) and 3 (>50 %). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final score was obtained for each case by the sum of the percentage and the intensity score. MiR-335 expression levels were further analyzed by classifying final scores as low (based on a score less than 3.21) and as high (based on a score greater than 3.21), because the cutoff value 3.21 was the median of the final scores of miR-335 expression.

EGFP-miR-335 Expression Vector

We got the commercial pGCMV/EGFP/Neo-Vector (GenePharma, *Shanghai*, China) with an over-expression of miR-335, and the same blank-vector as control. Two human prostate carcinoma cell lines (LNCaP and DU145) were respectively transfected through Fugene transfecting agents (Roche) with hsa-miR-335 vector, blank control (NC) following the manufacturer's instructions.

Cell Viability Assay

Cell viability assay was performed to evaluate the proliferation ability of the transfected LNCaP and DU145 cell lines, respectively. Briefly, 5×10^3 cells were seeded into 96-well plates for 24, 48, 72 and 96 h. Cells were then incubated with 20 μl of 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma, St Louis, MO) for 4 h at 37 °C. The supernatant was then discarded and 200 μl of dimethyl sulfoxide (DMSO) was added to solubilize the crystals for 30 min at room temperature. Absorbance was measured at a wavelength of 495 nm using a spectrophotometer (Multiskan MK3; Thermo, Shimadzu, Japan). Data were expressed as $\overline{X} \pm s$ of three independent experiments.

Cell Invasion Assay

The transwell cell invasion assay was performed to evaluate the invasion ability of transfected DU145 cell lines. Serum-free RPMI1640 medium was mixed with Matrigel (1:10; BD Biosciences, Bedford, MA, USA). The bottom of the culture inserts (8-μm pores) in 24-well tissue culture plates (Transwell, Corning, Corning, NY, USA) was coated with 50 μl of the mixture, and the Matrigel was allowed to solidify at 37 °C for 4 h. After solidification, 5×10^4 cells were harvested by trypsinization, washed with serum-free medium to 25×10^4/ml and placed in the upper chamber. The lower chamber contained 10 % fetal bovine serum for use as a chemoattractant. Following 48 h of incubation at 37 °C with 5 % CO_2, the number of cells that had migrated through the pores was fixed with 10 % formalin and stained with 0.05 % Crystal Violet, which was solubilized with methanol. The absorbance (540 nm) of the solution was measured by a kinetic microplate reader (Spectra MAX 190; Molecular Devices Co., Sunnyvale, CA). Data were expressed as $\overline{X} \pm s$ of three independent experiments.

Cell Migration Assay

Scratch wound-healing motility assay was performed to evaluate the migration ability of transfected DU145 cell lines. A scratch was set with a pipette tip running though the dish when DU145 cells were seeded and grown to confluence. After cultured under standard conditions for 48 h, plates were washed twice with fresh medium to remove nonadherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge. Data were expressed as $\overline{X} \pm s$ of three independent experiments.

Statistical Analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. Statistical analyses of miRNA microarray and qRT-

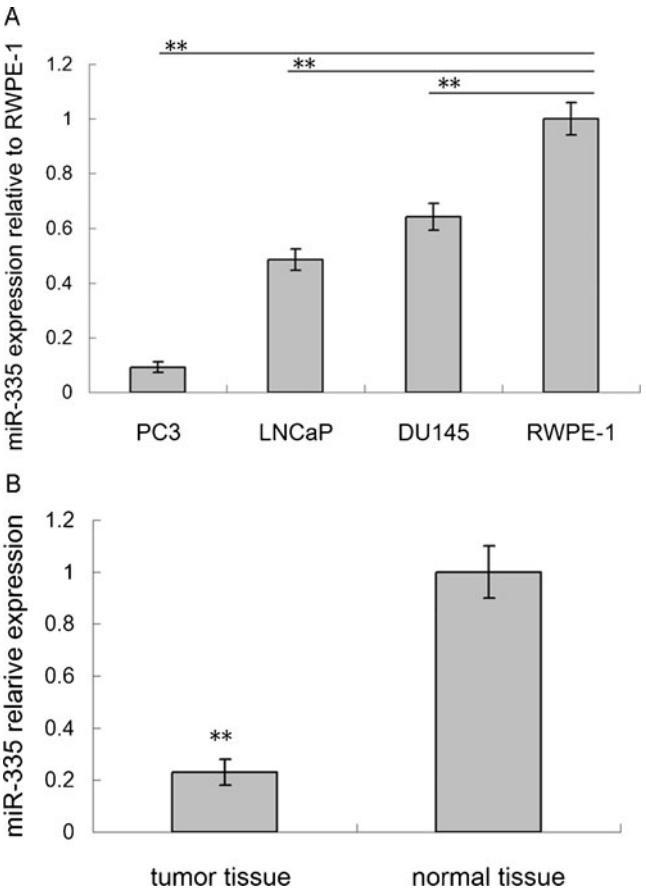

Fig. 1 MiR-335 expression in prostate cancer (PCa) cell lines and tissues. **a** Relative expression of miR-335 in three PCa cell lines (LNCaP, DU145 and PC-3) and a non-malignant epithelial prostate cell line (RWPE-1) was detected by qRT-PCR. Data were expressed as $\overline{X} \pm s$. '*' refers to statistical significance between groups ($P<0.05$); '**' refers to statistical significance between groups ($P<0.01$). **b** Relative expression of miR-335 in PCa tissues and their pair-matched adjacent non-tumor tissues was detected by qRT-PCR. Data were expressed as $\overline{X} \pm s$. '*' refers to statistical significance between groups ($P<0.05$); '**' refers to statistical significance between groups ($P<0.01$)

PCR were conducted using paired *t* test. For in situ hybridization assay, 2-way ANOVA with the Bonferroni posttest was conducted. Statistical analyses were performed with Kaplan-Meier method for the question of survival and Cox regression analysis for the multivariate analysis. All the other results were analyzed using paired *t* test. Differences were considered statistically significant when *p* was less than 0.05.

Results

Down-Regulation of miRNA-335 in Human Tumorigenic Prostate Cell Lines

The expression levels of miR-335 in 3 human PCa cell lines (LNCaP, DU145 and PC-3) and a non-malignant epithelial prostate cell line (RWPE-1) were detected by qRT-PCR. The miR-335 levels were down-regulated in all 3 PCa cell lines compared with a normal prostate epithelial cell line (LNCaP, 2.05-fold, $P<0.01$; DU145, 1.56-fold, $P<0.01$; PC-3, 10.85-fold, $P<0.01$; Fig. 1a).

Down-Regulation of miRNA-335 in Human PCa Tissues

We nextly examined miR-335 expression in 20 primary PCa tissues relative to their pair-matched adjacent benign prostate tissues using qRT-PCR and found that miR-335 expression in PCa tissues was significantly lower than that in adjacent benign prostate tissues ($P=0.01$, Fig. 1b).

Down-Regulation of miRNA-335 Associates with Aggressive Clinicopathological Characteristics in PCa

To determine whether miR-335 expression is associated with the clinicopathological characteristics of PCa, we detected miRNA-335 expression by in situ hybridization. Figure 2 shows the representative images of miRNA-335 expression by in situ hybridization from 104 PCa and 20 adjacent benign prostate tissues. The expression levels of miRNA-335 in PCa tissues were lower than those in adjacent benign prostate tissues significantly (staining score: PCa=3.27±0.99 vs. Benign=4.55±1.34, $P=0.04$). In addition, in prostate cancer sample, the reduced expression of

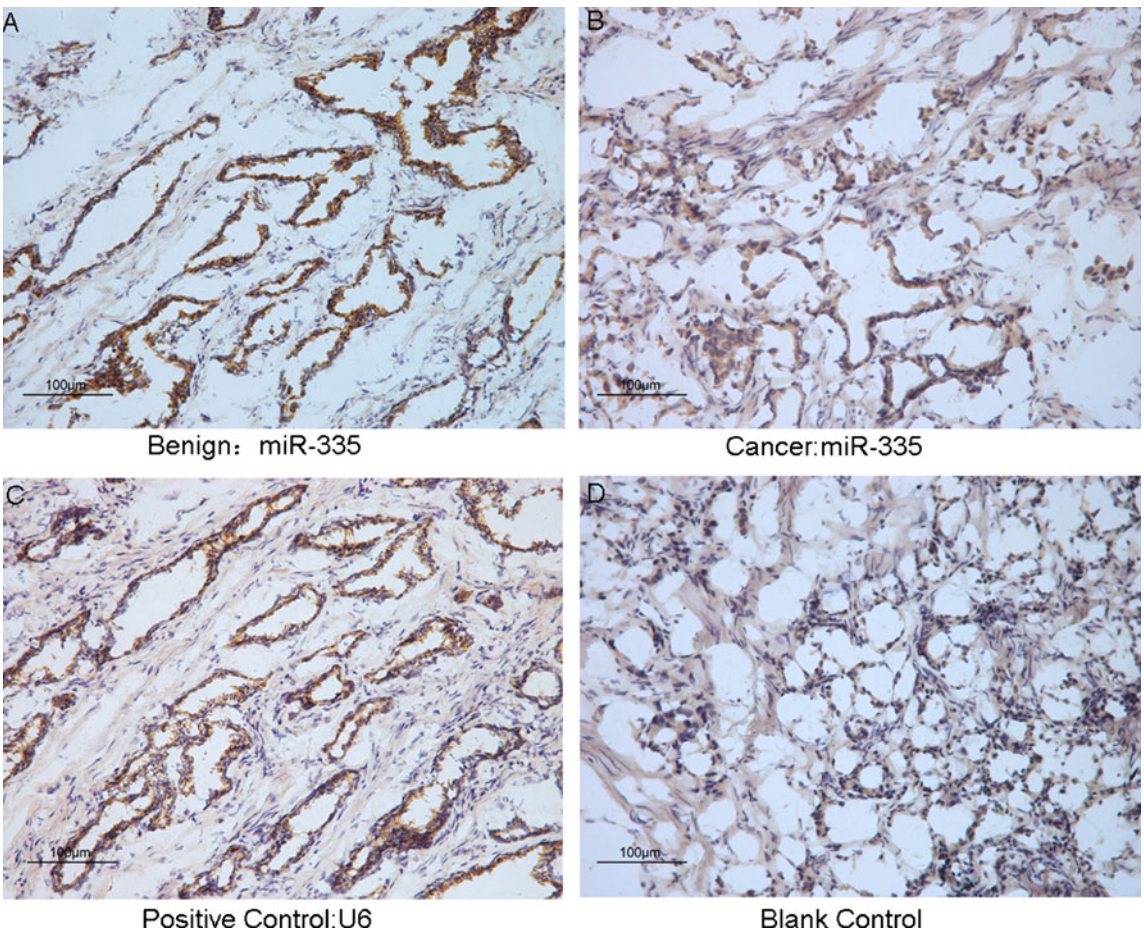

Fig. 2 MiRNA-335 expression was verified by in situ hybridization (Original magnification×400). **a** The miRNA-335 signal was strong in the adjacent benign prostate tissues; **b** The miRNA-335 signal was weak in the PCa tissues; **c** Positive control (U6); **d** Blank control

Table 3 Association of miRNA-335 expression with the clinicopathological features of PCa patients

Clinical features	hsa-miR-335		
	Numbers	$\overline{X} \pm s$	P
Age (years)			
<60	62	3.22±0.71	
≥60	42	3.35±0.69	0.79
Serum PSA levels (ng/ml)			
<4	21	3.51±0.70	
≥4	83	3.22±0.64	0.28
Gleason score			
<8	86	3.54±0.69	
≥8	18	1.98±0.75	0.04
Clinical stage			
<T2A	64	3.69±0.68	
≥T2A	40	2.61±0.63	0.04
Pathological stage			
T2A-T2C	78	3.35±0.66	
T3A-T4	26	3.06±0.69	0.38
Metastasis			
No	89	3.63±0.70	
Yes	15	1.80±1.03	0.02

miR-335 was significantly associated with high Gleason Score (P=0.04, Table 3), advanced clinical stage (P=0.04, Table 3), and positive metastasis (P=0.02, Table 3), but not with other clinicopathological features of PCa tissues, including patients' age, serum PSA Levels and pathological stage (all P>0.05, Table 3).

We further investigated the prognostic implication of miRNA-335 expression in PCa. However, the statistics results indicated that there were no significant differences in overall survival, biochemical recurrence-free, metastasis-free survival, metastasis-free biochemical relapse rates between high expression group and low expression group of miRNA-335 (all p>0.05, Fig. 3).

MiR-335 Suppresses Cell Proliferation of PCa Cell Lines in Vitro

To determine whether the overexpression of miR-335 in PCa cells can affect cell proliferation, LNCaP and DU145 cell lines were transiently transfected with hsa-miR-335 vector, respectively. MiR-qRT-PCR analysis was performed to detect the expression level of miR-335. Our results showed that the expression levels of miR-335 in miR-335-transfected PCa cells were significantly higher than those in control-miR-transfected cells (LNCaP: P=0.001; DU145: P=0.001; Fig. 4a). The MTT assay indicated that the cell viability of miR-335 transfected LNCaP and DU145 cells were both significantly less than that of negative control at 48 h after transfection (Fig. 4b and c, LNCaP and DU145: P<0.001).

MiR-335 Suppresses Cell Invasion and Migration of PCa Cell Lines in Vitro

The transwell invasion assay using Matrigel was performed to determine the effect of miR-335 on the invasive ability of the miR-335-transfected DU145 cells. The results clearly revealed that the overexpression of miR-335 could significantly reduce the invasion of miR-335-transfected DU145 cells compared with that of controls (55±6 cells/field vs. 102±10 cells/filed, P<0.01; Fig. 5). Wound-healing assay also showed that miR-335 markedly reduced the migration of DU145 cells (38±7 vs. 119±10 migrated cells, P<0.01; Fig. 6).

Discussion

This is the first study of the investigation on the functional and clinical significance of miR-335 in PCa. Advanced PCa is currently incurable because of the metastasis to bone and lymph nodes, and the progression from an androgen-dependent to an androgen-independent disease [16]. Therefore, it is necessary to diagnose the local tumor at an early stage, which needs further understanding of the biological mechanisms underlying the aggressive progression of PCa. Previous studies identified several specific and unique miRNAs which contribute to the initiation, progression, and metastasis of PCa. The main findings of the present study are as following three points. Firstly, miR-335 was dramatically downregulated in both PCa cell lines and clinical tissues, suggesting that the low expression of miR-335 is significantly associated with PCa. Secondly, the low expression of miR-335 was significantly associated with advanced tumor progression of PCa, but not with the prognosis in PCa patients; and finally, the overexpression of miR-335 could inhibit the cell proliferation, invasion and migration of PCa cell lines.

MiR-335, encoded by the second intron of the maternally imprinted gene MEST (human chromosome 7q32), is normally expressed in a variety of human tissues and deregulated in several types of tumors, suggesting its complex biological roles during tumorigenesis [17]. Wang et al. [10] found the down-regulation of miR-335 in breast cancer tissues and sera by a RT-PCR analysis; Marcucci et al. [18], Soon et al. [19] and Xu et al. [20] also detected the low expression of miR-335 in adrenocortical carcinomas, acute myeloid

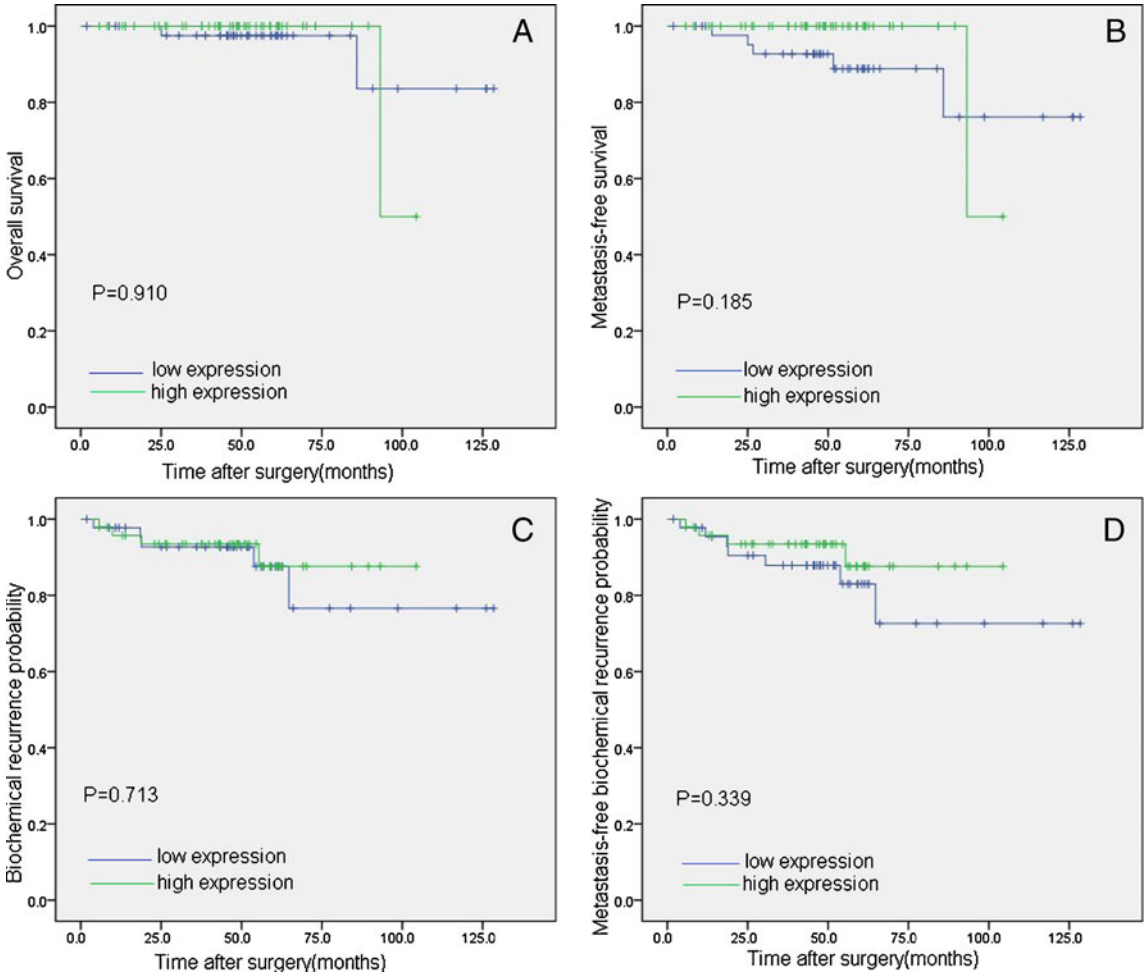

Fig. 3 Kaplan-Meier survival curves of overall survival (**a**), metastasis-free survival (**b**), biochemical recurrence-free (**c**), metastasis-free biochemical relapse (**d**) for miRNA-335 and PRDX3 expression in PCa

leukemia and gastric cancer cell lines, suggesting that the reduced expression of miR-335 is significantly associated with these malignancies. Tavazoie et al. [9] identified miR-335 as a metastasis suppressor in breast cancer cells, since its reduced expression could be associated with the ability to form breast cancer metastasis in mice. Its association with tumor chemoresistance was further validated in ovarian cancer [11]. In the present study, we showed the similar results that the expression levels of miR-335 were dramatically decreased in PCa cell lines and clinical PCa tissues. Its overexpression could suppress cell proliferation, invasion and migration of PCa cell lines. However, several previous studies also detected the increased expression of miR-335 in some

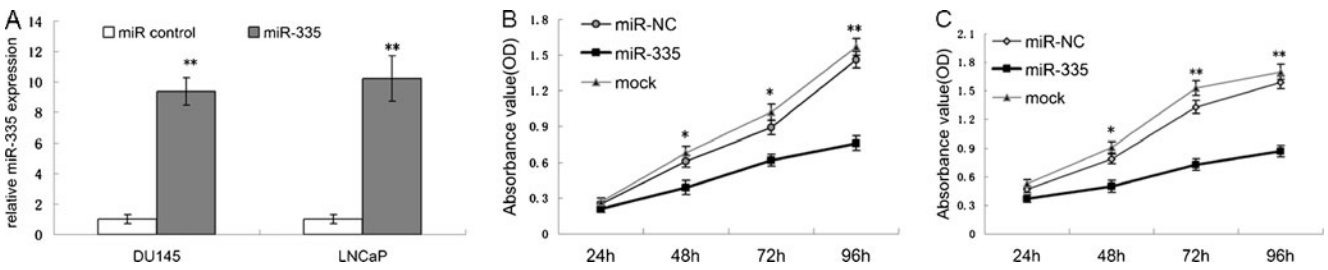

Fig. 4 MiR-335 overexpression suppresses cell proliferation. **a** Relative miR-335 expression in LNCaP and DU145 cells after miR-335 transfection. **b** Cell proliferation analysis of DU145 cells after miR-335 transfection by MTT assay. **c** Cell proliferation analysis of LNCaP cells after miR-335 transfection by MTT assay. Data were expressed as $\overline{X} \pm s$. '*' refers to statistical significance between groups ($P<0.05$); '**' refers to statistical significance between groups ($P<0.01$)

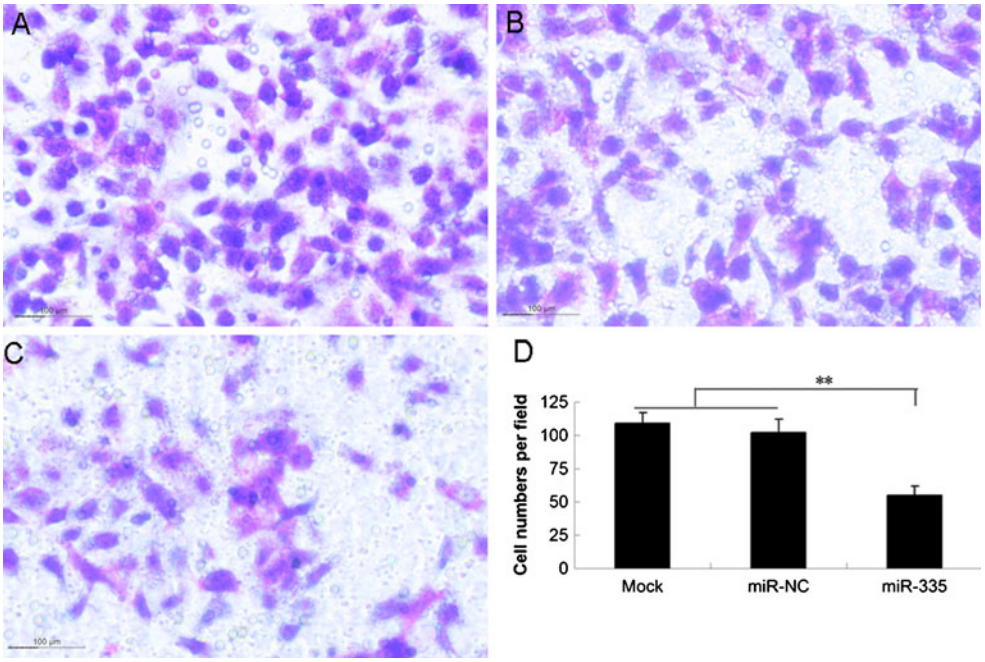

Fig. 5 MiR-335 suppresses invasion of DU145 cells. Representative images of the invaded DU145 cells transfected with mock-blank control (mock, **a**), negative control (miR-NC, **b**) or EGFP-miR-335 (miR-335, **c**) for 48 h. The cells were harvested and subjected to transwell invasion assay. **d** A graphical representation of the number of cells in three groups. Data were expressed as $\overline{X} \pm s$. '**' refers to statistical significance between groups ($P<0.01$)

malignant cells. Shu et al. [12] report that the miR-335 level is highly elevated in C6 astrocytoma cells and human malignant astrocytomas, and this microRNA may act as a tumor promoter in conferring tumorigenic features such as growth and invasion on malignant astrocytoma; The overexpression of miR-335 also plays an important role in the development of colonic cancer [21], pediatric acute leukemia [22] and multiple myeloma [23]. This difference may be caused by tissue specificity or ethnic diversity.

With the similar results with our data, the previous study of Wang et al. [24] found that the treatment of pomegranate juice, which was shown in a clinical trial to inhibit progression of PCa, could upregulate the expression of miR-335 suggesting that this miRNA should be an anti-invasive factor in this disease. However, the gene transcripts/functions potentially influenced by miR-335 in PCa have not been elucidated. Further experimental studies are required to investigate these problems.

In conclusion, our data demonstrated for the first time the inhibitory effect of miR-335 on cell proliferation and invasion for PCa cells. The loss of this microRNA might be associated with clinical progression of PCa patients.

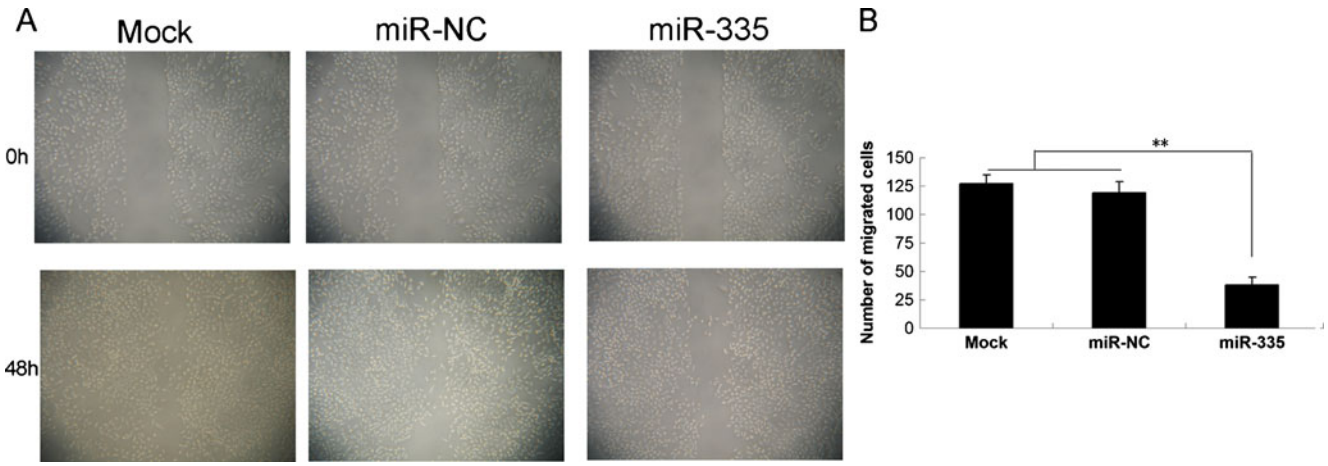

Fig. 6 MiR-335 suppresses migration of DU145 cells. **a** Representative images of the wound healing assay. **b** miR-335 inhibits woundhealing of DU145 cells. DU145 cells were transiently transfected with mock-blank control (mock), negative control (miR-NC) or EGFP-miR-335 (miR-335) for 48 h. The cells were harvested and subjected to wound-healing assay. The width of the remaining open wound calculated in relation to separation at time 0 h. Data were expressed as $\overline{X} \pm s$. '**' refers to statistical significance between groups ($P<0.01$)

Furthermore, this study is hypothesis generating, and that further prospective analysis would be worth doing.

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Competing Interests The authors declare that they have no competing interests.

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