MicroRNA 363 mediated positive regulation of c-myc translation affect prostate cancer development and progress

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Prostate cancer (CaP) is the sixth most significant cancer killer of men in China. In this study, the potential role of micro-363 (miR-363) in CaP development and progression was investigated. Pri-miR-363 or anti-miR-363 was transfected into the CaP cells line PC-3 cells. Cell proliferation, transformation property, and epithelial-to-mesenchymal transition (EMT) were evaluated by MTT, clonogenic assay, colony formation in soft agar and western blotting, respectively. The expression and involvement of c-myc, a downstream target of miR-363 were also determined. The results showed that endogenous expression of miR-363 was significantly increased in CaP cells compared with normal prostate cells. High expression of miR-363 in PC-3 cells through transfection induces cell proliferation and positively regulates cell transformation property as well as promotes EMT of PC-3 cells. Through knockdown of c-myc, the results also showed that c-myc was involved in the regulation of biological function of PC-3 cells by miR-363. Taken together, this study adds support to the potential role of miR-363 in the diagnosis and treatment of CaP.

Key words: prostate cancer, transformation property, proliferation, micro-363, epithelial-to-mesenchymal transition

Prostate cancer (CaP) is the sixth most significant cancer killer of men in China. In 2012, the incidence of CaP in the registration area of China was 9.92/100000 [1]. CaP is highly heterogeneous, ranging from asymptomatic to rapidly fatal malignancy [2]. Early diagnosis of cancer and timely detection of disease progression following either radical prostatectomy or radiation therapy are crucial for effective treatment of CaP and for a beneficial clinical outcome [3]. Recently, interest in alternative biomarkers for CaP, such as microRNAs (miRNAs) has been rapidly growing [4].

miRNAs are made up of 22 endogenous nucleotides [5]. These small, non-coding RNAs are important regulators of gene expression at the post-transcriptional level through repressing target proteins. The repressive effect is mainly achieved via RNA-RNA binding at imperfect complementary sequences within the 3'-untranslated region (3'-UTR) of the target mRNA, subsequently causing either mRNA degradation or translational inhibition [6]. It has become increasingly clear that aberrant expression of miRNAs is closely associated with proliferation, invasion, metastasis, and prognosis of various cancers, including CaP, gastric cancer, breast cancer, glioma and lung cancer [7-10]. Studies have shown that in CaP, 30% of epigenetic silencing regions contain the loci of miRNAs, and differences in the expression patterns of many miRNAs in CaP have been reported in different studies [8, 11-13]. miRNAs in CaP tissues might be promising biomarkers that might be a useful marker for the prognosis of CaP.

Several studies have revealed the possible regulatory functions of miR-363 in diverse biological processes. In human uterine leiomyoma and CD4+ and CD8+ cells of cord blood, miR-363 is significantly up-regulated. However, it is down-regulated in CD4+ T cells of patients with rheumatoid arthritis. miR-363 is down-regulated in human papillomavirus (HPV)-transfected keratinocyte HaCaT cells, but up-regulated in HPV-positive pharyngeal squamous cancers [14]. miR-363 is also associated with many cancers, including gastric cancer, hepatocarcinoma, and neuroblastoma [14-16]. In aggressive gastric cancer, miR-363 affects cell adhesion and migration through targeting disintegrin and metalloproteinase 15 (ADAM15) [17]. In HPV-positive squamous cell carcinoma of the head and neck, it has also been shown to
regulate myosin-1b (MYO1B), which modulates cell motility and migration[18].

However, to the best of our knowledge, the potential role of miR-363 in CaP development and progression is still unclear. In this study, pri-miR-363 or anti-miR-363 was transfected into the CaP cells PC-3 cells. Cellular activities including proliferation, transformation property, and epithelial-to-mesenchymal transition (EMT) were all evaluated. We also determined the downstream target of miR-363 in CaP cells. This study adds support to the potential use of miR-363 as a biomarker or target for the diagnosis and treatment of CaP.

**Materials and methods**

**Cell culture.** Human CaP cell lines 22Rv1, LNCap, DU145, and PC-3 and normal prostate epithelial cell line RWPE-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and penicillin (100 U/ml, TaKaRa, Dalian, China) and were maintained under an atmosphere containing 5% CO₂.

**Total RNA extraction and miRNA quantitative real-time reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted from cultured cells using Trizol Reagent (Invitrogen, CA, USA). cDNA was synthesized using M-MLV MicroRNA Reverse Transcription Kit (Promega, Madison, WI, USA) from 10ng of total RNA. Real-time RT-PCR was performed with SYBRH Premix Ex TaqTM (TaKaRa, Biotech Co., Ltd, Dalian, China) and specific primer designed for miR-363/c-myc to detect mature miR-363 or c-myc. miRNA expression was normalized with the level of U6. PCR was performed under the following conditions: 94°C for 4min, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 40 s. Each sample was run in triplicate. The primers used in this study were as following: GAPDH: sense 5’-ACC CAC TCC TCC ACC TTT G-3’, antisense:5’-CTC TTG TGC TCT TGC TGG G-3’; miR-363: sense: 5’-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTG GCA CTG GAT ACG ACT ACA GA-3’, antisense: 5’-TCG AAT TGC ACG GTA TCC ATC-3’; U6: 5’- AAC GCT TCA CGA ATT TGC GT-3’, antisense: 5’- CTC GCT TCG GCA GCA CA-3’; c-myc: sense: 5’-TGG TGC TCT ATG AGG AGA CA-3’, 5’-GTT TCG TTT CAA CTG TTT GCG TCC-3’.

**Cell transfection.** The recombinant miR-363-expressing adenoviral plasmid (and recombinant synthetic negative control miRNAs, synthetic anti-miR-363 sequence and synthetic anti-miR-363 negative control adenoviral plasmid) were purchased from Genechem Ltd. (Shanghai, China). Transfections PC-3 cells was performed according to the manufacturer’s protocol as previously described [19]. Briefly, PC-3 cells were plated in six-well plates and grown to 60–80 % confluence. Each well received 10 μL of lipofectamine reagent (Amersham, Piscataway, N, USA) and pri-miR-363, synthetic negative control miRNAs (miR-363-NC), synthetic anti-miR-363 sequence (anti-miR-363), or synthetic anti-miR-363 negative control (anti-miR-363-NC), to a final concentration of 50 nM. For other experiments, c-myc-shRNA vectors were transfected into cells to produce c-myc siRNA stable expression cells. (c-myc shRNA: 5’-CCA TAA TGT AAA CTG CCT CAA CTC GAG TTG AGG CAG TTT ACA TGA TGG TTT TT-3’).

**Western blot analysis.** Whole cell lysates were prepared as described previously [20]. PC-3 cells in different groups were lysed with RIPA buffer and the protein concentration of cell lysates was determined using the BCA assay. Forty micrograms of total protein per well were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. After blocking with 0.5% non-fat milk, the target proteins were probed with 1:1000 mouse anti-human c-myc, E-cadherin, plakoglobin, N-cadherin or β-actin antibody overnight at 4°C. After washing thrice (10 min/time) with 1× PBS-Tween 20, the membranes were incubated with HRP-conjugated sheep anti-mouse IgG antibody (1:4000 diluted in PBS) at room temperature for 1 h and washed again. The reactive bands were detected by enhanced chemiluminescence (ECL) according to the manufacturer’s protocol.

**MTT assays.** PC-3 cells transfected with different miRNAs (or together with c-myc-specific siRNA) were seeded onto 24-well plates at a density of 1×10⁴ cells per well in triplicate. Cell viability was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich, St. Louis, MO, USA) method. After incubation for 48 h, 10 μL of MTT (0.5 mg/ml) were added to each well for an additional 3 h of incubation. Then, 250 μL of dimethylsulf oxide (DMSO) were added to each well and the absorbance at 490 nm was measured using a microplate enzyme-linked immunosorbent assay reader (Biochrom EZ Read 400, Cambridge, UK).

**Clonogenic assay.** Clonogenic assays were performed as previously described [21]. PC-3 cells suspended in RPMI 1640 media with 10% fetal bovine serum (FBS) were plated in six-well plates (1×10⁴ cells/well) and incubated for 10 days. Colonies were fixed and stained with 0.005% crystal violet in 70% methanol overnight. Images of the colonies were taken with the Bio-Rad Gel Doc XR+ Imaging System, and quantitated using Bio-Rad Quantity One Gel Doc version 4.6.9 software. All cultures were performed in triplicate and the experiments for each miRNA were repeated three times.

**Anchorage-independent colony growth.** Anchorage-independent growth was measured by assessing colony formation in soft agar, as previously described [22]. PC-3 cells were seeded (1×10⁵ cells/well) in 0.35% agar mixed with RPMI1640 medium in a six-well plate and cultured at 37°C for 10days. The ability to form colonies in soft agar was measured by counting the number of colonies under a stereoscopic microscope in triplicate of a six-well plate. Data shown represent means ± standard deviations (SDs) of three independent experiments.
**Statistical analysis.** The two-tailed Student's t-test was used to evaluate the significance of the differences between two groups; P values<0.05 were considered significant.

**Results**

**miR-363 expression in CaP cells.** To check whether miR-363 participates in tumor progression of CaP, we first determined the relative levels of endogenous miR-363 using miRNA quantitative real-time PCR in four human CaP cell lines including 22Rv1, LNCap, DU145, and PC-3. Normal prostate epithelial cell line RWPE-1 was used as control. As shown in figure 1, the expression of miR-363 in four CaP cells was significantly increased compared with the normal cell line RWPE-1. PC-3 cells showed the highest endogenous miR-363 expression. Since PC-3 cell is androgen-receptor negative, in this study, PC-3 and LNCap (androgen-sensitive human prostate cancer cell line) cells were chosen for subsequent investigation.

**High expression of miR-363 in PC-3 cells induces cell proliferation.** To determine the potential biological function of miR-363 in CaP, pri-miR363, anti-miR363 or scrambled control was stably transfected into PC-3 cells to overexpression or knockdown in PC-3 cells. miRNA quantitative RT-PCR was performed 24h, 48h, 72h after transfection. As shown in figure 2A, 48h after transfection, miR-363 expression was significantly increased in pri-miR363-transfected PC-3 cells and was significantly reduced in anti-miR363-transfected PC-3 cells compared with the control group. Similar results were observed 72h after transfection.

We then determined cell proliferation of different transfection groups by the MTT assay 72h after transfection. As shown in figure 2B, compared with cells at resting and cells transfected with pre-miR-363-NC, overexpression of miR-363 significantly increased PC-3 cell proliferation. While cell proliferation of anti-pre-miR-363 transfected PC-3 cells were significantly decreased.

**miR-363 positively regulates transformation property of PC-3 cells.** To better elucidate the effect of miR-363 on division property of PC-3 cells, we further performed clonogenic assays to determine the ability of each cell to undergo "unlimited" division. As shown in figure 3A, overexpression of miR-363 significantly increased the number of colonies to 187% of that at resting. On the other hand, knockdown of miR-363 significantly reduced the number of colonies to 59% of that at resting. All these results suggested that the pro-tumor property of miR-363.

Furthermore, to examine anchorage-independent growth ability, we performed soft agar colony formation assays, which are an accepted in vitro assay for detecting cell malignancy. PC-3 cells transfected with different miRNAs were plated in soft agar and incubated for 2 weeks. As shown in figure 4, transfection of pri-miR-363 significantly increased anchorage-independent growth. Compared with the control group, the number of colonies was significantly increased by about 48%. Similar results were also observed in anti-miR-363 transfected cells.

[Figure 1: Endogenous expression of miR-363 in four human CaP cell lines by miRNA quantitative real-time PCR. *P<0.05 vs RWPE-1 cells.]

[Figure 2: High expression of miR-363 in PC-3 cells induces cell proliferation. PC-3 cells were transfected with different miRNAs: (A) miR-363 expression in PC-3 cells of different transfection groups were detected at 24h, 48h and 72h after transfection, (B) Proliferation of PC-3 cells transfected with different miRNAs were detected by the MTT assay 72h after transfection. *P<0.05, significant differences were observed compared with cells at resting or cells transfected with controls.]
Figure 3 Overexpression/knockdown of miR-363 significantly increased/reduced the number of colonies by clonogenic assays. (A) the number of colonies of different group. (B) a: cells at resting, b: PC-3 cells transfected with pri-miR-363, c: PC-3 cells transfected with anti-pri-miR-363. *P<0.05, significant differences were observed compared with cells at resting.

Figure 4 Overexpression/knockdown of miR-363 significantly increased/reduced anchorage-independent growth of PC-3 cells by soft agar colony formation assays. (A) anchorage-independent growth of PC-3 cells. (B) a: PC-3 cells at resting, b: PC-3 cells transfected with pri-miR-363, c: PC-3 cells transfected with anti-pri-miR-363. *P<0.05, significant differences were observed compared with cells at resting.
Experiments only in PC-3 cells which were androgen receptor that miR-363 participated in the EMT event. However, we did in expression of E-cadherin and plakoglobin also suggested and anchorage-independent cell growth assay. The change of PC-3 cells had pro-tumor function using clonogenic formation as also demonstrated that high expression of miR-363 in PC-3 cells could induce PC-3 cell proliferation. We supposed that high expression of miR-363 could induce PC-3 cell proliferation and transformation property, we determined the expression of c-myc, a downstream target oncogene of miR-363. As shown in figure 6A and figure 6B, mRNA and protein levels of c-myc in pri-miR363-transfected PC-3 cells were significantly increased. To further confirm the role of c-myc, c-myc-specific siRNAs were transfected into pri-miR363-transfected PC-3 cells and c-myc levels were significantly decreased (figure 6C). In normal cells transfected with c-myc-specific siRNAs, cell proliferation were decrease. However, no significant difference was found. c-myc silencing significantly deceased miR-363 overexpression induced PC-3 cell proliferation and colony formation. All these results suggested that c-myc was a downstream target of miR-363 in regulating the biological function of PC-3 cells.

Discussion

The various genetic alterations in CaP play important roles in the specific activity of miRNAs, as 30% of epigenetic silenced regions contained the loci of miRNAs. Recent studies have demonstrated that aberrant expression of miRNAs is closely associated with the development, invasion, metastasis and prognosis of CaP [27]. The exact function of miR-363 in CaP progression, however, has not been elucidated. We detected the expression of miR-363 in different CaP cells and normal prostate cells, and found that the CaP cells showed significantly increased miR-363 expression. We supposed that alterations in miR-363 might contribute to the development and progression of CaP. In this study, we found that miR-363 played critical roles in activities of PC-3 cells. Using miRNA quantitative real-time RT-PCR, we showed that miR-363 was significantly increased in PC-3 cells, and high expression of miR-363 in PC-3 cells could induce PC-3 cell proliferation. We also demonstrated that high expression of miR-363 in PC-3 cells had pro-tumor function using clonogenic formation assay and anchorage-independent cell growth assay. The change in expression of E-cadherin and plakoglobin also suggested that miR-363 participated in the EMT event. However, we did experiments only in PC-3 cells which were androgen receptor negative. Further studies were expected to investigate the role of miR-363 in other different kinds of cells.

MicroRNAs regulate gene expression by affecting the stability or translation efficiency of target mRNAs. Up to 50 miRNAs have been reported to be significantly expressed in human CaP [24]. Overexpression of the oncomirs epigenetically silences apoptosis-related genes, inducing tumor growth and metastasis, while loss of tumor suppressor miRNAs is another mechanism related to the progression of CaP [25, 26]. In this study, miR-363 expression was measured in CaP cells. miRNA-363 shows different expression patterns in different diseases and different cells, and thus performs diverse functions. Through a recombinant miR-363-expressing adenoviral plasmid, high miR-363 was stably expressed in PC-3 cells. High overexpressed miR-363 significantly increased cell proliferation. Clonogenic assay showed that high overexpression of miR-363 promoted the ability of each PC-3 cell to undergo “unlimited” division. Meantime, we found that miR-363 overexpression promoted PC-3 cell malignancy in vitro which was assessed using soft agar colony formation assay. The decreased E-cadherin and plakoglobin and increased N-cadherin also suggested that miR-363 was involved in the EMT event. Collectively, these findings prove the involvement of miR-363 in the progression, invasion, and metastasis of CaP.

In this study, using co-transfection of miR-363 and c-myc-specific siRNAs, we also found that c-myc, a downstream oncogene of miRNAs, mediated the function of miR-363. C-myc is critical for development and survival and is well-known for its regulation of proliferation, differentiation, and oncogenesis [27]. Deregulation of c-myc acts as an oncogenic driver
in many cancers, including CaP [28]. For example, inhibition of c-myc translation causes significant growth inhibition and apoptosis in CaP cells and in subcutaneous tumor xenografts. Another study showed that antisense c-myc retroviral vector suppressed established human CaP [29]. Through microRNA.org (http://www.microrna.org/microrna/getMirnaForm.do), we found that c-myc was not a direct target of miR-363. Studies from Han Han et al. have shown that in human HCC, miR-363-3p destabilizes myc indirectly by directly targeting and inhibiting USP28, which promotes the proteasome-mediated degradation of myc protein[16]. However, in prostate cancer, how miR-363 affected c-myc expression were barely studied. Further studies are need to investigate the exact signaling pathways involving miR-363 and c-myc.

Figure 6 miR-363 regulates biological function of PC-3 cells through c-myc. (A/B): mRNA/protein levels of c-myc in PC-3 cells with different transfections. (C): relative mRNA levels of c-myc in c-myc shRNA stable expression cells; (D/E): c-myc silencing abolished miR-363 overexpression induced PC-3 cell proliferation (D) and colony formation (E).
Our data here provide evidence that the expression of miR-363 is increased in CaP cells. The increased miR-363 promotes PC-3 cell activities including proliferation, transformation ability and EMT, and the oncogene c-myc is involved. A better understanding of miR-363-mediated molecular mechanisms in CaP could potentially provide novel therapeutic strategies against CaP.

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