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MIR-150 promotes prostate cancer stem cell development via suppressing p27Kip1

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Abstract. – OBJECTIVE: Our previous study found that high miR-150 expression was positively correlated with prostate tumor recurrence or metastasis. In this work, we investigated the expression of miR-150 in prostate cancer stem cells (CSCs) and explored its regulation over p27 in the development of CSCs.

MATERIALS AND METHODS: MiR-150 expression in CD144 or CD44 positive primary prostate cells and in DU145 cell line was measured. It regulation over CSCs was measured using tumor sphere assay and qRT-PCR analysis of CSC related Oct4, Nestin and Nanog genes. The direct binding between miR-150 and 3'UTR of p27 mRNA was verified using dual luciferase, qRT-PCR and western blot assay. The influence of miR-150-p27 axis on prostate CSC properties was further investigated.

RESULTS: Findings of this study found miR-150 expression was significantly upregulated in CD44+ or CD133+ subgroups of prostate cancer cells. MiR-150 could directly target 3'UTR of p27 and decrease its expression, through which it increased the number and volume of tumor sphere formed by DU145 cells, as well as the expression of CSC related Oct4, Nestin and Nanog genes.

CONCLUSIONS: Increased miR-150 expression might participate in the development and progression of human prostate CSC by suppressing p27. This supported our previous study which found miR-150 was positively correlated with prostate tumor recurrence or metastasis.

Key words:
Prostate cancer; Cancer stem cell; miR-150; p27Kip1.

Introduction

Prostate cancer is the second leading cause of male malignancy related deaths in the developed countries[1,2]. Although current therapies based on androgen ablation can generate very positive effects in the initial phase of treatment, most of the cases eventually progress within 2 years to form invasive and drug-resistant metastatic cancers[3-5]. There are emerging studies in recent years showed that there is a small proportion of prostate cancer stem cells (CSCs)[6,7]. They are more resistant to toxic injuries and chemoradiation therapy and can drive tumor growth and metastasis than the differentiated daughter cells[6,7]. The proportion of CSCs also correlates with a poor prognosis[8]. Therefore, the prostate CSCs might be a promising target for new therapy of prostate cancer. Increasing evidence showed that the CD44 or/and CD133 positive subpopulations contains a large proportion of prostate CSCs[9-11].

Recently, some studies found that miRNAs participate in regulation of human prostate CSCs. For example, miR-34a can inhibit prostate CSCs and metastasis by directly repressing CD44[12]. MiR-320 can suppress the CSC properties of prostate cancer cells by downregulating the Wnt/beta-catenin signaling pathway[13]. MiR-143 and miR-145 can suppress tumor sphere formation and expression of CSC markers and 'stemness' factors including CD133, CD44, Oct4, c-Myc and Klf4 in PC-3 cells[14]. Our previous investigation found that high miR-150 expression was positively correlated with tumor recurrence or metastasis[15]. In addition, PC patients with high miR-150 expression had significantly poorer overall survival[15]. One recent work found that miR-142 can promote the breast CSC properties at least by activating the WNT signaling pathway and miR-150 expression, suggesting miR-150 might also be an important molecule involved in regulation of CSCs[16]. Cyclin-dependent kinase inhibitor 1B (p27Kip1 or p27) is an enzyme inhibitor that is encoded by the CDKN1B gene[17]. It is a cell-cycle regulatory protein that interacts

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with cyclin-CDK2 and -CDK4, inhibiting cell cycle progression at G1[17]. Previous researches[18,19] reported that p27 is involved in regulation of prostate cancer stem cells. However, its upstream regulation in prostate cancer is not fully revealed.

In this study, we investigated the expression of miR-150 in prostate CSCs and explored its regulation over p27 in the development of CSCs.

Materials and Methods

Human tissue collection

The study protocol was proved by the Ethics Committee of the Second Artillery General Hospital of PLA and all participants were recruited from the hospital. 20 patients received radical prostatectomy due to histologically confirmed prostate cancer and did not receive any anti-tumor treatment before the resection were recruited. Informed consent was obtained from the patients before tissue collection. Cancerous tissues were obtained during the surgery. To prepare primary prostate cancer cells for flow cytometry study, the tumor specimens were treated according to the method introduced in one previous study[20]. Briefly, the tumor tissues were washed, minced into small pieces (about 1 mm^3) and, then, subjected to enzymatic dissociation by using Trypsin/EDTA and collagenase I for 20 minutes at 37°C. The digested solution was filtered using sterile 40-micron cell strainer (BD Falcon; Bedford, MA, USA) to get single-cell suspension.

Cell culture and transfection

Human prostate cancer cell line DU145 and human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection. DU145 and HEK 293T cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium and Dulbecco's Modified Eagle Medium (DMEM), respectively. The medium was supplemented with 10% fetal bovine serum (Gibco; Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. Both cells were cultured in a humidified air with 5% CO_2 at 37°C.

The pLV-miR-150 expression plasmid and the lentiviral packaging vector mix were purchased from Biosettia (San Diego, CA, USA). P27 wide type (p27-wt, with 3'-UTR region) or mutant (p27-mut, without 3'-UTR region) lentiviral expression vectors were purchased from GENECHEM (Shanghai, China). To produce lentiviral particles, the lentiviral vectors and the correspondent packaging mix were co-transfected to HEK 293T cells using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to manufacturer's instruction. The ready-to-use human p27 shRNA lentiviral particles (sc-29429-V) were purchased from Santa Cruz Biotech (Santa Cruz; CA, USA). To overexpress miR-150 or p27 or to knockdown of endogenous p27 in DU145 cells, the cells were infected with the lentiviral particles with the presence of 8 μg/ml polybrene (Sigma-Aldrich; St Louis, MO, USA).

Flow cytometry analysis and cell sorting

The primary prostate cancer cells or the DU145 cells were fixed with 4% paraformaldehyde, washed three times with PBS and then suspended in PBE (PBS containing 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2) in a volume of 80 μl for antibody labeling. Anti-human CD44-FITC and anti-human CD133-PE and the respective isotype controls were purchased from eBioscience (San Diego, CA, USA). The cells were incubated with the antibodies for 30 min at 4°C. Then, the $CD133^+$ or $CD44^+$ primary prostate cancer cells were isolated with a Beckman Coulter MoFlo XDP flow cytometer. The DU145 cells expressing CD44 or/and CD133 ($CD44^+$, $CD133^+$ or $CD44^+/CD133^+$ cells) were isolated using magnetic cell sorting (MACS). DU145 cells were labeled with primary CD44 or 133 antibody conjugated with microbeads (mouse IgG1, Miltenyi Biotec; 1 μL per million cells) and separated on MACS LS column (Miltenyi Biotec; Auburn, CA, USA). All procedures were carried out according to the manufacturer's instructions. The purity of sorted cells was evaluated by a Beckman Coulter FC 500 MCL/MPL counter (Beckman; Fullerton, CA, USA).

QRT-PCT analysis

Total RNA was extracted from the tissue or cell samples using TRIzol reagent (Invitrogen; Ontario, Canada), while total miRNA was extracted using the miRVana miRNA Isolation Kit (Ambion; Austin, TX, USA) according to manufacturer's instruction. MiR-150 expression was quantified using TaqMan MicroRNA Assay Kit (Applied Biosystems; Foster City, CA, USA).

To quantify the expression of CSC related genes and p27, cDNA was reversely transcribed using the SuperScript™ First-strand Synthesis System (Invitrogen, Paisley, UK). The mRNA level of Oct4, Nestin, Nanog and p27 were quantified using the gene specific primers (Table I)

Table I. Primer sequences for qRT-PCR.

Gene	Primer sequences
OCT4	F: 5'-TCAGTGATGCTGTTGATCAGG-3' R: 5'-GCTATCTACTGTGTGTCCCAGTC-3′
Nestin	F: 5'-CAGCTGGCGCACCTCAAGATG-3' R: 5'-AGGGAAGTTGGGCTCAGGACCTGG-3'
Nanog	F: 5'-TGAACCTCAGCTACAAACAGGTG-3' R: 5'-AACTGCATGCAGGACTGCAGAG-3′
P27Kip1	F: 5'-GCAGTGTCCAGGGATGAGGA-3' R: 5'-ATTTTCTTCTGTTCTGTTGGCCC-3′

and SYBR® Premix Dimmer Eraser kit (TaKaRa; Dalian, China) in an ABI Prism 7500 (Applied Biosystems; Foster City, CA, USA). GAPDH was used as the endogenous control. The results were presented using $2^{-\Delta\Delta CT}$ method.

CCK-8 assay of cell proliferation

Cell viability of DU145 cells with indicated treatments was determined using WST-8 assay with the Cell Counting Kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA). Briefly, 10 μL prepackaged CCK-8 solutions were added to the cell culture. The plate was further incubated at 37°C in a cell incubator for 2 hours. Then, absorbance at 450 nm of the supernatant was measured using a spectrophotometry.

Sphere formation assay

Briefly, 1×10^3 cells were plated onto a 6-well ultra-low attachment plate (Corning; Corning, NY, USA) in serum-free DMEM/F-12 supplemented with N-2 supplement, 10 ng/ml EGF, and 10 ng/ml bFGF (Invitrogen, Paisley, UK). After 14 days of culture, the number of tumor spheres formed was counted using an inverted microscope. The radius of each tumor spheroid was measured using NIS-Elements Microscope Imaging Software (Nikon; Tokyo, Japan) and used to calculate the volume ($V = 4/3 \times \pi \times r^3$).

Dual luciferase assay

Putative binding sites between miR-150 and 3'UTR of p27 were predicted using TargetScan 6.2. The online prediction showed that there were two possible binding sites between miR-150 and p27. Therefore, two pairs of widetype and mutant (without miR-150 binding sites) p27 3'UTR sequences with flanking *SacI* and *XhoI* restriction enzyme digestion sites were chemically synthesized. Then the sequences were interested between *SacI* and *XhoI* sites of pGL-3 promoter vector respectively. The recombinant plasmids were named as pGL3-p27-WT1, pGL3-p27-WT2, pGL3-p27-MUT1 and pGL3-p27-MUT2 respectively. Then, HEK 293T cells were co-transfected with 200 ng recombinant plasmids and 50 nM miR-150 mimics or negative control using Lipofectamin 2000 (Invitrogen, Paisley, UK). 24h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla luciferase.

Western blot analysis

Cells were lysed using a RIPA buffer (Beyotime; Shanghai, China) and the protein concentration was measured using a BCA Protein Assay Kit (Beyotime, Haimen, China). The protein samples were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were incubated with primary anti-p27 (1:1000, ab137736, Abcam, Cambridge, MA, USA) overnight at 4°C. Membranes were washed and incubated with HRP-labeled secondary antibodies (1:5000, Goat Anti-Rabbit IgG H&L (HRP), ab97080). The band signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Statistical Analysis

Paired Wilcoxon test was performed to compare miR-150 expression between $CD44^+$ and $CD44^-$ and between $CD133^+$ and $CD133^-$ primary prostate tumor cells obtained from the 20 patients. Other group comparison was performed using unpaired *t*-test. A two-sided *p* value of <0.05 was regarded as statistically significant.

Results

MiR-150 is significantly upregulated in CD133+ or CD44+ primary prostate cancer cells

The primary tumor cells obtained from the patients were firstly subjected to flow cytometry analysis of CSC markers. The results showed that the primary tumor samples all have a small proportion of $CD44^+$ or $CD133^+$ cells (Figure 1A). To identify the expression level of miR-150 in the CSCs and the non-CSCs, the $CD44^+$ or $CD133^+$ primary prostate cancer cells were then sorted. QRT-PCR analysis based on the sorted

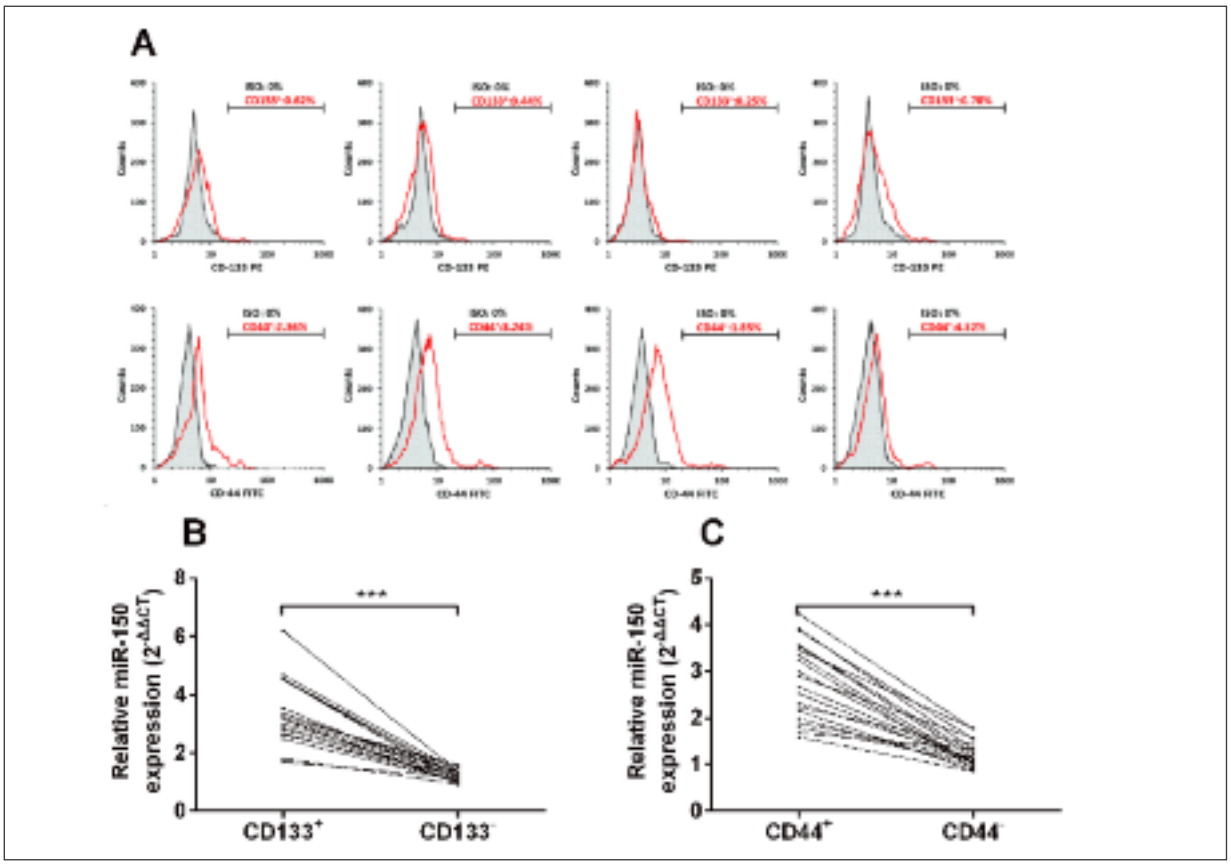

Figure 1. MiR-150 expression was significantly upregulated in CD44$^+$ or CD133$^+$ subgroups of primary prostate cancer cells. ***A,*** Representative images of flow cytometry analysis of primary prostate cancer cells stained with CD133 (up) or CD44 (down) from fours randomly selected prostate cancer patients. (**B-C**) QRT-PCR analysis and paired comparison of miR-150 expression in the primary tumor cells sorted into CD133 positive and negative (**B**) or CD44 positive or negative group of the 20 prostate cancer patients. *p<0.05, **p<0.01, ***p<0.001.

cell samples showed that both CD133$^+$ and CD44$^+$ primary prostate cancer cells had significantly higher miR-150 expression than the correspondent negative cell groups (Figure 1B).

MiR-150 overexpression increases CSC properties of prostate cancer cells

Since the expression of miR-150 is significantly increased in CSCs of prostate tumor we, then, decided to investigate its functions in the in-vitro cell model. DU145 cells were sorted into CD44$^+$, CD133$^+$ and CD44$^+$/CD133$^+$ groups (Figure 2A and B). Compared with the correspondent negative group, CD44$^+$, CD133$^+$ and CD44$^+$/CD133$^+$ groups all had significantly higher expression of miR-150 (Figure 2C). Then, we enforced miR-150 expression in DU145 cells by transfection of pLV-miR-150 expression plasmid (Figure 2D). The cancer cells with miR-150 overexpression had significantly higher proliferation rate (Figure 2E) and significantly stronger ability to form tumor sphere in terms of number and volume (Figure 2 F, G and H). Since the ability to form mammospheres in suspension culture is one of the key character of CSCs[21] we, then, detected whether miR-150 overexpression influenced the expression of CSC markers of prostate cancer, including Oct4, Nestin and Nanog. QRT-PCR analysis showed that miR-150 overexpression was associated with signifciantly increased Oct4, Nestin and Nanog expression (Figure 2I).

MiR-150 directly targets 3'UTR of p27 and decreases its expression

Our preliminary study observed that there are two possible binding sites between miR-150 and 3'UTR of p27 (Figure 3A). To verify these two

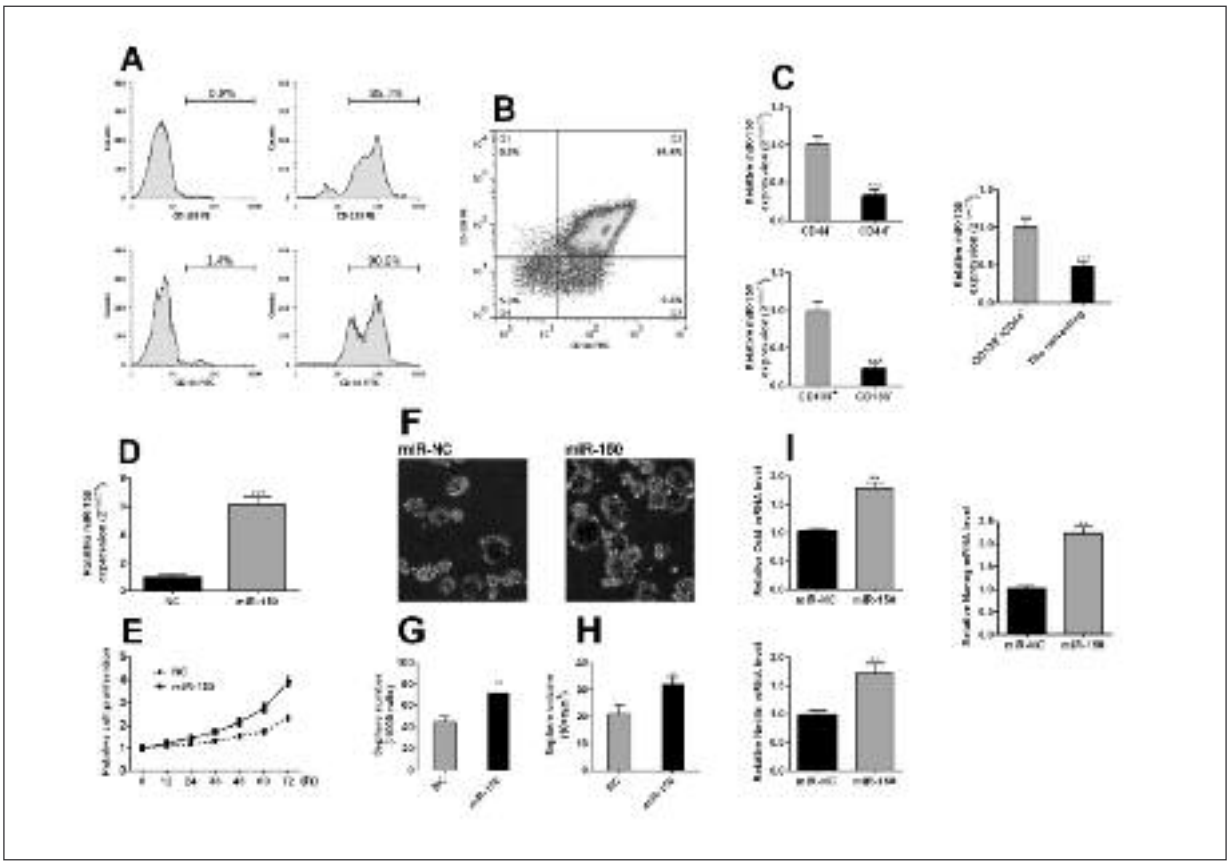

Figure 2. MiR-150 overexpression inceases CSC properties of prostate cancer cells. ***A-B,*** Representative images of flow cytometry analysis of the DU145 cells sorted into $CD44^+$, $CD133^+$ (**A**) and $CD44^+/CD133^+$ (**B**) groups. **C**, QRT-PCR analysis of miR-150 expression in DU145 cells sorted into $CD44^+$, $CD133^+$ and $CD44^+/CD133^+$. **D**, QRT-PCR analysis of miR-150 expression in DU145 cells infected with the pLV-miR-150 expression plasmid or the negative control. **E**, CCK-8 assay of relative cell viability of the DU145 cells with or without enforced miR-150 overexpression at the indicated time point. **F**, Tumorsphere cultures. Free-floating, viable mammospheroids formed by DU145 cells with or without enforced miR-150 overexpression. **G-H**, Quantitation of the number (**G**) and volume (**H**) of mammospheroids formed (mean ±SD). **I**, QRT-PCR analysis of Oct4, Nestin and Nanog expression levels in DU145 cells with or without enforced miR-150 overexpression. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

binding sites, we designed correspondent mutant sequences (Figure 3A). Then, we performed dual luciferase assay to verify whether miR-150 could influence the expression of luciferase via binding to the predicted sequences. The results showed that miR-150 could suppress the luciferase activity of the reporters carrying any one of the wide-type sequence (Figure 3 B and C). However, it had no inhibiting effect on the reporters with mutant sequences (Figure 3 B and C). Then, we studied whether miR-150 could decrease p27 expression in DU145 cells. QRT-PCR and Western blot analysis showed that miR-150 overexpression could decrease the expression of p27 at both mRNA and protein level (Figure 3D and E), the effect of which was similar to p27 shRNA.

MiR-150 increases CSC properties of prostate cancer cells partly through inhibiting p27

P27 is an important molecule modulating the acquired CSC properties in prostate cancer[15]. Therefore, considering the regulative role of miR-150 over p27, we hypothesized that miR-150 can increase CSC properties of prostate cancer cells through suppressing p27. DU145 cells were firstly infected with p27-wt or p27-mut lentiviral particles (Figure 4 A and B). In *in-vitro* proliferation assay, we observed that miR-150 partly revered p27-wt, but not p27-mut induced lower proliferation rate (Figure 4C). Then, we investigated whether miR-150 affect the CSC properties of DU145 cells via p27. In tumor

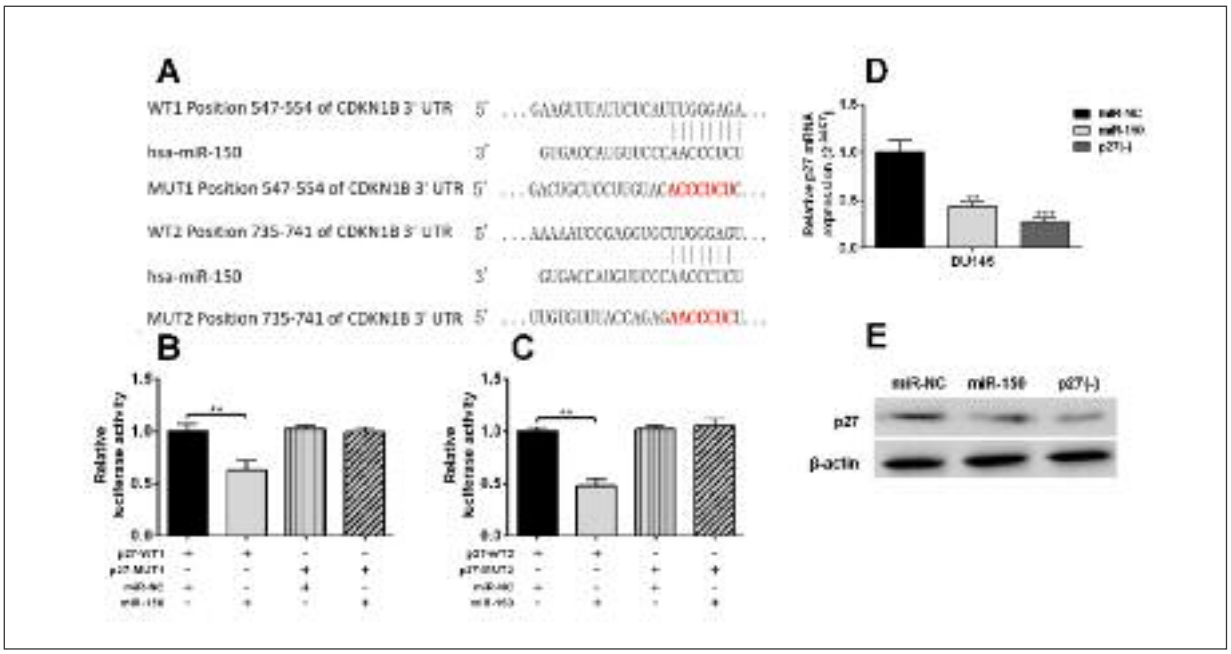

Figure 3. MiR-150 directly targets 3'UTR of P27 and decreases its expression. **A,** Predicted binding sites between miR-150 and 3'UTR of p27. WT: widetype sequence; MUT: designed mutant sequence. (**B-C**) Dual luciferase assay of relative luciferase activity between pGL3-p27-WT1 (p27-WT1) and pGL3-p27-MUT1 (p27-MUT1) (**B**) or between pGL3-p27-WT2 (p27-WT2) and pGL3-p27-MUT2 (p27-MUT2) (**C**) transfected with 50 nM miR-150 mimics or the negative control. Firefly luciferase activity was normalized to that of Renilla luciferase. (**D-E**) QRT-PCR (**D**) and western blot analysis (**E**) of p27 expression in DU145 cells infected with the pLV-miR-150 expression plasmid or p27 shRNA lentiviral particles. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

sphere assay, we observed that p27 overexpression decreased the number and volume of tumor sphere formed by DU145 cells (Figure 4 D and E). Enforced expression of miR-150 partly abrogated the effects of p27-wt. However, it had no effect on P27-mut (Figure 4 D and E). In addition, miR-150 overexpression also partly canceled p27-wt, but not p27-mut induced lower expression of OCT4, Nestin and Nanog (Figure 4 D, E and F). These results suggest that miR-150 increases CSC properties of prostate cancer cells partly through inhibiting p27.

Discussion

MiR-150 is significantly upregulated and acts as an oncogene in several types of cancers, including breast cancer[22], gastric cancer[23], and lung cancer[24]. In prostate cancer, our previous study found that miR-150 was overexpressed in PC cell lines[15]. In addition, we also observed that miR-150 expression was positively correlated with tumor recurrence or metastasis. In addition, patients with high miR-150 expression had poorer overall survival and disease free survival[15]. Although the oncogenic role of miR-150 has been gradually recognized in these cancers, whether it is related to CSC in these types of cancers was not fully understood. One recent study in breast CSCs found that the expression of miR-150 could be activated by miR-142 via the canonical Wnt signaling pathway in breast CSCs[16]. Enforced expression of miR-150 in normal mouse mammary stem cells resulted in generation of hyperproliferative mammary glands[16]. Therefore, miR-150 might be involved in regulation of CSCs.

In this study, based on 20 primary prostate tumor tissues, we observed that miR-150 expression was significantly higher in $CD133^+$ and $CD44^+$ subgroups compared with the correspondent negative groups. In DU145 cells, we also observed that the $CD133^+$, $CD44^+$ or $CD44^+/CD133^+$ subgroups had significantly higher expression of miR-150. Interestingly, through increasing miR-150 expression in DU145 cells, the cells showed a higher proliferation rate and increased CSC properties. Notably, miR-150 not only aberrant expressed in the CSC subgroups of prostate cancer, its overexpression also enhanced the expres-

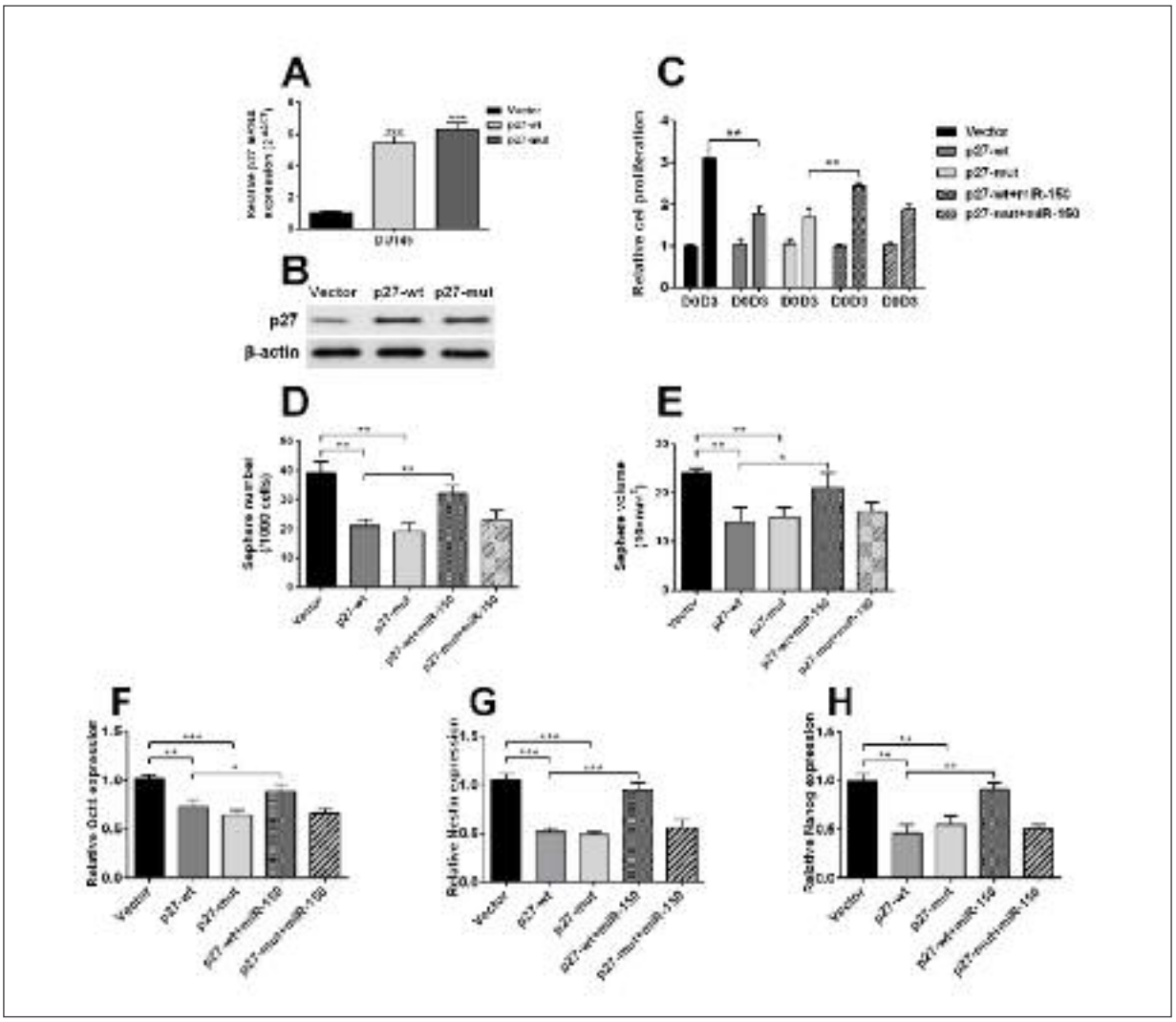

Figure 4. MiR-150 increases CSC properties of prostate cancer cells partly through inhibiting p27. DU145 cells were infected with infected with p27 wide type (p27-wt) or mutant (p27-mut) lentiviral expression vectors alone or in combination with pLV-miR-150 expression plasmid. (**A**-**B**) QRT-PCR (**A**) and Western blot (**B**) analysis of p27 expression at mRNA (**A**) and protein (**B**) level. **C**, CCK-8 assay of cell viability of indicated treatment at day 0 and day 3. (**D**-**E**) Quantification of sphere number (D) and volume (**E**) of free-floating, viable mammospheroids formed by the cells with indicated treatment. **F**-**G**-**H**, QRT-PCR analysis of Oct4 (**F**), Nestin (**G**) and Nanog (**G**) expression levels in the cells with indicated treatments. *p<0.05, **p<0.01, ***p<0.001.

sion of CSC markers, such as Oct4, Nestin and Nanog that support 'stem cell-like properties'. These results suggest that miR-150 might have an important role in development and maintenance of prostate CSCs. However, what are the downstream targets involved in the regulation is not clear. Therefore, we decided to further explore its functions and downstream regulations.

Through our preliminary studies, we found p27Kip1 is a potential target of miR-150 in prostate cancer. During the past decades, a series of studies reported that reduced expression of p27 might play an important role in development of prostatic neoplasia[18,25,26]. The frequent down-regulation of p27 protein expression is correlated with poor clinical outcome[25]. In mouse model, it was found that the low p27 expression contributed to prostate tumorigenicity and prostate cancer progression[27]. Some antitumor reagents mediated anti-proliferative signaling mechanism also involves up-regulation of p27[28,29]. However, its upstream regulation in prostate cancer and its role in prostate CSC are not quite clear. Some recent studies reported that several onco-miRNA

can downregulate p27 expression and promote cancer development. For example, increased miR-429 expression can promote human prostate cancer cell proliferation by targeting p27[30]. MiR-148a can promote gastric cancer cell proliferation by targeting p27[31]. MiR-181b is elevated in the serum of cirrhosis patients and can promote hepatic stellate cells proliferation by targeting p27[32]. In this study, by using dual luciferase assay and western blot analysis, we verified that miR-150 can directly target p27 and suppress its expression. By suppressing p27 expression, miR-150 increased proliferation of DU145 cells. In addition, we also observed that via decreasing p27 expression, miR-150 enhanced CSC properties of the cancer cells, including tumor sphere number and size, as well as the CSC markers including Oct4, Nestin and Nanog.

Conclusions

Taken together, we demonstrated that increased miR-150 expression may participate in the development and progression of human prostate CSC by suppressing p27. This supported our previous study which found miR-150 is positively correlated with prostate tumor recurrence or metastasis.

Conflict of interest

The Authors declare that they have no conflict of interests.

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