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MiR-150 promotes the cell invasion of prostate cancer cells by directly regulating the expression of p53

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Received April 28, 2016, accepted May 27, 2016

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Pharmazie 71: 533–536 (2016) *doi: 10.1691/ph.2016.6645*

Therapeutic targeting of p53 has been implicated as a promising strategy for cancer treatment. MiRNAs are emerging as important modulators of invasion, regulation of p53. Resent reports have shown that miR-150 is involved in the growth, invasion and metastasis in numerous tumor types. However, the role of miR-150 in prostate cancer pathology is unclear. In this study, we firstly determined the miR-150 expression levels of prostate cancer cell lines by quantitative real-time PCR (qRT-PCR). The effects of miR-150 on prostate cancer cell proliferation and invasion were evaluated using MTT, colony and transwell assays. The target of miR-150 was identified and confirmed using a luciferase activity assay. The results revealed that miR-150 was significantly upregulated in prostate cancer cells compared with RWPE-1 normal prostate epithelial cells. The ectopic expression of miR-150 significantly promoted prostate cancer cell proliferation, colony formation and invasion. In addition, p53 was confirmed as a downstream target of miR-150 in the prostate cancer cells by western blot and qRT-PCR analysis as well as luciferase activity assays. Together, these findings show that miR-150 promotes prostate cancer cell proliferation and invasion by targeting p53, suggesting that targeting miR-150 may be a potential therapeutic strategy in prostate cancer patients.

1. Introduction

The incidence of prostate cancer steadily increases in the People's Republic of China, especially in some developed areas such as Shanghai with an annual increase of 8% and is the fifth most common male malignancy in this region after 2010 (Zhu et al. 2016). The tumorigenesis of prostate cancer is a complicated process that involves the deregulation of a variety of genes. Distant metastases are responsible for the failure of prostate cancer therapy and the poor prognosis of prostate cancer (Zhang et al. 2016). Therefore, investigation of the underlying molecular mechanism of prostate cancer progression is required.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs, which negatively regulate the expression of downstream genes through direct interaction with the 3'-untranslated region (3'-UTR) of the corresponding target mRNAs (Peng et al. 2015). MiRNAs are crucial for the proliferation, migration, invasion and metabolism of different types of tumor cells including liver, breast, lung, ovarium and prostate cancer (Xu et al. 2016; Abba et al. 2016). MiRNAs could function as oncogenes or tumor suppressor genes, depending on the target genes regulated. MiRNAs that are overexpressed in many tumors often target tumor suppressors associated with various steps of the tumor progression; thus, identifying these miRNAs may be vital in the management of disease.

Functional miR-150 is 22 nucleotides long, and its gene is located at chromosome 19q13.33 (Sun et al. 2015). It has been reported that miR-150 expression regulation provides a promising novel candidate used as a tumor biomarker, targeted therapeutic strategy and index of prognosis in cancer. MiR-150 promotes the proliferation of gastric cancer and lung cancer cells through negative regulation of the pro-apoptotic gene early growth response factor 2 (EGR2) and by targeting p53, respectively, suggesting its pro-tumorigenic function (Wu et al. 2010). *Xie et al. (2010)* demonstrated a negative correlation between the expression levels of miR-150 and p53 following treatment of K562 cells with cisplatin, indicating that cisplatin induced apoptosis in the K562 cells by inhibiting miR-150 expression, which then upregulated p53 expression. The biological role of miR-150 in prostate cancer remains unclear. To date, however, the mechanism behind the involvement of the miRNA-dependent p53 pathway in the prostate cancer has not been investigated. The aim of the present study was to investigate the biological role and clinical significance of miR-150 in prostate cancer in order to explore its potential application as a prognostic marker and therapeutic target for prostate cancer patients.

2. Investigations and results

2.1. *MiR-150 is significantly up-regulated in prostate cancer cell lines*

MiR-150 expression levels were measured in the prostate cancer cell lines LNCap, PC3 and DU145 as well as the normal prostate epithelial cells RWPE-1. MiR-150 was significantly overexpressed in the prostate cancer cell lines compared with the normal prostate epithelial cell line. Of all three prostate cancer cell lines, DU145 possessed the highest levels of miR-150 expression and PC3 showed the lowest expression of miR-150 (Fig. 1).

2.2. *MiR-150 promotes proliferation in prostate cancer cells*

As shown in Fig. 2, PC3 cells were transfected with miR-150 mimics to overexpress miR-150. We found that the expression of miR-150 significantly increased PC3 cell viability (p<0.01). In addition, miR-150 expression could markedly increase the numbers of PC3 cell colonies formed compared with the negative control (p<0.01).

2.3. *MiR-150 promotes invasion and migration in prostate cancer cells*

As miR-150 is upregulated in prostate cancer cell lines, miR-150 may function as a tumor promoter in prostate cancer. PC3 cells

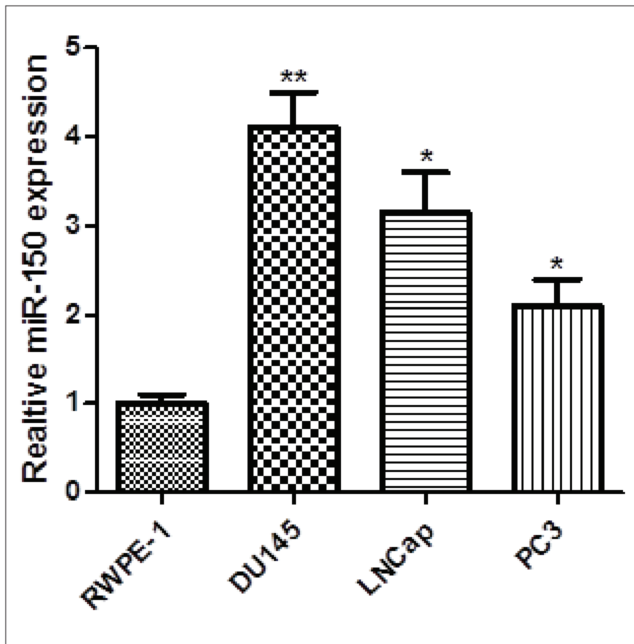

Fig. 1: MiR-150 mRNA is overexpressed in PC cell lines. Expression of miR-150 mRNA in PC cell lines (DU145, PC-3, LNCaP) and RWPE-1 were examined by quantitative RT-PCR (*p<0.05, **p<0.01).

were transfected with miR-150 mimics to overexpress miR-150. To assess the effect of miR-150 on the invasive and migrant abilities of prostate cancer cells, a Transwell assay was performed. Overexpression of miR-150 significantly promoted the invasive and migrant abilities of the PC3 cells (Fig. 3).

2.4. *P53 is a direct downstream target of miR-150 in prostate cancer cells*

To determine how the downregulation in miR-150 expression might promote tumor progression, western blot and qPCR analyses were performed after 24 h post-transfection (Figs. 3B, C). The enforced expression of hsa-miR-150 led to a marked decrease in the expression levels of endogenous p53 mRNA and protein compared to PC12 cells transfected with NC.

To check whether a direct interaction is involved between miR-150 and its target oncogene p53, we performed luciferase reporter assays (Fig. 3D). We found that co-transfection of miR-150 along with the wild type 3'UTR of p53 caused a significant decrease in luciferase activity compared to controls.

3. Discussion

MiRNAs are implicated in the post-transcriptional regulation of gene expression in diverse cellular processes. Some miRNAs have been found to be frequently dysregulated in prostate cancer progression by regulating cell proliferation, apoptosis, migration and invasion, and certain miRNAs have been reported to be correlated with clinical characteristics and outcome (Chen et al. 2008). MiRNAs have shown to be potential regulators of cancer metastasis. In addition, recent work has shown that miRNAs are closely linked to tumor progression and metastasis (Subramani et al. 2013). While we were unable to validate the role of miRNAs as a prognostic biomarker in prostate cancer, we demonstrated several novel findings regarding their role in prostate cancer progression. Thus, identifying the specific miRNAs involved in prostate cancer progression may provide novel therapeutic targets in prostate cancer treatment.

In our study, we examined the expression levels of miR-150 in prostate cancer cells and the normal prostate epithelial cells. Compared to the normal prostate epithelial cells, miR-150 expression was significantly upregulated in the prostate cancer cells. The abnormal expression of miRNAs in prostate cancer has been previously evalu-

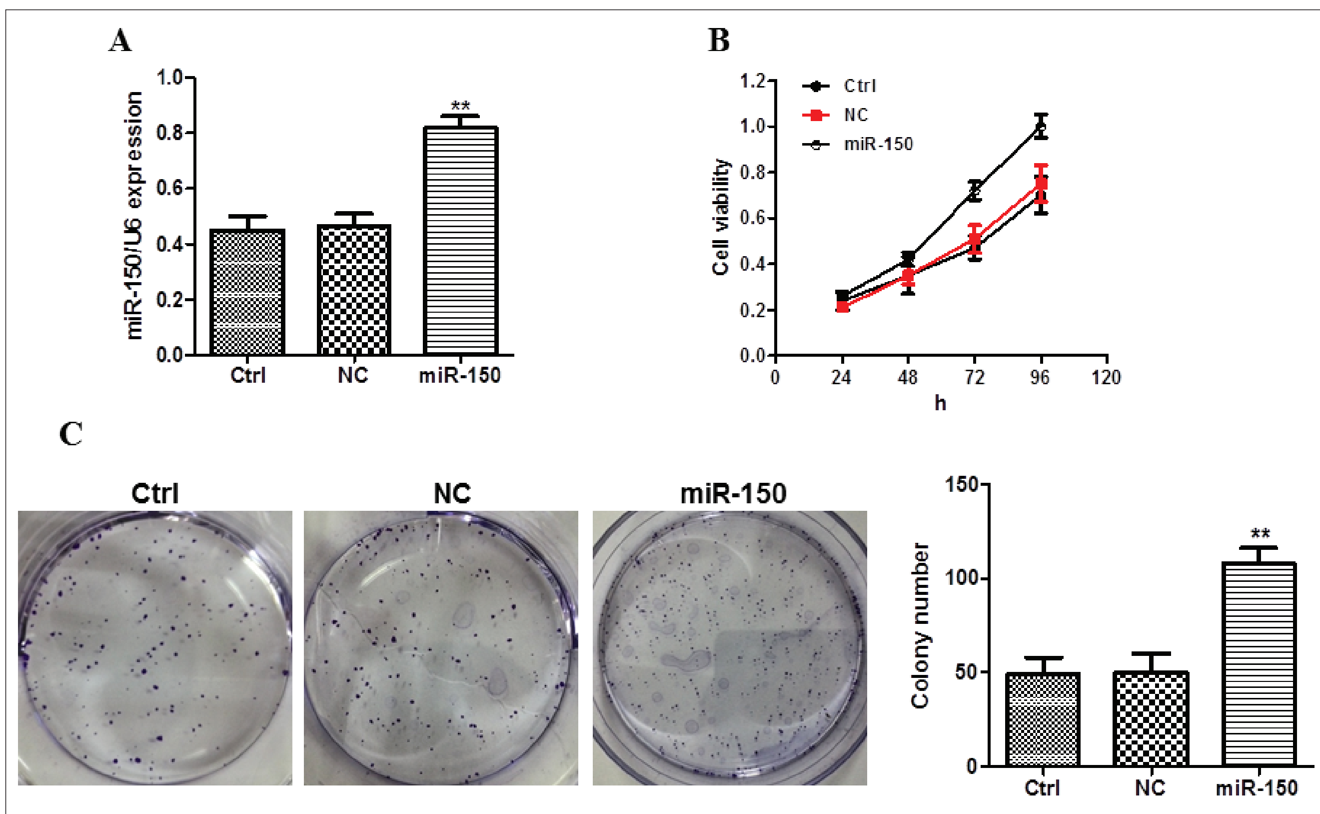

Fig. 2: Ectopic miR-150 expression in PC3 prostate cancer cells activities cell growth, colony formation. (A) The efficiency of transfection was confirmed by reverse transcription quantitative polymerase chain reaction (*p<0.01). (B) Ectopic miR-150 expression significantly promoted cell viability, as demonstrated by MTT assay. (C) Ectopic miR-150 expression significantly increased the cell colony formation numbers (*p<0.01).

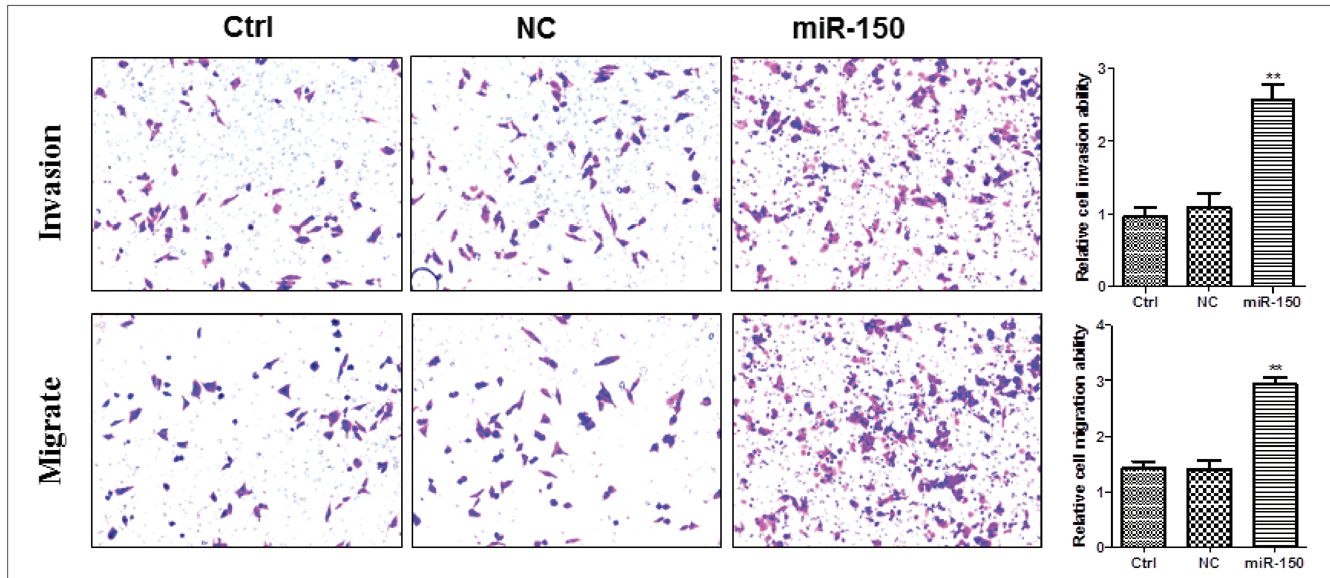

Fig. 3: Ectopic miR-150 expression in PC3 prostate cancer cells promotes cell invasion and migrate. (A) Ectopic miR-150 expression significantly stimulated cell invasiveness (**p*<0.01). (B) Ectopic miR-150 expression significantly stimulated cell migration (**p*<0.05).

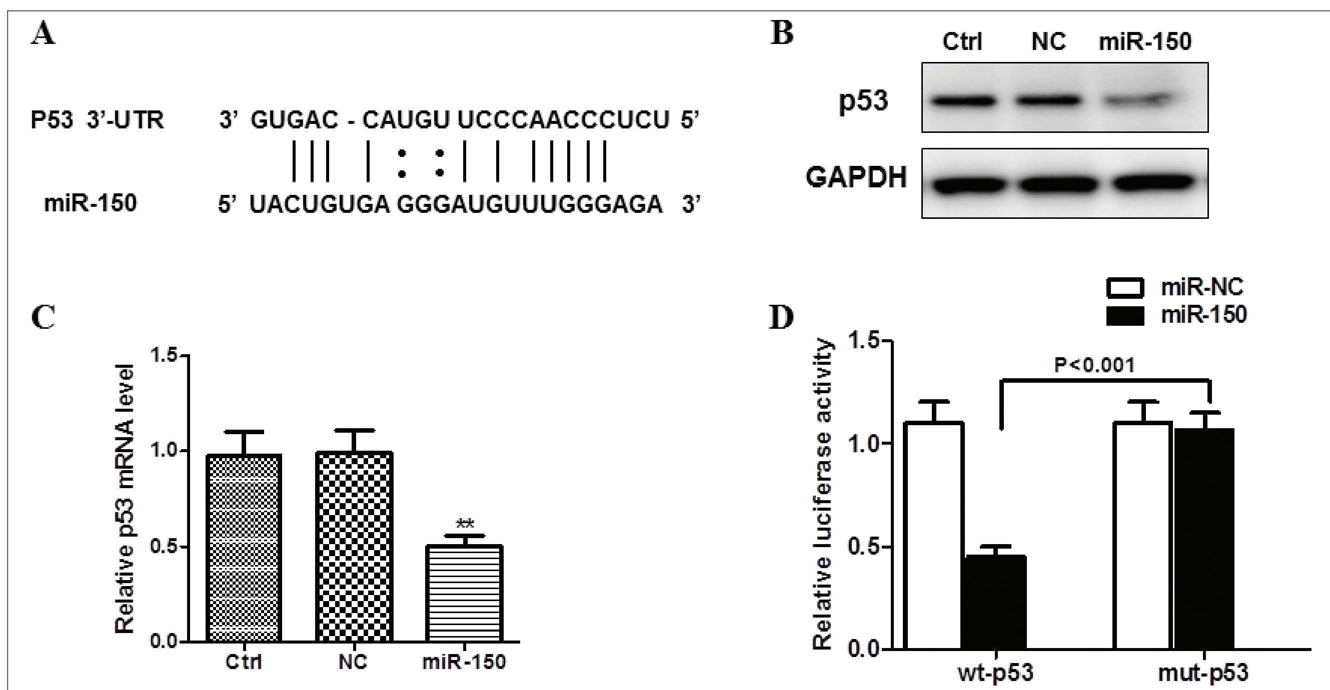

Fig. 4: Ectopic miR-150 expression in PC3 prostate cancer cells promotes cell invasion and migrate. (A) Ectopic miR-150 expression significantly stimulated cell invasiveness (**p*<0.01). (B) Ectopic miR-150 expression significantly stimulated cell migration (**p*<0.05).

ated (Song et al. 2015). Thus, we hypothesized that miR-150 may function as a tumor promoter. Next, we transfected miR-150 mimics into PC3 cells to induce overexpression. Exogenous overexpression of miR-150 significantly promoted the cell proliferation and colony formation ability of PC3 cells. Using the transwell migration assay, we found that the overexpression of miR-150 in prostate cancer cells could activate their invasion and migratory ability.

To ascertain why miR-150 exhibited these effects on cell function, we investigated putative targets of miR-150 and identified p53, which has been reported to play a very important role in many cancers. To confirm whether p53 was a real target of miR-150, a luciferase activity assay was performed which revealed that miR-150 overexpression reduced p53 3'-UTR wt luciferase activity, but not that of the mt. Both p53 mRNA and protein levels decreased after transfection of PC3 cells with miR-153. Together, these data indicate that miR-150 directly interacts with p53 mRNA and suppresses p53 protein expression. These findings support the hypothesis that p53 is a new target of miR-150 in prostate cancer cells.

In conclusion, the present study demonstrated that miR-150 is significantly upregulated in prostate cancer cell lines. MiR-150 expression levels were also associated with invasive and proliferative parameters in the prostate cancer cell line. Ectopic miR-150 expression resulted in promotion of prostate cancer cell proliferation, colony formation and invasion. Further investigation revealed that p53 may be a target of miR-150. Therefore, miR-150 may serve as a predictor for prognosis and as a therapeutic target in prostate cancer patients.

4. Experimental

4.1. Cell culture

RWPE-1 normal prostate epithelial cells, and LNCap, PC3 and DU145 prostate cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured according to the manufacturer's instructions. All transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

4.2. RNA isolation and mRNA expression analysis

For the RT-PCR, the total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. MiR-150 expression levels were measured using a mirVana qRT-PCR miRNA Detection kit (Ambion Inc., Austin, TX, USA). U6 small RNA served as an internal reference. And the p53 mRNA expression levels were normalized to GAPDH served as an endogenous control. Their relative expression levels were measured using a Prism 7500 Real-Time PCR monitor (Applied Biosystems). The sequences of the primers were as follows (Jin et al. 2014): p53 forward, 5'-CAC GTA CTC TCC TCC CCT CA-3' and reverse, 5'-CTT CTG TAC GGC GGT CTC TC-3'; and GAPDH forward, 5'-ATG GGG AAG GTG AAG GTC G-3' and reverse, 5'-TAA AAG CAG CCC TGG TGA CC-3'; miR-150 forward, 5'-CAG TAT TCT CTC CCA ACC CTT GTA-3' and reverse 5'-AAT GGA TGA TCT CGT CAG TCT GTT-3', U6 forward, 5'-CTC GCT TCG GCA GCACA-3', and U6 reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'.

4.3. Western blot analysis

The cells were washed twice with ice PBS and lysed using RIPA buffer. Samples of proteins (50 μg) were separated using SDS-PAGE and transferred to the PVDF membranes (Sterlitech Corporation, Kent, WA, USA). The membranes were then blocked with 5% skimmed milk for 1 h at room temperature and then incubated with specific antibodies against miR-150, p53 and GAPDH in 1:1,000 dilution overnight at 4 °C. Following three washes with TBST, the membranes were incubated with a goat anti-rabbit secondary antibody (Boster Systems, Inc., Pleasanton, CA, USA) for 1 h. The bands were then visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Amersham, UK) following the manufacturer's instructions.

4.4. Oligonucleotide transfection

The hsa-miR-150 mimic and negative control (NC) oligonucleotides were obtained from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). PC3 cells were harvested and plated in a six-well plate with 70-80% confluence the day prior to transfection. The hsa-miR-150 mimic or NC (50 nmol/l) was transfected using Lipofectamine 2000. After 24 h, the cells were collected to perform in vitro assays.

4.5. Cell proliferation assay

Cell proliferation was measured by using MTT assay. At 24 h post-transfection, the cells were plated into 96-well plates at a density of $2x10^3$cells/well. The viability of the cells was examined by MTT assay (Sigma-Aldrich). Five independent experiments were performed.

4.6. Colony formation assay

For the colony formation assay, 500 transfected cells were placed in a six-well plate and cultured for 14 days using RPMI 1640 medium (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum.
Cell colonies were fixed with methanol and then stained with 0.1% crystal violet. Colonies were observed using a microscope and colonies.

4.7. In vitro migration assay

The migration ability of PC3 cells transfected with hsa-miR-150 vector and NC vector was tested in Matrigel coated cell culture chambers (8-μm pore; BD Biosciences, Franklin Lakes, NJ, USA). PC3 cells were transfected and cultured to confluence or near (90%) confluence in 24-well dishes. Then, PC3 cells were resuspended in 200 ml serum-free 1640 medium were placed into the upper chamber of the insert with Matrigel. Medium with 5% FBS was added into the lower chambers as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were carefully removed. Cells that had invaded through the membrane were manually counted at 200x magnification from ten different fields of each filter. The mean of triplicate assays for each experimental condition was analyzed.

4.8. In vitro invasion assay

The invasion ability of PC3 cells transfected with hsa-miR-150 vector and NC vector was tested in Matrigel coated cell culture chambers (8-μm pore; BD Biosciences, Franklin Lakes, NJ, USA). PC3 cells were transfected and cultured to confluence or near (90%) confluence in 24-well dishes. Then, PC3 cells were resuspended in 200 ml serum-free 1640 medium and were placed into the upper chamber of the insert with Matrigel. Medium with 5% FBS was added into the lower chambers as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane were carefully removed. Cells that had invaded through the membrane were manually counted at 200x magnification from ten different fields of each filter. The mean of triplicate assays for each experimental condition was analyzed.

4.9. Vector construction and dual-luciferase reporter assay

To analyze the underlying mechanism of miR-150 in prostate cancer, TargetScan (www.targetscan.org) and miRanda (www.microrna.org) were used to search for potential targets of miR-150 in prostate cancer. Luciferase assays. The 3'-UTR of p53 and a mutant reporter (mut-p53), in which the predicted miR-150 binding site on p53 was mutated, was cloned into luciferase reporters and co-transfected with either a miR-150 mimic or a control. Twenty-four h afer transfection, luciferase activities were measured by a dual-luciferase assay (Promega, USA).

4.10. Statistical analysis

The data are expressed as the mean ± SEM at least three independent determinations. Statistical differences between groups were determined by one-way ANOVA by using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, United States). $P<0.05$ was considered to indicate a statistically significant difference.

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