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# Inhibition of invasion and migration of prostate cancer cells by *miRNA-509-5p* via targeting *MDM2*

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**ABSTRACT.** Prostate cancer is a common malignancy of the male reproductive-urinary system. *MDM2* is an oncogene, whose expression can be regulated by microRNA (miRNA). The present study investigated the expression and correlation of *miRNA-509-5p* and *MDM2* to determine the mechanism of their function in invasion and migration of prostate cancer cells. RT-PCR was performed to detect the expression of *miRNA-509-5p* and *MDM2* in tumor, tumor-adjacent, and normal tissues, obtained from prostate cancer patients, using the HGC-27 cell line as an *in vitro* model. Cultured HGC-27 cells were transfected with *miRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, and mimic control. Expression levels of *miRNA-509-5p* and *MDM2* were quantified by RT-PCR. Cell proliferation and invasion/migration were examined by the MTT and transwell assays, respectively. *MiRNA-509-5p* was

significantly down-regulated in prostate cancer cells exhibiting high *MDM2* mRNA levels. MiRNA mimic transfection elevated miRNA levels and suppressed *MDM2* expression. With prolonged incubation time, the proliferation ratio and OD values of *miRNA-509-5p* mimic transfected cells decreased, along with decrease in cell migration and invasion. These results suggested that *miRNA-509-5p* negatively regulates *MDM2* expression via targeting the 3'-UTR of genes. As a novel tumor suppressor, *miRNA-509-5p* in prostate cancer HGC-27 cells can suppress *MDM2* expression and inhibit cell proliferation, invasion, and migration. Therefore, *miRNA-509-5p* could be used as a novel therapeutic agent in the treatment of prostate cancer.

**Key words:** *MicroRNA-509-5p*; *MDM2*; Prostate cancer; Cell proliferation; Cell invasion; Migration

## INTRODUCTION

Prostate cancer is the most common male-specific cancer and majorly responsible for cancer-related mortality in men. The incidence of prostate cancer depends on the geographic location and ethnicity of individuals. Susceptibility to the disease has been observed to be higher in men in Western countries than in China. However, due to population aging and life style transitions, the incidence of prostate cancer in China has been increasing rapidly (Ferlay et al., 2010; Jemal et al., 2011). Some studies have reported the association of prostate cancer occurrence and progression with oncogene/tumor suppressor gene imbalances. Increase in cell proliferation and decrease in apoptosis levels contribute to increased tumor cell numbers, leading to the occurrence of prostate cancer (Siegel et al., 2012).

MicroRNA (miRNA), a type of non-coding single stranded RNA with 19~25 nucleotide acids, is widely distributed in eukaryotes and regulates the occurrence and progression of malignant tumors via negative regulation of gene expression at the post-transcriptional level (Ding et al., 2010; Lovat et al., 2011). In 1991, Fakharzadeh et al. (1991) purified *MDM-1*, *MDM-2*, and *MDM3* for the first time. They observed that over-expression of only *MDM-2* led to tumor formation in 100% of nude mice, suggesting that *MDM2* worked as an oncogene to facilitate the malignant transformation of cells. The overexpression of *MDM2* has been observed in sarcomas and prostate, blood, breast, and colon cancers (Weng et al., 2005; Liu et al., 2008; Carry and Garcia-Echeverria, 2013). Therefore, this study analyzed the expression of *miRNA-509-5p* and *MDM2* in the prostate cancer cell line HGC-27 to reveal the effect of *miRNA-509-5p* on invasion and migration of prostate cancer cells and illustrate the related mechanisms.

## MATERIAL AND METHODS

### General information

A total of 20 prostate cancer patients, who were admitted to the Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital for surgery from May 2014 to May 2016, were recruited for this study. Prostate cancer diagnosis had been confirmed

by pathological examination in all patients. There were 5 patients at stage I, 9 at stage II, and 6 at stage III of prostate cancer. Based on the tumor differentiation grade, there were 11 high-grade, 7 moderate-grade, and 2 low-grade cases. The patient age ranged from 19 to 62 years with an average age of 43.1 ± 6.2 years. Prostate cancer tissue specimens, after biopsy, were stored at -70°C for further use. No patient had received chemo- or radio-therapy before surgery. Another cohort of 20 patients with benign prostate disease, admitted to Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital for biopsy or surgery, were recruited as the control group (aged between 28 to 55 years, average = 40.8 ± 5.3 years). There were no significant differences based on age among the patients ($P > 0.05$).

The study protocol was approved by the Research Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. All patients provided informed consent before study commencement.

## Cells and reagent

The cells and reagents used were as follows: Human prostate cancer cell line HGC-27 (Department of Cell Biology, Chinese Medical University); *MiRNA-509-9p* mimics and inhibitor (Gene Pharma, Shanghai, China); Lipofectamine TM 2000 (Invitrogen, Waltham, MA, USA); *MiRNA-509-5p*, *MDM2*, *β-actin*, and RT-PCR test kit (Takara Shuzo, Kusatsu, Shiga, Japan); PCR cycler PTC-100TM (MJ Research Inc., San Fransisco, CA, USA,); Trizol reagent (Gibco BRL, Grand Island, NY, USA); and RPMI 1640 medium and MTT (Sigma, St. Louis, MO, USA).

## RT-PCR in tissues

The expression of *miRNA-509-5p* and *MDM2* in prostate cancer tumor tissues (N = 20), tumor-adjacent tissues (N = 20), and normal prostate tissues (N = 20) were detected by RT-PCR. Briefly, mRNA was extracted from patients' prostate tissues using the Trizol kit as per the manufacturer's instructions. The total RNA concentration was determined by the D260 nm/D280 nm ratio. RNA (200 ng) was used to synthesize cDNA based on its polyA ends. The cDNA product was amplified by PCR using specifically designed primers (Table 1). In a 20-μL reverse transcription reaction system, 2 μL RNA, 3 μL cDNA, 0.2 μL Taq DNA polymerase, and 1 μL primer were added for PCR amplification. The PCR conditions were as follows: pre-denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min, ending with 72°C for 5 min. After gel electrophoresis and scanning, PCR products were analyzed to obtain relative values against *β-actin*. Quantity One software was used to calculate the optical densities of all test groups, while the internal reference gene was used to obtain their proliferation ratios.

**Table 1.** Primer design.

| Target gene | Sense primer | Anti-sense primer |
|---|---|---|
| *miR-509-5p* | 5'-AAATCACCACCTTCACAGCC-3' | 5'-GTTGTAATGGTTCTCCTCCAGC-3' |
| *MDM2* | 5'-ATGGCAGCCGGG AGCATCACC-3' | 5'-CACACACTCCTTTGATAGACACAA-3' |
| *β-actin* | 5'-GAAACTACCTTCAACTCCATC-3' | 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3' |

## Cell culture and transfection

Human prostate cancer cell line HGC-27 was inoculated in RPMI 1640 medium and incubated in a 37°C chamber with 5% $CO_2$. Cells to be transfected were then transferred to Dulbecco's modified Eagle's complete medium, containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin, and incubated in a 37°C chamber with 5% $CO_2$. Tumor cells were then inoculated in a cell culture plate and incubated overnight. When the growth of the culture was close to 60% confluence, cells were transfected with *miRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, or mimic control by Lipofectamine 2000. The culture medium was replaced after 4~6 h. The cells were used for further assay after incubation for 48 h. Simultaneous control procedures were performed using un-transfected HGC-27 cells.

## RT-PCR in HGC-27 cells

Total RNA was extracted from the cells using Trizol reagent, 48 h after transfecting them with *miRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, or mimic control. RNA concentration was detected and then quantified using the D260 nm/D280 nm ratio. Total RNA (200 ng) was used to synthesize cDNA based on its polyA tails. PCR amplification was performed using the cDNA product as template and designed primers (*miRNA-509-5p* - forward: 5'-AGCCG TCAAG AGCAA TAACG AA-3'; *miRNA-509-5p* - reverse: 5'-GTGCA GGGTC CGAGG T-3'; *MDM2* - forward: 5'-TGGTG GGCAG TATGT TGT-3'; *MDM2* - reverse: 5'-GCTAT TGGCA TTGGT GAA-3'; *U6* - forward: 5'-CTCGC TTCGG CAGCA CA-3'; *U6* - reverse: 5'-AACGC TTCAC GAATT TGCGT-3'). PCR conditions were as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. *U6* was used as the internal reference control.

## MTT assay

Cells transfected with *MiRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, or mimic control along with blank control cells at log phase were adjusted to a density of 8 x $10^4$ cells/mL and seeded into a 12-well plate, which was incubated at 37°C with 5% $CO_2$. Cell viability was monitored continuously for 24, 48, and 72 h to ensure that the cells grew while attached to the well. MTT solution (5 mg/mL) was then added and the plate was incubated for 4 h. After quenching the reaction, 150 μL dimethyl sulfoxide was added to each well, and the plate was vortexed for 10 min to dissolve crystals. Values obtained on measuring the absorbance (A) at 570 nm by the enzyme-linked immunosorbent assay apparatus were used to plot the growth curve.

## Transwell assay

### *Invasion assay*

Matrigel, an artificial basement membrane, was added to the transwell chamber and the membrane was kept at 4°C overnight. The chamber was first hydrated using a serum-free medium for 1 h at 37°C. After transfection, the cells were seeded into the upper compartment, while the bottom was filled with RPMI 1640 medium. Giemsa staining was performed to observe cell morphology and for cell quantification.

### *Migration assay*

Cells were directly added into the chamber without prior addition of the artificial basal membrane.

### *Luciferase reporter gene*

The 3'-untranslated region (3'-UTR) of *MDM2* containing the *miRNA-509-5p* binding sites was amplified by PCR, and the products were inserted into control vectors. The amplification specificity can be demonstrated by control vectors carrying mutated *MDM2* 3'-UTR. Co-transfection of target vectors and *miRNA-509-5p* was performed as per the manufacturer instructions accompanying Lipofectamine TM 2000. Dual luciferase reporter gene assay was employed as per the manufacturer's instructions, 24 h after HGC-7 cell transfection, to determine the gene expression. Three replicates of this assay were performed.

## Statistical analysis

SPSS 17.0 software was used for statistical analysis. All data are presented as means ± SD. Calculated data were tested by chi square test. Analysis of variance was used to analyze the measured data. Values obtained were significant when $P < 0.05$.

## RESULTS

## *MiRNA-509-5p* and *MDM2* levels in tumor tissues

Using RT-PCR, we detected levels of *miRNA-509-5p* and *MDM2* in prostate cancer tumor tissues, tumor-adjacent tissues, and normal prostate tissues. Results showed significantly reduced *miRNA-509-5p* levels and elevated *MDM2* levels in prostate carcinoma tissues than in tumor-adjacent tissues or normal prostate tissues ($P < 0.05$). No significant difference has been detected between tumor-adjacent and normal prostate tissues (Table 2, Figure 1). This data suggested that *miRNA-509-5p* might be involved in the pathogenesis of prostate cancer.

**Table 2.** *MiRNA-509-5p* and *MDM2* mRNA levels.

| Group | N | *miRNA-509-5p* | *MDM2* mRNA |
|---|---|---|---|
| Prostate carcinoma | 20 | 0.314 ± 0.048*# | 1.419 ± 0.075*# |
| Tumor-adjacent | 20 | 1.606 ± 0.012 | 0.608 ± 0.015 |
| Normal prostate | 20 | 1.532 ± 0.015 | 0.545 ± 0.013 |

*$P < 0.05$ compared to tumor adjacent tissues; #$P < 0.05$ compared to normal prostate tissues.

## Effect of *miRNA-509-5p* on *MDM2* expression and proliferation of HGC-27 cell line

RT-PCR revealed significantly elevated *miRNA-509-5p* levels and reduced *MDM2* levels in HGC-27 cells transfected with *miRNA-509-5p* mimics ($P < 0.05$) compared to cells transfected with *miRNA-509-5p* inhibitor or mimic control. With increasing culture time, *miRNA-509-5p* levels gradually increased, while *MDM2* levels decreased in the *miRNA-509-5p* mimic transfection group ($P < 0.05$). In cells transfected with *miRNA-509-5p* inhibitor, *miRNA-509-5p* levels were reduced, while *MDM2* levels were elevated ($P < 0.05$, Table 3).

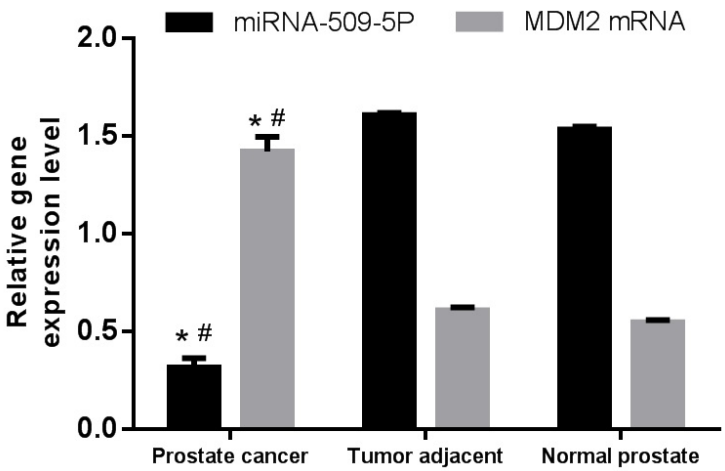


**Figure 1.** Expression of *miRNA-509-5p* and *MDM2* in prostate cancer tissues (N = 20). *P < 0.05 compared to tumor-adjacent tissues (N = 20); #P < 0.05 compared to normal prostate tissues (N = 20). Data are reported as means ± SD.

**Table 3.** *MiRNA-509-5p* and *MDM2* mRNA levels in HGC-27 cell line.

| Gene | *miR-509-5p* mimic | *miR-509-5p* inhibitor | Mimic control | Blank control |
|---|---|---|---|---|
| *miR-509-5p* | | | | |
| 24 h | 1.205 ± 0.027[ab] | 0.171 ± 0.007[abc] | 0.602 ± 0.010 | 0.523 ± 0.006 |
| 48 h | 1.602 ± 0.041[abd] | 0.026 ± 0.002[abcd] | 0.610 ± 0.011 | 0.536 ± 0.008 |
| *MDM2* mRNA | | | | |
| 24 h | 1.061 ± 0.016[ab] | 1.384 ± 0.076[abc] | 1.720 ± 0.070 | 1.765 ± 0.081 |
| 48 h | 0.812 ± 0.007[abd] | 1.872 ± 0.085[abcd] | 1.700 ± 0.067 | 1.674 ± 0.068 |

[a]P < 0.05 compared to mimic control group; [b]P < 0.05 compared to blank control group; [c]P < 0.05 compared to *miR-509-5p* mimics group.

To evaluate the role of *miRNA-509-5p* on the proliferation of prostate cancer cells, HGC-27 cells were transfected with *miRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, or mimic control followed by MTT assay analysis. We observed remarkably decreased OD values and proliferation ratios in the *miRNA-509-5p* mimic group compared to those in the *miRNA-509-5p* inhibitor, mimic control, or blank control groups. With increasing incubation time, the proliferation ratio and OD values gradually decreased in the *miRNA-509-5p* mimics group and increased in the *miRNA-509-5p* inhibitor, mimic control, and blank control groups (P < 0.05, Table 4).

## Invasion and migration of HGC-27 cells

To evaluate whether *miRNA-509-5p* affects the HGC-27 cell invasion and migration abilities, the transwell assay was performed. Results showed that cells transfected with *miRNA-509-5p* inhibitor or mimic control possessed significantly effective abilities of invasion and migration, while those transfected with *miRNA-509-5p* mimic had lower invasion and migration abilities (P < 0.05, Figure 2). Therefore, *miRNA-509-5p* was observed to cause a decrease in cell invasion and migration.

**Table 4.** Proliferation activity of human HGC-27 cells after transfection.

| Index | *miR-509-5p* mimic | *miR-509-5p* inhibitor | Mimic control | Blank control |
|---|---|---|---|---|
| OD value | | | | |
| 24 h | $0.781 \pm 0.011^{ab}$ | $1.029 \pm 0.018^{abc}$ | $1.010 \pm 0.012$ | $1.001 \pm 0.016$ |
| 48 h | $0.392 \pm 0.011^{abd}$ | $1.214 \pm 0.042^{abcd}$ | $1.120 \pm 0.031^{d}$ | $1.283 \pm 0.030^{d}$ |
| Proliferation rate (%) | | | | |
| 24 h | $109^{ab}$ | $128^{abc}$ | 123 | 120 |
| 48 h | $82^{abd}$ | $169^{abcd}$ | $157^{d}$ | $168^{d}$ |

$^{a}P < 0.05$ compared to mimic control group; $^{b}P < 0.05$ compared to blank control group; $^{c}P < 0.05$ compared to *miR-509-5p* mimics group; $^{d}P < 0.05$ compared to 24 h.

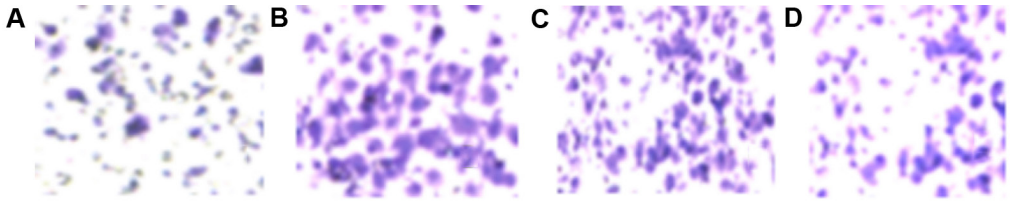


**Figure 2.** Invasion and migration of HGC-27 cells. **A.** *miR-509-5p* mimic, **B.** *miR-509-5p* inhibitor, **C.** mimic control, **D.** blank control.

## Targeted interaction of *miRNA-509-5p* with the 3'-UTR of *MDM*

To investigate whether *MDM* was a target gene of miRNA-509-5p, the luciferase reporter assay was performed. We observed decreased luciferase activity in *MDM2* 3'-UTR and *miRNA-509-5p* co-transfected HGC-27 cells ($P < 0.05$). Co-transfection with *MDM2* 3'-UTR and *miRNA-509-5p* bearing mutated binding sites resulted in significantly elevated luciferase activity, indicating obstruction in the binding of *miRNA-509-5p* to 3'-UTR of *MDM2* (Figure 3) Therefore, *MDM* is a potential target gene of *miRNA-509-5p*.

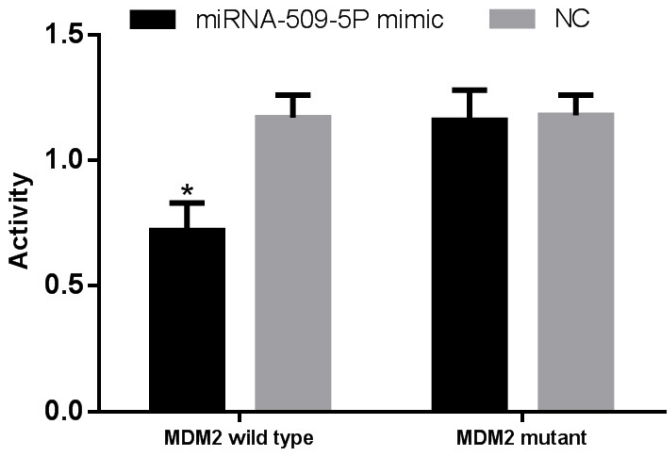


**Figure 3.** Targeted action of *miR-509-5p* on 3'-UTR of *MDM2* by luciferase assay. *$P < 0.05$ compared to control (NC) group. Data are reported as means ± SD. At least three replicates of the assay were performed.

## DISCUSSION

As the most common malignancy in male-specific urinary and reproductive systems, prostate cancer ranks fifth among all cancer incidences worldwide, with regional and ethical group-specific distribution patterns (Shen and Abate-Shen, 2010). In developing countries, the cumulative incidence of prostate cancer in males aged between 0 and 74 years is 1.4% (6th most common cancer). In developed countries, the rate of incidence is 7.8%, making prostate cancer the leading cause of cancer death (Center et al., 2012). Both occurrence and progression of prostate cancer can be regulated by multiple factors.

MiRNA is a type of single-stranded RNA that can regulate gene expression. One miRNA molecule can modulate the transcription of hundreds of mRNAs (Farh et al., 2005). MiRNA participates in various physiological and pathological processes for regulating gene expression and affecting cell proliferation and apoptosis (Farazi et al., 2011). *MDM2* is over-expressed in various cancers such as cancers of the prostate, blood, breast, and colon (Popowicz et al., 2010; Wade et al., 2010; Rew and Sun, 2014). *MDM2* is closely associated with the pathogenesis and progression of prostate cancer as it can transform cells in the prostate for the generation of xenograft tumors (Ma et al., 2014).

We recruited prostate cancer patients, who had undergone surgeries for the disease in Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, for this study. RT-PCR was used to detect the *miRNA-509-5p* and *MDM2* mRNA levels in prostate cancer tumor, tumor-adjacent, and normal prostate tissues. Results showed significantly lower *miRNA-509-5p* levels and higher *MDM2* mRNA levels in prostate cancer tissues compared to those in tumor-adjacent or normal prostate tissues. These results indicated that there is down-regulation of *miRNA-509-5p* and over-expression of *MDM2* in prostate cancer patients. MiRNA participates in the expression of various genes such as those of cell growth, differentiation, and apoptosis. Therefore, the expression profile of miRNA can reflect the state of progression and differentiation of tumors. Certain miRNAs can work as specific markers for prostate cancer by contributing towards the deduction of its classification, tumor grade, or differential diagnosis against benign prostate disease (Peter, 2009; Donnem et al., 2012). *MDM2* was initially cloned from BALB/3T3 cells, and is highly expressed in pulmonary, breast, and colon cancers (Okoro et al., 2013).

To further study the mechanisms of *miRNA-509-5p* and *MDM2* in prostate cancer and the correlation between them, we selected the prostate cancer cell line HGC-27 and transfected the cells with *miRNA-509-5p* mimics, *miRNA-509-5p* inhibitor, or mimic control. RT-PCR results showed lower *miRNA-509-5p* levels and elevated *MDM2* mRNA levels in *miRNA-509-5p* inhibitor transfected cells. In cells transfected with the *miRNA-509-5p* mimic, *miRNA-509-5p* levels were elevated, while *MDM2* mRNA levels were reduced. With increasing incubation time, *miRNA-509-5p* levels gradually increased, while *MDM2* mRNA levels decreased. These results indicate that *MDM2* was down-regulated in HGC-27 cells after transfection with *miRNA-509-5p*. Therefore, *MDM2* might be a target gene of *miRNA-509-5p*.

To investigate the role of *miRNA-509-5p* and *MDM2* in proliferation, invasion, and migration of HGC-27 cells, we used the MTT assay. Results showed a gradual increase in the OD values and proliferation ratio in cells transfected with the *miRNA-509-5p* inhibitor, and a decrease in the OD values and proliferation ratio in cells transfected with *miRNA-509-5p* mimics. With increasing incubation time, the magnitude of lower OD values or proliferation ratio became more potent. Collectively, these results showed that the transfection of *miRNA-*

*509-5p* effectively inhibits the proliferation activity of HGC-27 cells. Additionally, the transwell assay demonstrated that cells transfected with *miRNA-509-5p* possessed remarkably potentiated invasion and migration abilities in contrast to the cells transfected with *miRNA-509-5p* mimics. These results indicate that *miRNA-509-5p* up-regulation could inhibit invasion and migration of HGC-27 cells. A previous study reported that the abnormal amplification and over-expression of *MDM2* in prostate cancer tissues led to the inactivation of tumor suppression activity of wild type *p53* (Vogel et al., 2012). *MDM2* could bind with *p53* to inhibit its transcription and block *p53*-induced activation via a negative feedback loop, resulting in the loss of *p53*-directed tumor suppressor effects (Lind et al., 2006; Miyazaki et al., 2013).

To substantiate the relationship between *miRNA-509-5p* and *MDM2* further, we performed the luciferase reporter assay. We observed reduced luciferase activity in HGC-27 cells co-transfected with *MDM2* 3'-UTR and *miRNA-509-5p*. Luciferase activity was elevated in cells co-transfected with *MDM2* 3'-UTR and *miRNA-509-5p* bearing mutated binding sites, indicating that the binding between *MDM2* 3'-UTR and *miRNA-509-5p* had been blocked. Therefore, we proposed that *miRNA-509-5p* could target the 3'-UTR of *MDM2* in a negative feedback manner. The up-regulation of *miRNA-509-5p* in HGC-27 cells could inhibit *MDM2* expression, thus inhibiting cell proliferation, invasion, and migration. Some studies reported that *MDM2* could work as a drug target for prostate cancer gene therapy, as the degradation of *MDM2* could elevate intracellular p53 levels and potentiate p53 restrictive effects during the G1/S and G2/M phases, arresting cells at the G1/G2 phase and inducing apoptosis (Yin et al., 2003; Li et al., 2010). However, the exact underlying mechanism of regulation of *MDM2* expression by *miRNA-509-5p* could not be elucidated and is the main limitation of the present study. We will conduct studies to investigate this mechanism in the future.

In conclusion, *miRNA-509-5p* is down-regulated, while *MDM2* levels are up-regulated in prostate cancer patients. *MiRNA-509-5p*, as a novel tumor suppressor, could target the 3'-UTR of *MDM2* via negative feedback loop affecting *MDM2* expression. The potentiation of *miRNA-509-5p* expression in HGC-27 cells inhibited *MDM2* expression levels, affecting cell proliferation, invasion, and migration and exerting inhibitory effects to a certain extent. Therefore, *miRNA-509-5p* may play an important role in the occurrence and progression of prostate cancer, and could be used as a novel therapeutic agent in the treatment of prostate cancer.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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## REFERENCES

Carry JC and Garcia-Echeverria C (2013). Inhibitors of the p53/hdm2 protein-protein interaction-path to the clinic. *Bioorg. Med. Chem. Lett.* 23: 2480-2485. http://dx.doi.org/10.1016/j.bmcl.2013.03.034

Center MM, Jemal A, Lortet-Tieulent J, Ward E, et al. (2012). International variation in prostate cancer incidence and mortality rates. *Eur. Urol.* 61: 1079-1092. http://dx.doi.org/10.1016/j.eururo.2012.02.054

Ding L, Xu Y, Zhang W, Deng Y, et al. (2010). MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. *Cell Res.* 20: 784-793. http://dx.doi.org/10.1038/cr.2010.79

Donnem T, Fenton CG, Lonvik K, Berg T, et al. (2012). MicroRNA signatures in tumor tissue related to angiogenesis in non-small cell lung cancer. *PLoS One* 7: e29671. http://dx.doi.org/10.1371/journal.pone.0029671

Fakharzadeh SS, Trusko SP and George DL (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.* 10: 1565-1569.

Farazi TA, Spitzer JI, Morozov P and Tuschl T (2011). miRNAs in human cancer. *J. Pathol.* 223: 102-115. http://dx.doi.org/10.1002/path.2806

Farh KK, Grimson A, Jan C, Lewis BP, et al. (2005). The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. *Science* 310: 1817-1821. http://dx.doi.org/10.1126/science.1121158

Ferlay J, Shin HR, Bray F, Forman D, et al. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127: 2893-2917. http://dx.doi.org/10.1002/ijc.25516

Jemal A, Bray F, Center MM, Ferlay J, et al. (2011). Global cancer statistics. *CA Cancer J. Clin.* 61: 69-90. http://dx.doi.org/10.3322/caac.20107

Li C, Pazgier M, Li C, Yuan W, et al. (2010). Systematic mutational analysis of peptide inhibition of the p53-MDM2/MDMX interactions. *J. Mol. Biol.* 398: 200-213. http://dx.doi.org/10.1016/j.jmb.2010.03.005

Lind H, Zienolddiny S, Ekstrøm PO, Skaug V, et al. (2006). Association of a functional polymorphism in the promoter of the MDM2 gene with risk of nonsmall cell lung cancer. *Int. J. Cancer* 119: 718-721. http://dx.doi.org/10.1002/ijc.21872

Liu G, Wheatley-Price P, Zhou W, Park S, et al. (2008). Genetic polymorphisms of MDM2, cumulative cigarette smoking and nonsmall cell lung cancer risk. *Int. J. Cancer* 122: 915-918. http://dx.doi.org/10.1002/ijc.23178

Lovat F, Valeri N and Croce CM (2011). MicroRNAs in the pathogenesis of cancer. *Semin. Oncol.* 38: 724-733. http://dx.doi.org/10.1053/j.seminoncol.2011.08.006

Ma Y, Lahue BR, Gibeau CR, Shipps GW, Jr., et al. (2014). Pivotal role of an aliphatic side chain in the development of an HDM2 inhibitor. *ACS Med. Chem. Lett.* 5: 572-575. http://dx.doi.org/10.1021/ml500019s

Miyazaki M, Naito H, Sugimoto Y, Kawato H, et al. (2013). Lead optimization of novel p53-MDM2 interaction inhibitors possessing dihydroimidazothiazole scaffold. *Bioorg. Med. Chem. Lett.* 23: 728-732. http://dx.doi.org/10.1016/j.bmcl.2012.11.091

Okoro DR, Arva N, Gao C, Polotskaia A, et al. (2013). Endogenous human MDM2-C is highly expressed in human cancers and functions as a p53-independent growth activator. *PLoS One* 8: e77643. http://dx.doi.org/10.1371/journal.pone.0077643

Peter ME (2009). Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. *Cell Cycle* 8: 843-852. http://dx.doi.org/10.4161/cc.8.6.7907

Popowicz GM, Czarna A, Wolf S, Wang K, et al. (2010). Structures of low molecular weight inhibitors bound to MDMX and MDM2 reveal new approaches for p53-MDMX/MDM2 antagonist drug discovery. *Cell Cycle* 9: 1104-1111. http://dx.doi.org/10.4161/cc.9.6.10956

Rew Y and Sun D (2014). Discovery of a small molecule MDM2 inhibitor (AMG 232) for treating cancer. *J. Med. Chem.* 57: 6332-6341. http://dx.doi.org/10.1021/jm500627s

Shen MM and Abate-Shen C (2010). Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev.* 24: 1967-2000. http://dx.doi.org/10.1101/gad.1965810

Siegel R, Naishadham D and Jemal A (2012). Cancer statistics, 2012. *CA Cancer J. Clin.* 62: 10-29. http://dx.doi.org/10.3322/caac.20138

Vogel SM, Bauer MR, Joerger AC, Wilcken R, et al. (2012). Lithocholic acid is an endogenous inhibitor of MDM4 and MDM2. *Proc. Natl. Acad. Sci. USA* 109: 16906-16910. http://dx.doi.org/10.1073/pnas.1215060109

Wade M, Wang YV and Wahl GM (2010). The p53 orchestra: Mdm2 and Mdmx set the tone. *Trends Cell Biol.* 20: 299-309. http://dx.doi.org/10.1016/j.tcb.2010.01.009

Weng MW, Lai JC, Hsu CP, Yu KY, et al. (2005). Alternative splicing of MDM2 mRNA in lung carcinomas and lung cell lines. *Environ. Mol. Mutagen.* 46: 1-11. http://dx.doi.org/10.1002/em.20118

Yin JQ, Gao J, Shao R, Tian WN, et al. (2003). siRNA agents inhibit oncogene expression and attenuate human tumor cell growth. *J. Exp. Ther. Oncol.* 3: 194-204. http://dx.doi.org/10.1046/j.1359-4117.2003.01092.x