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

X.M. Tian¹, Y.Z. Luo², P. He², J. Li², Z.W. Ma² and Y. An²

¹Operation Room,
Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital,
Chengdu, Sichuan, China
²Department of Urology,
Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital,
Chengdu, Sichuan, China

Corresponding author: Y. An
E-mail: anyu888688@163.com

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-509-5p 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.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-509-5p</td>
<td>5'-AAATCACCACCTTCACAGCC-3'</td>
<td>5'-GTTGTAATGGTTCTCCTCCAGC-3'</td>
</tr>
<tr>
<td>MDM2</td>
<td>5'-ATGGCAGCCGGGAGCATCACC-3'</td>
<td>5'-CACACACTCCTTTGATAGACACAA-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GAAACTACCTTCAACTCCATC-3'</td>
<td>5'-CTAGAAAGCATTGGGGGACATG6GAGGGGCC-3'</td>
</tr>
</tbody>
</table>

Genetics and Molecular Research 16 (1): gmr16019195
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% CO₂. 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% CO₂. 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 TGATGT TGT-3'; MDM2 - reverse: 5'-GCTAT TGAAA TTCGC 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 10⁴ cells/mL and seeded into a 12-well plate, which was incubated at 37°C with 5% CO₂. 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.
Correlation between miRNA-509-5p and MDM2 in prostate cancer

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>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>miRNA-509-5p mRNA</th>
<th>MDM2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate carcinoma</td>
<td>20</td>
<td>0.314 ± 0.048*</td>
<td>1.419 ± 0.075*</td>
</tr>
<tr>
<td>Tumor-adjacent</td>
<td>20</td>
<td>1.606 ± 0.012</td>
<td>0.608 ± 0.015</td>
</tr>
<tr>
<td>Normal prostate</td>
<td>20</td>
<td>1.532 ± 0.015</td>
<td>0.545 ± 0.013</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>miR-509-5p mimic</th>
<th>miR-509-5p inhibitor</th>
<th>Mimic control</th>
<th>Blank control</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-509-5p</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>1.205 ± 0.027**</td>
<td>0.171 ± 0.007***</td>
<td>0.602 ± 0.010</td>
<td>0.523 ± 0.006</td>
</tr>
<tr>
<td>48 h</td>
<td>1.602 ± 0.041**</td>
<td>0.026 ± 0.002**</td>
<td>0.610 ± 0.011</td>
<td>0.536 ± 0.008</td>
</tr>
<tr>
<td>MDM2 mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>1.061 ± 0.016**</td>
<td>1.384 ± 0.076***</td>
<td>1.720 ± 0.070</td>
<td>1.765 ± 0.081</td>
</tr>
<tr>
<td>48 h</td>
<td>0.812 ± 0.007***</td>
<td>1.872 ± 0.085****</td>
<td>1.700 ± 0.067</td>
<td>1.674 ± 0.068</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to mimic control group; **P < 0.05 compared to blank control group; ***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.
Correlation between miRNA-509-5p and MDM2 in prostate cancer

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

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-
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

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES

X.M. Tian et al.


Genetics and Molecular Research 16 (1): gmr16019195