MiR-505 inhibits prostate cancer cell invasion, metastasis and epithelial-to-mesenchymal transition through targeting HMGB-1

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Summary

Purpose: It has been proved that miR-505 expression was changed in prostate cancer (PC) tissues. However, its role and molecular mechanism in PC cells remains unclear. Our study aimed to study the microRNA (miR)-505 potential role and potential mechanism in PC cells.

Methods: miR-505 and HMGB-1 expression in PC tissues and cells was measured by RT-PCR and western blot, respectively. MiR-505 mimic or inhibitor was applied to increase or decrease miR-505 expression in DU145 cells separately. Invaded cells and migrated cells were detected by transwell assay. Epithelial-mesenchymal transition (EMT) was evaluated using western blot. Moreover, Luciferase reporter assay was carried out to confirm miR-505’s target gene.

Results: miR-505 expression was declined while HMGB-1 expression was raised in PC tissues and cells. Furthermore, increasing miR-505 expression suppressed, whereas decreasing miR-505 expression promoted cell invasion, migration and EMT in DU145 cells. Moreover, miR-505 could target HMGB-1 in regulating PC progression. Knockdown of HMGB-1 inhibited cell invasion and migration and re-expression of HMGB-1 reversed miR-505 mimic inhibitory effect on PC cell invasion and migration.

Conclusion: We conclude that miR-505 suppressed cell invasion, metastasis and EMT through targeting HMGB-1, which provided a potential target for PC treatment.

Key words: EMT, HMGB-1, invasion, metastasis, miR-505, prostate cancer

Introduction

Prostate cancer (PC) is an epithelial malignancy that occurs in the prostate gland. The incidence rate of PC is high, ranking sixth in the incidence of male malignant tumors in our country [1] and the incidence increases with age. Despite the progression of advanced PC treatment, the relative survival rates are still low [2]. Therefore, it is a challenge to understand the molecular mechanism of PC progression for performing anti-metastatic therapies. Strong evidence has shown that some miRNAs played pivotal roles in most tumors metastasis [3,4]. In PC, miR-129 [5], miR-181c [6], miR-141[7] and miR-19a [8] had been verified to play suppressive effects on cell metastasis. In addition, miR-505 was also proved to be tumor suppressor in multiple cancers. For example, re-expression of miR-505 inhibited cervical cancer cell proliferation and invasion [9]. Mudduluru et al showed that miR-505 acted as a tumor suppressor in colorectal cancer [10]. MiR-505 effect on hepatoma cells proliferation and invasion was also suppressive [11]. Recently, it has been reported that miR-505 expressional level was down-regulated in PC tissues [12]. However, the details of miR-505 role on PC cell invasion and metastasis and its potential mechanism has not been clarified to date.
It is well known that the role of epithelial-mesenchymal transition (EMT) in cancer cell metastasis is very important and it is a critical biological process to study the ability of cancer cells to migrate and invade, including PC [13,14]. So, it is very important to understand how miR-505 regulates PC cell invasion and metastasis by EMT.

The putative target of miR-505 predicted by TargetScanHuman 7.1 was HMGB-1 (high mobility group box1), which was reported to be involved in many cellular biological processes, such as tumor cell proliferation, invasion and metastasis [15,16]. HMGB-1 role in different tumors was targeted by different miRs. For example, HMGB-1 expression was raised and modulated by miR-410 in pancreatic ductal adenocarcinoma [17]. Li et al showed that HMGB-1 was a target of miR-34a in inhibiting cutaneous squamous cell carcinoma growth, invasion and migration [18]. Furthermore, miR-129/HMGB-1 axis took part in hepatocellular carcinoma invasion and metastasis [19]. A previous study showed that HMGB-1 expression level was up-regulated in PC and was related to cell progression [20]. However, how HMGB-1 regulated PC cell invasion and migration and whether it was a target of miR-505 in regulating PC development has not been reported until now.

Our study intended to investigate miR-505 and HMGB-1 role in PC cell invasion and metastasis and the molecular mechanism of miR-505 in PC.

**Methods**

**Tissue samples**

The ethics committee of Weifang People’s Hospital approved this project and all patients with PC signed the informed consent before tissues acquisition. Seventy pairs of tissue specimens and normal tissues were collected from PC patients who were operated in Weifang People’s Hospital. The specimens were instantly put into liquid nitrogen when the tumor tissues were removed by a cotton swab, and the migrated cells were removed by a cotton swab, and the migrated cells were counted under a microscope. We chose ten random fields to count the cells at 100x magnification. For migration, the transwell chamber with 8 μm polycarbonate pore size was inserted into the upper and lower chambers. 2×10^5 PC cells were grown in the upper chambers with serum-free DMEM and the lower chambers were filled with DMEM containing 10% FBS and then incubated at 37°C for 48 h, to allow the cells to migrate from the upper chambers to the lower chambers. Then, cells were fixed with methanol for 10 min and stained with 0.1% crystal violet for another 15 min. The non-migrated cells in the upper surface were removed by a cotton swab, and the migrated cells were photographed through a microscope. We chose ten random fields to count the cells at 100x magnification. For invasion assay, the chambers were coated with matrigel and the cells were grown in the upper chamber with serum-containing DMEM and the lower chamber was covered with matrigel and then incubated at 37°C for 48 h. For the invasion assay, the cells were counted in ten random fields using a microscope.

**Cell invasion and migration assay**

Cell invasion and migration were detected by transwell assay. For migration, the transwell chamber with 8 μm polycarbonate pore size was inserted into the upper and lower chambers. 2×10^5 PC cells were grown in the upper chambers with serum-free DMEM and the lower chambers were filled with DMEM containing 10% FBS and then incubated at 37°C for 48 h, to allow the cells to migrate from the upper chambers to the lower chambers. Then, cells were fixed with methanol for 10 min and stained with 0.1% crystal violet for another 15 min. The non-migrated cells in the upper surface were removed by a cotton swab, and the migrated cells were photographed through a microscope. The proteins from PC tissue specimens and cells were extracted using RIPA lysis buffer (Beyotime, Beijing, China) containing 1% PMSF. Bicinchoninic acid (BCA) kit was used to determine the protein concentration. An equal number of proteins were added on 12% SDS-PAGE to electrophorese and transferred to NC membrane. Then, 5-10% skimmed milk was added to block the membranes for 2 h at room temperature. Subsequently, anti-HMGB-1 (Abcam, London, UK) and anti-GAPDH (Santa Cruz, MA, USA) primary antibodies were added to probe with the blots at 4°C overnight, and then added the anti-HP-conjugated secondary antibodies (Santa Cruz, USA) for incubation at room temperature for 2h. Finally, the enhanced chemiluminesence kit (ECL, Millipore, MA, USA) was used to detect the signals of GAPDH. The target protein/GAPDH level was used to evaluate the relative target protein expression.

**Western blotting**

Total RNAs were extracted from tissues or cells using TRIZOL reagent (Invitrogen, NY, USA). Nanodrop 2000 (Thermo, MA, USA) was applied to detect the purity and concentration of RNAs. SuperScript III reverse transcriptase (Invitrogen, NY, USA) and TaqMan Human miRNA Assay Kit (Thermo Fischer Scientific, USA) were used to synthesize the cDNA, amplify PCR and quantify miR-505 and U6, respectively. U6 was used as a control to normalize the expression of miR-505. SYBR Premix Ex Taq™ II Kit (Takara, Shanghai, China) was applied to amplify the PCR and quantify HMGB-1 and GAPDH miRs. GAPDH was used as a control of HMGB-1. Relative expression level was calculated by the 2^ΔΔCT method. The sequences of the primers were as follows: miR-505-F: 5’-CGUCAACACUUUGGCUGUUCU-3’, miR-505-R: 5’-GGGAGCC AGGAAGUAUUGAUGU-3’; HMGB-1-F: 5’-CTTCTTGGAGGGAAAGCTAGT-3’, HMGB-1-R: 5’-TTTGGTAGTTTATGTTAGG-3’; U6-F: 5’-GCTCGGCTCG- GCAG CACA-3’, U6-R: 5’-ACGGTTCAGAATTTGCGT-3’; GAPDH-F: 5’-CCAGAAGA CTGTTGG ATGG-3’, GAPDH-R: 5’-CAGTGAGCITCCCCGTTCAG-3’.
and incubated for 30 min at 37°C. Other experimental steps were similar to the migration assay.

**Luciferase reporter assay**

Luciferase reporter assay was applied to measure the HMGB-1 luciferase ability. Lipofectamine 2000 (Invitrogen, NY, USA) was used to co-trasfect miR-505 mimic, miR-505 inhibitor or negative control and HMGB-1 3’UTR-psiCHECK-2™ Vector into DU145 cells. Dual luciferase assay was used to measure the firefly luciferase activity after transfection for 24h. Renilla luciferase was applied to normalize the results.

**Statistics**

Experiments in this study were conducted three times separately. Data was displayed as mean ± SD and analyzed using SPSS 17.0 statistical software (SPSS, Chicago, Ill, USA) and GraphPad Prism 5. Comparison of the differences between two groups was performed using Student’s t-test and the differences between more than two groups were evaluated by Tukey’s post hoc test after one-way ANOVA. P<0.05 was regarded as statistically significant.

**Results**

**Decrease of miR-505 and increase of HMGB-1 in PC**

Firstly, we tested miR-505 expression and its clinical values in 70 paired PC tissues to explore the relationship between miR-505 expression and clinicopathological characteristics of PC. RT-PCR results showed that miR-505 average expression in 70 cases of PC tissues was obviously lower than in normal tissues (Figure 1A). Besides, miR-505 expression in three PC cell lines (DU145, PC3 and LAPC4) was reduced significantly in comparison with normal cell line RWPE-1 (Figure 1B). Because of the lowest expression of miR-505 in DU145 cells, it was selected for further study.

To explore whether miR-505 expression was related to the clinicopathological characteristics of PC, the relative mean expression of miR-505 (0.53) was used to divide 70 PC patients into two groups: low expression miR-505 group (n=32) and high expression miR-505 group (n=38). The relationship between miR-505 and PC patients’ clinical features are shown in Table 1 which shows that miR-505 expression was closely associated with Gleason score and it was similar to distant metastasis and pathological stage.

Secondly, we measured HMGB-1 expression in PC by RT-PCR. As Figure 1C shows that HMGB-1 expression level was elevated in PC tumors in comparison with normal tissues, as well as in PC cells too (Figure 1D). The relationship between miR-505 expression and HMGB-1, as shown in Figure 1E, was inversely related.

The prognostic role of miR-505 and HMGB-1 in PC

Survival analysis was performed to reveal the relationship between miR-505 or HMGB-1 and the survival among PC patients. As shown in Figure 2A, the cases with high expression of miR-505 showed a high overall survival, which was similar to disease-free survival (Figure 2B). The opposite

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Table 1. miR-505 expression in relation to clinicopathological characteristics of 70 PC patients
Figure 1. Decreased miR-505 and increased HMGB-1 in PC. A: miR-505 expression measured in PC tissues and matched normal tissues. B: miR-505 expression measured in PC cells (DU145, PC3 and LAPC4) and normal cells (RWPE-1). C: HMGB-1 expression measured in PC tissues and matched normal tissues. D: HMGB-1 expression measured in PC cells and normal cells. E: The relevance of miR-505 and HMGB-1 expression (r= -0.6564, p<0.0001) (*p<0.05, **p<0.01).

Figure 2. The prognostic value of miR-505 and HMGB-1. A: The relationship between PC patients overall survival and miR-505 high expression (n=52) or miR-505 low expression (n=38) by Kaplan–Meier analysis (p<0.01). B: Comparison of the disease-free survival of PC patients with miR-505 high expression and miR-505 low expression (p<0.05). C: The relationship between PC patients’ overall survival and HMGB-1 low expression or high expression (n=38) by Kaplan–Meier analysis (p<0.05). D: Comparison of the disease-free survival of PC patients with HMGB-1 low expression and HMGB-1 high expression.
MiR-505 attenuates prostate cancer progression

Figure 3. miR-505 inhibitory effect on PC cell invasion, metastasis and EMT. A: Measurement of miR-505 expression in DU145 cells after treatment with miR-505 mimic or inhibitor. B: N-cadherin, E-cadherin and vimentin protein level measured by western blot in DU145 cells after increasing or inhibiting miR-505. C: Relative cell migration detected in DU145 cells after increasing or inhibiting miR-505. D: Relative cell invasion detected in DU145 cells after increasing or inhibiting miR-505. (**p<0.01, ***p<0.001; ##p<0.01).

Figure 4. HMGB-1 was the miR-505’s target. A: TargetScan analysis revealed the prediction binding sites of miR-505 and HMGB-1 3’-UTR. B: Luciferase activity tested in DU145 cells after treatment with HMGB-1 and miR-505 mimic or control mimic. C: HMGB-1 protein expression measured in DU145 cells after increasing or inhibiting miR-505 expression. D: HMGB-1 mRNA expression measured in DU145 cells after increasing or inhibiting miR-505 expression (**p<0.01; ***p<0.001).
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A trend was found in the cases with high expression of HMGB-1 (Figure 2C,2D). The mean relative expression of HMGB-1 <3.95 was considered as low expression of HMGB-1 group and >3.95 as high expression of HMGB-1 group. These results suggested that high miR-505 expression presented a better prognosis while high HMGB-1 expression showed a poor prognosis.

Suppression effect of miR-505 in PC cell invasion, metastasis and EMT

To study the effect of miR-505 on PC progression, miR-505 mimic or inhibitor were transfected into DU145 cells to achieve miR-505 re-expression or inhibition, separately. As expected, the transfection efficiency was very successful, miR-505 expression was raised after treatment with miR-505 mimic, but decreased after treatment with miR-505 inhibitor in DU145 cells (Figure 3A). Western blot was used to explore how miR-505 modulated EMT in DU145 cells, using 3 common EMT markers. As shown in Figure 3B, mesenchymal markers (N-cadherin and vimentin) expression level was reduced in miR-505 mimic group, but increased in miR-505 inhibitor group in comparison with control cells. The epithelial marker E-cadherin protein expression showed an opposite effect on PC cells. In addition, transwell assay was carried out to check cell invasion and migration. The results showed that the invaded and migrated cells in miR-505 mimic group decreased while they increased in miR-505 inhibitor group (Figure 3C,3D). The above results indicated that miR-505 was tumor-suppressive in regulating PC invasion, metastasis and EMT.

MiR-505 targeted HMGB-1 in PC cells

Then, we explored miR-505 molecular mechanism in regulating PC progression. Firstly, we used TargetscanHuman 7.1 to analyze the putative target of miR-505. As Figure 4A shows, it was predicted that HMGB-1 might be a possible target of miR-505. The luciferase reporter assay was carried out to further clarify whether HMGB-1 was the miR-505’s target gene in PC. The results showed that co-transfection of miR-505 mimic with HMGB-1 3’-

Figure 5. Inhibitory effect of HMGB-1 siRNA on PC cell invasion, metastasis and EMT. A: HMGB-1 expression measured in DU145 cells after inhibiting HMGB-1. B: N-cadherin, E-cadherin and vimentin protein level measured by western blot in DU145 cells after silencing HMGB-1. C: Relative cell migration detected in DU145 cells after silencing HMGB-1. D: Relative cell invasion detected in DU145 cells after silencing HMGB-1 (**p<0.01, ***p<0.001).
UTR wild type (WT) revealed a declined expression of luciferase activity in DU145 cells compared with control. However, there were no significant differences in mutated (MUT) type of HMGB-1 (Figure 4B). Subsequently, we studied whether miR-505 level affected HMGB-1 expression in DU145 cells by western blot and RT-PCR. Data showed that re-expression of miR-505 could reduce HMGB-1 protein and mRNA expression, while inhibition of miR-505 raised HMGB-1 expression (Figure 4C,4D). The above results suggested that HMGB-1 was a target of miR-505 in PC.

**Suppressive effect of HMGB-1 siRNA in PC cell invasion, metastasis and EMT**

We then investigated the HMGB-1 role in PC progression. HMGB-1 siRNA was used to inhibiting HMGB-1 due to its high expression in PC. As expected, HMGB-1 expression was reduced in DU145 cells transfected with HMGB-1 siRNA (Figure 5A). N-cadherin, vimentin and E-cadherin expression was tested by western blot and Figure 5B showed N-cadherin and vimentin expression level was lower, but E-cadherin protein expression was higher in the HMGB-1 siRNA group than in the control. Cell migratory and invasive ability in DU145 cells was tested by transwell assay. The results showed that HMGB-1 siRNA suppressed cell migration and invasion (Figure 5C, 5D). In short, HMGB-1 siRNA played suppressive role on PC cells invasion, migration and EMT.

**MiR-505 inhibitory effect was reversed by HMGB-1 on PC cell progression**

To investigate the HMGB-1 impact on miR-505’s inhibitory effect on PC, we first over-expressed HMGB-1 in DU145 cells and evaluated it with RT-PCR and western blot. Both methods showed that the transfection efficiency was successful (Figure 6A) and HMGB-1 could reverse miR-505 inhibitory effect on PC cell invasion and migration (Figure 6B,6C).

![Figure 6](image-url)
Discussion

In the present study we stated that miR-505 expression level was markedly decreased in PC tissue specimens and cells, which was in line with reports that miR-505 is down-regulated in PC tissues [12]. Furthermore, increasing miR-505 inhibited PC cell invasion, metastasis and EMT, whereas inhibiting miR-505 promoted these functions. Importantly, HMGB-1 was verified as a specific target of miR-505. Our study further revealed that re-expression of miR-505 inhibited HMGB-1 expression, while silencing miR-505 enhanced HMGB-1 expression, which further suppressed or promoted PC cell invasion and migration. In addition, the inhibitory effect of miR-505 mimic on cell invasion and migration was reversed by over-expression of HMGB-1. Therefore, these results suggested a tumor suppressive effect of miR-505 on PC cell progression.

MiR-505 was reported to participate in the proliferation and migration of multiple tumor cells, including cervical cancer, colorectal cancer, hepatocellular carcinoma, chronic myeloid leukemia, colon adenocarcinoma and breast cancer [9, 10, 21-24] by different mechanisms. MiR-505 functioned either as tumor-suppressive or tumor-promoter on cancer cell progression depending on the type of tumors. Although miR-505 is involved in the progression of many cancers, its biological role and mechanism in PC invasion, metastasis and EMT remains unclear until now. In our study, we showed that miR-505 expression level was reduced in PC tissues and cells. Increasing miR-505 expression curbed PC cell invasion, migration and EMT via targeting HMGB-1. Moreover, our results revealed that miR-505 suppressive effect on PC cell invasion and metastasis was reversed by HMGB-1.

EMT refers to the biological process of epithelial cells transformation into interstitial cells through a specific mechanism. As an important mechanism for tumor cell metastasis, EMT is increasingly concerned by prostate cancer researchers [25]. In recent years, it is becoming more and more obvious that miRs are the key regulators of EMT and tumor occurrence. MiR-203 was reported to modulate tumor cell invasion and metastasis by regulating EMT in head and neck cancer [26]. Our study revealed that mesenchymal markers (N-cadherin and vimentin) were reduced after increasing miR-505 expression but increased after inhibiting miR-505 expression. However, the epithelial cell marker E-cadherin showed an opposite trend in DU145 cells, which suggested that miR-505 suppressed EMT in PC.

HMGB-1 was reported to participate in many tumor cell biological processes. Wu et al reported that miR-193-HMGB-1 participated in lung cancer cell proliferation and migration [27]. Moreover, a research by Xiong et al stated that HMGB-1 was up-regulated in cutaneous squamous cell carcinoma and played important role in cell progression regulated by miR-34a [18]. In PC, Wu and his colleagues reported that HMGB-1 was up-regulated in PC and over-expression of HMGB-1 correlated with poor prognosis in PC [28]. Moreover, Gnanasekar et al found that silencing HMGB-1 inhibited PC cell growth and showed that HMGB-1 was critical for PC cells survival [29]. In our study, as we expected, HMGB-1 expression was higher in PC tissues and cells than in normal controls. Down-regulation of HMGB-1 suppressed PC cell invasive and migratory ability. We also demonstrated that HMGB-1 acted as a target of miR-505 in regulating PC cell invasion and migration and miR-505 mimic inhibitory effect was attenuated by inhibiting HMGB-1 expression.

In short, our results showed that miR-505 played a tumor-suppressive role on PC cell invasion and metastasis via targeting HMGB-1. Therefore, better understanding of miR-505 role in PC cell invasion and metastasis would help know PC metastasis development, which might provide a new therapeutic strategy for PC.

Conflict of interests

The authors declare no conflict of interests.

References

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MiR-505 attenuates prostate cancer progression


