Silencing of HMGA2 promotes apoptosis and inhibits migration and invasion of prostate cancer cells

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The high mobility group protein A2 (HMGA2) has been demonstrated as an architectural transcription factor that is associated with pathogenesis of many malignant cancers; however, its role in prostate cancer cells remains largely unknown. To explore whether HMGA2 participates in the development and progression of prostate cancer, small interfering RNA (siRNA) targeted on human HMGA2 was transfected to suppress the HMGA2 expression in prostate cancer PC3 and DU145 cells, and then the cellular biology changes after decreased the expression of HMGA2 was examined. Our results showed that knockdown of HMGA2 markedly inhibited cell proliferation; this reduced cell proliferation was due to the promotion of cell apoptosis as the Bcl-xl was decreased, whereas Bax was up-regulated. In addition, we found that HMGA2 knockdown resulted in reduction of cell migration and invasion, as well as repressed the expression of matrix metalloproteinases (MMPs) and affected the occurrence of epithelial-mesenchymal transition (EMT) in both cell types. We further found that decreased HMGA2 expression inhibited the transforming growth factor-β (TGF-β)/Smad signalling pathway in cancer cells. In conclusion, our data indicated that HMGA2 was associated with apoptosis, migration and invasion of prostate cancer, which might be a promising therapeutic target for prostate cancer.

1. Introduction

Prostate cancer is the most common frequent malignancy form of non-skin cancer in men, with an estimated 220,080 men will be diagnosed annually, although the death rates for male prostate cancer has been decreasing as a result of the improvements in early detection and treatments, it is still the second leading cause of cancer death, and approximately 27,540 will die from the disease in 2015 in the Unites States (Siegel et al. 2015). Nowadays, most patients are diagnosed with clinically localized cancer that can be treated effectively with radical prostatectomy, radiotherapy or androgen deprivation therapy. In spite of that, it is reported that about 15% of patients will develop locally recurrent or metastatic disease (DiBlasio et al. 2009). Moreover, the disease almost invariably progressed to castration-resistant prostate cancer (CRPC), the treatment for those who underwent the CPRC was limited and death occurred much more with shorter survival time (Ramsay et al. 2011; Maruyama et al. 2014). Therefore, it is extremely urgent to develop effective novel targets for therapeutic intervention of prostate cancer.

HMGA2 is a member of the HMGA family of nonhistone chromatin proteins. By binding to AT-rich regions in DNA, it alters chromatin architecture to either promote or inhibit the action of transcriptional enhancers. It is an oncofetal protein that is at high levels during embryonic development and many malignant neoplasms, including of gastric (Zha et al. 2012), nasopharyngeal (Liu et al. 2015) and hepatocellular carcinoma (Luo et al. 2013), but is either very low or has no expression in normal adult tissues. The oncogenic properties of HMGA2 are

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shown to be involved in tumour cell differentiation, stem cell self-renewal, DNA damage response, as well as to be participating in EMT (Venkatesan et al. 2012). Nowadays, increasingly more studies have shown that HMGA2 serves an important role in tumour development and progression through regulating tumour cell metastasis, stemness of cancer stem cell, and EMT (Liu et al. 2015; Morishita et al. 2013). Additionally, Zhu et al. (2013) found that miR-154 inhibited EMT by targeting HMGA2 in prostate cancer. Based on these findings, we proposed that the HMGA2 might be involved in the progression of prostate cancer.

In this study, we demonstrated that silencing of HMGA2 by siRNA suppressed the prostate cancer cell proliferation, migration and invasion, and decreased the expression of MMPs and the occurrence of EMT, the underlying mechanism during the process might be associated with the TGF-β/Smad signalling pathway.

2. Materials and methods

2.1 Cell culture and antibodies

The human prostate cancer cell lines PC3 and DU145 were purchased from the Chinese Academy of Science (Shanghai, China). Cells were grown in RPMI-1640 (Gibco, NY, USA) medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China) under standard cell culture conditions of 5% CO₂ in an incubator at 37°C. Polyclonal rabbit antibodies against HMGA2, E-cadherin, and N-cadherin were purchased from the Abcam, MA, USA; Polyclonal rabbit antibodies against Bax and Bel-xl were purchased from Cell Signaling Technology, MA, USA; Polyclonal rabbit antibodies against MMP-2, MMP-9 were purchased from the Santa Cruz Biotechnology, CA, USA; Polyclonal rabbit antibodies against Vimentin, TGF-β RI, Smad2, Smad3, phospho-Smad2/3 and β-actin were purchased from Bioworld Technology, CA, USA. The appropriate peroxidase-conjugated goat anti-rabbit IgG secondary antibodies were obtained from Zhongshan Biotech, Beijing, China.

2.2 Small interfering RNA and transfection

Small interfering RNA that targeting HMGA2 and scrambled non-specific sequence, which served as negative controls were all designed, synthesized and purified by GenePharma (Shanghai, China). 24 h prior to transfection, cells were seeded on six-well plates and then transfected with 30 pmol HMGA2-siRNA or negative control-siRNA using 3 μL siLentFect reagent (Bio-Rad, CA, USA) according to the manufacturer’s protocol. 6 h later, the medium was changed to fresh RPMI-1640 with 10% FBS, and continued to culture for 48 h, cells were then harvest for the following assays.

2.3 Western blotting

For Western blotting, the total proteins of cancer cells were extracted by lysis buffer containing phosphatase and protease inhibitors, then centrifuged at 4°C at 12,000g for 5 min. The protein concentration was determined by the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, Nantong, China). Equal amounts of protein were loaded onto 15% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE, Beyotime, Nantong, China) for electrophoresis and then transferred to nitrocellulose filter membranes (Millipore, MA, USA). The membrane was blocked in a solution of washing buffer containing 5% non-fat milk for 2 h, and then incubated with the following diluted primary antibodies overnight at 4°C (HMGA2, 1:200 dilution; Vimentin, TGF-β RI, Smad2, Smad3, phospho-Smad2/3 1:500 dilution; E-cadherin, N-cadherin, Bax, Bel-xl, MMP-2, MMP-9 1:1,000 dilution; and β-actin 1:10,000 dilution). After washed in washing buffer 3 times for 15 min, membrane was incubated for 2 h with secondary antibodies at a 1:10,000 dilution at room temperature and then washed again in washing buffer containing Tween-20. At last, the membrane was visualized using an enhanced chemiluminescence (ECL) reagent (Tanon, Shanghai, China) and analysed by Image J software. The β-actin was used as an internal reference.

2.4 Cell proliferation assay

Cell proliferation was analysed using Cell Counting Kit-8 (CCK-8, Beyotime, Nantong, China). The prostate cancer cells were placed onto 96-well plates at a density of 5×10³ cells per well in 100 μL of growth medium and allowed to adhere overnight. At different time points (24, 48, 72, and 96 h), the culture medium was removed and replaced with 100 μL RPMI-1640 culture medium containing 10 μL of CCK-8 solutions. After incubation at 37°C for 1 h, the optical density (OD) values were measured at 450 nm using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA). This assay was conducted in triplicate, and six wells were used per condition each time.

2.5 Cell apoptosis assay

Cell apoptosis was detected by an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (KeyGen, Nanjing, China) according to the supplier’s protocols. In brief, 48 h post-transfection, cells
were collected, centrifuged, and resuspended in 500 μL of 1× binding buffer, Annexin V-FITC (5 μL) and PI (5 μL) were then added to each tube. The tubes were incubated in the dark at room temperature for 15 min. Cell apoptosis assay was then performed within 1 h on a flow cytometry (FACScan, BD Biosciences, NJ, USA).

2.6 Transwell migration assay

The migration capacities of PC3 and DU145 cells were determined using transwell chambers (8μm pore size, Corning, NY, USA). 5×10^4 cells were plated into the chamber with 200 μL serum-free medium per well. The outer chambers were filled with 500 μL medium containing 10% FBS. After 12 h, the top surface of the membrane was scrubbed gently with a cotton swab, whereas, cells migrated to the undersurface were then fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet prior to undergoing a series of washes. The cells passing to the undersurface of each filter were then photographed and counted (200× magnifications), the mean number of migrated cells was determined by counting five random fields of each well.

2.7 Matrigel invasion assay

For invasion assays, each chamber was coated freshly with 50 μg Matrigel (BD Bioscience, NJ, USA) before starting the assay, then cells were seeded into the upper transwell chamber. Briefly, the bottom chambers were filled with RPMI-1640 media with 10% FBS and the top chambers were seeded with 5×10^4 cells that transfected with negative control or HMGA2 siRNA per well in RPMI-1640 media. After incubation for 24 h, the noninvasive cells that remaining on the upper chambers were removed by cotton swabs, and the invaded cells on the lower surface of the filter were fixed and stained followed by microscopic examination. The number of cells in five random optical fields was averaged.

2.8 Statistical analysis

All experiments were carried out at least three times. Data were expressed as mean± standard deviation (SD). Statistical analyses were performed by independent samples t-test using the software SPSS version 16.0 (SPSS Inc, IL, USA). P <0.05 was used to determine the statistical significance when interpreting the results (*P<0.05, **P<0.01, ***P<0.001).

3. Results

3.1 HMGA2-targeted siRNA reduces the expression of HMGA2 in both PC3 and DU145 cells

The gene-silencing effect mediated by siRNA specifically against HMGA2 in PC3 and DU145 cells was confirmed by Western blot analysis. The results showed that approximately 48 h after transfection, the protein levels of HMGA2 were decreased after HMGA2 knockdown in both cell lines (figure 1).

3.2 Knockdown of HMGA2 inhibits the proliferation and induces the apoptosis of cancer cells

To address whether HMGA2-siRNA had an effect on cell growth, the CCK-8 assay was performed. Compared to control cells, cells transfected with HMGA2-siRNA showed significant inhibition of cell proliferation (figure 2A). To elucidate whether the decreased proliferation was due to the high-regulation of cell apoptosis, we conducted the flow cytometry assay to detect Annexin V/PI double staining and found that HMGA2 knockdown could induced higher apoptosis (figure 2B). In addition, it is well known that the Bcl-xl and Bax are critical regulators of cell apoptosis, so we continued to detect the protein levels by a series of Western blot assays, data revealed that HMGA2
knockdown led to the increasing of Bax, and the low-expression of Bcl-xl (figure 2C).

3.3 **Knockdown of HMGA2 represses the migration and invasion capacities of prostate cancer cells**

Metastasis is the principal cause of cancer death, and cell migration and invasion are the hallmarks of cancer metastasis. Thus, we carried out the transwell migration and invasion assay to assess whether HMGA2 had effects on the abilities of migration and invasion of prostate cancer cells. As shown in the figure 3A, the migration in experimental group cells was remarkably decreased compared with those control cells. Meanwhile, matrigel invasion assay also showed that less cells penetrated through the matrigel-coated chambers in HMGA2-siRNA transfected cells (figure 3B). It is acknowledged the extracellular matrix degradation induced by MMPs

![Figure 2](image_url)

**Figure 2.** Effects of HMGA2-siRNA on prostate cancer cell proliferation and apoptosis. (A) Cell proliferation was analysed by CCK-8 assay. Growth curves of PC3 and DU145 cells were shown for each treatment at 24, 48, 72, and 96 h. Viable cell numbers were significantly different compared with negative control group. (B) Cell apoptosis was detected by Annexin V-FITC/PI double staining and FCM analysis. The results showed that down-regulation of HMGA2 led to a higher apoptosis rates. (C) Apoptosis-associated proteins were measured by western blotting. The protein levels of key molecules in Bcl-2 family such as Bax were increased, while Bcl-xl decreased significantly. The results were represented the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.
is required for cell invasion; we then conducted Western blotting to investigate the effects of HMGA2 down-regulation on the expression of MMPs, and our results demonstrated that when compared to the respective corresponding controls, the expressions of MMP-2 and MMP-9 were vastly down-regulated in PC3 and DU145 cells after HMGA2 down-expression (figure 4A), which might be associated with the suppression of migration and invasion.

3.4 Knockdown of HMGA2 suppresses EMT in part through down-regulation of TGF-β/Smad signalling pathway in prostate cancer cells

EMT is an invasive phenotype of cancer cells and involved in the events of tumour progression and metastasis. To confirm whether silencing of HMGA2 could prevent the EMT in prostate cancer cells, Western blotting was conducted. Here, we validated that HMGA2 could regulate the process of EMT, in which, with the reduction of HMGA2, the expression of epithelial marker E-cadherin was increased, while mesenchymal markers N-cadherin and Vimentin were significantly decreased (figure 4B). Previous studies have revealed that the TGF-β/Smad pathway is a major signalling pathway that involved in EMT, so we further investigated the related protein levels of TGF-β/Smad signal pathway; the Western blot analysis showed that the TGF-β RII, Smad2, Smad3 and p-Smad2/3 protein expressions were much lower than that in control cells (figure 5). These data indicated that EMT could be suppressed by reduced HMGA2 in part through the TGF-β/Smad signalling pathway in prostate cancer cells.
4. Discussion

HMGA2 belongs to the HMGA family of architectural transcription factors, which plays an important role in chromatin organization. It has been reported that HMGA2 was closely related to the progression, invasion, metastasis and prognosis of a number of malignant tumours (Zou et al. 2012). However, the biological effects of HMGA2 on prostate cancer remain limited. Here, we demonstrated that HMGA2 was likely to regulate the proliferation, migration, invasion, and EMT in both PC3 and DU145 cells, and affect the expression of MMPs and the activation of TGF-β/Smad signalling pathway. Our present studies suggested that HMGA2 played a crucial role in prostate cancer development and progression.

It has been reported that HMGA2 plays an important role in the process regulating apoptosis and proliferation in various cancers (Li et al. 2014a, b). Thus, we supposed that whether HMGA2 could play a significant role as a tumour promoter to regulate cell apoptosis and proliferation in prostate cancer. Our preliminary study showed that the optical density (OD) value obtained with CCK-8 increased with the increase in cell density and it correlated very well to the cell number counted with a hemocytometer, demonstrating that CCK-8 kit can be used to determine the cell proliferation in prostate cancer cells treated with or without HMGA2-siRNA. Consistent with previous studies, the CCK-8 assay showed that the lost expression of HMGA2 led to the reduction of cell proliferation. In addition, we found here that by using siRNA

Figure 4. Effects of HMGA2-siRNA on the expression of proteins associated with MMPs and EMT. (A) Western blotting results showed that the protein levels of MMP-2 and MMP-9 were down-regulated after transfected with HMGA2-siRNA. (B) The expression of the epithelial phenotype marker E-cadherin was significantly increased and of N-cadherin and Vimentin, which were the mesenchymal phenotype markers were decreased in PC3 and DU145 si-HMGA2 cells compared with the control group cells respectively. Experiments were repeated three times, data in this study were expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Effects of HMGA2-siRNA on the expression of proteins associated with TGF-β/Smad signalling pathway. The results showed that the related proteins, including of TGF-β RII, Smad2, Smad3, p-Smad2/3 were lower compared with those treated with control siRNA. Experiments were conducted in triplicate. Data were expressed as mean ± SD. *P<0.05, **P<0.01.
to silence HMGA2 expression, the pro-apoptotic genes Bax expression was increased, while the Bel-1 was decreased, which in turn induced cell apoptosis in prostate cancer.

EMT is a well-recognized mechanism that involved in embryonic development, wound healing, and cancer progression. It is often marked by decreased expression of E-cadherin (epithelial marker) and increased expression of N-cadherin and Vimentin (mesenchymal markers). The alternations of these proteins have been demonstrated to contribute to impair cell–cell adhesion, thereby allowing the spread of cancer cells from the primary sites (Tiwari et al. 2012; Yilmaz and Christofori 2009; Thiery et al. 2009). Many reports have suggested that EMT has a high correlation with the generation of cancer stem cells, the malignancy of tumour cells and the resistance to anticancer therapies (McConkey et al. 2009; Zhang et al. 2011; Kayastha et al. 2015). It has been demonstrated that HMGA2 has an effect on the EMT in many cancer cells, such as colon (Li et al. 2014a, b), gastric (Lu et al. 2015) and hepatocellular cancer (Li et al. 2014a, b). In addition, numerous studies have verified that invasive ability of cancer cells can be regulated by MMPs (Kessenbrock et al. 2010, in which MMP-2 and MMP-9 were reported to be closely associated with advanced tumour stage, increased invasion and metastasis, and shortened survival time (Slattery et al. 2013). In this study, we have revealed that HMGA2 knockdown effectively repressed the migration and invasion of PC3 and DU145 cells. However, whether the changes is associated with the expressions of MMPs and the process of EMT in different prostate cancer cells is not yet known. Therefore, we checked the different expressions of MMP-2 and MMP-9 in cells transfected with HMGA2-siRNA compared to control, and found that HMGA2 knockdown led to a significant decrease in protein levels of MMP-2 and MMP-9. In addition, our study identified that HMGA2 knockdown reversed the progression of EMT via increasing the expression of E-cadherin and decreasing the N-cadherin and Vimentin protein levels in PC3 and DU145 cells.

A large number of studies have indicated that many molecular and microenvironmental factors could induce EMT in a variety of cell types, including TGF-β (De Craene and Berx 2013). During the process, TGF-β1 binds to heterodimeric complexes of TGF-β receptors, and then phosphorylates Smad2 and Smad3, which then form trimers with Smad4. Then, the trimer translocates into the nucleus, in which it associates with transcription factors to regulate gene transcription (Yang et al. 2015). Xia et al. (2015) reported that HMGA2 knockdown attenuated EMT-induced migration and invasion through the TGF-β/Smad3 signalling pathway in nasopharyngeal cancer cells. Consistent with these findings, we found that the protein levels of TGF-β RII, Smad2, Smad3, phospho-Smad2/3 markedly decreased in HMGA2-siRNA transfected cells, which suggested that down-regulation of HMGA2 inhibited the activation of TGF-β/Smad pathway in prostate cancer cells. Additionally, the core EMT regulatory factors, such as Snail, Slug and Twist have been proved to be inversely correlated with the expression of E-cadherin (De Craene and Berx 2013; Dalvi et al. 2009; Lander et al. 2011; Diaz et al. 2014). Therefore, further studies are needed to address whether HMGA2 has an effect on those transcriptional repressors.

In conclusion, we identified that silencing of HMGA2 effectively suppressed the proliferation, migration, invasion and EMT process, and promoted the apoptosis of prostate cancer cells. Therefore, targeting HMGA2 might be a beneficial therapeutic approach for prostate cancer treatment.

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