Expression of oncogenic HMGN5 increases the sensitivity of prostate cancer cells to gemcitabine

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Abstract. Prostate cancer is a leading cause of cancer-related death among men. Early diagnosis and treatment are successful against prostate cancer, yet the clinical treatment of advanced prostate cancer remains a challenge. Gemcitabine is used to treat a broad spectrum of solid tumors; however, the clinical response of prostate cancer patients to gemcitabine is limited. In the present study, we showed that HMGN5, a nucleosome binding protein that can unfold chromatin by binding to histone (H1), is overexpressed in prostate cancer cells and plays an oncogenic role in prostate cancer tumorigenesis and development by activating the MAPK signaling pathway. We also found that sensitivity of prostate cancer cells to gemcitabine was positively correlated with HMGN5 expression. Knockdown of HMGN5 expression reduced the sensitivity of PC-3 cells to gemcitabine, and ectopic HMGN5 expression in DU145 cells enhanced the sensitivity to gemcitabine. Gemcitabine decreased HMGN5 expression, consequently leading to inactivation of the MAPK signaling pathway and cleavage of the PARP protein. Finally, we showed that PC-3 cells acquire gemcitabine resistance by gradual loss of HMGN5 expression. The present study suggests that HMGN5 is a potential biomarker for treating prostate cancer, and patients with a high level HMGN5 will benefit from gemcitabine treatment.

Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related death among American men. In 2012, an estimated 241,740 men were diagnosed with prostate cancer, and 28,170 died of the disease (1). Radical prostatectomy, radiation therapy or hormonal therapy is often effective for newly diagnosed prostate cancer patients. However, a number of patients will progress after two to three years, and the growth of cancer will resume despite hormone therapy (2,3). Unfortunately, the clinical treatment of advanced prostate cancer still remains a challenge.

Gemcitabine is a nucleoside analog that inhibits DNA biosynthesis, and is active against a broad spectrum of solid tumors (4), such as non-small cell lung cancer, pancreatic, bladder and breast cancer. Although gemcitabine significantly inhibits the growth of prostate cancer cell lines (5,6), clinical data suggest only modest activity when gemcitabine is used as a single agent for metastatic androgen-independent prostate cancer (AIPC), and the efficacy of gemcitabine for AIPC varies widely between patients (7). Therefore, it is imperative to investigate the mechanism of gemcitabine inhibition of prostate cancer, and define the gemcitabine-sensitive patient subgroups.

HMGN5, also known as nucleosome binding protein 1 (NSBP1), is a new member of the high mobility group N (HMGN) protein family (8). HMGN5 is localized to the nucleus by a nucleosome-binding domain, and binds to histone proteins by its negatively charged C-terminus, which unfolds chromatin and counteracts linker histone-mediated chromatin compaction and affects transcription and DNA repair (9-11). HMGN5 plays an important role during development and tumorigenesis (8,12,13), and HMGN5 expression is upregulated in prostate (14), bladder (15), clear cell renal cell carcinoma (16), breast (17), cutaneous squamous (18) and ovarian cancer (19), suggesting an association between high HMGN5 expression and tumorigenesis. In our previous studies, we found that HMGN5 was overexpressed in prostate cancer tissues and prostate cancer cell lines, and downregulation of HMGN5 with shRNA caused cell cycle arrest, growth inhibition and apoptosis *in vitro* and *in vivo* (20,21). We also found that gemcitabine downregulated the expression of HMGN5 (22). This finding motivated us to investigate whether gemcitabine exerts antitumor effects through inhibition of the HMGN5 pathway in prostate cancer.

In the present study, we demonstrated that HMGN5 promotes prostate cancer development through activation of the MAPK signaling pathway, and we also found that gemcitabine suppressed growth of prostate cancer cells by

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inhibiting HMGN5, and that prostate cancer cells expressing a high level of HMGN5 are more sensitive to gemcitabine. These findings indicate that HMGN5 is a potential treatment marker for prostate cancer, and that patients expressing a high level of HMGN5 will benefit from gemcitabine treatment.

Materials and methods

Cell culture. The human prostate cancer cell lines, LNCaP, DU145, 22RV1 and PC-3, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The PC-3M cell line was purchased from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). Cells were cultured in RPMI-1640 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) with 1% antibiotics. The human prostate epithelial cell line RWPE-1 was purchased from ATCC, and was maintained in keratinocyte serum-free medium supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37˚C in a humidified incubator with 5% CO_2 and 95% O_2.

shRNA lentiviral vector and HMGN5-expressing lentiviral vector infection. HMGN5 shRNA sequences and construction of the lentivirus were identical to those used in a previous study (23). PC-3 cells were seeded into 6-well plates and grown to 70% confluency. On the day of infection, the purified lentivirus (shCtrl or shHMGN5) was added to the cells at a multiplicity of infection (MOI) of 20 for 6 h, and cells were washed twice with medium. Successful knockdown of HMGN5 was analyzed by western blotting and real-time quantitative PCR (qPCR) after 72 h.

Human HMGN5 cDNA was obtained from the German Science Centre for Genome Research (RZPD), and the fragments containing the HMGN5 coding sequence were subcloned between the *Age*I and *Nhe*I sites of the GV205 lentiviral vector (synthesized by GeneChem Corporation, Shanghai, China). The lentiviral construct was verified by standard DNA sequencing.

qPCR. Total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. We reverse-transcribed total RNA (3 μg) using the reverse transcription system (Promega, Madison, WI, USA). qPCR was performed using SYBR-Green PCR Mix (Roche, Indianapolis, IN, USA) in an Applied Biosystems 7300 Fast Real-Time PCR system. The primer sequences for real-time qPCR were as follows: HMGN5, GCAGTCAGGCAGT GACTGCCTTCG (forward) and CCCTTTTCTGTGGCATC TTC (reverse); GAPDH, CAGTCAGCCGCATCTTCTTTT (forward) and GTGACCAGGCGCCCAATAC (reverse). Gene expression analysis was performed using the comparative ΔΔCt method; expression was normalized to GAPDH.

Western blot analysis. Protein lysate was prepared by homogenization in RIPA lysis buffer containing phosphatase and protease inhibitors. Western blot assay was performed according to previously described protocols (24). Anti-HMGN5 (Sigma, St. Louis, MO, USA), -ERK1/2, -pERK1/2, -GAPDH and -PARP (Cell Signaling Technology, Inc., Beverly, MA, USA) were used as primary antibodies, and either goat anti-mouse IgG or goat anti-rabbit IgG (Sigma) was used as secondary antibody. The membrane was visualized using the ECL detection system (GE Healthcare Biosciences, Piscataway, NJ, USA).

Cell proliferation assay. Cell proliferation was assessed using the CellTiter-Blue reagent (Promega) according to the manufacturer's instructions. Cells (2,000-5,000/well) were seeded into a 96-well plate and cultured for 12 h before new medium containing different doses of gemcitabine was added; cells were cultured for an additional 48 h, then 20 μl of CellTiter-Blue reagent was added to each well. Plates were incubated for 4 h at 37˚C, then fluorescence was recorded at an excitation wavelength of 560 nm and an emission wavelength range of 590 nm using a Labsystems Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Boston, MA, USA).

Cell invasion assay. The cell invasion assay was performed with 8-μm cell culture inserts (Millipore Corporation, Billerica, MA, USA) coated with 60 μl ECM gel (BD Biosciences, Bedford, MA, USA) mixed with RPMI-1640 serum-free medium in 1:4 dilution for 5 h at 37˚C. Infected PC-3 cells ($5x10^4$) were resuspended in 100 μl serum-free RPMI-1640 and placed into Matrigel, and the lower chamber of the Transwell was filled with 600 μl of RPMI-1640 containing 10% FBS before incubating cells at 37˚C for 24 h. The Transwells were removed from the 24-well plates and fixed with 100% methanol for 30 min, and then stained with 0.1% crystal violet for 30 min. Non-invaded cells on the top of the Transwell were removed with a cotton swab. Five visual fields were chosen randomly under a light microscope at x200 magnification, and invaded cells were counted. The data are expressed as means ± SD (standard deviation).

Colony formation assay. After infection for 24 h, cells (1,000/well) were seeded into a 6-well plate, and culture medium was changed every 3 days for 2 weeks. Colonies (50 or more cells/colony) were counted after staining with gentian violet.

Apoptosis assay. The apoptosis assay was performed using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega) according to the manufacturer's instructions. Following infection for 24 h, the PC-3 cells (5,000/well) were seeded into a 96-well plate and cultured for 48 h, and then 100 μl of Apo-ONE Caspase-3/7 reagent was added to each well, and the plate was incubated at room temperature for 2 h before measuring the fluorescence of each well at an excitation wavelength of 480 nm and an emission wavelength range of 530 nm.

Statistical analysis. All data groups were analyzed by one-way ANOVA using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA), and the graphs were generated in MS Excel. Data are expressed as means ± SD. A probability (P) value of <0.05 was assigned to indicate a statistically significant difference when compared with the control and is indicated by an asterisk in the figures.

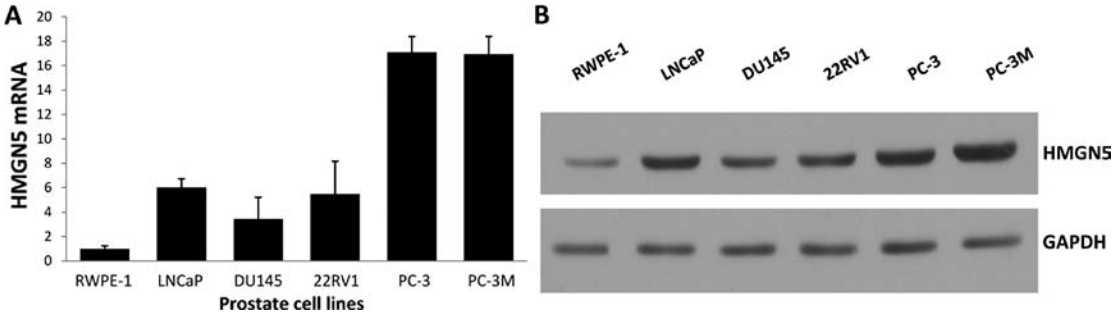

Figure 1. Overexpression of HMGN5 in the prostate cancer cell lines. (A) qRT-PCR analysis of the HMGN5 mRNA levels in the RWPE-1, LNCaP, DU145, 22RV1, PC-3 and PC-3M cells showed that HMGN5 was overexpressed in all prostate cancer cells when compared with the level in the normal prostate epithelial cells (RWPE-1). (B) Western blot analysis demonstrated that the HMGN5 protein level was elevated in the prostate cancer cells.

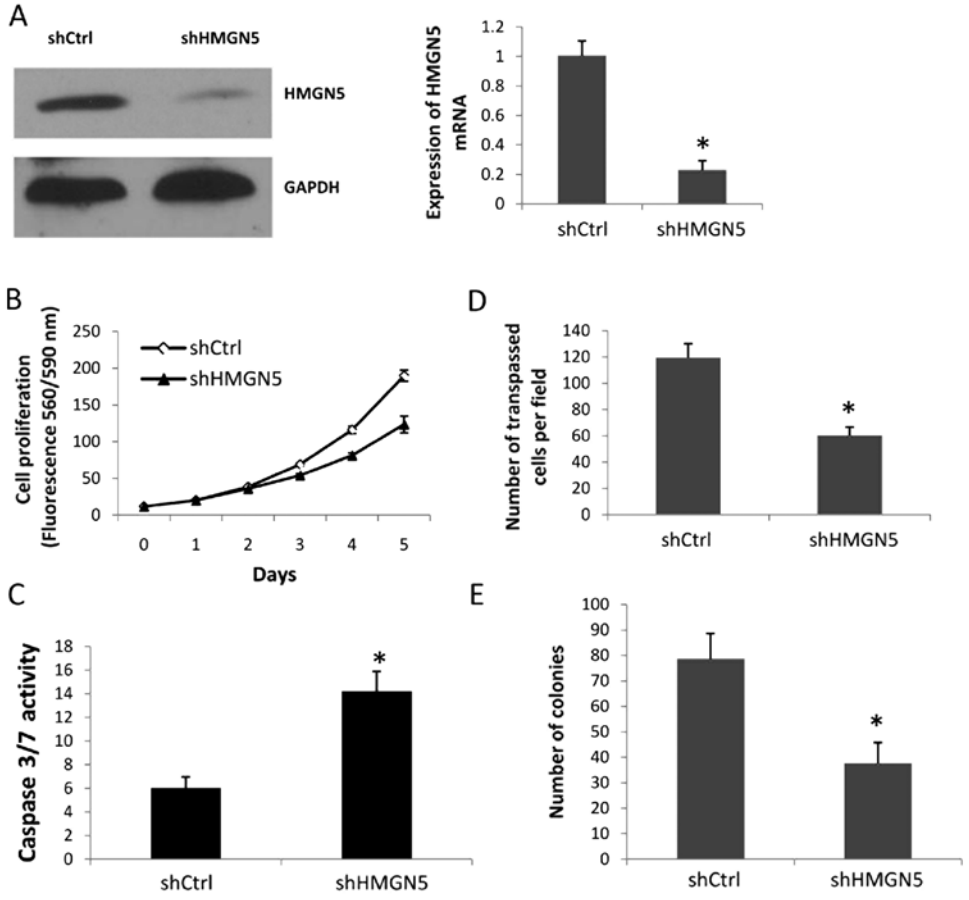

Figure 2. HMGN5 knockdown decreases prostate cancer cell growth and invasion, and promotes prostate cancer cell apoptosis. (A) Western blot and qRT-PCR analyses demonstrated that HMGN5 expression in the PC-3 cells infected with shHMGN5 for 72 h was reduced at both the protein and mRNA levels when compared with these levels in the shCtrl-infected PC-3 cells. (B) Cell proliferation assay showed that HMGN5 knockdown significantly reduced the proliferation of the PC-3 cells over a 5-day period. (C) Knockdown of HMGN5 induced apoptosis in the PC-3 cells. Apoptosis was detected by homogeneous caspase-3/7 assay. (D) Knockdown of HMGN5 inhibited the invasion of the PC-3 cells. Matrigel invasion assay showed the invasion of PC-3 cells/field (magnification, x200). (E) Colony formation assay indicated that knockdown of HMGN5 inhibited PC-3 colony formation. Data are shown as the average number of colonies/1,000 cells plated. The results are expressed as the means ± SD of triplicate values for each sample. All P-values were obtained from the t-test. *P<0.05 vs. the shCtrl-infected control group.

Results

HMGN5 expression is high in prostate cancer cells. HMGN5 expression was determined using real-time qPCR and western blot analysis in the prostate cancer cell lines, including LNCaP, DU145, 22RV1, PC-3 and PC-3M, as well as human prostate epithelial cell line RWPE-1. Both the mRNA and protein levels of HMGN5 were higher in the prostate cancer cell lines than the levels in the control human prostate epithelial cell line, and the HMGN5 protein level was correlated with its mRNA level (Fig. 1A and B).

HMGN5 knockdown decreases prostate cancer cell growth and invasion, and promotes prostate cancer cell apoptosis. To

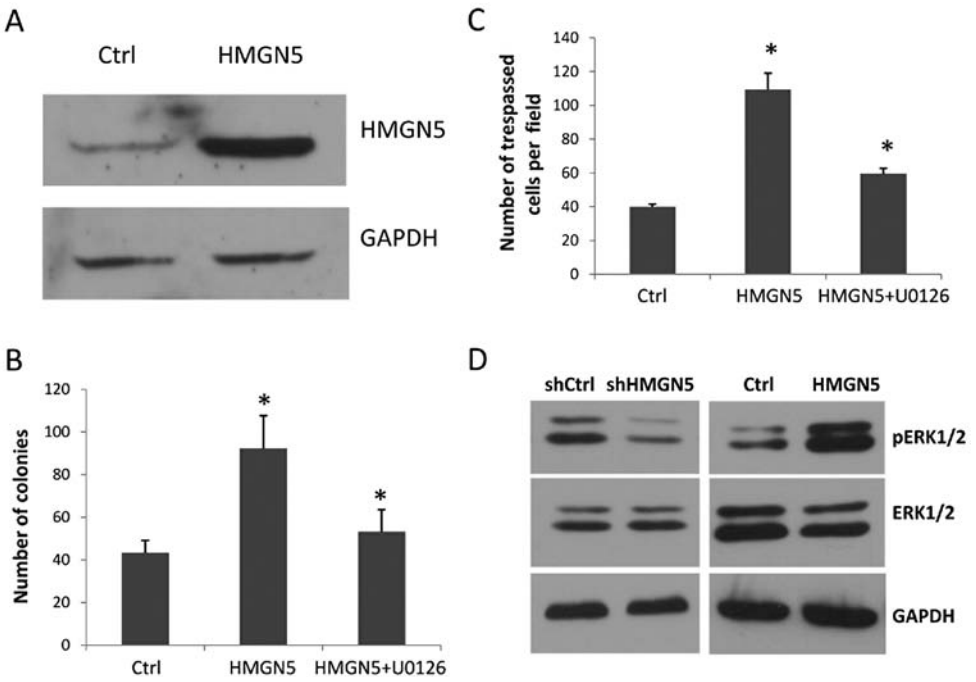

Figure 3. Ectopic HMGN5 expression induces normal prostate epithelial cell colony formation and invasion via the MAPK signaling pathway. (A) Western blotting demonstrated that HMGN5 protein expression in the RWPE-1 cells with the HMGN5-expressing lentiviral vector was significantly increased when compared with that in the RWPE-1 cells with the control lentiviral vector after 72 h. (B) Ectopic HMGN5 expression promoted RWPE-1 cell colony formation, which was abrogated by 10 μM U0126. The RWPE-1 cell colony formation is presented as the average number of colonies/1,000 cells plated ± SD; n=3 biological replicates. (C) Treatment with 10 μM U0126 also abrogated the HMGN5-mediated invasion. (D) Knockdown of HMGN5 in the PC-3 cells significantly decreased pERK1/2, which is a MAPK signaling pathway marker, and ectopic HMGN5 expression in RWPE-1 increased the pERK1/2 level; no obvious change for ERK1/2, a precursor of pERK1/2, was observed in either cell line. All P-values were obtained from the t test. *P<0.05 vs. the control-infected group.

explore the function of HMGN5 in prostate cancer, we used a loss-of-function approach. PC-3 cells were infected with the shHMGN5 or shCtrl lentiviral vectors for 72 h; the shHMGN5 reduced HMGN5 expression at both the mRNA and protein levels (Fig. 2A). Cell proliferation assay results showed that the PC-3 cells with shHMGN5 exhibited a consistent decrease over the time course examined, with a decrease in proliferation of 30.03% at day 4 and 34.91% at day 5, compared with the PC-3 cells with shCtrl (Fig. 2B). To determine whether the downregulation of HMGN5 promoted PC-3 cell apoptosis, we used a homogeneous caspase-3/7 analysis, in which caspase-3/7 activity denoted the apoptosis level. The results showed that shHMGN5 induced the activity of caspase-3/7 compared with shCtrl in the PC-3 cells (Fig. 2C). Next, we assessed the role of HMGN5 in PC-3 cell invasion. The cell invasion assay indicated that there were fewer PC-3 cells in the shHMGN5-infected group when compared with the shCtrl group; the number of cells crossing the Matrigel was 60.3±6.4 in the shHMGN5 group vs. 119.4±10.8 in the shCtrl group (Fig. 2D). To further assess the tumor promoter functions of HMGN5 in prostate cancer cells, infected PC-3 cells were assessed with anchorage-dependent colony formation assays. After 2 weeks of culture, the number of colonies in the PC-3 cells infected with shHMGN5 was significantly less than that in the PC-3 with the shCtrl (Fig. 2E). These data provide evidence that HMGN5 promotes cell proliferation, invasion and clonogenicity, and antagonizes cell apoptosis.

HMGN5 overexpression induces colony formation and invasion. To further investigate the function of HMGN5 in prostate cancer tumorigenesis, we constructed an HMGN5-expressing lentiviral vector, and the human prostate epithelial cell line RWPE-1 was engineered to stably express HMGN5. Western blot analysis showed that the HMGN5-transfected clones (RWPE-1-HMGN5) had a high level of HMGN5 protein compared with the GV205 (empty vector)-transfected controls (RWPE-1-Ctrl) (Fig. 3A). Then, the colony formation assay and cell invasion assay were performed, which mimic crucial events in tumorigenesis and cancer progression. We found that the RWPE-1-HMGN5 cells formed more, larger colonies compared with the RWPE-1-Ctrl cells in monolayer culture for 14 days (Fig. 3B). Furthermore, ectopic HMGN5 expression significantly increased the migration of the RWPE-1 cells in the cell invasion assay (Fig. 3C).

HMGN5 activates the MAPK signaling pathway. Abnormal activation of the MAPK signaling pathway plays an important role in prostate cancer tumorigenesis and progress; thus, we studied whether HMGN5 contributes to prostate cancer development by activating the MAPK signaling pathway. Western blot results showed that the level of pERK1/2 protein, which indicates the activity of the MAPK signaling pathway, was significantly increased in the RWPE-1 cells ectopically expressing HMGN5, and was strongly decreased in the PC-3 cells with HMGN5 knockdown when compared with the control (Fig. 3D). However, for ERK1/2, there was no difference in expression between the RWPE-1-HMGN5 and RWPE-1-Ctrl cells or the PC-3 with shHMGN5 and PC-3 with shCtrl cells (Fig. 3D). We observed the reverse effect following treatment with the ERK inhibitor U0126. As shown

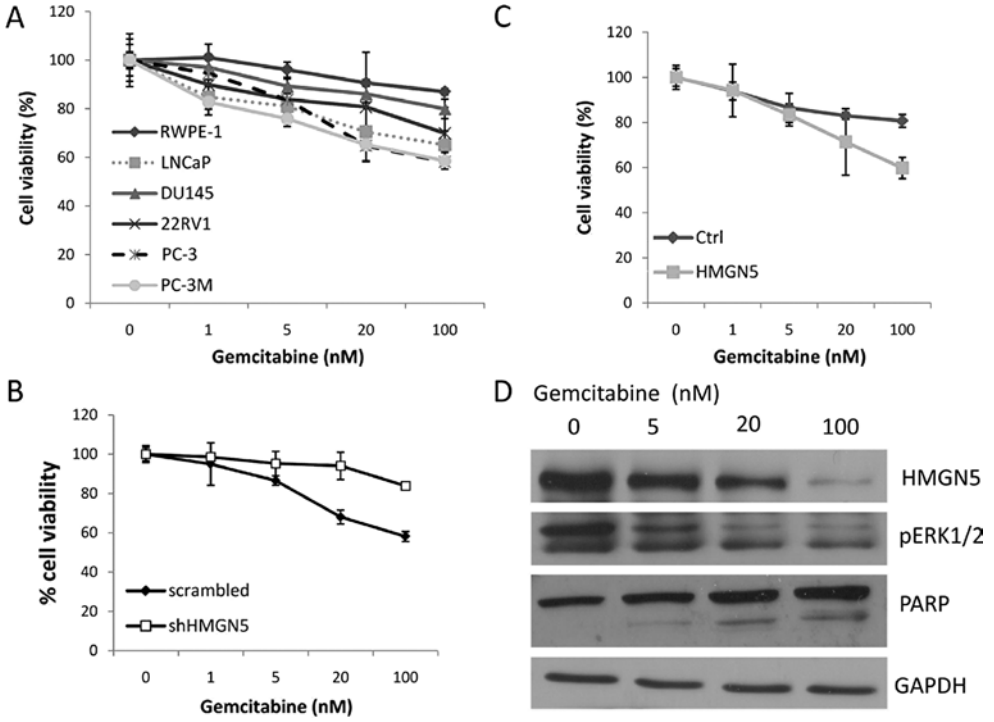

Figure 4. HMGN5 level determines the response of prostate cancer cells to gemcitabine. (A) Sensitivity of prostate cells to gemcitabine was consistent with the HMGN5 expression level in the prostate cancer cells. Prostate cancer cells (PC-3, PC-3M and LNCaP) with a high level of HMGN5 expression were sensitive to gemcitabine, yet RWPE-1, with a low level of HMGN5 expression, was resistant to gemcitabine. The sensitivity to gemcitabine of DU145 and 22RV1 cells, which expressed an intermediate level of HMGN5, were at a level between the prostate cancer cells and RWPE-1. (B) Knockdown of HMGN5 in the gemcitabine-sensitive PC-3 cells reduced sensitivity to gemcitabine. (C) Ectopic HMGN5 expression in the DU145 cells increased their sensitivity to gemcitabine. (D) Gemcitabine increased expression of HMGN5, pERK1/2 and PARP in the PC-3 cells. The PC-3 cells were treated with different doses of gemcitabine for 48 h. HMGN5, pERK1/2 and PARP expression was detected by western blotting; GAPDH was used as an internal control.

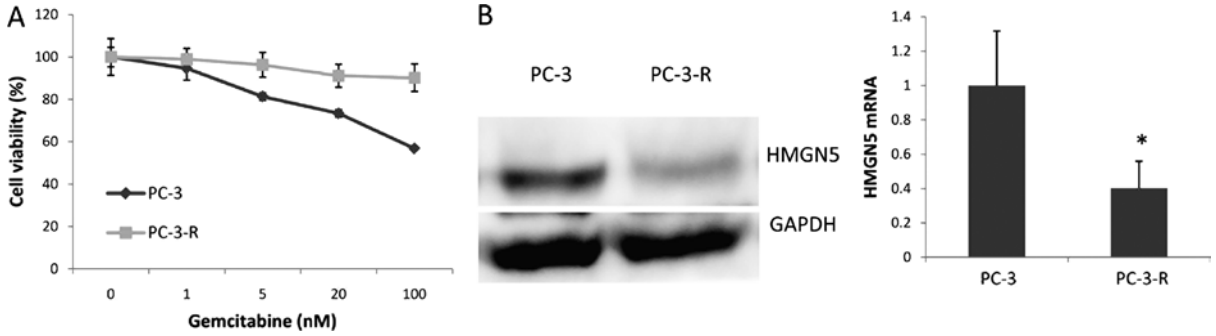

Figure 5. Loss of HMGN5 expression conferred secondary gemcitabine resistance. (A) Gemcitabine-resistant PC-3 cells (PC-3-R) were established by culturing PC-3 cells with a low level of gemcitabine for 3 months; the sensitivity of PC-3 and PC-3-R cells to gemcitabine was determined by cell proliferation analysis. (B) Compared with the PC-3 cells, PC-3-R cells expressed a lower level of HMGN5 at both the protein and mRNA levels.

in Fig. 3B and C, the invasion and clonogenicity mediated by ectopic HMGN5 expression in the RWPE-1 cells was attenuated by U0126. These results demonstrated that HMGN5 contributes to prostate cancer tumorigenesis and progression by activating the MAPK signaling pathway.

HMGN5 expression determines the response of prostate cancer cells to gemcitabine. Our previous study showed that gemcitabine downregulates HMGN5 mRNA levels, thus this next study focused on the relationship between the HMGN5 expression level and the response to gemcitabine in prostate cancer. First, we assessed the effect of gemcitabine on prostate cell lines with various levels of HMGN5 (Fig. 4A). Cell viability was detected by the CellTiter-Blue assay after 48 h of treatment with different doses of gemcitabine. Notably, cells with a higher level of HMGN5 expression (PC-3, PC-3M and LNCaP) showed more sensitivity to gemcitabine, and cells with a lower level of HMGN5 expression (DU145, 22RV1) showed less sensitivity to gemcitabine; cells with the lowest level of HMGN5 expression (RWPE-1) showed resistance to gemcitabine (Fig. 4A). Furthermore, downregulation of HMGN5 in the PC-3 cells with shHMGN5 resulted in the resistance of PC-3 cells to gemcitabine. PC-3 cells with shCtrl were sensitive to gemcitabine (Fig. 4B). Upregulation

of HMGN5 in DU145 cells induced their sensitivity to gemcitabine (Fig. 4C). Next, we explored the mechanism of HMGN5-mediated sensitivity to gemcitabine. We first tested whether gemcitabine treatment downregulated the HMGN5 protein level in PC-3 cells. After 48 h of treatment with different doses of gemcitabine, western blotting showed that gemcitabine downregulated HMGN5 expression in a dose-dependent manner (Fig. 4D), meanwhile, consistent with HMGN5 expression, the MAPK signaling pathway marker, pERK1/2, was also downregulated by gemcitabine. Cleavage of PARP was upregulated by gemcitabine (Fig. 4D).

We established a gemcitabine-resistant PC-3 cell line (PC-3-R) by culturing PC-3 cells with a low level of gemcitabine for 3 months. Notably, the PC-3-R cell line expressed less HMGN5 protein and mRNA compared with its parental cells (Fig. 5). Taken together, these results indicate that HMGN5 may be a potential biomarker for the treatment of prostate cancer patients with gemcitabine.

Discussion

In the present study, we found new evidence to prove that HMGN5 plays an oncogenic role in prostate cancer, and we demonstrated that knockdown of HMGN5 suppressed cell proliferation, reduced cell invasion and induced apoptosis in PC-3 cells, which express the highest level of HMGN5 among prostate cancer cell lines. Conversely, ectopic expression of HMGN5 in human prostate epithelial cell line RWPE-1 promoted cell colony formation and induced cell invasion.

We demonstrated that HMGN5 exerted its function by activating the MAPK signaling pathway, which plays a vital role in prostate cancer tumorigenesis and progression (25). Upregulation of HMGN5 activated the MAPK signaling pathway in RWPE-1 cells, and PC-3 cells with HMGN5 knockdown showed less pERK1/2 expression, indicating that the MAPK signaling pathway was less active. In addition, the HMGN5-mediated malignant phenotype was partially reversed by U0126, which is an ERK inhibitor. The mechanism by which HMGN5 activates the MAPK signaling pathway is unclear. HMGN5 is a nucleosome-binding protein. By binding to histone H1, HMGN5 can unfold chromatin and regulate transcription; therefore, HMGN5 may activate the MAPK signaling pathway by elevating the transcription of genes upstream of the MAPK signaling pathway.

Although gemcitabine has been clinically proven to act against a broad spectrum of solid tumors and inhibit prostate cancer cell lines effectively, the clinical effect of gemcitabine on AIPC is limited. Gemcitabine is usually used to treat AIPC in combination with other chemotherapy drugs, such as docetaxel or prednisone (26-28). In the present study, we found that there was a variation in the response of prostate cancer cell lines to gemcitabine; prostate cancer cells with higher HMGN5 expression showed more sensitivity to gemcitabine, and cells with lower expression of HMGN5 showed less sensitivity to gemcitabine. Knockdown of HMGN5 with shHMGN5 reduced the sensitivity of PC-3 cells to gemcitabine while ectopic expression of HMGN5 in DU145 cells promoted sensitivity to gemcitabine. Furthermore, gemcitabine was found to downregulate HMGN5 and pERK1/2 expression and upregulated cleaved-PARP. The mechanism of this result is unclear; gemcitabine may have killed the subpopulation of PC-3 cells with a high level HMGN5, and the remaining cells expressed a low level of HMGN5. Another explanation for this may be that gemcitabine causes downregulation directly in prostate cancer cells.

Moreover, we also found a decrease in HMGN5 in secondary gemcitabine-resistant PC-3 cells, indicating that prostate cancer cells may gain gemcitabine resistance by downregulating HMGN5 expression. Collectively, we found that HMGN5 may be a biomarker which predicts the response of prostate cancer to gemcitabine.

HMGN5 is a nucleosome-binding protein. It can bind to histone protein H1 by its negatively charged C-terminus, and unfold chromatin and counteract linker histone-mediated chromatin compaction, as gemcitabine exerts its antitumor activity by inhibiting DNA biosynthesis. HMGN5 may promote the effect of gemcitabine by loosening chromatin and facilitating gemcitabine to combine and react with DNA.

Based on our data, we conclude that HMGN5 is an oncogene and plays an important role in prostate cancer tumorigenesis and progression. HMGN5 has the potential to be a therapeutic target for prostate cancer treatment. Moreover, the level of HMGN5 may be used as a biomarker to predict the patients that would benefit from gemcitabine treatment.

Acknowledgements

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