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High Mobility Group A2 (HMGA2) Promotes EMT *via* MAPK Pathway in Prostate Cancer

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Abstract

Studies have shown that High mobility group A2 (HMGA2), a non-histone protein, can promote epithelial-mesenchymal transition (EMT), which plays a critical role in prostate cancer progression and metastasis. Interestingly, full-length or wild-type HMGA2 and truncated (lacking the 3'UTR) HMGA2 isoforms are overexpressed in several cancers. However, there are no studies investigating the expression and differential roles of WT *vs* truncated HMGA2 isoforms in prostate cancer. Immunohistochemical staining of prostate tissue microarray revealed low membrane expression in normal epithelial prostate cells, and that expression increased with tumor grade as well as a switch from predominantly cytoplasmic HMGA2 in lower tumor grades, to mostly nuclear in high grade and bone metastatic tissue. LNCaP cells stably overexpressing wild-type HMGA2 displayed nuclear localization of HMGA2 and induction of EMT associated with increased Snail, Twist and vimentin expression compared to LNCaP Neo control cells, as shown by immunofluorescence and western blot analyses. This was associated with increased cell migration on collagen shown using boyden chamber assay. Conversely, LNCaP cells overexpressing truncated HMGA2 showed cytoplasmic HMGA2 expression that did not induce EMT yet displayed increased cell proliferation and migration compared to LNCaP Neo. Both wild-type and truncated HMGA2 increased levels of phospho-ERK, and interestingly, treatment with U0126, MAPK inhibitor, antagonized wild-type HMGA2-mediated EMT and cell migration, but did not affect truncated HMGA2-mediated cell proliferation or migration. Therefore, although both wild-type and truncated HMGA2 may promote prostate tumor progression, wild-type HMGA2 acts by inducing EMT *via* MAPK pathway.

Keywords

Epithelial Mesenchymal Transition; Prostate Cancer; MAPK/ERK

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Conflict of interest

The authors declare no conflict of interest.

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1. INTRODUCTION

Prostate cancer is the most common malignant cancer in men in several countries [1]. Epithelial-mesenchymal transition (EMT) plays a critical role in cancer progression and metastasis. Cancer cells downregulate epithelial markers such as E-cadherin, while mesenchymal markers such as SNAIL1 (Snail), Twist, and some cytokeratin filaments like vimentin are upregulated, [2, 3]. Therefore, cancer cells become invasive, migratory and able to breakdown the basement membrane in order to metastasize [4].

High mobility Group A2 (HMGA2) is a member of non-histone chromatin protein. HMGA2 is highly expressed during the embryogenesis while expression decreases or is absent in the adults. HMGA2 has a role in regulating the development and differentiation [5]. HMGA2 is known as an architecture transcription factor which alters the chromatin structure to either promote or inhibit gene expression [6]. Overexpression of HMGA2 has been reported in many cancers such as lung carcinomas [7], ovarian cancer [8], breast cancer [9], gastric cancer [10]. With regards to prostate, HMGA2 overexpression in tissue has only been demonstrated using real-time polymerase chain reaction in a canine prostate tissue model [11]. HMGA2 up-regulation mediated by TGF- β signaling pathway led to EMT and tumor metastasis *in vitro* and *in vivo* as shown using colorectal and breast cancer mouse models [12]. A study demonstrated that HMGA2 co-associates with SMAD to bind to Snail promoter, which upregulates Snail expression, consequently inducing EMT [13]. Let-7 miRNA has seven complementary sites in 3'UTR HMGA2 which regulates protein expression [14]. HMGA2 protein expression increases by losing the 3'UTR region due to the chromosomal rearrangements in the region 12 q13–15, where HMGA2 gene is located [15]. Both full length (wild-type) and truncated HMGA2 are upregulated in several cancers [16] however, it has never been investigated in prostate cancer.

The aim of this study is to investigate HMGA2 expression in human prostate patient tissue and begin to dissect the differential mechanism(s) of action of wild-type *vs* truncated HMGA2 isoform in prostate cancer. The study suggested that HMGA2 shows low membrane expression in normal epithelial prostate cells, and that expression increases with tumor grade as well as a switch from predominantly cytoplasmic HMGA2 in lower tumor grades, to mostly nuclear in high grade and bone metastatic tissue. Overexpression of wild-type but not truncated HMGA2 induced EMT in LNCaP prostate cancer cells through activation MAPK pathway. Furthermore, overexpression of wild-type HMGA2 increased cell migration, while truncated HMGA2 increased cell proliferation and migration.

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

Prostate cancer cells panel RWPE1, DU145, PC3, 22Rv1 and LNCaP cells were from American Type Culture Collection (Manassas, VA). C4–2, ARCaP-E, an epithelial cell line and ARCaP-M the mesenchymal cell line were a kind gift from Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA, USA. All these cells were grown in RPMI-1640 from (Lonza, Alpharetta, GA) supplemented with 10% (v/v) FBS from (Atlanta biological, Flowery Branch, GA), 50 μ g/ml penicillin, and 100 μ g/ml streptomycin, except for RWPE1

cells, which were grown in Keratinocyte-SFM media supplemented with keratinocyte supplements (ThermoFisher Scientific, Grand Island, NY). The African American prostate cancer cell line E006AA parental and its more aggressive subline E006AA-hT were a kind gift from Dr. Shahriar Koochekpour of Roswell Park Cancer Institute (Buffalo, NY). These cells were grown in DMEM from (Lonza, Alpharetta, GA) with 10% (v/v) FBS, 1X penicillin-streptomycin (Mediatech, Herndon, VA). All cells were maintained at 37°C with 5% CO₂ in a humidified incubator. Nitrocellulose membrane was from Bio-Rad (Hercules, CA). Anti-mouse β -actin, anti-rabbit Twist, and anti-rabbit ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rat monoclonal Snail antibody and anti-rabbit p44/42 MAPK (Erk1/2) were from Cell Signaling Technology, Danvers, MA. Goat monoclonal anti-HMGA2, and goat monoclonal anti-vimentin antibody were purchased from R&D Systems (Minneapolis, MN). Anti-mouse monoclonal E-cadherin was from B.D Biosciences. Antimouse Occludin was from life technologies. Luminata and the protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN. Rat tail collagen I was from BD Biosciences (Franklin Lakes, NJ). UO126 (MEK1/2 inhibitor) was purchased from EMD Callbiochem (Billerica, MA).

2.2 Stable Overexpression of HMGA2

LNCaP parental cells were plated in 6 well culture dish at 80% confluence without antibiotics overnight. Next day, cells were transfected with either HMGA2 wild-type plasmid, HMGA2 truncated plasmid, or empty vector pcDNA 3.1 Neo (provided from Addgene, Cambridge, MA) using Lipofectamine 2000 (Invitrogen, San Diego, CA) for 72 h. Stable transfectants were selected using 800 μ g/ml G418 and maintained in 400 μ g/ml G418 followed by selection of single colonies; HMGA2 wild-type (WT4, WT9, WT21), HMGA2 truncated (TR2, TR18, TR23), and empty vector Neo (Neo8, Neo19). Selected clones were tested for HMGA2 expression by western blot analysis.

2.3 Western blot analysis

Cells were lysed in a modified RIPA buffer as described previously [17]. Supernatants were quantified using a micro BCA assay (Promega, Madison, WI). 20–30 μ g of cell lysate was resolved using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by trans-blotting onto nitrocellulose membrane. Membranes were incubated with appropriate primary and secondary antibody, followed by visualization using Luminata Forte ECL reagent. The membranes were stripped using Restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL) prior to reprobing with a different antibody.

2.4 Treatments with MAPK inhibitor (U0126)

2×10^6 cells were plated in T-75 flask overnight; next day cells were serum starved for 2 h followed by treatment with 20 μ M U0126 or 0.005% DMSO for 72 h, prior to collecting cell lysate or performing cell migration/proliferation assays.

2.5 Immunofluorescence

5×10^3 cells were plated into 16 well chamber slides (Bio-Tek, Nunc, Winooski, VT), the cells were fixed with methanol/ethanol 1:1 volume and blocked with 5% goat serum.

Subsequently, slides were incubated with primary antibody at 1:50 or 1:100 dilutions in Dako antibody diluent solution for 1 h at room temp, washed with 1× TBS-T (Dako, Camarillo, CA), then incubated with secondary antibody in the dark for 1 h at room temp. Secondary antibodies used were goat anti-rabbit Oregon green 488, anti-mouse Alexa red 594, and rabbit anti-Goat IgG Alexa Fluor 594 (Invitrogen, Carlsbad, CA). Slides were washed prior to counterstaining with DAPI (1 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA). Slides were mounted using Fluoro gel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Fluorescence microscopy was performed using Zeiss microscope and Axiovision Rel 4.8 software.

2.6 Immunohistochemistry

We examined the expression of HMGA2 in normal human prostate tissue and different stages of prostate cancer by immunohistochemistry (IHC) utilizing tissue microarray (US Biomax; PR8011a). IHC was performed using the Avidin-biotin method. Tissue was deparaffinised in xylene and rehydrated using alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, followed by 10% serum to avoid non-specific binding. Sections were incubated with 1:200 primary antibody against HMGA2 at 4°C overnight followed by biotinylated secondary antibody, and incubation with avidin-biotin complex (Vector, Burlingame, CA). Immunoreactivity was visualized using diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). The slide was subsequently counterstained with hematoxylin and mounted with xylene solution. Images were acquired using the Leica Aperio VERSA.

2.7 Cell Migration

We utilized Costar 24-well plates containing a polycarbonate filter insert with an 8- µm pore size, to coat with 4.46 µg/ µl rat tail collagen I (BD Bioscience, Bedford, MA) on the outside for 24 h at 4°C. 1×10^4 cells were plated in the upper chamber containing RPMI phenol red free media supplemented with 0.5% Charcoal Stripped Fetal Bovine Serum (DCC), whereas the lower chamber contained RPMI supplemented with 10% FBS. After 48 h, cells that migrated to the bottom of the insert were fixed, stained with 0.05% crystal violet, and counted to obtain the relative cell migration. Each experiment was done in triplicate, and the graphs represent an average of the 3 wells. The experiments were repeated at least three times.

2.8 Cell viability assay

LNCaP Neo, LNCaP HMGA2 WT, and HMGA2 TR cells were plated at a density of 2,000 cells per well in 96-well plates and allowed to attach overnight. Viability was assessed daily using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the supplier's protocol.

2.9 Statistical analysis

Data were analyzed by a paired student's t-test or ANOVA using GraphPad Prism software.

3. RESULTS

3.1 HMGA2 expression increases with prostate cancer progression

Initially, we wanted to evaluate the basal expression of the HMGA2 protein in human prostate cell lines and patient tissue. Western blot analysis showed highest HMGA2 expression in PC3, ARCaP-E, and ARCaP-M aggressive prostate cancer cells (Fig. 1A). Interestingly, PC3 cells showed two bands that might be due to the truncated isoform or post-translation modification as has been observed for HMGA1 protein in PC3 cell lines [18]. Immunofluorescence analysis illustrated that PC3 and ARCaP-M (mesenchymal phenotype) showed both cytoplasmic and nuclear localization of HMGA2, however, ARCaP-E (epithelial phenotype) showed predominantly cytoplasmic expression of HMGA2 (Fig. 1B). In order to examine HMGA2 expression in prostate cancer patients, we utilized a prostate tissue microarray to analyze the expression by IHC staining. This revealed expression of HMGA2 was detectable with very low expression in normal tissue that was mainly localized within the cell membrane (Fig. 2). The HMGA2 levels and the translocation from cytoplasmic toward nuclear expression were increased with the stage of prostate cancer as well as bone metastasis sample (Fig. 2). Therefore, HMGA2 expression may increase with prostate cancer progression.

3.2 Overexpression of WT HMGA2 induces EMT prostate cancer, while both WT and TR HMGA2 increase cell viability and migration

To test the differential role of HMGA2 isoforms in prostate cancer cells, we stably overexpressed HMGA2 wild-type (WT), HMGA2 truncated (TR), or Neo (empty vector) cDNA into LNCaP cells, which has low endogenous levels of HMGA2. From several colony selections, we chose three representative colonies for WT (HMGA2 WT4, WT9, WT21) and TR (HMGA2 TR2, TR18, and TR23), and two representative controls (Neo8, Neo19). The exogenous WT HMGA2 protein in LNCaP cells was at the same molecular weight as the endogenous expression of HMGA2 in ARCaP and PC3 (upper band) prostate cancer cell lines at about 20 KDa, however, HMGA2 TR in LNCaP cells was detected at 16KDa (Fig. 3A), which corresponds to loss of part of the C-terminus that contains the nuclear localization sequence, as well as the 3' untranslated region [19]. Immunofluorescence revealed that WT HMGA2 displayed nuclear and cytoplasmic localization while TR HMGA2 was exclusively cytoplasmic (Fig. 3B). Next, we examined expression of EMT markers by western blot analysis. Overexpression of HMGA2 WT but not TR increased the expression of mesenchymal markers, Snail, vimentin, Twist, compared to LNCaP Neo (Fig. 3A). Immunofluorescence analysis confirmed western blot results and further showed that although western blot showed that epithelial markers E-cadherin and Occludin did not decrease but in fact seemed higher for both WT and TR overexpression (Fig. 3A), the localization was no longer at the membrane as seen for LNCaP Neo, but cytoplasmic for WT and nuclear for TR (Fig. 3B). Functionally, we detected a significant increase in cell viability with cells overexpressing HMGA2 TR compared with LNCaP Neo control, while no significant change in cell viability was observed with cells overexpressing HMGA2 WT (Fig. 3C). However, LNCaP cells overexpressing HMGA2 WT and TR both significantly increased the cells' migratory potential across collagen compared to the LNCaP Neo control,

with HMGA2WT having a higher effect (Fig. 3D). Therefore, HMGA2 WT promotes cell migration and EMT while HMGA2 promotes cell viability and cell migration but not EMT.

3.3 HMGA2 WT induces EMT via ERK pathway

HMGA2 and Smads bind to Snail promoter to increase the expression of Snail followed by EMT transition [13]. Previously we have been demonstrated that Snail can regulate the ERK pathway in breast cancer cells to promote EMT [20]. We sought to examine whether HMGA2 WT can regulate EMT via the ERK pathway. We showed by western blot analysis, an increase in phospho-ERK (p-ERK) expression after we overexpressed both WT and TR HMGA2, as compared to LNCaP Neo (Fig. 4A). Western blot analysis showed that treatment with MAPK inhibitor, U0126, effectively inhibited p-ERK in cells overexpressing HMGA2 WT and TR (Fig. 4B). Interestingly, we detected a decrease in the mesenchymal markers, Snail and vimentin, and an increase in E-cadherin in HMGA2 WT cells treated with U0126, compared to untreated cells or cells treated with DMSO control, while HMGA2 TR cells were unaffected (Fig. 4B). Treatment with U0126 decreased cell migration in LNCaP cells overexpressing HMGA2 WT compared with control DMSO ($p=0.267$), but functionally had no effect on cell viability (Fig. 4C) or migration for cells overexpressing HMGA2 TR (Fig. 4D). Therefore, wild-type HMGA2 induces EMT in part via the MAPK signaling pathway.

4. DISCUSSION

High Mobility Group A2 (HMGA2) expression has been reported in cancers such as pancreatic cancer [21], lung cancer [22], and recently in a canine prostate cancer model [11]. Due to chromosomal rearrangements within HMGA2 gene, loss of the 3'UTR regulatory region can occur, which leads to overexpression of HMGA2 protein due to deletion of the Let-7 miRNA suppressor binding sites [23]. Both HMGA2 isoforms, wild-type (WT) [24] and truncated (TR) have been reported to up-regulated in cancers including uterine leiomyoma [15], bone and soft tissue chondromas [25], and lipomas [26]. Studies also show that HMGA2 promotes EMT [27, 28] and induces metastasis in several cancers [12, 29]. However, HMGA2 expression in human prostate tissue and the role of HMGA2 isoforms in prostate cancer has not been reported. In this study, we sought to investigate the differential roles of HMGA2 isoforms in prostate cancer.

Here, we demonstrate that HMGA2 is highly expressed in the aggressive prostate cancer cell lines, PC3, ARCaP-E and ARCaP-M. Furthermore, immunofluorescence shows HMGA2 is cytoplasmic in ARCaP-E or nuclear/cytoplasmic localization in ARCaPM and PC3 cells. PC3 displayed two bands might be due to post translation modification or truncated protein. In patient tissue, we found an increase in HMGA2 expression along with translocation into the nucleus with prostate cancer progression while in normal prostate tissue, there was low HMGA2 expression expressed at the cell membrane. This is similar to a study by Li et al., which found HMGA2 translocated from the membrane into the nucleus in a high passage of keratinocytes (passage 14) and during carcinoma development [30]. In our study, lower grade prostate cancer tissues showed cytoplasmic expression of HMGA2 along with positive expression in stroma. Studies showed combined expression HMGA2 and AR in the stroma

increases the invasiveness of prostate cancer [31]. We observed nuclear expression of HMGA2 in later grade 3 and in bone metastatic tissue. When we stably overexpressed HMGA2 truncated or wild-type cDNA in LNCaP cells, we noted cytoplasmic localization of HMGA2 truncated isoform, while HMGA2 wild-type was nuclear/cytoplasmic localization. The exclusion of truncated HMGA2 from the nucleus can be explained by the construct also lacking part of the C-terminus that contains the nuclear localization sequence. We also showed that wild-type HMGA2 but not the truncated HMGA2 was able to induce the EMT by increasing expression of mesenchymal markers, Snail, Twist and vimentin. Although epithelial marker expression was not decreased, it was no longer at the membrane but cytoplasmic for HMGA2 WT and interestingly, nuclear for HMGA2 TR. We have previously shown that Snail overexpression in LNCaP cells induced EMT without significantly changing E-cadherin expression, but its translocation from the membrane to the nucleus [32]. Additionally, studies have reported E-cadherin cleavage at its cytoplasmic domain *via* p120 which leads to translocation of E-cadherin into the nucleus and gene transcription[33]. It is therefore possible that truncated HMGA2 may mediate oncogenic activity via nuclear E-cadherin by mechanisms that are unknown at this time.

We found that exogenous overexpression of HMGA2 WT and TR increases the levels of p-ERK. Furthermore, inhibiting the MAPK signaling pathway using U0126 antagonized HMGA2 WT-mediated EMT and cell migration, without affecting TR-mediated cell viability or migration. That suggested HMGA2 WT utilizes MAPK signaling pathway to induce EMT transition in prostate cancer. Our study shows that overexpression of HMGA2 TR increases cell proliferation and migration, which was unaffected by inhibiting MAPK signaling with U0126 inhibitor. Previous studies have shown that overexpression of truncated HMGA2 in transgenic mice increased proliferative hematopoiesis along with hypercellular bone marrow and splenomegaly with extramedullary erythropoiesis [34]. However, others showed that full-length HMGA2 has the oncogenic activity while its truncated form has no critical role in cellular transformation in mesenchymal tumors [24]. Therefore, future studies are needed to delineate the functional significance of the increase in ERK phosphorylation by HMGA2 TR for better understanding of the role of TR HMGA2 in cancer.

In conclusion, HMGA2 expression increases with prostate cancer progression. Full-length HMGA2 induces the EMT and cell migration in prostate cancer cells *via* the MAPK pathway, while truncated HMGA2 promotes cell migration and viability *via* as yet unknown mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

EMT	Epithelial Mesenchymal Transition
HMGA2	High mobility group A2
ERK	Extracellular signal-regulated kinase-1

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Highlights

- HMGA2 expression increases with prostate cancer progression.
- Wild-type HMGA2 promotes EMT via MAPK.
- Truncated HMGA2 increases cell viability and migration via non-EMT pathway.

Figure 1: HMGA2 expression in prostate cancer cell lines.

A) Western Blot analysis was used to detect the protein expression of HMGA2 in prostate cancer cell lines. Actin was utilized as a loading control. B) Immunofluorescence illustrates nuclear/cytoplasmic localization of HMGA2 in PC3 and ARCaP-M (Mesenchymal phenotype), and cytoplasmic localization in ARCaP-E (epithelial phenotype) cells.

Figure 2: HMGA2 increases with prostate cancer progression.

Immunohistochemical (IHC) staining of prostate cancer adenocarcinoma tissue microarray sections was performed. Representative images of HMGA2 in various stages of prostate cancer show that HMGA2 localization becomes more nuclear with tumor progression. Magnification 20X, Inset 40X.

Figure 3: Overexpression of HMGA2 WT induces EMT and cell migration, while HMGA2 TR increases cell migration and viability.

A) LNCaP cells were stably transfected with wild-type (WT), truncated (TR) HMGA2 or Neo (empty vector). EMT markers tested by western blot analysis included mesenchymal markers (Snail, Twist, and vimentin) and epithelial markers (E-cadherin and Occludin). Actin was utilized as a loading control. B) Immunofluorescence was performed with antibodies to HMGA2, Snail, vimentin and E-cadherin. It showed that expression of HMGA2 WT is nuclear/cytoplasmic while HMGA2 TR is predominantly cytoplasmic. HMGA2 WT displayed high expression of Snail, vimentin, and cytoplasmic E-cadherin compared with empty vector Neo, therefore HMGA2 WT promotes EMT. C) Cell migration across transwells coated with collagen in boyden chamber or D) Cell viability using MTS assay was performed with LNCaP cells overexpressing HMGA2 WT, TR or Neo control. Results are representative of 3 independent experiments. ($n = 3$, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$). Values were normalized to LNCaP Neo and the mean $\pm$ SEM of data were obtained from three independent replicate experiments. Statistical analysis was done with Student *t* test.

Figure 4: HMGA2 induces EMT via ERK pathway.

A) LNCaP cells overexpressing HMGA2 WT or TR were utilized to analyze phospho-ERK (p-ERK) expression by western blot and compared to LNCaP Neo. Total-ERK levels were also analyzed and actin was utilized as a loading control. B) LNCaP cells overexpressing HMGA2 WT or TR were treated with 20 μ M U0126 (MAPK inhibitor) or 0.005% DMSO control for 72 h followed by analysis of B) EMT markers by western blot analysis. C) Relative cell migration across transwells coated with collagen after 48 h showed a decrease in cell migration of cells overexpressing HMGA2 WT (although not statistically significant; $p=0.267$) compared to DMSO control with no significant change in migration with cells overexpressing HMGA2 TR. D) Cell viability (MTS) assay performed for up to 3 days with LNCaP cells overexpressing HMGA2 WT and TR HMGA2 showed no significant changes after treating with U0126.