Abstract. High-mobility group AT-hook 2 (HMGA2), a member of the high mobility group family, has been reported to correlate with cancer progression. However, there is no report concerning the correlation between HMGA2 and metastasis in renal cell carcinoma. In the present study, we found that HMGA2 was highly expressed in five renal cell carcinoma cell lines compared with that in the normal renal tubular epithelial HK2 cell line. Additionally, HMGA2 facilitated cell migration and invasion of renal cell carcinoma cells, as evidenced by wound healing and Transwell assays. Subsequently, our results revealed that the E-cadherin level was upregulated, while N-cadherin, Twist1 and Twist2 expression were downregulated in HMGA2-depleted ACHN cells. In contrast, overexpression of HMGA2 in 786-O cells enhanced epithelial-mesenchymal transition (EMT). In addition, analysis of the database Cancer Browser further validated the positive correlation between HGMA2 and Twist1 or Twist2 in renal cell carcinoma patients. To confirm the underlying mechanism of HMGA2-regulated EMT, our results revealed that silencing of HMGA2 downregulated the mRNA and protein levels of TGF-β and Smad2, while HMGA2 overexpression had the opposite effect. Furthermore, TGF-β overexpression could partially reverse the anti-metastatic effect and mesenchymal-epithelial transition (MET) by HMGA2 loss, while TGF-β deficiency impeded the pro-metastatic phenotype and high expression of EMT markers induced by HMGA2 overexpression. In summary, our results demonstrated that HMGA2 facilitated a metastatic phenotype and the EMT process in renal cell carcinoma cells in vitro through a TGF-β-dependent pathway. In addition, these data strongly suggest that HGMA2 may serve as a potential therapeutic target and prognostic biomarker against renal cell carcinoma in the future.

Introduction

Renal cell carcinoma is the most common malignant tumor of the kidney, accounting for almost 3% of all human malignancies (1). Insensitivity to radiotherapy and chemotherapy are a great obstacle for the treatment of metastatic renal cell carcinoma. Epithelial-mesenchymal transition (EMT), a hallmark of metastasis, is a complicated process by which cells lose epithelial characteristics and acquire a mesenchymal phenotype (2). In the process of EMT, cancer cells escape from the primary site and invade to distant tissues through blood and lymphatic vessels. In addition, an EMT phenotype is often accompanied by the downregulation of epithelial markers E-cadherin and zonula occludens 1 (ZO-1), and upregulation of mesenchymal markers such as N-cadherin, Twist1 and Twist2, resulting in enhanced motility (3,4). Furthermore, the expression of E-cadherin or vimentin has been reported to be associated with tumor progression and overall survival (OS) in a variety of cancers, including lung cancer and nasopharyngeal carcinoma (5,6). Additionally, Slug and Snail are found to downregulate E-cadherin by binding to the promoter of E-cadherin (7). In view of this, there is an urgent need to identify potential therapeutic targets against renal cell carcinoma.

High-mobility group AT-hook 2 (HMGA2), a member of the high mobility group family, is a small non-histone nuclear-binding protein, which contains three AT-hook structural domains and an acid C-terminal tail. It has been reported that HMGA2 participates in proliferation and differentiation during embryonic development (8). In addition, HMGA2 was found to be overexpressed in various types of tumors, including ovarian cancer, hepatocellular carcinoma and gliomas (9-11). Moreover, a high level of HMGA2 was correlated with poor prognosis in breast cancer and lung cancer patients (12,13). Although numerous studies have validated that HMGA2 may play a crucial role in tumor progression, only few studies have shown a correlation between HMGA2 and renal cell carcinoma.
carcinoma. In addition, the underlying role of HMGA2 in renal cell carcinoma has not yet been elucidated.

In the present study, we focused on the role of HMGA2 in cell migration, invasion and EMT of renal cell carcinoma and revealed the possible mechanism by which HMGA2 regulates EMT in vitro.

Materials and methods

Reagents. Rabbit monoclonal antibodies against HMGA2 (8179), E-cadherin (E-Ca; 3195), N-cadherin (N-Ca; 13116), Twist1 (46702), TGF-β (3709), phosphorylated-Smad2 (p-Smad2; 3108), Smad2 (5339), Gli1 (3538), phosphorylated-β-catenin (p-β-catenin; 4176) and β-actin (4970) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibody against Twist2 (ab66031) was purchased from Abcam (Cambridge, UK). The appropriate peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were purchased from Zhongshan Biotech (Beijing, China). ACHN stably transfected with sh-HMGA2, 786-O stably transfected with HMGA2 (OE-HMGA2), and their respective corresponding empty vector control sublines (ACHN-scramble, 786-O-vector) were previously constructed.

Western blotting. For western blot analysis, cells were washed with phosphate-buffered saline (PBS) after the specific treatment, and proteins were extracted using a lysis buffer [10 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l ethylene glycol tetraacetic acid, 0.3 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l sodium orthovanadate, 5'-GCTTGAGGGTCTGAATCTTGCT-3'; Twist2 (200 bp) forward, 5'-GTCCGCAGTCTTACGAGGAG-3' and reverse, 5'-ATGCACATCCTTCGATAAGACTG-3'; Twist1 (156 bp) forward, 5'-CATGTACGTTGCTATCCAGGC-3'; and reverse, 5'-GTGGGTTTCCACCATTAGCAC-3'; Gli1 (3538), phosphorylated-β-catenin (p-β-catenin; 4176) and β-actin (4970) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). All cells were maintained in RPMI-1640 medium and supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO2.

Cell lines and culture. Human renal tubular epithelial HK2 cell line, and five renal cell carcinoma cell lines 786-O, 769-P, OSRC-2, ACHN and Caki-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained in RPMI-1640 medium and supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO2.

Cell migration assay. The cell migration ability of renal cell carcinoma cells following different treatments was assessed using Transwell migration assay. Cells (ACHN, 2x105; 786-O, 1.5x105) in 200 µl serum-free medium were seeded into the upper chambers, and 800 µl of 10% fetal calf serum-containing medium was used as a chemoattractant in the lower chamber. After incubation for 24 h, the cells which migrated onto the bottom of the filter were then fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet (Beyotime, Shanghai, China). Cells were then washed and photographed in five independent visual fields using an inverted microscope (Olympus IX50; Olympus) at x100 magnification.

Matrigel invasion assay. Cell invasive ability was detected by Matrigel invasion assay using a Millicell chamber (Millipore, Billerica, MA, USA). The Transwell chambers were precoated with 50 µl mixture (Matrigel:serum-free medium 1:5) and the stable clone cell lines (ACHN, 5x104; 786-O, 4x104) in 200 µl serum-free medium were seeded onto the upper chamber for 24 h according to the instructions of the Transwell migration assay. The difference between two groups was analyzed by Student’s t-test (two-sided).

Quantitative real-time PCR assay. Total cellular RNA was extracted using TRIZol reagent (Invitrogen). The RNA sample was reversely transcribed by PrimerScript RT reagent kit (Takara, Dalian, China). Then the relative levels of target gene messenger RNA (mRNA) were evaluated by quantitative real-time PCR assay (qPCR) using FAST SYBR-Green Master Mix with the gene-specific primers: E-cadherin (119 bp) forward, 5'-CGAGAGCTCACGCTTCAGG-3'; and reverse, 5'-GGGTGTGTCAGGGGAAAAATAGG-3'; N-cadherin (94 bp) forward, 5'-TCAGGGCGTCTGTAGGAGGCTT-3' and reverse, 5'-ATGCACACCTCCTTCGATAGACTG-3'; Twist1 (156 bp) forward, 5'-GTCCCGAGCTTCAAGGAGG-3' and reverse, 5'-GCTTGAAGGTCATTAATCTCGT-3'; Twist2 (200 bp) forward, 5'-GGAGTGCGCCGTCTCTACAGG-3' and reverse, 5'-TCTGGAGGAGCTGTAGGAGG-3'; TGF-β (201 bp) forward, 5'-GGCCAGATCCGTGCAACG-3' and reverse, 5'-GTGGGTTTCCACCATTAGCAC-3'; Smad2 (182 bp) forward, 5'-CGTCCTACCTTGCTTAGACG-3' and reverse, 5'-CTCAAGCTCATCATATGTGCTCTG-3'; and β-actin (250 bp) forward, 5'-CATGTACGGTGTGTCAGGCAGG-3' and reverse, 5'-TAAGTCGCAATACGCAAC-3'. The n-fold change in expression was calculated according to the method of 2-∆∆Ct.

Plasmid transfection. TGF-β cDNA was cloned into the pcDNA3.1 vector. The plasmid was transfected into renal cell carcinoma ACHN cells with sh-HMGA2 or 768-O cells with HMGA2 overexpression by Bo Kou using X-treme Gene HP DNA transfection reagent (Roche, Germany) for 48 h according to the manufacturer’s instructions, and subsequently prepared for the mechanistic research.
Statistical analysis. All experimental data were analyzed by GraphPad Prism (San Diego, CA, USA) software and presented as means ± SD. Differences between two groups were analyzed by Student's t-test (two-sided). The correlations between HGMA2 and Twist1 or Twist2 were analyzed by Spearman's rank test. In addition, overall survival (OS) was assessed by log-rank test, while a Kaplan-Meier curve was generated. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of HMGA2 in human renal epithelial cells and renal cell carcinoma cell lines. Firstly, we detected the expression of HMGA2 in several renal cell carcinoma cell lines using western blotting. The results demonstrated that HMGA2 was elevated in all renal cell carcinoma cell lines, compared with that noted in the human renal tubular epithelial HK2 cell line (Fig. 1A). Among these cell lines, HMGA2 was the most highly expressed in the renal cell carcinoma ACHN cell line, whereas it was lowly expressed in the 786-O cell line. In view of this, we constructed stable clone cell lines with knockdown or overexpression of HMGA2. In addition, we explored the HMGA2 expression level in ACHN cells transfected with negative control, scramble, sh-HMGA2 (B and C), and 786-O cells transfected with the negative control, vector, OE-HMGA2 (D and E) were determined by qPCR and western blotting. Quantification from three independent experiments is shown with error bars representing standard deviation (SD). ***P<0.001 and ****P<0.0001.

HMGA2 regulates the metastatic phenotype of renal cell carcinoma. Studies showed that HMGA2 is associated with cancer metastasis in a variety of cancers (14,15). Thus, we hypothesized that HMGA2 is implicated in the migration and invasion of human renal cell carcinoma. To verify this hypothesis, wound healing and Transwell migration assays with statistical quantification were used. As expected, our results indicated that HMGA2 knockdown had a much wider scratch width and restrained cell migration when compared to the negative control or scramble in ACHN cell line (Fig. 2A and C). In contrast, HMGA2 overexpression promoted cell migration of renal cell carcinoma (Fig. 2B and D). Next, we determined whether HMGA2 affects invasive ability in vitro. As shown in Fig. 2C, the silencing of HMGA2 expression decreased the number of invaded cells, while overexpressing HMGA2 increased the invasive property of 786-O cell line compared with negative control (Fig. 2D). These data indicated that HMGA2 knockdown inhibited the metastatic phenotype of human renal cell carcinoma cells in vitro.

HMGA2 regulates the EMT of renal cell carcinoma. Several studies have stated that HMGA2 is involved in the process of EMT (16,17). To validate the function of HMGA2 in the EMT process of renal cell carcinoma cells, we evaluated the levels of both epithelial and mesenchymal markers by qPCR and western blotting. The deficiency in HMGA2 presented an elevated level of E-cadherin, while a reduction in N-cadherin, Twist1 and Twist2 (Fig. 3A and C). Conversely, 786-O cells with HMGA2 overexpression exhibited a significant decline in E-cadherin level, while an increase in N-cadherin, Twist1 and Twist2. (Fig. 3B and D). These results indicated that knockdown of HMGA2 reversed the EMT of human renal cell carcinoma.

Correlation between HMGA2 and EMT markers based on Cancer Browser database. To further explore the association between HMGA2 and EMT in renal cell carcinoma, the clinical data and gene expression from database Cancer Browser (TCGA_KIRC_exp_HiSeqV2-2015-02-24) were
extracted. The results demonstrated that high expression of HMGA2 was correlated with increased Twist1 expression (R=0.0.4013, P<0.0001) (Fig. 4A). Meanwhile, HMGA2 expression was positively correlated with the Twist2 level in renal cell carcinoma (R=0.4122, P<0.0001) (Fig. 4B). Then, we used Kaplan-Meier analysis to evaluate the prognostic value of HMGA2 in renal cell carcinoma. Importantly, the low HMGA2 patient group had a better OS than that of the
high-expression group (Fig. 4C), indicating that high HMGA2 may be a poor prognostic predictor of renal cell carcinoma.

Silencing of HMGA2 decreases TGF-β and Smad2 expression in renal cell carcinoma cells. qPCR was used to detect the expression of TGF-β, Smad2 and β-actin in HMGA2-depleted ACHN (A) and HMGA2-overexpressing 786-O cells (B). Quantification from three independent experiments is shown as mean ± standard deviation (SD). ***P<0.001 and ****P<0.0001. Western blotting was used to detect the protein levels of HMGA2, TGF-β, phosphorylated-Smad2 (p-Smad2), Smad2, Gli1, phosphorylated-β-catenin (p-β-catenin) and β-actin in HMGA2-depleted ACHN cells (C) and HMGA2-overexpressing 786-O cells (D). Representative protein bands from three experiments are shown.

The EMT process is governed by various regulatory networks, such as TGF-β, Wnt and Hedgehog signaling. To clarify the correlation among HMGA2 and several signals in renal cell carcinoma, we firstly evaluated that the change in TGF-β-, Wnt- and Hedgehog-related markers in HMGA2-knockdown
ACHN cells or HMGA2-overexpressing 786-O cells. The mRNA levels of TGF-β and Smad2 were downregulated by silencing of HMGA2 (Fig. 5A), and upregulated in the 786-O cells with HMGA2 overexpression (Fig. 5B). To further examine the protein levels of the above markers, we found a marked decrease of TGF-β and phosphorylated-Smad2 in the HMGA2-depleted ACHN cells, and a marked increase in TGF-β and phosphorylated-Smad2 in the HMGA2-overexpressing 786-O cells (Fig. 5C and D). Meanwhile, the protein level of total Smad2, Gli1 and p-β-catenin had no significant change following HMGA2 knockdown or over-expression. These findings suggest that HMGA2 regulated TGF-β/Smad2 signaling in renal cell carcinoma.

**Discussion**

Accumulating evidence indicates that HMGA2 is highly expressed in solid tumors and is regulated by complicated regulatory systems (18-20). Studies have shown that in renal cell carcinoma, HMGA2 expression is significantly higher than that in benign and normal renal tissues (21). Moreover, there is a positive correlation between HMGA2 and clinical staging and lymph node metastasis. Also, another study reported that the expression of HMGA2 was significantly associated with tumor size and Fuhrman grade in patients with clear cell renal cell carcinoma (ccRCC) (22). In the present study, we firstly confirmed that HMGA2 was highly expressed in five renal cell carcinoma cell lines compared with that in the normal renal tubular epithelial HK2 cell line. Recent studies have shown that HMGA2 may play an essential role in cancer proliferation, migration and metastasis (23-25). It was reported that deficiency of HMGA2 reduced the metastatic potential of breast cancer cells (26). Additionally, HMGA2 seems to have the potential of enhancing self-renewal capacity in cancer stem cells (27). The present study demonstrated that HMGA2 knockdown inhibited cell migration and invasion, while over-expression of HMGA2 facilitated the metastatic phenotype in renal cell carcinoma cells in vitro, as evidenced by wound healing and Transwell assays. These results emphasize the critical role of HMGA2 in renal cell carcinoma metastasis.

EMT is a complex process during which tumor cells gain more aggressive and metastatic ability (28). Previous
achn cells. In contrast, overexpression of HMGA2 in 786-O
Twist1 and Twist2 were downregulated in HMGA2-depleted
Wnt/β-catenin signaling pathway is reported to promote EMT
in oral squamous carcinoma stem cells (32). Studies have
tested that TGF-β induces PLOD2 expression to promote
EMT in cervical cancer (33). In the present study, there was no signif-
cant change in Gli1 and p-β-catenin by HMGA2 deple-
tion or overexpression. In addition, our results showed that
silencing of HMGA2 downregulated the mRNA and protein
levels of TGF-β and Smad2, while HMGA2 overexpression
had the opposite effect. In addition, TGF-β overexpression by
transient transfection partially abolished the anti-metastatic
phenotype and mesenchymal-epithelial transition (MET)
by HMGA2 loss, whereas TGF-β deficiency reversed the
pro-metastatic effect and high expression of TGF-β- and
EMT-related markers by overexpression of HMGA2. These
results confirmed the vital role of the TGF-β signaling pathway
in HMGA2-mediated EMT.

In conclusion, our results revealed, for the first time, that
HMGA2 facilitated a metastatic phenotype and the EMT
process in renal cell carcinoma in vitro through a TGF-β-
dependent pathway. In addition, our findings strongly suggest
that HGMA2 may serve as a potential therapeutic target and
prognostic biomarker against renal cell carcinoma in the
future, although further investigation is needed.

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References

1. Fayaz MS, Al-Qaderi AE and El-Sherify MS: Metastatic renal
carcinoma with undetectable renal mass presenting as
lymphadenopathy. CEN Case Rep 6: 36-38, 2017.
2. Liu Y, Zeng S, Jiang X, Lai D and Su Z: SOX4 induces tumor
invasion by targeting EMT-related pathway in prostate cancer.
Tumour Biol 39: 1010428317698373, 2017.
3. Kong FF, Qu ZQ, Yuan HH, Wang JY, Zhao M, Guo YH, Shi J,
Gong XD, Zhu YL, Liu F, et al: Overexpression of FOXM1 is
associated with EMT and is a predictor of poor prognosis in
non-small cell lung cancer. Oncol Rep 31: 2660-2668, 2014.
4. Luo W, Fang W, Li S and Yao K: Ablent expression of nuclear
vimentin and related epithelial-mesenchymal transition markers
in nasopharyngeal carcinoma. Int J Cancer 131: 1863-1873, 2012.
5. Naber HP, Drabsch Y, Snaar-Jagalska BE, ten Dijke P and van
Laar T: Snail and Slug, key regulators of TGF-β-induced EMT,
are sufficient for the induction of single-cell invasion. Biochem
Biophys Res Commun 433: 58-63, 2013.
6. Kumar MS, Armenteros-Monterroso E, East P, Chakravorty P,
Matthews N, Winslow MM and Downing J: HMGA2 functions
as a competing endogenous RNA to promote lung cancer
progression. Nature 505: 212-217, 2014.
7. Wang X, Liu X, Li AX, Chen L, Lai L, Lin HH, Hu S, Yao L,
Peng J, Loera S, et al: Overexpression of HMGA2 promotes
metastasis and impacts survival of colorectal cancers. Clin
Cancer Res 17: 2570-2580, 2011.
8. Xi XN, Yin XY and Ye HM: Effects of HMGA2 on malignant
degree, invasion, metastasis, proliferation and cellular
morphology of ovarian cancer cells. Asian Pac J Trop Med 7:
289-292, 2014.
9. Zou Q, Wu H, Fu F, Yi W, Pei L and Zhou M: KKIP sup-
presses the proliferation and metastasis of breast cancer cells
through up-regulation of miR-185 targeting HMGA2. Arch Biochem
Biophys 610: 25-32, 2016.
10. Chen J, Shen G, Liu S and Meng Q: Downregulation of HMGA2
inhibits cellular proliferation and invasion, improves tumour
induced apoptosis in prostate cancer. Tumour Biol 37: 699-707, 2016.
11. Liu Y, Fu QZ, Pu L, Meng OG, Liu XF, Dong SF, Yang JX and
Lv GY: HMGA2 expression in renal carcinoma and its clinical
significance. J Med Biochem 34: 338-343, 2015.
12. Na N, Si T, Huang Z, Mao B, Hong L, Li H, Qi J and Qiu J: High
expression of HMGA2 predicts poor survival in patients with
clear cell renal cell carcinoma. Onco Targets Ther 9: 7199-7205,
2016.
13. Shi Z, Wu D, Tang R, Li X, Chen R, Xue S, Zhang C and Sun X:
Silencing of HMGA2 promotes apoptosis and inhibits migra-
tion and invasion of prostate cancer cells. J Biosci 41: 229-236, 2016.
14. Sun M, Song CX, Huang H, Frankeberger CA, Sankarasharma D,
Gomes S, Chen P, Chen J, Chada KK, He C, et al: HMGA2/
TET1/HOXA9 signaling pathway regulates breast cancer growth
and metastasis. Proc Natl Acad Sci USA 110: 9920-9925, 2013.
15. Natarajan S, Hombach-Klonisch S, Driege P and Klonisch T:
HMGA2 inhibits apoptosis through interaction with ATR-CHK1
signaling complex in human cancer cells. Neoplasia 15: 263-280,
2013.
26. Yang E, Cisowski J, Nguyen N, O’Callaghan K, Xu J, Agarwal A, Kuliopulos A and Covic L: Dysregulated protease activated receptor 1 (PAR1) promotes metastatic phenotype in breast cancer through HMGA2. Oncogene 35: 1529-1540, 2016.

27. Kaur H, Ali SZ, Huey L, Hüttinger-Cabezás M, Taylor I, Mao XG, Weingart M, Chu Q, Rodriguez FJ, Eberhart CG, et al: The transcriptional modulator HMGA2 promotes stemness and tumorigenicity in glioblastoma. Cancer Lett 377: 55-64, 2016.

28. Micalizzi DS, Haber DA and Maheswaran S: Cancer metastasis through the prism of epithelial-to-mesenchymal transition in circulating tumor cells. Mol Oncol 11: 770-780, 2017.

29. Xia YY, Yin L, Jiang N, Guo WJ, Tian H, Jiang XS, Wu J, Chen M, Wu JZ and He X: Downregulating HMGA2 attenuates epithelial-mesenchymal transition-induced invasion and migration in nasopharyngeal cancer cells. Biochem Biophys Res Commun 463: 357-363, 2015.

30. Li Y, Zhao Z, Xu C, Zhou Z, Zhu Z and You T: HMGA2 induces transcription factor Slug expression to promote epithelial-to-mesenchymal transition and contributes to colon cancer progression. Cancer Lett 355: 130-140, 2014.

31. Ke Z, Caiping S, Qing Z and Xiaoqing W: Sonic hedgehog-Gli1 signals promote epithelial-mesenchymal transition in ovarian cancer by mediating PI3K/AKT pathway. Med Oncol 32: 368, 2015.

32. Qiao B, He BX, Cai JH, Tao Q and King-Yin Lam A: MicroRNA-27a-3p modulates the Wnt/β-catenin signaling pathway to promote epithelial-mesenchymal transition in oral squamous carcinoma stem cells by targeting SFRP1. Sci Rep 7: 44688, 2017.

33. Xu F, Zhang J, Hu G, Liu L and Liang W: Hypoxia and TGF-β1 induced PLOD2 expression improve the migration and invasion of cervical cancer cells by promoting epithelial-to-mesenchymal transition (EMT) and focal adhesion formation. Cancer Cell Int 17: 54, 2017.