Expression of HMGA2 in bladder cancer and its association with epithelial-to-mesenchymal transition

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Abstract

Objectives: High mobility group protein2 (HMGA2) and epithelial-to-mesenchymal transition are both related to progress of bladder cancer, however, the relationship between HMGA2, E-cadherin and vimentin in bladder cancer is not yet known. Thus, this study has examined expression of HMGA2, E-cadherin and vimentin in bladder cancer and investigated their relationship.

Materials and methods: The 5637 bladder cancer cell line and SV-HUC-1 normal uroepithelial cells were used to study expression of HMGA2, E-cadherin and vimentin using RT-PCR and western blotting. Paraffin wax-embedded bladder cancer tissues were used to study protein expression using immunohistochemistry and χ^2 analysis and Kendall's correlation were utilized statistical methods.

Results: Overexpression of HMGA2 was associated with down-regulation of E-cadherin and up-regulation of vimentin in the 5637 bladder cancer line. A total of 49 paraffin wax-embedded tissues of transitional cell bladder cancer were used. Positive expression levels of HMGA2 protein and vimentin were 41 and 43% in bladder tissues, respectively. No expression of E-cadherin was found in 43%. Expression of HMGA2, loss of E-cadherin and expression of vimentin are all significantly correlated with bladder cancer grade and stage. Loss of E-cadherin and expression of vimentin both correlated with recurrence of the bladder cancer.

Conclusions: Expression of HMGA2 was closely associated with occurrence of epithelial-to-mesenchymal transition. Expression of HMGA2, loss of E-cadherin and expression of vimentin may indicate high degree malignancy of bladder cancer. Loss of E-cadherin expression and positive expression of vimentin may predict recurrence of bladder cancer.

Introduction

Epithelial-to-mesenchymal transition (EMT) is an important process in tumour invasion and metastasis (1). In EMT, carcinoma cells lose their epithelial properties and gain mesenchymal ones, thereby enhancing their ability to migrate and invade. The hallmark change that characterizes EMT is loss of epithelial protein E-cadherin and up-regulation of the mesenchymal proteins such as vimentin (2,3). However, underlying molecular mechanisms of these conditions are poorly understood.

High mobility group protein2 (HMGA2) is an architectural transcription factor that belongs to the HMGA protein family (4). It is a non-histone nuclear-binding protein and important regulator of cell proliferation and differentiation. Normally, HMGA2 protein is highly expressed during embryonic development, whereas expression is almost undetectable in most adult, differentiated tissues. Overexpression of HMGA2 has been observed in a variety of cancers, including of the lung (5), breast (6), thyroid (7), stomach (8), ovary (9) and retinoblastoma (10). Recently, Morishita *et al.* (11) reported that HMGA2 loss of function in a mouse model of cancer reduced tumour multiplicity, and found that HMGA2 played a critical role in EMT by activating the TGF- β signalling pathway, thereby inducing invasion and metastasis of human epithelial cancers. Yang *et al.* (12) recently reported that HMGA2 was overexpressed in bladder cancer, and that its expression levels were strongly correlated with tumour grade and stage.

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However, whether expression of HMGA2 is associated with exchange of E-cadherin for vimentin is not yet known. In this study, we explored expression of HMGA2, E-cadherin and vimentin in bladder cell lines and transitional cell bladder cancers.

Material and methods

Cell lines culture and maintenance

Cell lines 5637 bladder cancer and SV-HUC-1 normal uroepithelial cells were obtained from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. 5637 line was derived from a grade II human transitional cell carcinoma of the urinary bladder and SV-HUC-1 was derived from ureteral uroepithelium. 5637 bladder cancer cell line was cultured in RPMI-1640 with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 µg/ml penicillin and 1 µg/ml streptomycin at 37 °C in air with 5% carbon dioxide. SV-HUC-1 normal uroepithelial cells was grown in Has's F12 medium with 7% foetal bovine serum, 4 mM L-glutamine, 100 µg/ml penicillin and 1 µg/ml streptomycin at 37 °C in air with 5% carbon dioxide.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cell RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Then, total RNA from each sample was reverse-transcribed (RT) using RevertAid First Strand cDNA Synthesis kit (Fermentas Inc., Hanover, MD, USA), by incubation at 70 °C for 10 min, followed by reverse transcription at 42 °C for 1 h and incubation at 70 °C for 10 min. Resulting cDNA was amplified by PCR using gene-specific primers, performed with primers for HMGA2 5'-CA GCCGTCCACTTCAGC-3' (sense) and 5'-TGCCTTTGG GTCTTCC-3' (anti-sense), E-cadherin 5'-GATAGAGAA CGCATTGCCACATAC-3' (sense) and 5'-CTGAT-GACT CCTGTGTTCTCTTA-3' (anti-sense), vimentin 5'-CAG AGAGAGGAAGCCGAAAA-3' (sense) and 5'-TCCTCT TCGTGGAGTTTCTT 3' (anti-sense), β-actin 5'-AGCG AGCATCCCCCAAAGTT -3' (sense) and 5'-GGGCACG AAGGCTCATCATT-3' (anti-sense) by 30 cycles of 45 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C. β-actin served as internal control to determine RNA quality of samples.

Western blotting

Protein extracts were prepared by washing cells in PBS and lysing in RIPA buffer containing protease inhibitor.

Equal amounts of total protein from each sample were loaded on 12.5% SDS-PAGE gel and transferred to PVDF membranes. Membranes were incubated overnight at 4 °C in TBST containing 5% bovine serum albumin with rabbit anti-human monoclonal antibody HMGA2, E-cadherin and vimentin (Cell Signaling Technology, Boston, MA, USA) (1:1000). Rabbit antihuman β-actin polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) (1:500) was used for detecting internal control protein. Secondary antibody was detected by Western ECL-enhanced luminol reagent (PerkinElmer Inc., Waltham, MA, USA).

Patients and tissue specimens

A total of 49 paraffin wax-embedded samples of transitional cell bladder cancer (31 males and 18 females, 32–81 years of age) and 5 specimens of adjacent normal bladder tissue were collected from the First Hospital of Jilin University, between April 2008 and October 2010. Patients were selected based on (i) who underwent transurethral bladder tumour resection, (ii) distinctive histopathological diagnosis of transitional cell carcinoma, (iii) availability of detailed clinicopathological data and (iv) patients who had undergone instillation of intravesical chemotherapy post-operatively. The protocol of this study was approved by the Ethics Committee of First Hospital of Jilin University (Changchun, China).

Tumours were staged according to the 6th edition of pTNM classification of the Union for International Cancer Control (UICC, 2002). A total of 41 patients were of stage Ta or T1, and 8 of stage T2. Histological grading of tumours was defined according to criteria of the WHO (G1, $n = 24$; G2–G3, $n = 25$). Median follow-up was 40 months (range 18–40). Seven patients were lost. Clinicopathological characteristics are summarized in Table 1.

Immunohistochemical staining

Formalin-fixed and paraffin wax-embedded tissue sections (5-µm) on glass slides were deparaffinized and rehydrated. Antigen retrieval was achieved by heating the samples in a microwave oven in 10 mM citrate buffer, then rinsed in buffer supplied by the manufacturer. After inhibition of endogenous peroxidase activity for 30 min with methanol containing 0.3% H₂O₂, sections were blocked with 2% bovine serum albumin for 30 min and incubated overnight at 4 °C with primary polyclonal rabbit anti-human antibody HMGA2, 1:50 (Cell Signaling Technology), E-cadherin, 1:200 (Cell Signaling Technology) and vimentin, 1:100 (Cell Signaling Technology). After washing three times in PBS, slides were

Table 1. Correlation between HMGA2, E-cadherin, vimentin expression and clinicopathological features of patients with bladder cancer

Parameter	Case	HMGA2 expression			<i>P</i> value	E-cadherin expression			<i>P</i> value	Positive	Negative	<i>P</i> value
		Positive	Negative			Positive	Negative					
Gender												
Male	31	14	17		0.417	17	14		0.669	14	17	0.669
Female	18	6	12			11	7			7	11	
Age												
≤60	9	3	6		0.613	7	2		0.166	3	6	0.523
>60	40	17	23			21	19			18	22	
Grade												
G1	24	6	18		0.027	18	6		0.013	6	18	0.013
G2–G3	25	14	11			10	15			15	10	
T stage												
Ta–T1	41	14	27		0.032	26	15		0.045	15	26	0.045
T2	8	6	2			2	6			6	2	
Follow-up*												
Recurrence	18	10	8		0.044	5	13		0.013	14	4	0.002
Non-recurrence	24	6	18			16	8			7	17	

*Seven patients were lost to follow-up. Figures in bold denote significant values.

incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG for 30 min, followed by the reaction with diaminobenzidine then counterstained with Mayer's haematoxylin. Negative control was performed by omission of the primary antibody and substituting it with non-specific rabbit IgG.

Two pathologists (Xiaobo Ma and Xu Yan) evaluated the immunostaining blind, with no knowledge of clinical outcomes or other clinicopathological data. Expression of proteins was evaluated by scanning each entire tissue specimen at low magnification ($\times 40$), then confirmed under high magnification ($\times 200$ and $\times 400$). Immunohistochemical staining intensity was scored as 0 (no staining), 1 (weakly stained), 2 (moderately stained) or 3 (strongly stained). Extent of staining was scored as 0 (negative), 1 ($<10\%$ of tumour area stained), 2 ($10\text{--}50\%$ of area stained), or 3 ($>50\%$ of area stained) (12,13). Total scores were evaluated by combining staining intensity and staining distribution. For HMGA2 and E-cadherin, scores of 0–2 were regarded as negative, while scores equal or above 2 were regarded as positive (12,13). For vimentin, 0–5 scores were considered negative, while scores equal or above 5 were considered positive (13).

Statistical analysis

All statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). Significance of expression of HMGA2, E-cadherin and vimentin proteins and clinicopathological parameters were evaluated using χ^2 analysis and Kendall's correlation was utilized to assess whether relationships could

be identified between expression of proteins and tumour recurrence. Values of $P < 0.05$ were considered statistically significant.

Results

Expression of HMGA2, E-cadherin and vimentin in cell lines

RT-PCR analysis revealed that HMGA2 and vimentin were weakly expressed and E-cadherin was strongly expressed in the SV-HUC-1 cell line. Conversely, HMGA2 and vimentin were strongly expressed and E-cadherin was weakly expressed in 5637 cell line (Fig. 1). Similar results were obtained by western blotting for detection of HMGA2, E-cadherin and vimentin in SV-HUC-1 and 5637 cell lines (Fig. 2).

Figure 1. mRNA expression of HMGA2, E-cadherin and vimentin in SV-HUC-1 and 5637 cell lines using RT-PCR. RT-PCR analysis showed that lower mRNA level of HMGA2 and vimentin was observed in SV-HUC-1 than the 5637 cell line. Conversely, mRNA level of E-cadherin in SV-HUC-1 cell line was much higher than that in 5637 cell line.

Figure 2. Protein expression of HMGA2, E-cadherin and vimentin in SV-HUC-1 and 5637 cell lines using western blotting. Western-blot analysis indicated that HMGA2 and vimentin were expressed at lower levels in SV-HUC-1 than 5637 cell line, and, E-cadherin exhibited much higher expression in SV-HUC-1 cell line.

Protein expression of HMGA2, E-cadherin and vimentin in bladder cancer samples

High mobility group protein2 immunohistochemical positive reaction product was mainly localized in the

nucleus and/or cytoplasm (Fig. 3b). In terms of E-cadherin, immunohistochemically positive reaction product was localized in membranes (Fig. 4a). Vimentin staining was assessed as positive if either cytoplasm or membranes were positively stained (Fig. 5b).

Compared to no HMGA2 staining in the 5 normal urothelial samples, a total of 41% (20/49) of bladder tumours had positive expression of HMGA2, which was statistically significant ($P < 0.001$). In contrast, E-cadherin was not expressed in 21 of 49 cases (43%) of bladder cancers, but was expressed in all 5 normal urothelial tissues ($P < 0.001$). Expression of vimentin was observed in 21 of 49 cases (43%) of bladder cancers, whereas it was negative in all 5 normal urothelia ($P < 0.001$).

Relationship of HMGA2, E-cadherin and vimentin expression to clinical variables of bladder cancer

Positive expression of HMGA2 correlated significantly with tumour grade and stage ($P = 0.027$ and $P = 0.032$,

Figure 3. Immunohistochemical staining of HMGA2 in normal bladder tissue (a) and bladder cancer sample (b). (a) No expression of HMGA2 in normal bladder tissue. (b) Positive expression of HMGA2 in bladder cancer (arrow: positive reaction in the cytoplasm) (magnification, 10×40).

Figure 4. Immunohistochemical staining of E-cadherin in normal bladder tissue (a) and bladder cancer sample (b). (a) Positive expression of E-cadherin in normal bladder tissue (arrow: positive reaction in the membrane). (b): No expression of E-cadherin in bladder cancer sample (magnification, 10×40).

Figure 5. Immunohistochemical staining of vimentin in normal bladder tissue (a) and bladder cancer sample (b). (a) No expression of vimentin in normal bladder tissue. (b): Positive expression of vimentin in bladder cancer sample (arrow: positive reaction in the cytoplasm and membrane) (magnification, 10×40).

respectively). Loss of E-cadherin expression was more evident in poorly differentiated tumours compared to well-differentiated tumours. Compared to grade1 and stage Ta–T1, positive vimentin expression was found in 60% patients with grade 2–grade 3 disease (15/25) and 75% patients with stage T2 disease (6/8) ($P = 0.013$ and $P = 0.045$, respectively). HMGA2, E-cadherin and vimentin expressions were all not associated with gender or age (Table 1).

Prognostic recurrence value of HMGA2, E-cadherin and vimentin protein in bladder cancer

We evaluated the ability of HMGA2, E-cadherin and vimentin staining to predict tumour recurrence in bladder cancer, by the Kendall method. Although positive level of HMGA2 among recurrent tumour patients was 55.6 (10/18) ($P = 0.044$), expression of HMGA2 did not correlate with recurrence of bladder cancer ($r = 0.229$, $P = 0.113$). However, loss of E-cadherin and expression of vimentin both correlated with recurrence of bladder cancer ($r = 0.539$, $P < 0.001$ and $r = 0.452$, $P = 0.002$, respectively).

Discussion

Expression of HMGA2 has been observed in a variety of cancers and it has been associated with aggressive tumour growth and poor prognosis (14). Historically, expression of HMGA2 in bladder cancer has not been clear; only a single paper was found by this group after searching articles in MEDLINE database using the key words 'HMGA2' and 'bladder cancer'. Yang *et al.* (12) examined HMGA2 expression in 44 frozen bladder cancer tissues and 18 adjacent normal bladder tissues and found that HMGA2 was up-regulated in bladder cancer at both transcriptional and translational levels compared to normal bladder tissue. In this study, we found that HMGA2 was expressed in cells of the 5637 bladder cancer cell line compared to SV-HUC-1 normal uroepithelial cell line. Compared to normal bladder tissues, we also confirmed positive expression of HMGA2 in bladder cancer tissues.

In addition to being essential for embryonic and heart development, and wound healing, EMT also has crucial roles in progression of carcinomas (15,16). It is involved in many critical events, such as cell migration and invasion, inducing stem cell properties, preventing apoptosis and senescence, and contributing to immunosuppression (17). Previous studies have reported that many genes induce occurrence of EMT, such as *Zeb1* (18), *ALKBH2* (19), *MALAT-1* (20) and *Id-1* (21). Until recently, HMGA2 has not been linked to EMT

regulation in bladder cancer. Wu *et al.* (22) however, showed that HMGA2 overexpression conferred powerful oncogenic signals in ovarian cancers by modulation of EMT genes. Zha *et al.* (8) indicated that overexpressing HMGA2 enhanced oncogenic properties of gastric epithelial origin cell *in vitro* and *in vivo* and that the Wnt/ β -catenin pathway activated by HMGA2 might be the underlying mechanism of EMT in gastric cancer cells.

Based on the theory that loss of epithelial protein E-cadherin and up-regulation of mesenchymal proteins such as vimentin were the hallmark change during EMT, we examined expression HMGA2, E-cadherin and vimentin. We found that overexpression of HMGA2 was associated with down-regulation of E-cadherin and up-regulation of vimentin in the 5637 bladder cancer line. We also demonstrated positive expression of HMGA2, no expression of E-cadherin and positive expression of vimentin in bladder cancer tissues; expression of HMGA2 was negatively correlated with loss of E-cadherin. Thus inhibition of HMGA2 may avoid transition of epithelial to mesenchymal cells.

Yang *et al.* (12) found that HMGA2 was up-regulated in bladder cancer at both transcriptional and translational levels compared to normal bladder tissue, and that HMGA2 was a potential prognostic marker for predicting tumour recurrence and progression. In this study, we also demonstrated that expression of HMGA2 correlated with recurrence of bladder cancer. In addition, loss of E-cadherin expression and positive expression of vimentin seemed to be able to predict bladder cancer recurrence.

In conclusion, positive expression of HMGA2 is accompanied by no expression of E-cadherin and positive expression of vimentin in bladder cancer. Inhibition of HMGA2 may avoid EMT. Expression of HMGA2, loss of E-cadherin and expression of vimentin were all significantly correlated with bladder cancer grade and stage. Loss of E-cadherin expression and positive expression of vimentin could predict bladder cancer recurrence.

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