HOXB2 is a Putative Tumour Promotor in Human Bladder Cancer

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Abstract. *Background/Aim:* The present study aimed to investigate the role of Homeobox B2 (HOXB2) in bladder cancer (BC). *Materials and Methods:* The Cancer Genome Atlas (TCGA) dataset was used to analyse HOXB2 expression in BC. The influence of HOXB2 on the cellular functions of BC cells was determined in both HOXB2 knockdown and HOXB2 overexpressed BC cell lines using in vitro assays. *Results:* HOXB2 mRNA was significantly upregulated in luminal infiltrated and luminal papillary subtypes of BC. Drug Metabolism Cytochrome P450 was significantly enriched in BCs expressing high levels of HOXB2. Knockdown of HOXB2 from EJ138 cells reduced growth, adhesion and invasion. In contrast, overexpression of HOXB2 in RT112 cells induced growth and adhesion of bladder cancer cells. *Conclusion:* Increased HOXB2 expression in papillary BC can promote cell growth and adhesion of BC cells. Drug Metabolism Cytochrome P450 pathway was enriched in BCs overexpressing HOXB2.

Bladder cancer (BC) is one of the most common urogenital carcinomas, and its incidence is the second highest, immediately after prostate cancer. Most bladder cancers are histologically characterised as transitional cell carcinomas (TCC). Worldwide, the age-standardised incidence rate for

males is 9.0 per 100,000 persons/year and 2.2 for females. In China, the incidence rate was 6.61 in 2012 (1). As a first-line therapy, surgery and chemotherapy are commonly used. Unfortunately, as this disease has a progression and recurrence rate of up to 50-70% (2), it is still difficult to predict the prognosis of BC. Aberrant chromosomal and genetic events have been reported in BC (3). However, understanding of the molecular mechanisms of BC remains poor and should be intensively explored.

Homeobox (HOX) genes commonly contain a characteristic 183-nucleotide sequence (homeobox). HOX genes encode transcription factors (4), which are involved in the regulation of differentiation, cell migration, cell growth, cell cycle, cellular identity and organogenesis (5). To date, 39 members have been identified in the HOX family and are divided into four groups, *i.e.* A, B, C and D, according to their chromosomal location. Aberrant expression and function of HOX genes have been evident in a number of tumours, such as breast, leukemia, stomach, lung, liver and prostate cancer (6-11). Deregulated HOX has also been observed in BC (12-14).

Homeobox B2 (HOXB2) is located on chromosome 17 (15). HOXB2 encodes a transcription factor and acts as one of the key regulators of the morphogenesis and differentiation of cells (16). Expression of HOXB2 in the genital tubercle plays an essential role in embryonic development of external genitalia and urogenital tract (17). However, the expression and involvement of HOXB2 in BC have not yet been disclosed. The expression of HOXB2 and its prognostic significance in BC were investigated in the current study.

Materials and Methods

Materials and cell lines. EJ138 and RT112 human bladder cancer cell lines were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). The cells were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin).

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Table I. Primer sequences for polymerase chain reaction (PCR).

Primer	Forward	Reverse
HOXB2	5' CCAAGAAACCCAGCCAATC 3'	5' GTGTTGGTGTAAGCCGTC 3'
GAPDH	5' ATGATATCGCCGCGCTCGTC 3'	5' GCTCGGTCAGGATCTTCA 3'

Construction of human HOXB2 expression vectors and transfection. Anti-human *HOXB2* hammerhead ribozymes were designed by targeting appropriate cleavage sites following an analysis of the secondary structure of *HOXB2* mRNA using an online RNA folding tool (Zuker's RNA Mfold program, <http://unafold.rna.albany.edu>). The ribozymes were generated using touch-down PCR followed by cloning into the pEF6/V5-His-TOPO plasmid vector (Invitrogen, Ltd., Paisley, UK). Full-length human *HOXB2* coding sequence was amplified from a cDNA library of normal human bladder tissues, and was subsequently cloned into the same plasmid vector. GenElute Plasmid Mini-Prep Kit (Sigma-Aldrich, Inc., Poole, UK) was used to isolate the plasmids. Anti-HOXB2 ribozyme transgenes were used to create a knockdown model in EJ138 cells. After 3 weeks of selection using G418 (250 µg/ml), the selected cells were subsequently maintained in a medium containing 50 µg/ml of G418. EJ138 cells with *HOXB2* knockdown were designated as EJ138-HOXB2-kd and used for the following experiments. *HOXB2* expression and control vectors were transfected into RT112 cells. The cells were subject to the similar selection using G418 and stably strains that overexpressed *HOXB2* were designated as RT112-HOXB2-exp and used for subsequent experiments.

RNA isolation, reverse transcription-PCR (RT-PCR) and western blot analysis. TRI Reagent (Sigma-Aldrich, Inc.) was used for RNA isolation. The extracted RNA was then used as a template for the following RT using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR amplification of the target genes was achieved by an initial denaturation at 95°C for 5 min, followed by 36 cycles of 30 s at 95°C, 30 s at 55°C and 40 s at 72°C, and a final extension at 72°C for 10 min.

Cells were lysed in a lysis buffer containing 50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, aprotinin, leupeptin and sodium vanadate followed by quantification using a DCTM protein assay kit (Bio Rad Laboratories, Inc.). Protein extracts (30 µg) were separated in 10% or 12% SDS-PAGE and then electrically blotted onto a 0.45 µm polyvinylidenedifluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were then blocked for 1 h at room temperature with 5% skimmed milk in PBST solution (0.05% Tween-20 in PBS). Target proteins were probed with the corresponding primary antibodies (1:500) overnight at 4°C, followed by incubation with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000) for 1 h at room temperature. Anti-HOXB2 (catalog no SJ2465545; Thermo Fisher Scientific Inc., Waltham, MA, USA), and anti-GAPDH (cat. no. sc 32233; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-mouse IgG secondary antibodies (cat. no. A5278, Sigma Aldrich) were used for the western blots. The protein bands were visualized using the EZ-ECL chemiluminescence kit (Biological Industries USA, Inc., Cromwell, CT, USA) and the Syngene G: BOX chemiluminescence imaging system (Syngene Europe, Cambridge, UK).

Cell proliferation assay. A standard procedure of crystal violet assay was used. EJ138 (2,000 cells/well) and RT112 (4,000 cells/well) cells were seeded on 96-well plates. The number of cells was determined at days 1, 3, and 5 by reading the absorbance after crystal violet staining. Briefly, the cells were fixed followed by staining with 0.1% crystal violet, which was then dissolved in 10% acetic acid. The absorbance was then quantified with a spectrophotometer (Elx800; Bio-Tek, Bedfordshire, UK) at 540 nm.

Cell-matrix adhesion assay. A 96-well plate was coated with Matrigel (5 µg/100 µl/well). The wells were rehydrated for 30 min at room temperature before 40,000 cells of each cell line per well were seeded. Six repeats were included for each cell line in each experiment. After an incubation of 45 min at 37°C, nonadherent cells were removed by two washes with balanced salt solution (BSS) buffer. Adhered cells were then determined following a fixation in 4% formaldehyde and staining with crystal violet.

In vitro migration and invasion assay. ThinCER™ 24-well plate inserts with 8 µm pores (Greiner Bio-One International GmbH, Kremsmünster, Austria) were used for the following migration and invasion assay. The upper chamber was filled with 100 µl DMEM containing 10% FBS, and the lower chamber was filled with 800 µl the same solution. 100,000 cells were seeded into each insert. After 24-h incubation, migrated cells were determined after a fixation in 4% formalin and staining with crystal violet.

Each transwell insert was pre-coated with 100 µg Matrigel (BD Matrigel Basement Membrane Matrix, Cat Number 354234; BD Bio-Science, Oxford, UK) before 20,000 cells for EJ138 or 40,000 cells for RT112 were seeded. After a culture of 48 hours, invaded cells were determined after fixation and staining.

Statistical analysis. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was employed for statistical analyses. Non-normally distributed data were analysed using Mann-Whitney *U*-test, and normally distributed data using a two-sample *t*-test. Differences were considered significant at $p < 0.05$.

Results

HOXB2 expression in bladder cancer from TCGA dataset. We analyzed *HOXB2* mRNA expression data of bladder cancer from the Cancer Genome Atlas (TCGA) dataset. UALCAN Dataset (18) indicated that *HOXB2* mRNA was significantly upregulated in papillary tumour subtype, compared with non-papillary tumor and normal tissues ($p=0.0037$ and 0.0042 , Figure 1) (19). According to molecular subtypes, *HOXB2* mRNA was significantly upregulated in both luminal infiltrated and luminal papillary

Figure 1. *HOXB2* expression in BC. A) Differential expression of *HOXB2* in different histological subtypes and molecular subtypes. B) Analysis of results from TCGA database showed that *HOXB2* expression is associated with the activation of P450 pathway. C) The mRNA expression (PCR) of *HOXB2* in EJ138 and RT112 cell lines before and after transfection. D) *HOXB2* protein in the BC cell lines was examined using western blot analysis. GAPDH was included as a control.

subtypes, compared with normal tissues, ($p=0.0249$ and 0.0068 , Figure 1A) (19).

Relationship between *HOXB2* gene expression and cytochrome P450 pathway. To investigate the relationship between *HOXB2* gene and some common pathogenic pathways, data from RNAseq results of the papillary subtype of bladder cancer in TCGA database (20) was used. This was followed by analyses using the Gene Set Enrichment

Analysis (GSEA) v3.0 (21). The high and low groups of TCGA papillary subtype of bladder cancer specimens were separated by the median value of *HOXB2* mRNA expression levels (22). The hallmark of Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets indicated that the signaling pathways were enriched mainly on biochemical metabolic pathways; especially the Cytochrome P450 pathway was significantly enriched in the BCs with high *HOXB2* expression (Figure 1B).

Figure 2. *HOXB2* regulates growth and adhesion of BC cells. A) Growth of EJ138 *HOXB2*-kd cells was reduced compared to the controls. B) Growth of RT112 cells overexpressing *HOXB2* (*HOXB2*-exp) over 3 days and 5 days culture was determined. C) Cell adhesion in EJ138 *HOXB2*-kd was remarkably reduced ($p=0.013$). D) RT112 *HOXB2*-exp cells exhibited increased adhesion ($p<0.001$).

Evaluation of *HOXB2* levels in cell lines. *HOXB2* transcripts in two different bladder cancer cell lines were detected using RT-PCR (Figure 1C). The expression of *HOXB2* was high in EJ138 and low in RT112 cells. *HOXB2* knockdown (*HOXB2*-kd) and overexpression (*HOXB2*-exp) were established in the EJ138 and RT112 cells, using anti-*HOXB2* ribozyme and expressing constructed plasmids, respectively. After the selection using G418, *HOXB2* expression in the transfected cells was determined using western blotting (Figure 1D). The *HOXB2*-modified EJ138 and RT112 cell lines were used in the following experiments.

Effects of *HOXB2* knockdown or overexpression on *in vitro* growth of bladder cancer cells. The effect of *HOXB2* on *in vitro* cell growth was assessed using two cell models, namely EJ138 cells with *HOXB2* knockdown and RT112 cells overexpressing *HOXB2*. EJ138 *HOXB2*-kd bladder cancer cells displayed a significantly reduced growth, $p<0.001$ compared with both control transfection and the wild type cells (Figure 2A). The RT112 *HOXB2*-exp cells, in which

HOXB2 expression had been up regulated, exhibited an increase of growth, $p<0.001$ compared with both control transfection and wild type RT112 cells (Figure 2B).

Impact on *in vitro* cell matrix adhesion of *HOXB2* Knockdown or overexpression. Influence of *HOXB2* on the adhesion of these bladder cancer cell lines was then evaluated. Down-regulation of *HOXB2* in EJ138 remarkably reduced the adhesion in comparison with the controls, $p=0.013$ (Figure 2C), while the overexpression of *HOXB2* resulted in an increase of adhesion in RT112 cells, $p<0.001$ (Figure 2D).

Effects of *HOXB2* on *in vitro* invasion of bladder cancer cells. We performed a transwell assay to examine the impact of *HOXB2* on invasiveness of the bladder cancer cell lines. Down-regulation of *HOXB2* in EJ138 resulted in a marked reduction in invasion, $p<0.001$ (Figure 3A). But *HOXB2* overexpression in RT112 cells had no effect on invasion compared with the control cells (Figure 3B).

Figure 3. Effect of altered *HOXB2* expression on invasion and migration of BC cells. A and C) Reduced invasiveness and migration were seen in the EJ138 *HOXB2*-kd cells. B and D) Invasion and migration of RT112 cells overexpressing *HOXB2* (*HOXB2*-exp) cells were not different from the control cells.

Effects of *HOXB2* on *in vitro* migration of bladder cancer cells. *In vitro* transwell chamber-based assay was also used to determine the migration and invasion of the BC cell lines following changes in *HOXB2* expression. The number of *HOXB2*-kd EJ138 cells that migrated through the diaphragm membrane was lower than that of control cells, $p < 0.001$ (Figure 3C). In contrast, *HOXB2* overexpression did not have a significant effect on the migration of RT112 cells (Figure 3D).

Discussion

Oncogenesis comprises numerous steps that lead to accumulation of genetic mutations and eventually an invasive phenotype. Aberrant epigenetic events are also critical for the tumorigenic process and subsequent evolution of neoplasia. This knowledge provides possibilities for developing novel therapeutic approaches (23).

HOXB2 is involved in embryonic development of the external genitalia and urogenital tract (17). Differential expression of HOXB2 has been observed in certain solid tumours. For example, overexpression of HOXB2 has been evident in cervical cancer (24), lung adenocarcinomas (25) and pancreatic cancer (26), and has been correlated with disease progression. Elevated amplification of the *HOXB2* gene has also been observed in cancerous samples (11/15) compared with healthy urothelium (4/15) (12). By analysing the data of the GEO database, we found that HOXB2 expression was increased in certain types of BC compared with the normal epithelium. Interestingly, this is only reflected in papillary urothelial cancer histological subtypes but not in non-papillary tumors, and has also been verified in molecular subtypes. It is well known that papillary urothelial carcinoma often shows non-invasive growth, and also predicts a better prognosis, while non-papillary urothelial carcinoma tends to show invasive growth and poor prognosis. These findings suggest that HOX is likely associated with the development of urothelial cancer of a specific type or period.

The Drug Metabolism Cytochrome P450 pathway, as part of Phase I metabolism in the detoxification of xenobiotics, has a possible involvement in tumorigenesis, disease progression and treatment of BC (27). In the present study, we used TCGA data retrieval and GSEA analysis to confirm the potential link between the *HOXB2* gene and the P450 pathway, which requires further study.

Knockdown of HOXB2 in EJ138 cell lines and over-expression in RT112, resulted in opposite effects in the respective bladder cancer cells on proliferation, adhesion, invasion, and migration. These results suggested that HOXB2 plays a role in coordinating aggressive traits of BC cells. This was consistent with the results regarding other malignancies indicating that HOXB2 is a putative tumour activator in BC (28).

However, other studies have obtained different results. For example, knockdown of HOXB2 promoted growth of breast cancer cells (16). Additionally, HOXB2 presented an inhibitory effect in FLT3-ITD driven acute myeloid leukemia (29). These results indicate that the effects of *HOXB2* gene may be tissue or tumour specific.

Intermediate filament proteins, cytokeratins (CKs) are involved in cell-cell communication and in the maintenance of cell shape. Deregulated CK8, CK18 CK19 and CK20 have been observed in BCs (30-32). CK genes are located closely to the 3' end of the HOX locus (17). Thus, the involvement of both HOX and keratin genes in the tumorigenic process and the consequent evolution to an invasive phenotype warrants further investigation.

Taken together, our present study revealed an increased expression of HOXB2 in papillary BC. HOB2 promoted cell growth and adhesion of BC cells. Drug Metabolism Cytochrome P450 pathway was enriched in the BCs when HOXB2 was highly expressed.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

JL, LS and XC carried out experimental work and data analyses, YY and WGJ conceived the study, PD and YY provided clinical samples. All Authors contributed to the preparation of the manuscript.

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