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Frequent expression of zinc-finger protein ZNF165 in human urinary bladder transitional cell carcinoma

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ABSTRACT

The aim of the study is to evaluate mRNA/protein expression of zinc finger protein 165 (ZNF165) in transitional cell carcinomas (TCCs) of urinary bladder and correlate its expression with the clinicopathological characteristics of patients. In this study, the methods of quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) were utilized to evaluate mRNA/protein expression of ZNF165 in TCC. Independent Student's *t* test, ANOVA and Chi-square (χ^2) were used to analyze the data statistically. We observed overexpression of ZNF165 mRNA in testis and majority (59.2%) of TCC patients. ZNF165 mRNA expression was also detected in adjacent noncancerous tissues (ANCTs) and some other normal tissues. Relative mean fold expression of ZNF165 mRNA was found to be significantly ($p < 0.01$) higher in muscle-invasive bladder cancer (MIBC) as compared to non-muscle-invasive bladder cancer (NMIBC) patients. (12.11 ± 9.57 vs. 5.72 ± 2.61, $p = 0.009$). ZNF165 protein expression was demonstrated on archival formalin-fixed, paraffin-embedded (FFPE) bladder tissues using IHC and nuclear staining pattern was detected. No significant difference was observed in protein expression of ZNF165 between the two groups (NMIBC and MIBC patients) (61.1% vs. 55.2%, $p = 0.629$). No significant protein expression of ZNF165 was observed among ANCTs and benign prostatic hyperplasia (BPH) used as control. Our study results suggest that ZNF165 mRNA/protein expression was observed in TCC of human urinary bladder and might be used as a novel diagnostic biomarker and as well a vaccine target in development of urinary bladder cancer specific immunotherapy.

Introduction

Urinary bladder cancer (UBC), which is reported more frequently in men than in women, is the second most common urological malignancy (Kulkarni et al., 2012). The American Cancer Society reported that about 72,570 new cases of UBC (about 54,610 in men and 17,960 in women) were diagnosed whereas 15,210 patients died (10,820 men and 4390 women) in 2013 (Siegel et al., 2013). Tumors of urinary bladder are generally categorized into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) (Schalken et al., 1992; Steinberg et al., 1992). Approximately 75% of UBC patients are initially diagnosed with non-muscle-invasive low-grade, papillary transitional cell carcinoma (TCC) (stage Ta–T1) (Dyrskjot et al., 2012). These NMIBC patients experience a high recurrence rate but progression to a muscle-invasive stage is relatively low, depending on the grade and stage of the disease (Dyrskjot et al., 2012; Millan-Rodriguez et al., 2000). The remaining 25% of patients presents with MIBC (stage ≥T2) at initial diagnosis. These bladder tumors are aggressive, poorly differentiated and show a poor treatment response and frequent development of metastases despite radical cystectomy (Dyrskjot et al., 2012).

Abbreviations: ZNF165, zinc finger protein 165; TCC, transitional cell carcinoma; CTA, cancer testis antigen; qRTPCR, quantitative real-time PCR; IHC, immunohistochemistry; TURBT, transurethral resection of bladder tumor; WHO, World Health Organization; BCG, bacillus Calmette–Guerin; BSA, bovine serum albumin; ANCT, adjacent non-cancerous tissue; UBC, urinary bladder cancer.

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NMIBC patients are often treated with a local, organ-sparing transurethral resection of bladder tumor (TURBT) followed by intravesical immunotherapy with attenuated bacillus Calmette–Guerin (BCG), which by an unknown mechanism reduces the risk of recurring tumors, suggesting that anticancer vaccines could be especially useful for the treatment of UBC (Dyrskjot et al., 2012; Simons et al., 2008). Thus human UBC propounds a stimulating opportunity to develop antigen specific immunotherapy based on targeting of cancer-testis antigens (CTAs). CTAs are encoded by genes that are normally expressed only in the human germline, but are also expressed in various tumor types including bladder (Kulkarni et al., 2012; Dyrskjot et al., 2012; Cogdill et al., 2012). More than 200 CTAs have been characterized with particular attention as attractive cancer biomarkers and target for cancer specific immunotherapy (Kim et al., 2013). CTAs represent ideal target molecules for specific immunotherapeutic intervention in cancer patients including UBC (Kulkarni et al., 2012; Shiraishi et al., 2012; Davis et al., 2003; Parmiani et al., 2002). In this quest, expression of various CTAs in UBC has been evaluated for potential immunotherapy purposes (Dyrskjot et al., 2012; Yin et al., 2012; Bergeron et al., 2009; Picard et al., 2007; Kanehira et al., 2007; Sharma et al., 2003, 2006; Kurashige et al., 2001; Patard et al., 1995). Accordingly, cancer vaccine trials employing CTAs, in particular M phase phosphoprotein 1 (MPHOSPH1) and DEP domain containing 1 (DEPDC1), as vaccination agents are being carried out in UBC patients and treatment with peptide vaccines were well tolerated in patients (Obara et al., 2012).

In the present study, we evaluated one of the CTAs, zinc finger protein 165 (ZNF165), expressed in human adult testis, which is a member of kruppel family of zinc-finger-containing transcription factors. ZNF165 mRNA is also expressed in the hepatocellular carcinoma (HCC), gastric cancer, colon cancer, non-small-cell lung carcinoma, and head and neck squamous cell carcinoma (HNSCC) (Dong et al., 2004; Atanackovic et al., 2006), but no study to date has reported expression pattern of ZNF165 mRNA and protein in TCC of human urinary bladder. Therefore, the aim of our study was to assess the ZNF165 mRNA/protein expression in tissue specimens of UBC patients by using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC), and correlate its expression with clinicopathological parameters of patients.

Materials and methods

Patients and clinical specimens

To evaluate mRNA expression of ZNF165, 76 freshly frozen cancerous tissues and 20 adjacent non-cancerous tissues (ANCTs) were collected from the UBC patients who underwent TURBT or radical cystectomy between March 2007 and April 2010 at Department of Urology, King George's Medical University (KGMU), Lucknow, India. The mean age of the UBC patients was 54.68 ± 11.51 years.

In addition, we collected 65 FFPE bladder tumor tissues, 12 ANCT specimens and 10 BPH tissues from the archives of the Pathology Department to evaluate IHC expression of ZNF165. The mean age of the BC patients was 53.83 ± 11.46 years whereas mean age of BPH patients was 64.14 ± 11.46 years.

Bladder tumor stage was determined using 2002 Tumor lymph Nodes and Metastasis (TNM) classification (Greene et al., 2002), and grading of tumors was done according to the World Health Organization (WHO, 2004) guide lines (Eble et al., 2004). The patients' clinical characteristics are summarized in Table 1. The consent was obtained from all UBC patients for the use of the tissue samples and records and the protocols were approved by the institutional ethics committee to perform study. All urothelial carcinomas were characterized as TCC. All tissue specimens were labeled with a unique code and the all assays were performed and interpreted blind of the any cytologic, histopathology and clinical characteristics. Clinical information related to diagnostic, surgical procedures and tumor characteristics were obtained from the patient's medical records.

Table 1
Patient clinicopathological characteristics.

Clinicopathological characteristics	(*n*) %	
	Real-time-PCR assay	Immunohistochemistry assay
No. of patients	(76)	(65)
Age in years (mean ± SD)	54.68 ± 11.51	53.83 ± 11.46
Age (years, %)		
≤45	24 (31.6%)	17 (26.2%)
>45	52 (68.4%)	48 (73.8%)
Sex		
Male	67 (88.2%)	63 (96.9%)
Female	9 (11.8%)	2 (3.1%)
Grade		
Low	33 (43.4%)	23 (35.4%)
High	43 (56.6%)	42 (64.6%)
Stage		
Ta	3 (3.9%)	6 (9.2%)
T1	33 (43.4%)	23 (35.4%)
T2–T4	40 (52.6%)	36 (55.4%)
Smoking		
No	34 (44.7%)	29 (44.6%)
Yes	42 (55.3%)	36 (55.4%)
Tobacco chewers		
No	36 (47.4%)	33 (50.8%)
Yes	40 (52.6%)	32 (49.2%)

Real-time PCR analysis

Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, after phenol treatment and drying, RNA was dissolved in RNase-free water and the quality of the RNA was evaluated by electrophoresis on a 1% agarose gel. Then cDNA synthesis was carried out using Quantitect® Reverse Transcription Reagent (Qiagen GmbH, Hilden, Germany) as per given instructions. The ZNF165 primers were 5′-CAAGATGGCTACAGAACCAAAGAAAGC-3′ and 5′-T CATATTGTGCCATTTCCTCAGCATTTAC-3′(Dong et al., 2004). GAPDH primers were 5′- GAAGGTGAAGGTCGGAGT-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

ZNF165 mRNA levels were expressed as *n*-fold differences relative to GAPDH (internal control) and the levels in the normal testis (calibrator). PCR was performed using SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen), and the cycling conditions comprised an initial denaturation step of 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 10 s, annealing at 55 °C for 20 s and extension at 72 °C for 45 s.

Immunohistochemistry

Tissue sections preparation, antigen retrieval and immunostaining were performed as described previously (Qin et al., 2009). The tissue sections were deparaffinized in xylene and rehydrated in gradedand solutions of ethanol. Antigen retrieval was done by autoclaving the sections in 0.01 M sodium citrate buffer (pH 6.0). Then endogenous peroxidase activity was blocked with the application of 3% hydrogen peroxide. After endogenous peroxidase blocking, non specific binding sites were blocked with 1% bovine serum albumin (BSA). A rabbit polyclonal antibody reactive for human ZNF165 (Abcam plc, Cambridge, UK) was applied, and slides were incubated for 16 h at 4 °C. After washing, slides were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody ("ready to use"; DAKO). Then visualization

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