Brief Report

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Frequent expression of a novel cancer testis antigen, protein kinase human monopolar spindle 1 (hMps1/TTK) in human urinary bladder transitional cell carcinoma

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SUMMARY Urothelial bladder cancer (UBC) is a frequently occurring malignancy of the urinary tract. The present study was undertaken to evaluate the mRNA and immunohistochemical (IHC) expression of protein kinase human monopolar spindle 1 (hMps1/TTK) gene in transitional cell carcinoma (TCC) of the bladder and correlate its expression with the clinicopathological characteristics of patients. In the present study, quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was used to evaluate TTK mRNA expression in TCC. IHC analysis of TTK was also evaluated. Independent Student's *t*, ANOVA and chi-square (χ^2) tests were used to analyze the data statistically. The frequency of TTK mRNA over expression was detected in 50% of UBC (38/76) by qRT-PCR. Relative mean fold expression of TTK mRNA was found significantly ($p < 0.05$) higher in muscle-invasive bladder cancer (MIBC) as compared to non-muscle-invasive bladder cancer (NMIBC) patients (8.96 ± 4.51 vs. 5.64 ± 3.53, $p = 0.03$). Moreover, IHC reveals heterogenous immunostaining pattern of TTK in TCC tissues. The frequency of TTK protein over expression was detected in 56.9% (37 of 65) UBC patients. No significant IHC expression of TTK was detected among adjacent noncancerous tissues (ANCTs) and benign prostatic hyperplasia (BPH) used as control. Collectively our study observations conclude that TTK is a novel cancer/testis antigen (CTA) as a diagnostic marker for early diagnosis of UBC.

Keywords TTK protein kinase, cancer/testis antigen, transitional cell carcinoma, urinary bladder cancer, immunotherapy

1. Introduction

Human urinary bladder cancer (UBC) is the one of the most common malignancies worldwide and occurs at a higher frequency in male individuals (*1*). It is a heterogeneous disease encompassing distinct biologic features that lead to extremely different clinical behaviors (*2*). Approximately 75% of patients with transitional cell carcinoma (TCC) present a disease at a non-invasive stage that involves only the inner lining of the bladder (*3*). Patients with non-muscle-invasive bladder cancer (NMIBC) are usually treated with transurethral resection with or without intravesical chemotherapy and have a favourable prognosis; however, some of these patients suffer from recurrence with grade progression (*4*).The remaining 25% of newly diagnosed UBCs present with muscle invasion and have a higher risk of cancer-specific mortality (*5*) with the need of aggressive radical surgery or radiotherapy, with or without chemotherapy.

The field of cancer vaccine therapy is currently expected to become the fourth option in the treatment of UBC after surgery, chemotherapy and radiation therapy and a large number of clinical and translational investigations are underway at present in this direction (*6*). To date, only few pilot clinical trials have been conducted to evaluate vaccine candidates for UBC (*7*). These trials were conducted on a limited number of study patients and only in the context advanced invasive UBC (*6,8-10*), and metastatic UBC after failure of platinum-based regimens (*7*). Thus, identification of additional

cancer/testis antigen (CTA) in TCC of urinary bladder with increased potential is need of hour. In this quest, we evaluated one of the protein kinase human monopolar spindle 1 (hMps1/TTK) gene (NM_003318) which encodes a dual serine/threonine and tyrosine protein kinase and locates on chromosome 6q13-q21. *TTK* gene also belongs to cancer-testis (CT) gene family that is hardly detected in normal tissue except the testis. TTK is expressed at relatively high levels in testis, thymus tissues and various malignant tumor tissues (*11-22*), but not detected in most other benign tissues. Moreover, TTK has been also identified as novel targets in human UBC using microarray (*6,21*). TTK is over expressed in various malignancies; however, detailed expression pattern of TTK in association with clinicopathological characteristics of TCC remain unclear. Therefore, the objective of the present study was to evaluate mRNA and protein expression in tissue specimens of TCC patients by using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC), and correlate its expression with clinicopathological parameters of patients.

Table 1. Patient clinciopathological parameters

Clinicopathological characteristics	Real-time-PCR Assay (76), *n* (%)	Immunohistochemistry Assay (65), *n* (%)
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%)

2. Materials and Methods

2.1. Clinical specimens

A total of 76 freshly frozen UBC tissues (male: 67; female: 9) were collected from the TCC of urinary bladder patients for the evaluation of TTK mRNA expression using qRT-PCR. All patients underwent transurethral resection of bladder tumour (TURBT) or radical cystectomy (RC) between March 2006 and April 2012 at Department of Urology, KGMU, Lucknow, India. The mean age of the UBC patients included in qRT-PCR assay was 54.75 ± 11.51 years. In addition to analyse IHC expression of TTK, 65 formalin-fixed, paraffin-embedded (FFPE) bladder tissues, 12 adjacent noncancerous tissue (ANCT) specimens and 10 benign prostatic hyperplasia (BPH) tissues were obtained from the archives of the Pathology Department, KGMU, Lucknow, India. The mean age of the UBC patients included in IHC assay was 53.83 ± 11.47 years whereas mean age of BPH patients was 64.14 ± 11.46 years. The participants gave informed consent before taking part and given consent to use their tissues in the experiments. None of the TCC of urinary bladder patients has received any therapy, including chemotherapy, radiotherapy or other treatment, prior to surgery. Bladder tumours were staged according to the 2002 tumour-lymph node-metastasis (TNM) classification system (*23*). Tumours were graded according to the 2004 World Health Organization (WHO) bladder tumour classification criteria (*24*). Detailed clinicopathological parameters of the UBC patients are given in Table 1. All UBC patients provided signed informed consent and the study design was approved by the research ethics committee of our institution.

All tumours were histologically proven as TCCs and UBC was confirmed via examination of cystoscopic biopsy. All tissue specimens were coded with unique code and the results of qRT-PCR and IHC assays were interpreted blind of the histopathological characteristics of the bladder tumours. Upon surgical resection, all tissue specimens were initially stored in RNAlater® (Ambion Inc., Austin, TX) and immediately frozen at -80°C in liquid nitrogen until further analysis.

2.2. RNA extraction and quantitative reverse transcription-PCR

Total RNA was extracted from tissue specimens using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA from different normal tissues was purchased (Clonetech, Palo Alto, CA, USA). First-strand cDNA was synthesized from total RNA using Quantitect® Reverse Transcription Reagent (Qiagen GmbH, Hilden, Germany) as per manufacturer's instructions. In brief, the expression of the housekeeping gene β-actin, as an internal standard, and the gene of interest was evaluated using SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen). Human Bladder Total RNA (Clontech, Palo Alto, CA, USA) was used as a reference for evaluation of TTK mRNA levels in bladder cancerous tissues. Among normal tissues, TTK mRNA levels were expressed as n-fold differences relative to β-actin (internal control) and the levels in the normal testis (calibrator). The primer sequences and PCR conditions were the same as described previously (*25*).

2.3. Immunohistochemistry

IHC assay was performed on FFPE tissue sections

Table 2. Correlation between relative mean fold TTK mRNA expression and clinicopathological parameters of UBC patients

Characteristics Age (years):	Non-muscle-invasive Mean ± SD	*p* value	Muscle-invasive Mean ± SD	*p* value	Total	*p* value
≤ 45	8.00 ± 2.83 (2)	0.320	7.78 ± 3.07 (9)	0.344	7.82 ± 2.89 (11)	0.875
> 45	5.11 ± 3.59 (9)		9.56 ± 5.06 (18)		8.07 ± 5.02 (27)	
Sex:						
Female	6.00 ± 0.00 (1)	0.921	8.00 ± 0.00 (1)	0.833	7.00 ± 1.41 (2)	0.750
Male	5.60 ± 3.72 (10)		9.00 ± 4.60 (26)		8.06 ± 4.59 (36)	
Grade:						
Low	4.29 ± 3.15 (7)	0.093	6.83 ± 5.23 (6)	0.195	5.46 ± 4.26 (13)	0.010
High	8.00 ± 3.16 (4)		9.57 ± 4.23 (21)		9.32 ± 4.06 (25)	
Smoking:						
No	5.60 ± 4.10 (5)	0.977	8.08 ± 4.70 (12)	0.375	7.35 ± 4.55 (17)	0.430
Yes	5.67 ± 3.39 (6)		9.67 ± 4.39 (15)		8.52 ± 4.45 (21)	
Tobacco:						
No	5.50 ± 2.89 (4)	0.929	8.27 ± 5.20 (15)	0.380	7.68 ± 4.88 (19)	0.669
Yes	5.71 ± 4.07 (7)		9.83 ± 3.49 (12)		8.32 ± 4.14 (19)	

Numbers in parenthesis indicate the number of UBC patients.

according to our previously published protocol (*26*). The TTK protein in UBC tissues was detected using a rabbit polyclonal antibody against TTK (C-19; sc-540; 1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The IHC expression of TTK was evaluated according to the following criteria. The immunostaining patterns were applied into scales on the percentage of cells with positive immunostaining: 0, complete absence or negative stained cells; 1, < 10% positive cells; 2, > 10% positive cells but ≤ 50% and 3, > 50% positive cells. The IHC expression of TTK was scored as being present or not. Heterogenous staining of urothelial and cancer cells were scored negative when tumour biopsies stained less than 10%, while those stained more than 10% was considered as positive on IHC.

2.4. Statistical analysis

Continuous data were defined as Mean ± SD, while categorical in %. Qualitative variables were illustrated as percentages and numbers. Independent Student's *t* test and ANOVA was used to analyse comparison between groups and significance of mean difference was analyzed by Tukey's post hoc test after adjusting the multiple contrasts for significance. Associations between categorical groups (*i.e.*, TTK expression and clinicopathological parameters) were analyzed using the chi-square (χ^2) test and $p < 0.05$ was considered to be statistically significant. All statistical analysis was implemented with the SPSS software (Windows version 18.0).

3. Results and Discussion

3.1. Quantitative expression of TTK mRNA

Quantitative analysis of TTK mRNA expression was analyzed using qRT-PCR in 16 normal tissues and 76 bladder tumour tissues. The frequency of TTK mRNA expression was observed in 30.6% (11 of 36) of NMIBC and 67.5% (27 of 40) MIBC patients. Thus, overall 50.0% (38 of 76) TCC of urinary bladder patients were found to be positive for TTK mRNA expression. TTK mRNA expression was observed in higher number of MIBC patients than NMIBC patients (67.5% *vs*. 30.6%, $p < 0.001$). In the MIBC patients, relative mean fold expression of TTK was significantly higher as compared to NMIBC (8.96 ± 4.51 *vs*. 5.64 ± 3.53, $p = 0.03$). We detected TTK mRNA expression in 39.3% patients with low-grade and 58.1% with high-grade bladder tumours respectively.

TTK mRNA expression was detected only in testis, and thymus using qRT-PCR. However, over expression was detected only in testis and relative mean fold expression level of TTK mRNA in thymus was 112-fold lower than that in the testis.

The relationship between TTK mRNA expression and clinicopathological parameters of the TCC patients is summarized in Table 2. No significant correlation was found between TTK mRNA expression and clinicopathological parameters such as patient's age, gender, grade, disease stage, cigarette smoking and tobacco chewing *etc*. in both NMIBC and MIBC patients. However, high relative mean fold expression of TTK mRNA was detected in older age patients *vs*. younger patients, males *vs*. females, advanced stage *vs*. early stage, high-grade *vs*. low-grade and with cigarette smoking and chewing tobacco habit.

3.2. Expression of TTK protein

IHC revealed heterogenous (TTK protein to be expressed and located to both nuclei and cytoplasm) positive immunostaining in bladder tumour tissues and were interpreted as IHC-positive. Heterogenous

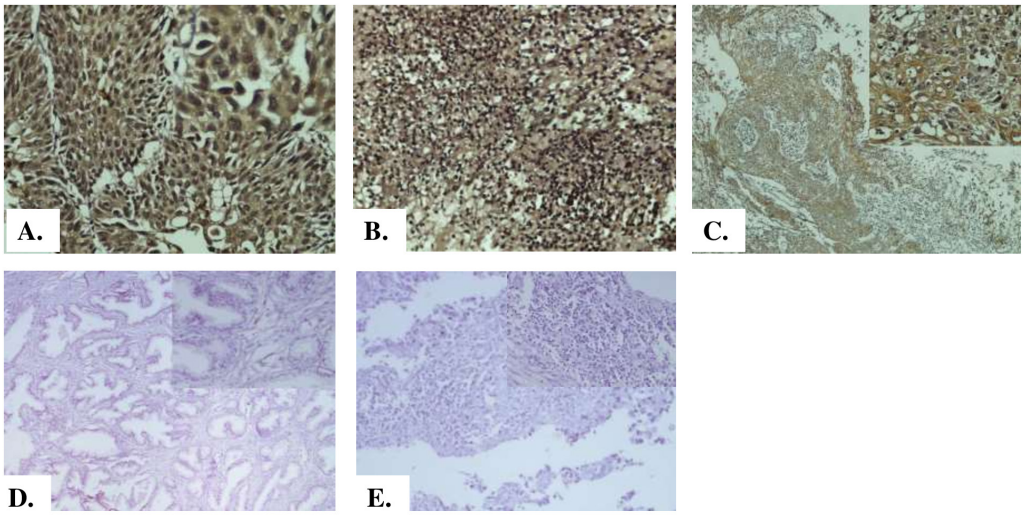

Figure 1. Immunohistochemical analysis of TTK in UBC. UBC tissues were stained with TTK specific rabbit polyclonal antibody. (A) NMIBC tissue showing strong positive expression; **(B)** MIBC showing strong positive expression; **(C)** MIBC showing positive expression; **(D)** BPH tissue showing negative expression; **(E)** NMIBC showing positive expression.

Table 3. Correlation between TTK protein expression and clinicopathological parameters of UBC patients

Characteristics	Non-muscle-invasive (n = 29)			Muscle-invasive (n = 36)			Total (n = 65)		
	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value
Age (years):									
≤ 45	4 (23.5%)	2 (16.7%)	0.653	4 (36.4%)	7 (28.0%)	0.616	8 (28.6%)	9 (24.3%)	0.700
> 45	13 (76.5%)	10 (83.3%)		7 (63.6%)	18 (72.0%)		20 (71.4%)	28 (75.7%)	
Sex:									
Female	0 (0.0%)	0 (0.0%)	NA	1 (9.1%)	1 (4.0%)	0.539	1 (3.6%)	1 (2.7%)	0.841
Male	17 (100%)	12 (100%)		10 (90.9%)	24 (96.0%)		27 (96.4%)	36 (97.3%)	
Grade:									
Low (G1)	12 (70.6%)	5 (41.7%)	0.119	1 (9.1%)	5 (20.0%)	0.418	13 (46.4%)	10 (27.0%)	0.105
High (G2-G3)	5 (29.4%)	7 (58.3%)		10 (90.9%)	20 (80.0%)		15 (53.6%)	27 (73.0%)	
Stage									
Ta	2 (11.8%)	4 (33.3%)	0.158	0 (0.0%)	0 (0.0%)	NA	2 (7.1%)	4 (10.8%)	0.28
T1	15 (88.2%)	8 (66.7%)		0 (0.0%)	0 (0.0%)		15 (53.6%)	8 (21.6%)	
T2 - T4	0 (0.0%)	0 (0.0%)		11 (100%)	25 (100%)	0	11 (39.3%)	25 (67.6%)	
Smoking:									
No	12 (70.6%)	7 (58.3%)	0.494	5 (45.5%)	5 (20.0%)	.116	17 (60.7%)	12 (32.4%)	0.023
Yes	5 (29.4%)	5 (41.7%)		6 (54.5%)	20 (80.0%)		11 (39.3%)	25 (67.6%)	
Tobacco chewing:									
No	8 (47.1%)	8 (66.7%)	0.296	8 (72.7%)	9 (36.0%)	0.042	16 (57.1%)	17 (45.9%)	0.371
Yes	9 (52.9%)	4 (33.3%)		3 (27.3%)	16 (64.0%)		12 (42.9%)	20 (54.1%)	

Numbers in parenthesis indicate the number of UBC patients.

expression of TTK was detected in 41.4% (12 of 29) of NMIBC and 69.4% (25 of 36) MIBC patients (Figure 1). Thus an overall frequency of TTK IHC expression was found to be positive in 56.9% (37 of 65) TCC patients. TTK protein expression was observed in significantly higher number of MIBC patients than NMIBC patients (69.4% *vs*. 41.4%, $p = 0.02$). Furthermore, heterogenous expression of TTK was detected in 43.4% patients with low-grade and 64.2% with high-grade urothelial tumours respectively. No significant expression of TTK protein was observed among ANCTs and BPH tissues. The relationship between TTK protein expression and clinicopathological parameters of the TCC patients is summarized in Table 3. The protein expression of TTK was not found to be significantly associated with the clinicopathological parameters as well as habits of NMIBC and MIBC patients. However, the protein expression of TTK was found to be significantly ($p = 0.042$) associated with the tobacco chewing habit in MIBC patients.

UBC is a heterogeneous disease which is specified by a high recurrence rate that necessitates continuous cystoscopic surveillance. Despite a better understanding of UBC biology and the use of adjuvant therapies, UBC remains one of the most expensive carcinoma to

treat due to its high recurrence rate, risk of progression, chemotherapeutic resistance and requirement of long-term follow-up strategies.

TTK over expression has been reported in many different types of cancers, and significantly correlated with specific clinicopathological tumour features. However, little is known about the expression status and clinical significance in TCC. Thus in the present study, we analyzed the detailed mRNA and protein expression pattern of TTK to evaluate its clinical significance in human TCC. In the present study, we have detected TTK mRNA expression in 50.0% UBC patients using qRT-PCR. Our study findings derived by qRT-PCR are in agreement with previous observations reporting TTK upregulation in a variety of tumours, such as breast, esophageous, lung, prostate, lung, anaplastic thyroid and bladder (*13-21*). Our study findings are also consistent with the previous studies of CTA expression in UBC that MAGE-A8 and MAGE-A9 expression was detected in 56% and 54% of bladder tumours (*27*), whereas NY-ESO-1 expression were observed in 35% and 45.1% tumours (*28,29*).

TTK mRNA expression characteristics were investigated in different tissues to determine whether TTK possess CTA expression characteristics. In present study TTK mRNA expression was detected in testis and thymus normal tissues using qRT-PCR. These findings were consistent with the previous report (*22*). Thus our study results imply that TTK mRNA expression plays an important role in bladder tumorigenesis. Our study further evaluated IHC expression of TTK in FFPE sections of bladder tissues, which affirm heterogenous expression pattern in TCC but no significant expression was observed among ANCTs. These results are consistent with the previous published reports in which high protein expression of TTK was observed in tumour specimens but not in ANCTs (*13*). In the present study, heterogenous immunostaining pattern of TTK was observed in FFPE sections of bladder tissues. These results are consistent with previous report that detected heterogenous expression pattern of various other CTAs in urothelial carcinoma (*30*). In our study no significant association was observed between TTK expression and clinicopathological parameters such as sex, age, stage, and grade of the UBC patients. These results are in accordance with findings from earlier published observations in TCC of bladder (*29*).

The present study had few limitations. The bladder tumor tissues used to evaluate mRNA and protein expression of TTK was obtained from different patient's cohort. In this quest, no association was observed between TTK mRNA expression and protein expression. Moreover, the relatively smaller sample size of TCC of urinary bladder patients did not granted to perform sub analysis.

In conclusion, the present study indicated upregulation of TTK mRNA expression and over expression of IHC staining in most of TCC patients irrespective of stage, grade and other clinicopathological parameters of TCC patients, indicative of its diagnostic potential as a molecular marker. However, it is further warranted to be compared with available gold standard diagnostic methods like biopsy and urine cytology to find out diagnostic efficacy of TTK over expression as a molecular marker in diagnosis of TCC. Our study findings also conclude that TTK might be a potential vaccine candidate for immunotherapy in TCC due to its expression among bladder tumours and restricted expression in normal tissues. However, findings remain to be verified in larger prospective clinical studies to evaluate its potential as a molecular biomarker and immunotherapeutic target for the TCC of urinary bladder.

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