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Original Article

The detection of PAX8 in human upper urinary tract urothelial carcinoma

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Abstract: Upper urinary tract urothelial carcinomas (UUT-UCs) are defined as malignant neoplasms of the urothelium from the upper urinary tract, including renal calyces, the renal pelvis and the distal ureter. The natural attributes of UUT-UCs differ from those of bladder cancer. The aim of the present study was to investigate PAX8 expression in the normal urothelium and in urothelial carcinomas (UCs) of the upper urinary tract. Immunohistochemistry was conducted in 35 cases of renal pelvic and 30 cases of ureteral papillary UCs and the adjacent normal urothelium respectively. PAX8 mRNA expression was evaluated by RT-PCR in a different set of normal urothelial mucosa of the urinary tract and UUT-UCs. In immunohistochemical studies, the positive rates of PAX8 staining in UCs of the renal pelvis and ureter were 17% and 6.6% respectively, presenting focally positive in most cases, while the positive rates in the adjacent normal epithelia of the pelvis and ureter were 100% and 93% respectively. PAX8 mRNA was detected in all of the tumors and adjacent normal urothelial mucosa specimens of the upper urinary tract. 4 types of PAX8 isoforms, PAX8a, PAX8b, PAX8c and PAX8e, were detected in UUT-UCs in this study. As in bladder cancer, PAX8 expression was highly heterogeneous in terms of the splicing mRNA isoforms, with the different isoforms differentially expressed in the UUT-UCs. Among the 4 types of PAX8 isoforms, the PAX8e isoform was found in almost all UUT-UCs tumor tissues, but the PAX8d isoform was not detected in UUT-UCs that were different from the transcriptional splicing patterns of PAX8 in bladder cancer reported in the literature. In addition, the above 4 types of PAX8 splicing isoforms were simultaneously detected in almost all of the normal mucosal epithelia of the upper urinary tract, which was very different from that of bladder mucosa. Further studies are suggested to reveal whether or not the differences in natural attributes between UCs of the upper and lower urinary tracts are related to their PAX8 transcriptional splicing patterns.

Keywords: Upper urinary tract urothelial carcinomas, PAX8, differential splicing

Introduction

Upper urinary tract urothelial carcinomas (UUT-UCs) are defined as malignant neoplasms of the urothelium from the upper urinary tract, including those from the renal calyces, renal pelvis, and distal ureter. UUT-UCs are relatively uncommon in adults and account for only 5-10% of urothelial carcinomas (UCs) or about 7% of all kidney tumors. The natural attributes of UUT-UCs are different from those of bladder cancers. The percentage of invasion in UUT-UCs is 60% at diagnosis compared with only 15-25% in bladder cancers [1]. There are also some similarities between UUT-UCs and bladder urothelial carcinomas in terms of epidemiology and risk factors. Many risk factors are attributed to the genesis of UUT-UCs, including environmental and occupational hazards, chemotherapeutic exposure, and previous history of urinary bladder or ureteral carcinoma [2]. The presence of UUT-UCs increases the risk of bladder cancer significantly.

PAX8 is nephric-lineage transcription factor which has important functions in renal organogenesis [3]. In the kidney, PAX8 has been previously demonstrated to be expressed in the majority of renal epithelial neoplasms [4]. Moreover, several studies have found that a small subset of UUT-UCs is also positive with PAX8 [5-7]. As for the expression of PAX8 in human bladder cancers, the results are contradictory in previous studies because of different

Table 1. Base sequence of the forward and reverse primers

Primer no.	5' to 3' base sequence	Reference
44	CTTCGCACGGATGCCTTCAGCCAG	Poleev *et al.* [15].
18	GGAACTCAGCAAGCTGG-AGTTGG	
19	AGCCGCTCGAGTGCCCATTTGAG	
45	CCAGGCCTCGCTGTAGGAGGAG	

primary antibodies being used (a positive rate ranging from 0 to 93%) [5, 8-10]. In addition, Pellizzari *et al.* reported that PAX8 expression was highly heterogeneous in terms of the splicing mRNA isoforms in human bladder cancer [8]. The transcriptional pattern of the PAX8 gene in human UUT-UCs remains unclear. Molecular genetic analysis in bladder UCs has been conducted in several studies [11-14], but extremely rare in UUT-UCs. In this study, the expression of PAX8 was detected in UUT-UCs and the normal epithelia adjacent to the neoplasms of the upper urinary tract by immunohistochemical staining and molecular analysis.

Materials and methods

Subjects and samplin

After approval from the ethics committee at our hospital, 35 cases of renal pelvic and 30 cases of ureteral primary papillary UCs were retrieved from the archive files for immunohistochemical studies. These patients underwent radical nephrectomy at our hospital between 2013 and 2017 and included 38 men and 27 women ranging in age from 42 to 83 years old. The normal urothelia adjacent to the neoplasms were evaluated concurrently in the 60 cases of tumor samples.

For the RT-PCR studies, 20 cases of primary papillary UCs from the renal pelvises and ureters in each group and the corresponding normal urothelial mucosa adjacent to the neoplasms of these UUT-UCs were collected immediately after surgery in sterile plastic containers, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis, and 1 case of normal urothelial mucosa adjacent to the neoplasm of bladder cancer was also collected as a control.

Immunohistochemical staining

Immunohistochemical staining was performed in a Dako autostainer with rabbit anti-PAX8 polyclonal antibody (1:100) (Proteintech, Inc, Chicago, IL, USA). In brief, 4 μm tissue sections were deparaffinized and incubated with 3% hydrogen peroxide for 15 to 20 minutes to quench the endogenous peroxidase activity. Antigen retrieval was performed using pressure cooker pretreatment in a citrate buffer (pH=6.0). Tissue sections were subsequently incubated with the primary antibody for 60 minutes at 25°C. After tris-buffered saline rinsing, the tissue was incubated using the Envision Plus secondary antibody for 30 minutes, followed by diaminobenzidine for 5 minutes. Appropriate positive (tonsil lymphocytes) and negative (incubation with secondary antibody only) controls were stained in parallel for each round of immunohistochemistry.

The evaluation of immunostaining included the extent and intensity of the staining, and only distinct nuclear staining of PAX8 was considered to be positive. Immunoreactivity with normal B lymphocytes was used as an internal positive control and as an intensity reference when present. Diffusely positive staining was considered more than 50% of tumor cells staining positive for PAX8; otherwise, the staining was considered as focally positive. When less than 10% or none of the tumor cells were stained positive for PAX8, it was considered as negative staining. The intensity of PAX8 staining in tumors was graded as weak (1+), moderate (2+), and strong (3+) [5].

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from tissue samples using a QIAamp RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instruction. The RNA concentration was measured by a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA), and the integrity was checked by electrophoresis on 1.0% (wt/vol) agarose gels visualized by 0.5 mg/ml ethidium bromide and UV-light photography. About 500 ng of total RNA was reverse transcribed using a PAX8-specific primer as reported by Poleev *et al.* [15]. RT-PCR was conducted using a PrimeScript RT reagent kit with a gDNA Eraser cDNA synthesis kit (Takara, Japan). The nested PCR method with primers no. 44/no. 18 and no. 19/no. 45 was used to amplify the target. In detail, the first round of PCR was carried out in 50 μl containing 5 μl 10 × TransFast Taq

Table 2. Summary of PAX8 immunostaining in UUT-UCs

Sites \ Intensity and extent	3+		2+		Total number of cases	
	Diffuse	Focal	Diffuse	Focal	Positive	Negative
UC, renal pelvis	1	2	1	2	6 (17%)	29
UC, ureter	1	0	0	1	2 (6.6%)	28
Total number of cases	2	2	1	3	8 (12%)	57

UUT-UCs, upper urinary tract urothelial carcinomas; UC, urothelial carcinoma.

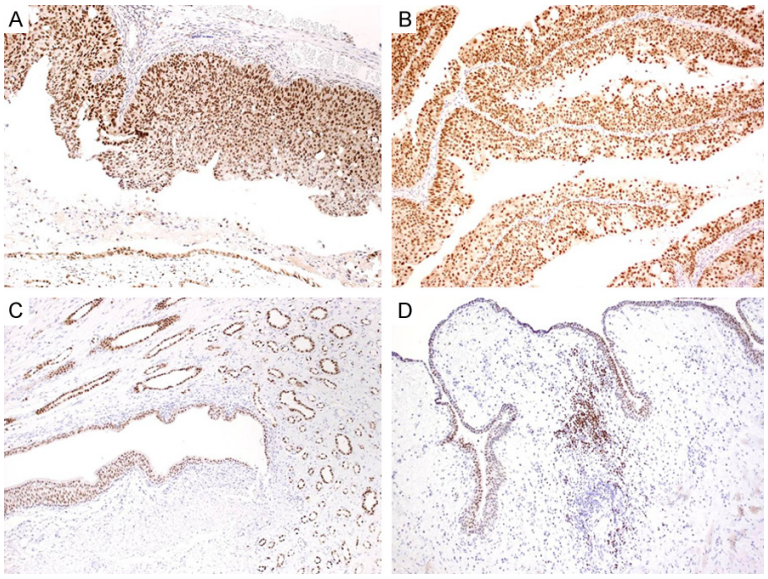

Figure 1. Immunohistochemical findings. Immunoreactive PAX8 was detected in the normal urothelial mucosa of the upper urinary tract and in a small subset of UUT-UCs. (A) UC of the renal pelvis; (B) UC of the ureter; (C) Normal urothelial mucosa of the renal pelvis; (D) Normal urothelial mucosa of the ureter. (A-D immunohistochemistry × 100).

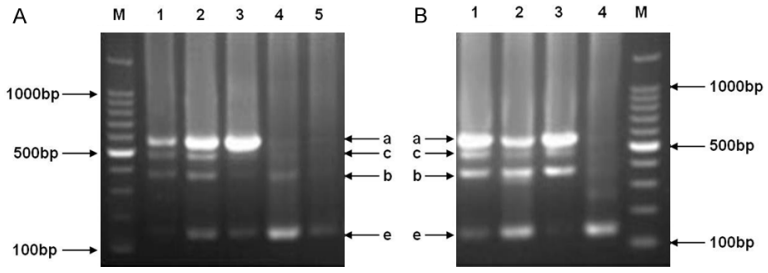

Figure 2. RT-PCR detection of PAX8 mRNA in normal urothelial mucosa and in UUT-UCs. A: Pax8 expression in UUT-UCs, lane 1 to 5 indicate UUT-UCs; B: Pax8 expression in the normal urothelial mucosa: lane 1 indicates urothelial mucosa of the ureter, lanes 2 and 3 indicate urothelial mucosa of the renal pelvis, and lane 4 indicates urothelial mucosa of the bladder.

Buffer, 4 μl 2.5 mM dNTPs, 10 μM primers of outer nest (no. 44 and no. 18, **Table 1**), 5 μl of the cDNA pool and 5 U TransFast Taq DNA polymerase (Transgene, China) in 30 cycles (94°C, 30 s; 55°C, 1 min; 72°C, 1 min). 5 μl of the first-round product was added to the 45 μl PCR premix containing primers of the inner nest (no. 19 and no. 45, **Table 1**) and PCR was carried out as above.

Clone and sequencing

The target bands of PCR products were purified using an Omega Gel DNA extraction kit (Omega Inc., USA). We then determined the concentration, and ligated them into a pEASY-T1 Cloning Kit (Transgen, China). The ligated mixtures were then transformed into the competent *Escherichia coli* DH5α cells (Transgen, China), and plated on LB agar plates. Finally, a total of 100 random positive clones were selected, PCR-amplified and sequenced.

Results

Expression of PAX8 in UUT-UCs and the normal urothelial mucosa adjacent to neoplasms

Immunohistochemistry for PAX8 was performed on whole sections of 65 UUT-UCs. We found that the positive rates of PAX8 immunoreactivity were 17% (6 of 35) and 6.6% (2 of 30) in the UCs of the renal pelvis and ureter respectively (**Table 2**; **Figure 1A**, **1B**). In the positive cases, most were focal in extent, as well as moderate to strong (2+~3+) in staining intensity. The positive rates in the corresponding normal epithelia adjacent to the

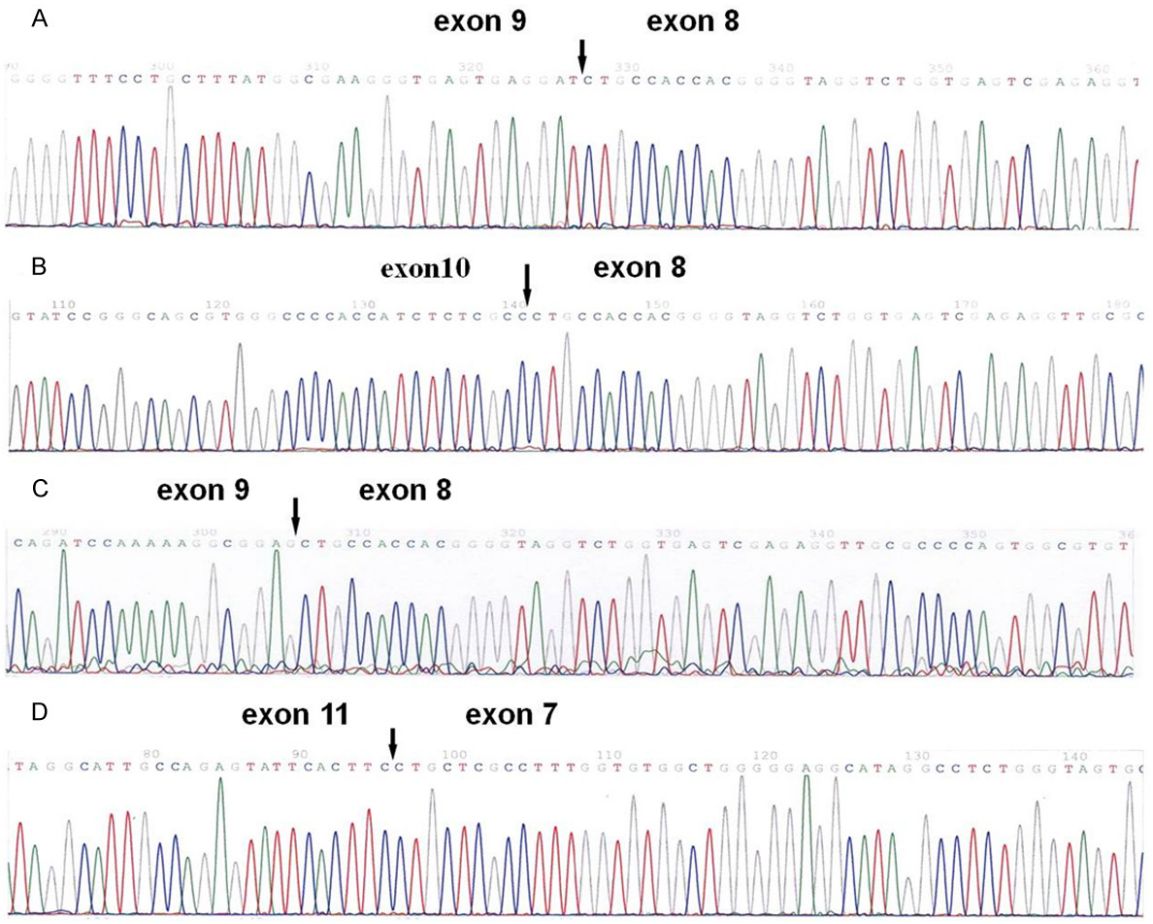

Figure 3. Gene sequence of RT-PCR products. A: PAX8a isoform; B: PAX8b isoform; C: PAX8c isoform; D: PAX8e isoform.

neoplasms of the pelvis and ureter were 100% and 93% respectively (**Figure 1C**, **1D**).

RT-PCR detection of PAX8 mRNA in the normal urothelium and UUT-UCs

In order to further extend the data obtained by immunohistochemistry, the presence of PAX8 mRNA in normal urothelial mucosa of urinary tract and in UUT-UCs was investigated by RT-PCR. A total of 4 kinds of band of 550, 361, 471 and 138 bp-long respectively were amplified in UUT-UCs in this study, which were confirmed by gene sequencing as PAX8a, PAX8b, PAX8c or PAX8e isoform. As in bladder cancer, PAX8 expression was highly heterogeneous in terms of the splicing mRNA isoforms, with the different isoforms differentially represented in the UUT-UCs. Among the 4 types of PAX8 isoforms, the PAX8e isoform was found in almost all UUT-UCs tumor tissues except for 1 case, while the PAX8d isoform (240 bp-long) was not detected in UUT-UCs. However, the PAX8d isoform was reported to be detected in several human bladder cancer cell lines and some bladder cancer tissues, but the PAX8e isoform was detected only in a few bladder cancer tissues or bladder cancer cell lines in the literature [8]. In addition, the above 4 types of splicing isoforms could be simultaneously detected in almost all of the normal mucosal urothelia of the upper urinary tract, except in 2 cases of mucosal urothelia of the renal pelvis where the PAX8e isoform was not detected. The PAX8 transcriptional splicing pattern of the mucosal urothelium of the upper urinary tract was very different from that of bladder mucosa, and only one 138 bp-long band (PAX8e isoform) was detected in the latter (**Figures 2A**, **2B**, **3A-D**).

Discussion

UCs in the upper urinary tract are relatively uncommon in adults, commonly occurring in the 60 to 80 year old patients. The UUT-UCs are well known for their multicentricity and/or high

incidence of recurrence, and the additional tumor lesions may involve the ureter, the bladder, or the contralateral side [2]. The histopathologic features presenting in UCs in the upper urinary tract are generally similar to those in the urinary bladder, but their natural attributes differ from those of bladder cancer [1, 2]. Due to the difficulty of conservative therapeutic regimen of UUT-UCs, radical nephroureterectomy with bladder cuff excision is the standard treatment for localized tumor. Unfortunately, the prognosis of the patients underwent surgery is still poor [16].

Cell lineage specific transcription factors are a group of regulatory proteins expressed in specific primordial tissues in the embryonic stages and in differentiated adult tissues of the same lineage [9, 17]. PAX8 is a nephric-lineage transcription factor which has important functions in renal organogenesis [3]. In the kidney, PAX8 has been shown to be expressed in the majority of renal epithelial neoplasms, including clear cell, papillary, chromophobe, and translocation (Xp11.2) renal cell carcinomas, collecting duct carcinomas, and in oncocytomas [4-6, 18]. However, interestingly, it has been found in several studies that a small subset of UCs, especially UCs involving the upper urinary tract, were positive for PAX8, and the positive rates were 23%, 9%, 17% and 12% [5-7, 19]. In addition, tissue microarray was constructed from 11 cases of sarcomatoid UCs of the upper tract by Chang *et al.* in 2013, and they found 2 (18%) cases were PAX8 positive [20]. In this study, we also observed that PAX8 expressed in a subset of UUT-UCs (about 12%). These findings suggest that a combined panel of markers (p63/PAX8/GATA3) should be used to improve the markers' performance in the diagnosis of epithelial neoplasms involving the renal sinus.

Although PAX8 has been extensively studied in renal epithelial neoplasms, there are few studies regarding PAX8 expression in UCs, and even less available data about ureteral cancer. In 2009, Tong *et al.* reported that PAX8 was positive in 23% (4/17) of UCs from the renal pelvis, but it was all negative in UCs from the ureter (2 cases) and bladder (40 cases) [5]. In the present study, the positive rates of PAX8 staining in UCs of the renal pelvis and ureter were 17% (6/35) and 6.6% (2/30) respectively. We also observed that PAX8 had strong staining in the urothelia of the renal papilla and the renal pelvis, but the positive cells were gradually decreased both in staining intensity and extent in the ureter. With regard to the expression of the PAX8 protein in bladder cancer, recent literature reports show that most cases are negative, with only a few found to be positive by immunohistochemical staining [5, 9, 10].

The human PAX8 gene generates at least five different alternatively spliced transcripts which encode different PAX8 mRNA isoforms, PAX8a to PAX8e, with different carboxy-terminal regions [15]. A large open reading frame of 450 amino acids, containing the 128-amino acids paired domain at its amino-terminal end, implicates in sequence-specific DNA binding [21]. The activating domain of Pax8a is encoded by exons 10, 11 and/or 12 and resides at the Carboxy-terminus of the protein. This activating domain is absent in human PAX8c, PAX8d and PAX8e isoforms due to a translational frame shifting that generates a novel proline- and arginine-rich domain [15]. Pellizzari *et al.* had reported that PAX8 mRNA expression was extremely heterogeneous, with the different isoforms differentially represented in the various noninvasive urothelial neoplasias of the bladder [8]. In this study, PAX8 expression was also highly heterogeneous in terms of the splicing mRNA isoforms in UUT-UCs. The PAX8e isoform was found in almost all UUT-UCs tumor tissues, but the PAX8d isoform was not detected in UUT-UCs that were different from the transcriptional splicing patterns of PAX8 in bladder cancer reported in the literature [8]. In addition, the above 4 types of splicing isoform could be simultaneously detected in almost all normal mucosal urothelia of the upper urinary tract, which was very different from that of bladder mucosa. To the best of our knowledge, this is the first report about the transcriptional pattern of PAX8 in papillary UCs and in the normal mucosal urothelia of the upper urinary tract.

Kozmik *et al.* have investigated the molecular natures of Pax-8 gene transcripts both in human kidney cell lines and during mouse ontogeny. The experiments revealed that alternative splicing generates Pax-8 isoforms with different transactivation properties. Moreover, alternative splicing of Pax-8 gene transcripts is temporally and spatially regulated during early mouse development [22]. Further studies are

suggested to reveal whether or not the differences in natural attributes between UCs of the upper and lower urinary tract are related to their PAX8 transcriptional splicing patterns.

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Disclosure of conflict of interest

None.

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