Significant Association of Cyclo-oxygenase 2 Genotypes with Upper Tract Urothelial Cancer

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Abstract. *Aim: Reliable biomarkers are in urgent need for diagnosis, outcome prediction and treatment-effect monitoring for upper tract urothelial carcinomas (UTUC). Although up-regulation of cyclo-oxygenase 2 (COX2) is found in stroma and tumor cells in more than half of the patients with UTUC investigated, the genomic contribution of COX2 to UTUC has not been studied. The study aimed to evaluate the association of six polymorphic genotypes of COX2 with UTUC within a Taiwanese population. Materials and Methods: A total of 218 patients with UTUC and 580 healthy controls were genotyped for six COX2 polymorphisms, namely A-1195G, G-765C, T+8473C, intron 1, intron 5 and intron 6, and examined for their association with UTUC risk. Results: The distribution of genotypes of COX2 G-765C and intron 5 were significantly different between patient and control groups (p=0.0001 and 0.0016, respectively), while others were not (p>0.05). The haplotype analysis showed that compared to the GG/TT haplotype of COX2 G-765C/intron 5, those carrying GG/AT variants have a significantly increased risk of UTUC (odds ratio=4.83, 95% confidence interval=1.79-13.06), while those carrying CG/TT variants have a decreased risk (odds ratio=0.26, 95% confidence interval=0.14-0.49). Conclusion: Our results suggest that individual and combined COX2 G-765C/intron 5 genotypes play a role in controlling COX2 expression and UTUC development.*

Upper tract urothelial cancer (UTUC) is a universal problem. However, the relative incidences of urothelial cancer of the renal pelvis, ureter and bladder for Western countries and Taiwan were 3:1:51 and 1:2.08:6.72, respectively (1). This higher incidence in Taiwan makes it worthwhile starting the genomic and proteomic studies for UTUC from Taiwan and then compare these with the counterpart findings in Western populations. Epidemiologically speaking, mounting evidence has shown that the elevated incidence of UTUC may be associated with arsenic exposure, smoking habit, analgesic abuse, occupational carcinogen exposure, hypertension, long-standing urinary obstruction, infection and Balkan nephropathy (2-6). Recently, mounting evidence has suggested that genetic polymorphic variations may also predispose to the development of UTUC (7, 8).

Cyclo-oxygenase-2 (COX2) is an inducible enzyme for the conversion of arachidonic acid to prostanoid, prostaglandin and thromboxane (9). Typically, COX2 is often undetectable in normal tissues. In 2012, it was found that COX2 was up-regulated in the stromal and tumoral sites from UTUC, which could serve as a prognostic marker for poor clinical outcome of UTUC (10). The samples included 128 paired tumoral and stromal specimens, and the up-regulation of COX2 was strongly associated with higher cancer-specific deaths and cancer recurrence rates (10). However, it is inconvenient to collect tissue from large numbers of healthy individuals for prediction and immuno-chemistry for protein expression is not as quick, convenient, and repeatable as DNA sequencing assays. At the DNA level, the contribution of *COX2* genotype to UTUC has never been

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Table I. *Primer sequences and restriction fragment length polymorphism conditions for cyclo-oxygenase 2 (COX2) genotyping analyses.*

Polymorphism (location)	Primer sequences (5' to 3')	Restriction enzyme	SNP sequence	DNA fragment size (bp)
A-1195G	F: CCCTGAGCACTACCCATGAT	Hha I	A	273
(rs689466)	R: GCCCTTCATAGGAGATACTGG		G	220+53
G-765C	F: TATTATGAGGAGAATTTACCTTTCGC	Pvu II	C	100
(rs20417)	R: GCTAAGTTGCTTTCAACAGAAGAAT		G	74+26
T+8473C	F: GTTTGAAATTTTAAAGTACTTTTGAT	Bcl I	T	147
(rs5275)	R: TTTCAAATTATTGTTTCATTGC		C	124+23
intron 1	F: GAGGTGAGAGTGTCTCAGAT	Taq I	G	439
(rs2745557)	R: CTCTCGGTTAGCGACCAATT		A	353+76
intron 5	F: GCGGCATAATCATGGTACAA	BsrG I	T	417
(rs16825748)	R: CAGCACTTCACGCATCAGTT		A	314+103
intron 6	F: ACTCTGGCTAGACAGCGTAA	Aci I	A	327
(rs2066826)	R: GCCAGATTGTGGCATACATC		G	233+94

F and R indicate forward and reverse primers, respectively.

studied as far as we are aware of. The overexpression of COX2 may contribute to carcinogenesis via increasing cell proliferation, suppressing apoptosis, enhancing invasiveness, and inducing chronic activation of immune responses and angiogenesis (11-13). Based on the evidence collected from animal and clinical models, COX2-specific inhibitors have both preventative and therapeutic effects as anticancer drugs for breast, bladder, lung and pancreatic cancer (14-18).

Following the rule of central dogma, subtle genetic variants of the *COX2* gene may affect the quantity of COX2 protein through altered self-regulated transcriptional activity or alternative splicing resulting from polymorphic variations at the promoter region or introns, respectively (19, 20). With the aim of clarifying the hypothesis that the polymorphic variants at promoter or intron regions of *COX2* may be associated with the risk of UTUC, the current study determined the genotypic frequency of six polymorphisms of *COX2* at A-1195G (rs689466), G-765C (rs20417), T+8473C (rs5275), intron 1 (rs2745557), intron 5 (rs16825748) and intron 6 (rs2066826), and their contribution to UTUC susceptibility in Taiwan.

Materials and Methods

Sample collection. A total of 218 patients with UTUC were recruited at the China Medical University and Kaohsiung Medical University medical Centers, all of whom were diagnosed by pathological examination of specimens obtained by biopsy or surgical resection. The clinical and histopathological information were collected from patient charts and pathological reports. The information was reviewed, and the data were entered into a database. The tumor stage was assigned according to the TNM staging system (21), and the pathological grade was determined according to the World Health Organization criteria (22). Five hundred and eighty healthy individuals, who had been matched with the patients by age, admitted to the same hospital for a health checkup and who had no previous diagnosis of neoplastic urological disease or other malignancy were enrolled as controls. All the participants enrolled provided their informed consent and Human Research Committees approved this study.

Genotyping conditions. The total genomic DNA for each participant was extracted from the leucocytes of peripheral blood and stored as previously described (23-25). The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s, and a final extension at 72˚C for 10 min. Pairs of PCR primer sequences and restriction enzyme for each DNA product of *COX2* genotyping work are all listed in Table I. The PCR products were cut by appropriate restriction enzymes and the reaction mixture was incubated for 2 h at 37˚C. Then 10 μl of each PCR product was loaded into a 3% agarose gel for electrophoresis.

Statistical analyses. Data for 218 UTUC cases and 580 controls were analyzed. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotypic frequencies of *COX2* single nucleotide polymorphism in the controls from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *COX2* genotypes between cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) and 95% confidence intervals (CIs) using unconditional logistic regression. All statistical tests were performed using SPSS for Windows (version 14.0; SPSS Inc., Chicago, IL, USA) on two-sided probabilities. The correlation between categorical variables was calculated for statistical significance using Pearson's chi-square test and the threshold for significance was $p<0.05$.

Results

The frequency distributions of clinical characteristics for the participants (218 UTUC patients and 580 healthy controls) are shown in Table II. Epidemiologically, there was no difference in the frequency distribution by the gender

Table II. *Frequency distributions of clinical characteristics among patients with upper tract urothelial cancer (UTUC) and healthy controls.*

Characteristics	UTUC (n=218), n (%)	Control (n=580), n (%)	*p*-Value
Gender			
Male	114 (52.3)	323 (55.7)	
Female	104 (47.7)	257 (44.3)	0.4256
Mean±SD age, years	65.4±4.7	62.9±3.9	0.8518
Location			
Renal pelvic tumor	84 (38.5)		
Ureter tumor	76 (34.9)		
Multiple tumors	58 (26.6)		
Grade			
Low	86 (39.4)		
High	132 (60.6)		
Stage			
I and II	168 (77.1)		
III and IV	50 (22.9)		

Table III. *Distributions of cyclo-oxygenase 2 (COX2) genotypic frequencies among the upper tract urothelial cancer (UTUC) cases and controls.*

Genotype	UTUC, n (%)	Controls, n (%)	Crude OR (95% CI)	*p*-Value
A-1195G (rs689466)				
AA	65 (29.8)	157 (27.1)	1.00 (reference)	
AG	102 (46.8)	279 (48.1)	0.88 (0.61-1.28)	0.5105
GG	51 (23.4)	144 (24.8)	0.86 (0.56-1.32)	0.5120
P_{trend}				0.7333
G-765C (rs20417)				
GG	202 (92.7)	465 (80.2)	1.00 (reference)	
CG	**16 (7.3)**	**115 (19.8)**	**0.32 (0.19-0.55)**	**0.0001**
T+8473C (rs5275)				
TT	153 (70.2)	383 (66.0)	1.00 (reference)	
CT	65 (29.8)	197 (34.0)	0.83 (0.59-1.16)	0.2730
intron 1 (rs2745557)				
CC	161 (73.9)	449 (77.4)	1.00 (reference)	
CT	50 (22.9)	117 (20.2)	1.19 (0.82-1.74)	0.3774
TT	7 (3.2)	14 (2.4)	1.39 (0.55-3.52)	0.4596
P_{trend}				0.5424
intron 5 (rs16825748)				
TT	204 (93.6)	570 (98.1)	1.00 (reference)	
AT	**14 (6.4)**	**10 (1.9)**	**3.91 (1.71-8.95)**	**0.0016**
intron 6 (rs2066826)				
GG	188 (86.2)	519 (89.5)	1.00 (reference)	
AG	25 (11.5)	51 (8.8)	1.35 (0.82-2.25)	0.2774
AA	5 (2.3)	10 (1.7)	1.38 (0.47-4.09)	0.5608
P_{trend}				0.4378

Significant data are shown in bold. OR: Odds ratio; CI: confidence interval.

(*p*=0.4256) or age (*p*=0.8518) since the populations were well-matched (Table II). From the clinical and pathological viewpoints, tumors were distributed in renal pelvic, ureter and multiple sites were 38.5%, 34.9% and 26.6%, respectively. Among the patients, 60.6% had high grade tumor, and 77.1% were of stages lower than pT3 (Table II).

The frequencies of the genotypes for *COX2* A-1195G, G-765C, T+8473C, intron 1, intron 5, and intron 6 among the patients with UTUC and healthy controls are summarized and analyzed in Table III. Among the six polymorphic genotypes investigated, two of them, G-765C (OR=0.32, 95% CI=0.19-0.55; *p*=0.0001) and intron 5 (OR=3.91, 95% CI=1.71-8.95; *p*=0.0016), were found to be differentially distributed between UTUC cases and control groups (Table III). The frequencies of GG and CG genotypes of *COX2* G-765C were 92.7% and 7.3% among UTUC cases, and 80.2% and 19.8% among healthy controls, respectively. The frequencies of TT and AT genotypes of *COX2* intron 5 were 93.6% and 6.4% among UTUC cases, and 98.1% and 1.9% among healthy controls, respectively (Table III). For other polymorphic sites of *COX2*, there was no difference in the distribution of genotypes among UTUC cases and controls (Table III).

In the next step, we performed the allelic frequency analysis, and the frequencies of the alleles for *COX2* A-1195G, G-765C, T+8473C, intron 1, intron 5, and intron 6 among the UTUC cases and healthy controls are summarized in Table IV. Consistent with the findings of Table III, G-765C and intron 5 of *COX2* were found to be associated with UTUC risk (Table IV). In detail, higher frequencies of G allele in G-765C and A allele in intron 5 in the UTUC case group than the control group were associated with higher risk of UTUC (Table IV; *p*=0.0001 and 0.0017, respectively). Regarding the other four *COX2* polymorphic sites, no distribution of their allelic frequencies was found to be significantly different between the control and UTUC case groups (Table IV).

Considering the possible interactions between the two determinant *COX2* genotypes for UTUC susceptibility, the haplotypic distributions of *COX2* G-765C and intron 5 were further analyzed (Table V). We set the most abundant genotypes for both G-765C and intron 5 genotypes as being wild-type for haplotypic combination. Under this criteria, the GG genotype for *COX2* G-765C and TT for *COX2* intron 5 were selected, resulting in the GG/TT combined genotype for G-765C/intron 5 as the reference haplotype. Compared to the reference haplotype of *COX2* G-765C/intron 5, the GG/AT group was found to be associated with a significantly higher risk of UTUC (OR=4.83, 95%CI=1.79-13.06; *p*=0.0014), while CG/TT carried a lower risk (OR=0.26, 95%CI=0.14-0.49; *p*=0.0001) (Table V). After adjusting for age and gender, the differences became more obvious for the GG/AT and CG/TT groups, with their individual ORs altered to 4.86 and 0.32, remaining highly statistically significant (Table V). The

Table IV. *Distributions of cyclo-oxygenase 2 (COX2) allelic frequencies among the upper tract urothelial cancer (UTUC) cases and controls.*

Allele	UTUC	%	Controls	%	Crude OR (95% CI)	*p*-Value
A-1195G (rs689466)						
Allele A	232	53.2%	593	51.1%	1.00 (reference)	
Allele G	204	46.8%	567	48.9%	0.92 (0.74-1.47)	0.4654
G-765C (rs20417)						
Allele G	420	96.3%	1045	90.1%	1.00 (reference)	
Allele C	**16**	**3.7%**	**115**	**9.9%**	**0.35 (0.20-0.59)**	**0.0001**
T+8473C (rs5275)						
Allele T	371	85.1%	963	83.0%	1.00 (reference)	
Allele C	65	14.9%	197	17.0%	0.86 (0.63-1.16)	0.3628
Intron 1 (rs2745557)						
Allele G	372	85.3%	1015	87.5%	1.00 (reference)	
Allele A	64	14.7%	145	12.5%	1.20 (0.88-1.65)	0.2789
Intron 5 (rs16825748)						
Allele T	422	96.8%	1150	99.1%	1.00 (reference)	
Allele A	**14**	**3.2%**	**10**	**0.9%**	**3.81 (1.68-8.66)**	**0.0017**
Intron 6 (rs2066826)						
Allele G	401	92.0%	1089	93.9%	1.00 (reference)	
Allele A	35	8.0%	71	6.1%	1.34 (0.88-2.04)	0.1768

Significant data are shown in bold. OR: Odds ratio; CI: confidence interval.

combination of CG/AT did not confer significantly altered cancer risk compared to the wild-type haplotype before or after adjusting for age and gender (Table V).

Discussion

Urothelial carcinoma is the second most common cancer which usually arises from the urothelium with transitional cell differentiation, including that of the renal pelvis, ureter and bladder. In literature, there are at least 600 articles investigating the contribution of individual genomic variations to bladder cancer (26-30), while few relate them to UTUC (7, 31, 32). Following the molecular central dogma, the single nucleotide variations of *COX2* may determine differential expression of COX2 and personal susceptibility to cancer. The supporting data for this come from the finding that COX2 is often undetectable in normal tissues, whereas overexpression of COX2 has been observed in neoplastic cells of canine (33) and human renal cell carcinoma (34-36). In a previous study, we found *COX2* to be up-regulated in both stromal and tumoral cells of more than half of the patients with UTUC and the positive expression of COX2 in stromal cells may be a biomarker for UTUC-specific death and recurrence (10). However, the contribution of *COX2* genotypes to UTUC has never been studied.

In this study, the *COX2* genotype of 218 patients with UTUC together with 580 controls (Table III) was examined. Statistically, the distributions of *COX2* genotypes for G-765C and intron 5 were differentially distributed among the UTUC and healthy control groups (Table III). In addition, the allelic frequencies of the two polymorphisms were also differentially distributed between the two groups (Table IV). The results showed that the G allele of G-765C and A allele of intron 5 were associated with higher risk for UTUC and the haplotype analysis suggested that individuals with GG/AT and CG/TT haplotypes at G-765C/intron 5 were at altered risk of UTUC before and after adjusting for gender and age (Table V). UTUC is not a common type of cancer in Taiwan, nor worldwide, which may be one of the limitation for genomic study of UTUC. Compared to previous findings, we enlarged the sample size from 56 cases and 436 controls in 2011 (37) to 218 cases and 580 controls (Table II). The strengthened sample size and the same trend for significant differences in genotypic distribution after age and gender adjustments highlight the value, accuracy and reliability of the overall findings (Table V).

This is the first genomic study of UTUC to show *COX2* G-765C and intron 5 genotypes being associated with UTUC risk. The G allele of *COX2* G-765C and A allele of *COX2* intron 5 were found to be genomic risk factors and may serve as early screening and predictive biomarkers for UTUC in Taiwan and elsewhere.

Table V. *Distribution of cyclo-oxygenase 2 (COX2) G-765C /intron 5 haplotypes among patients with upper tract urothelial cancer (UTUC) and controls.*

G-765C/intron 5	UTUC		Controls		OR (95% CI)	Adjusted OR (95% CI)[a]	*p*-Value[b]
haplotype	n	%	n	%			
GG/TT	190	87.2%	459	79.1%	1.00 (Reference)	1.00 (Reference)	
GG/AT	12	5.5%	6	1.1%	4.83 (1.79-13.06)	4.86 (1.69-11.80)	0.0014
CG/TT	12	5.5%	111	19.1%	0.26 (0.14-0.49)	0.32 (0.19-0.58)	0.0001
CG/AT	4	1.8%	4	0.7%	2.41 (0.60-9.76)	2.53 (0.67-7.81)	0.2441

Significant data are shown in bold. OR: Odds ratio; CI, confidence interval; [a]adjusted for age and gender; [b]based on Fisher's exact two-tailed test.

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