The Genetic Role of *Cyclooxygenase 2* in Ureter Cancer in Taiwan

Chao-Hsiang Chang[1,2*], Hsi-Chin Wu[1,2,*], Hung-Lung Ke[3,4,*], Hui-Hui Lin[4], Wu-Chung Shen[5], Shung-Shung Sun[1,5], Chia-Wen Tsai[1], Ru-Yin Tsai[1], and Da-Tian Bau[1,6]

[1]Terry Fox Cancer Research Laboratory and [2]Department of Urology, China Medical University Hospital, Taichung, Taiwan.

[3]Department of Urology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan.

[4]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

[5]Department of Biomedical Imaging and Radiological Science, and [6]Graduate Institutes of Clinical Medical Science, China Medical University, Taichung, Taiwan.

* These authors contributed equally to this work

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Correspondence to: Da-Tian Bau and Ru-Yin Tsai, Terry Fox Cancer Research Lab, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan.

Tel: +886 422053366 Ext 3312, Fax: +886 422053366,

e-mail: datian@mail.cmuh.org.tw/artbau1@yahoo.com.tw

Abstract

The association between cyclooxygenase 2 (Cox-2) and ureter cancer is never investigated, and in this study the association of *Cox-2* genotypic polymorphisms with ureter cancer is firstly examined. Fifty six ureter cancer patients and 436 non-cancer controls recruited from the China Medical Hospital in central Taiwan were genotyped and analyzed. We investigated up to six polymorphic variants of *Cox-2*, including G-1195A, G-765C, T+8473C, intron 1, 5, and 6, to analyze the association of the genotypes with susceptibility to ureter cancer. At the first step, no significant difference in the distribution between the ureter cancer and control groups was found in each of the polymorphism site investigated. In the second step, the analysis of joint effect for *Cox-2* G-765C and intron 6 showed that individuals with GC at G-765C and AG+AA at intron 6 present a higher potential for developing ureter cancer than other groups. There is no obvious association between *Cox-2* genotypes and ureter cancer stage or grade. Our findings suggest that the C allele of *Cox-2* G-765C together with A allele of intron 6 may be responsible for ureter carcinogenesis and may be useful in early detection and prediction of ureter cancer.

Key Words: Cox-2, polymorphism, ureter cancer, carcinogenesis.

Introduction

Urothelial carcinoma is the most common malignancy in the urinary tract, and the worldwide ratio of urothelial carcinoma in renal pelvis, ureter, and bladder was reported to be 3:1:51 (7). Although the renal pelvis and ureter cancers are relatively uncommon and accounting for about 7.2% of all renal malignanciescompared with bladder cancer, the incidence keeps increasing and becomes a threaten to human health. In Taiwan, the ratios of pelvis, ureter, and bladder have shifted from 3:1:51 in the world to 1.1:0.9:8.0, showing that the Taiwanese population may be of typical and specific genetic and environment factors for ureter cancer (11). Our previous immunochemistry reports have revealed that osteopotin (14) and hypoxia-induced factor-1α (15) may play a role in urothelial carcinoma of the upper urinary tract, and were potential as poor outcome predictors for the ureter cancer patients in Taiwan. However, there is no literature investigating the role of any gene in ureter cancer from the genomic angle. This may be due to the rareness of patients and difficult to collect the samples.

Accumulating evidence has shown that up-regulation of Cox-2 favors malignant progression (9, 19, 29, 32). However, mounting evidence from the investigations of the mRNA and protein levels of *Cox-2* showed that the levels may vary dramatically among the individuals, and the variation may be partially determined under different molecular mechanisms, which may depend on single nucleotide polymorphisms (SNPs) of *Cox-2*

itself (6, 22). In the literature, the associations of the SNPs of *Cox-2* with bladder (10, 13) and prostate cancer susceptibilities (5, 8, 21, 25) have been revealed. Therefore, it is very possible that *Cox-2* may also play an important role in ureter carcinogenesis.

The main purpose of this work is to investigate the role of *Cox-2* in ureter cancer from the genomic viewpoint. To do that, the genetic polymorphisms of six *Cox-2* SNPs, including G-1195A (rs689466), G-765C (rs20417), T+8473C (rs5275), intron 1 (rs2745557), intron 5 (rs16825748), and intron 6 (rs2066826), were analyzed in a Taiwanese population (control/case: 436/56).

Materials and Methods

Study Population and Sample Collection

Fifty six patients diagnosed with ureteral urothelial carcinoma by Dr. Chang and Dr. Wu, were recruited between 2005-2009 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. All the controls and patients are Taiwanese, and the genetic background of the population is very conservative. All the patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. The pathological grade was classified according to World health Organization (WHO) histologic criteria determined by the Bladder Consensus Conference Committee in 1998. Four hundred and thirty six of non-ureter cancer

healthy volunteers as controls were selected by matching for age, gender and smoking habits after initial random sampling from the Health Examination Cohort of the hospital. The exclusion criteria of the control group included previous malignancy, metastasized cancer from other or unknown origin, and any familial or genetic diseases. Both groups completed a short questionnaire which included habits and they were recorded. Subjects who had smoked more than one pack of cigarettes per day for at least 6 months were defined as smokers, no matter they are former users, recent quitters, or current users. Those who did fit the criteria above were defined as non-smokers. Our study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all participants.

Genotyping Assays

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed as previous genotyping studies (1-3, 18, 31). Briefly, all the DNA samples were packaged into aliquots immediately after being extracted from the blood, and storied in -70℃ refrigerator. The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 30 sec, and a final extension at 72^{o}C for 10 min. Then the PCR products were digested with the restriction enzymes for 4 h, separated with 3% agarose electrophoreses, stained with

ethidium bromide and observed under UV exposure, taken pictures and identified of the individual genotype. Genotypes were identified by non-digestible homozygous, half-digestible heterozygous and full-digestible homozygous. Each experiment was performed by at least two researchers double-blindly, and the concordance rate was about 99.7%. Pairs of PCR primer sequences, restriction enzymes, and the enzyme digestion codes for the relative genotypes for each DNA product are all listed in Table I.

Statistical Analyses

Only those individuals with both genotypic and clinical data (control/case=436/56) were selected for final analysis. To ensure that the controls used were representative of the general population, and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *Cox-2* SNPs 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 number in any cell was less than five) was used to compare the distribution of the genotypes between cases and controls. Data were deemed to be significant when the *P-value* was less than 0.05. Cancer risk associated with the genotypes was estimated as odds ratios (ORs) and 95% confidence intervals (95%CIs) using unconditional logistic regression.

Results

The frequency distributions of the age, gender and smoking habits of the 56 ureter cancer patients and 436 controls are shown in Table II. The characteristics of the patients and controls were all well matched. None of the differences in these characteristics between both groups were statistically significant (P>0.05) (Table II). Since age, gender and smoking status are reported to be environmental factors for ureter cancer, the well matched population may exclude the possible confounding effects of this investigation.

The frequencies of the genotypes for the *Cox-2* SNPs in controls and ureter cancer patients are shown in Table III. The genotype distributions of the genetic polymorphisms of *Cox-2* of the six polymorphisms investigated were not significant between the two groups (P>0.05) (Table III). The frequencies of the alleles for *Cox-2* SNPs in controls and ureter cancer patients are shown in Table IV. None of the allele of the *Cox-2* of the SNPs was found to be associated with ureter cancer (P>0.05). We have also analyzed the correlation between the *Cox-2* genotyping and the stages and grades among the ureter cancer patients, however, no significant correlation was found (data not shown).

To further investigate the association of *Cox-2* genotype and ureter cancer, the interactions among the SNPs were investigated by genotype analysis. Each of the frequencies of combined genotypic polymorphisms was analyzed, and here only the

results of G-765C and intron 6 genotypes (with least *P*-values) were shown in Table V, while other combinations were not significant (data not shown). There was no significant difference in the genetic frequency of any combined genotypes between the two groups for each combined genotype. The odds ratios (ORs) of the GG/AG+AA, GC/GG, GC/AG+AA combined genotypes compared with common GG/GG reference genotype were 1.63 (95%CI=0.64-4.15; *P*=0.279), 1.45 (95%CI=0.68-3.06; *P*=0.305), and 2.99 (95% CI=0.78-11.54; *P*=0.121), respectively.

Last, we have performed the haplotype analysis of the six SNPs to investigate the haplotypic effects of Cox-2 on ureter cancer risk. Compared with the haplotype of all wild-type major alleles of "WWWWWW", all the odds ratios were statistically significant for the variant haplotypes listed in Table VI, which carried the minor alleles of "V".

Discussion

There is still no reliable genetic biomarker for upper urinary tract carcinoma, and clinical stage and pathological grade are the only indexes for the prediction of ureter cancer prognosis. However, various cancer behaviors are frequently observed among patients of the same stage and with the same grade of ureter cancer (23). In order to find a potential and convenient biomarkers for ureter cancer early detection, six SNPs of the *Cox-2* gene were selected from the National Center for Biotechnology Information website and their associations with the susceptibility for ureter cancer in a

population of Taiwan was firstly examined.

In the situation that the cases are rare and not easy to collect within limited time period, we have enrolled more than 7.7-fold of controls to strengthen the analyzing power of the case-control study. In our results, it is found that neither of the individual genotype of *Cox-2* in the six SNPs investigated was significantly associated with the susceptibility for ureter cancer (Tables III and IV). In further combinative analysis of combined G-765C and intron 6 genotypes, the people with GC/AG+AA were of 2.99-fold odds ratio compared with common GG/GG, much larger than those of GG/AG+AA (1.63) and GC/GG (1.45) (Table V). The lack of significance at borderlines from the analysis of both odds ratios and *P*-value here encourage us to confirm this preliminary finding in a larger case samples in the future, and also the studies in other countries are warranted. We have also analyzed the gene-environment interactions, such as smoking status which is reported to be associated with ureter cancer (12, 16, 17), and the association of *Cox-2* genotypes with important clinical indexes, such as cancer stages and grades, but no statistical significant was found. This may be caused by the small sample of the cases.

In the literature, COX-2 was reported to play a key role in the pathogenesis of several human cancers, including breast (26), gastric (27), prostate (21), colorectal (28), lung (18, 30), esophageal (33), and bladder cancers [doi:10.1016/j.surg.2010.04.004]. In this study, the influence of the *COX-2* polymorphisms on ureter cancer was investigated. Our study revealed that carriers

of –765 C allele combined with intron 6 A allele, were potential to have higher risks of ureter cancer. This is similar to a finding that in bladder cancer reporting that –765 C allele of *Cox-2* is a bladder cancer risky genetic factor (10). There is a previous in vitro study showed that the production of prostaglandins is much higher (>10-fold) in –765CC homozygotes than in –765GG homozygotes, and the –765GC genotype was associated with an intermediate level of prostaglandin production (24). COX-2 has been shown to play a role in the proliferation of in vitro studies and the G-765C change creates a binding element for a cyclin-dependent E2F transcription factor, which regulates the expression of several genes in breast (26) and prostate (21) cancer studies. These results suggested that the presence of the *COX-2* –765C carrier genotype may lead to overexpression of COX-2 and enhance the production of prostaglandin, as stated previously, and may subsequently facilitate tumor progression by acting on differentiation and growth factors or by acting as immune suppressors (4). The roles of all the other five *COX-2* polymorphisms need further studies to evaluate their role in cancers.

Sanak and his colleagues found that instead of genotypes, haplotypes of *COX-2* could correlate better with prostaglandins biosynthetic capacity (24). Previous studies have reported an association of the A-1290/A-1195/C-765 haplotype with higher risk of esophageal cancer (20). Therefore, we have also performed the haplotype analysis. However, as shown in Table IV, all the other haplotypes seems to contribute to the risk of ureter cancer. This may be explained by the bias caused by small sample size, and the *COX-2* polymorphisms undertaken in the present study indeed were functionally related to each other. The variant combinative alleles among some of these six SNPs may result in a higher expression of COX-2, which enhances the synthesis of prostaglandin and, creates a higher risk of ureter cancer because of the joint effect of alleles. Therefore, it may affect the process of ureter carcinogenesis

through escape from immune surveillance mechanisms via a modulating expression of specific cytokines.

One limitation of the study is its sample size, for in the cases the sample size is quite less bases on the low incidence of disease. To enhance the analyzing power of this study, we have recruited more than 7.7-fold of non-cancer controls of the 56 ureter cancer cases to 436 cases. Although the analyzing power of this study is still not reaching the acceptable level 80%, which is commonly required, we have done already our best. In the future, a continuous enlargement of the investigation population is the fundamental work for our cancer research lab. Another limitation of this hospital-based case-control study is that the limited sample size might lead to the results which could not be generalized to populations in overall Taiwan. So we are going to collect samples from Southern Taiwan, where the incidence of ureter cancer is as high as central Taiwan to enlarge the sample size and to perform a comparison between different areas.

To sum up, this is the first study which investigated that genetic variation in *Cox-2* influences the risk of ureter cancer in Taiwan. The presence of the C allele at the promoter G-765C together with A allele at intron 6 was found to be associated with a higher risk of ureter cancer. We have provided preliminary data not only for potential biomarkers for Taiwan ureter cancer early detection, but also for the genetic basic background for further gene-gene and gene-environment interactions, or

genotype-phenotype correlation studies of ureter cancer in Taiwan.

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Table I. The primer sequences, PCR and restriction fragment length polymorphism (RFLP) conditions for *Cox-2* gene polymorphisms.

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

*F and R indicate forward and reverse primers, respectively.

Table II. Demographic characteristics of the ureter cancer patients and controls.

Characteristic	Controls (n = 436)			Patients (n = 56)			*P*-value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			63.9 (6.6)			61.4 (5.7)	
<60	223	51.1%		30	53.6%		0.778
≥60	213	48.9%		26	46.4%		
Gender							
Male	249	57.1%		33	58.9%		0.886
female	187	42.9%		23	41.1%		
Stage							
I and II				37	66.1%		
III and IV				19	33.9%		
Grade							
Low				32	57.1%		
High				24	42.9%		
Habit							
Cigarette smokers	336	77.1%		39	69.6%		0.2432
Non-smokers	100	22.9%		17	30.4%		

[a] Based on 2 X 2 Chi-square test.

Table III. Distribution of *Cox-2* genotypes among ureter cancer patient and control groups.

Genotype	Controls	%	Patients	%	*P*-value[a]	OR (95% CI)[b]
A-1195G (rs689466)						
AA	122	28.0%	15	26.8%	0.890	1.00 (reference)
AG	210	48.2%	26	46.4%		1.01 (0.51-1.97)
GG	104	23.8%	15	26.8%		1.17 (0.55-2.51)
G-765C (rs20417)						
GG	365	83.7%	43	76.8%	0.191	1.00 (reference)
GC	71	16.3%	13	23.2%		1.55 (0.80-3.04)
CC	0	0%	0	0%		ND
T+8473C (rs5275)						
TT	298	68.3%	36	64.3%	0.546	1.00 (reference)
TC	138	31.7%	20	35.7%		1.20 (0.67-2.15)
CC	0	0%	0	0%		ND
intron 1 (rs2745557)						
GG	320	73.4%	38	67.9%	0.596	1.00 (reference)
AG	107	24.5%	16	28.6%		1.26 (0.67-2.35)
AA	9	2.1%	2	3.5%		1.87 (0.39-8.98)
intron 5 (rs16825748)						
TT	433	99.3%	55	98.2%	0.384	1.00 (reference)
AT	3	0.7%	1	1.8%		2.62 (0.27-25.67)
AA	0	0%	0	0%		ND
intron 6 (rs2066826)						
GG	394	90.4%	47	83.9%	0.141	1.00 (reference)
AG	37	8.5%	9	16.1%		2.03 (0.93-4.49)
AA	5	1.1%	0	0%		ND

[a] Based on 2 X 3 Chi-square test.

[b] ND, not determined for the observed count(s) in case or control is zero; OR, odds ratio; 95% CIs, 95% confidence interval.

Table IV. *Cox-2* allelic frequencies among the ureter cancer patient and control groups.

Allele	Controls	%	Patients	%	*P*-value[a]	OR (95% CI)
A-1195G (rs689466)						
Allele A	454	52.1%	56	50.0%	0.681	1.00 (reference)
Allele G	418	47.9%	56	50.0%		1.09 (0.73-1.61)
G-765C (rs20417)						
Allele G	801	91.9%	99	88.4%	0.247	1.00 (reference)
Allele C	71	8.1%	13	11.6%		1.48 (0.79-2.77)
T+8473C (rs5275)						
Allele T	734	84.2%	92	82.1%	0.582	1.00 (reference)
Allele C	138	15.8%	20	17.9%		1.16 (0.69-1.94)
intron 1 (rs2745557)						
Allele G	747	85.7%	92	82.1%	0.322	1.00 (reference)
Allele A	125	14.3%	20	17.9%		1.30 (0.77-2.18)
intron 5 (rs16825748)						
Allele T	869	99.7%	111	99.1%	0.390	1.00 (reference)
Allele A	3	0.3%	1	0.9%		2.61 (0.27-25.31)
intron 6 (rs2066826)						
Allele G	825	94.6%	103	92%	0.255	1.00 (reference)
Allele A	47	5.4%	9	8%		1.53 (0.73-3.22)

[a] Based on 2 X 2 Chi-square test.

Table V. Frequencies of combined *Cox-2* -765 and intron 6 genotypes among the ureter cancer and control groups.

Cox-2 -765/intron 6 genotype	Control		Patients		OR (95% CI)	*P*-value[a]
	n	%	n	%		
All	436	100.0	56	100.0		
GG/GG	332	76.1	37	66.1	1.00	
GG/AG+AA	33	7.6	6	10.7	1.63 (0.64-4.15)	0.2787
GC/GG	62	14.2	10	17.9	1.45 (0.68-3.06)	0.3047
GC/AG+AA	9	2.1	3	5.3	2.99 (0.78-11.54)	0.1209

[a] Based on Fisher's exact test. OR, Odds ratio; CI, Confidence interval.

Table VI. Estimated haplotype frequencies of Cox-2 in ureter cancer patients and controls and haplotypic specific risks.

Haplotype[a]	Case (%)	Control (%)	OR (95% CI)[c]
WWWWWW	32.59	27.16	1.00 (Ref.[c])
WMWWWW	2.87	3.56	**1.49 (1.26–1.75)***
WWMWWW	6.11	5.92	**1.16 (1.03–1.32)***
WWWMWW	5.44	5.92	**1.31 (1.15–1.48)***
MWWWWW	29.96	27.16	**1.08 (1.01–1.17)***
MMWWWW	2.64	3.56	**1.62 (1.37–1.91)***
MWMWWW	5.62	5.92	**1.26 (1.11–1.43)***
MWWMWW	5.00	5.92	**1.42 (1.24–1.62)***
Others	9.77	14.86	**1.83 (1.66–2.01)***

[a] The SNPs are shown in order of their 5’ to 3’ location, as listed in Table I; W, wildtype major genotype, M, mutant minor genotype.

[b] OR, odds ratio; CI, confidence interval.

[c] Ref., reference.

* Statistical significant