The Role of *XRCC6* T-991C Functional Polymorphism in Renal Cell Carcinoma

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Abstract. *Background: The DNA non-homologous end-joining repair gene XRCC6 (Ku70) plays a key role in both the DNA double-strand break (DSB) repair and cell cycle arrest. Defects in DSB repair capacity can lead to genomic instability. We hypothesized that a variant in the XRCC6 gene was associated with susceptibility to renal cell carcinoma (RCC). Materials and Methods: In a hospital-based case–control study of 92 patients with RCC and 580 cancer-free controls, the frequency matched by age and sex, the associations of XRCC6 promoter T-991C (rs5751129), promoter G-57C (rs2267437), promoter A-31G (rs132770), and intron 3 (rs132774) polymorphisms with RCC risk were investigated in a Taiwanese population. At the same time, 30 adjacent renal tissue samples were tested to estimate the XRCC6 mRNA expression by real-time quantitative reverse transcription. Results: Compared with the TT genotype, the TC genotype had a significantly increased risk of RCC [adjusted odds ratio=2.24, 95% confidence interval=1.25-4.08, p=0.0175]. The in vivo mRNA expression in renal tissues revealed a statistically significant lower XRCC6 mRNA expression in samples with TC/CC genotypes compared to those with the TT genotype (p=0.0039). Conclusion: These evidence suggests that the XRCC6 T-991C genotype together with its mRNA expression are involved in the etiology of RCC and may be a marker for susceptibility to RCC in the population of Taiwan.*

Renal cell carcinoma (RCC) is the predominant form of malignancy of the kidney (>80%), and its frequency is increasing in both men and women. RCC occurs worldwide, with the highest incidence observed in developed countries (1, 2). After Japan, Taiwan has the second-highest prevalence rate of end-stage renal disease in the world. Although the exact causes of RCC have not been yet identified, recent epidemiological investigations have shown that cigarette smoking, hypertension, obesity, occupational exposure, diet, and family history of cancer are associated with RCC (1, 3, 4). However, only few exposed individuals develop RCC in their lifetime, suggesting that genetic susceptibility may be involved in the etiology of RCC.

The human DNA repair system protects the genome from various insults caused by endogenous and exogenous agents (5), and mutations or defects in the DNA repair system are thought to be essential for tumorigenesis (6, 7). Therefore, mutations of DNA repair genes might have an important role in the initiation of RCC. DNA double-strand breaks (DSBs) are repaired by the DNA DSB repair system (8), which consists of two subpathways, homologous recombination (HR) and non-homologous end-joining (NHEJ) (9). In humans, NHEJ is the predominant repair system. To date, several proteins involved in the NHEJ pathway have been identified, namely, ligase IV and its associated protein the X-ray cross complementing group 4 (XRCC4), three components of the DNA-dependent protein kinase complex, XRCC5, XRCC6, and the DNA-dependent protein kinase,

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catalytic subunit (DNA-PKcs) (10). Genetic variations in NHEJ genes influence DNA repair capacity and confer predisposition to several types of cancer, including those of the skin (11), breast (12-14), stomach (15), bladder (16), oral cancer (17) and RCC (18).

Because genetic polymorphism in DNA DSB repair genes have been shown to confer predisposition to many types of cancer, Hirata *et al*. investigated the association between some polymorphisms of DNA repair genes, such as *XRCC1*, xeroderma pigmentosum group C (*XPC*), excision repair cross complementation group 1 (*ERCC1*), *XRCC3*, and *XRCC7* and the risk for RCC (18). However, no study has yet confirmed the association between the polymorphisms of *XRCC6*, which is the most important gene in the human DNA repair system, and the risk of RCC. Some epidemiological studies have investigated the association between the *XRCC6* polymorphism and the risk for other types of cancer, including gastric (19), oral (17) and breast cancer (20). We hypothesized that the *XRCC6* T-991C polymorphism may also contribute to RCC risk. To test this hypothesis, the present study was designed to investigate the association of *XRCC6* T-991C polymorphism with risk for RCC in a hospital-based case–control study, in a Taiwanese population. In addition, we investigated the association of the *XRCC6* mRNA expression with RCC risk by reversed transcript PCR, to assess the potential functional effect of *XRCC6* T-991C polymorphism in RCC risk. To the best of our knowledge, this is the first study to evaluate the association between the *XRCC6* T-991C polymorphism and RCC susceptibility and to explore the potential function of this single-nucleotide polymorphism (SNP) in RCC at the same time.

Materials and Methods

Study population. The hospital-based case–control study recruited 92 patients with RCC and 580 cancer-free controls frequency matched by age and sex. RCC in all the patients was diagnosed and histopathologically confirmed as RCC by Drs. Wu, Chen, Lien and Chang, and patients were without any prior history of other cancer types. All the age- and gender-matched cancer-free controls were genetically unrelated to the patients with RCC and had no individual history of cancer. Another exclusion criterion for the controls was symptoms suggestive of RCC, such as hematuria. Each patient donated 3-5 ml venous blood after providing a written informed consent. The study was approved by the Institutional Review Board of China Medical University.

Genotyping protocol. Total genomic DNA of each patient was extracted from the leucocytes of peripheral blood using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and stored as previously published (15, 16, 21-24). The primers used for *XRCC6* promoter T-991C were: forward 5'-AACTCATGGACCCACGGTTGTGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-3'; for promoter G-57C were: forward 5'-AAACTTCAGACCACTCTCTTCT-3', and reverse 5'-AAGCCGCTGCCGGGTGCCCGA-3'; for promoter G-31A were: forward 5'-TACAGTCCTGACGTAGAAG-3', and reverse 5'-AAGCGACCAACTTGGACAGA-3'; for intron 3 were forward 5'-GTATACTTACTGCATTCTGG-3', and reverse 5'-CATAAGTGC TCAGTACCTAT-3'. The following cycling conditions were performed as previously described (25-31): 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.

mRNA XRCC6 expression pattern. To evaluate the correlation between the *XRCC6* mRNA expression and the *XRCC6* polymorphism, 30 surgically-removed renal tissue samples adjacent to tumors with different genotypes were subjected to extraction of total RNA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The total RNA was measured by real-time quantitative RT-PCR using the FTC-3000 real-time quantitative PCR instrument series (Funglyn Biotech Inc., Canada). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal quantitative control. The primers used for amplification of *XRCC6* mRNA were 5'-CGA TAA TGA AGG TTC TGG AAG-3' (forward) and 5'-CTG GAA GTG CTT GGT GAG-3' (reverse), and the primers for *GAPDH* were 5'-GAA ATC CCA TCA CCA TCT TCC AGG-3' (forward) and 5'-GAG CCC CAG CCT TCT CCA TG-3' (reverse). Fold changes were normalized by the level of *GAPDH* expression, and each assay was carried out in at least triplicate.

Restriction fragment length polymorphism (RFLP) conditions. For the *XRCC6* promoter T-991C, the resultant 301 bp PCR product was mixed with 2 U *Dpn*II. The restriction site was located at -991 with a C/T polymorphism, and the C-form PCR products could be further digested, while the T-form could not. Two fragments of 101 bp and 200 bp were present if the product was digestible C-form. The reaction mixture was incubated for 2 h at 37˚C. Then, 10 μl of product were loaded into a 3% agarose gel, containing ethidium bromide for electrophoresis. The polymorphism was categorized as either C/C homozygote (digested), T/T homozygote (undigested), or C/T heterozygote. For the *XRCC6* promoter G-57C, the resultant 298 bp PCR products were mixed with 2 U *Hae*II. The restriction site was located at -57 with a C/G polymorphism, and the G-form PCR products could be further digested, while the C-form could not. Two fractions 103 and 195 bp were present if the product was the digestible G-form. The reaction mixture was incubated for 2 h at 37˚C. Then, 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either G/G homozygote (digested), C/C homozygote (undigested), or C/G heterozygote. For the *XRCC6* promoter A-31G, the resultant 226 bp PCR products were mixed with 2 U *Mnl*I. The restriction site was located at -31 with an A/G polymorphism, and the A-form PCR products could be further digested, while the G-form could not. Two fractions of 80 and 146 bp were present if the product was the digestible A-form. The reaction was incubated for 2 h at 37˚C. Then, 10 μl of product were loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either A/A homozygote (digested), G/G homozygote (undigested), or A/G heterozygote. For the *XRCC6* promoter intron 3, the resultant 160 bp PCR products were mixed with 2 U *Msc*I. The restriction site was located at intron 3 with a TGG/CCA polymorphism, and the CCA form PCR products could be further digested, while the TGG form could not. Two fractions of 46 and 114 bp were present if the product was the digestible CCA-form. The reaction was incubated for 2 h at 37˚C. Then, 10 μl of product were loaded into a 3% agarose gel

Table I. *Distributions of selected characteristics between renal cell carcinoma cases and controls.*

Characteristic	Cases (n=92)		Controls (n=580)		*p*-Value
	N	%	N	%	
Age (years) (mean±SD)	58.8±11.7		58.3±11.5		0.8971
≤60	47	51.1%	307	52.9%	0.8223
>60	45	48.9%	273	47.1%	
Gender					
Male	59	64.1%	371	64.0%	1.0000
Female	33	35.9%	209	36.0%	
Smoking status					
Smokers	41	44.6%	220	37.9%	0.2499
Non-smokers	51	55.4%	360	62.1%	
Alcohol drinking status					
Drinkers	37	40.2%	209	36.0%	0.4848
Non-drinkers	55	59.8%	371	64.0%	
Hypertension					
Yes	61	66.3%	302	52.1%	0.0130
No	31	33.7%	278	47.9%	
Diabetes					
Yes	21		104		0.2523
No	71		476		
Family cancer history					
Yes	6	6.5%	17	2.9%	0.1125
No	86	93.5%	563	97.1%	

containing ethidium bromide for electrophoresis. The polymorphism was categorized as either CCA/CCA homozygote (digested), TGG/TGG homozygote (undigested), or CCA/TGG heterozygote.

Statistical analyses. To ensure that the used controls were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *XRCC6* single-nucleotide polymorphisms 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) were used to compare the distribution of the *XRCC6* genotypes between cases and controls. The associations between the *XRCC6* polymorphisms and RCC risk were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for possible confounders. $p<0.05$ was considered statistically significant, and all statistical tests were two-sided.

Results

Basic comparisons between the case and control groups. The characteristics of the controls and cases are summarized in Table I. There were no differences between the cases and controls in age, sex, smoking alcohol or drinking status, diabetes or family history of cancer ($p>0.05$). However, there were more individuals with hypertension among the RCC cases than among the controls (66.3% *versus* 52.1%), and these differences were found to be statistically significant ($p=0.0130$).

Association of XRCC6 genotypes and RCC risk. The genotypic distributions of the *XRCC6* polymorphisms in the cases and controls are shown in Table II. The ORs after adjusting those confounding factors (age, gender, smoking and alcohol drinking status) for those carrying TC and CC genotypes were 2.24 (95% CI=1.25-4.08) and 3.61 (95% CI=0.88-15.24) respectively, compared to those carrying TT wild-type genotype. The *p*-value for trend was significant ($p=0.0065$). In the dominant model (TC plus CC *versus* TT), the association between *XRCC6* promoter T-991C polymorphism and the risk of RCC was also statistically significant (adjusted OR=2.38, 95% CI=1.34-4.22). As for the *XRCC6* promoter C-57G, promoter A-31G, and intron 3 polymorphisms, their distributions were in Hardy-Weinberg equilibrium, but there was no difference between RCC and control groups in the distribution in the genotype frequency of these SNPs (Table II). To sum up, these data indicated that individuals carrying a variant C allele at the promoter T-991C may have a higher risk of RCC.

Association of the XRCC6 T-991C polymorphism with expression levels of XRCC6 mRNA. We collected 30 surgically-removed normal renal tissue samples adjacent to tumors. These were obtained from the patients with RCC before any therapy; the frequencies of the TT, TC, and CC genotypes of the *XRCC6* T-991C were 23, 5, and 2, respectively. The effects of these three genotypes regarding

Table II. *Distributions of genotypic and allelic frequencies among renal cell carcinoma cases and controls.*

	Cases (%)	Controls (%)	Adjusted OR[a] (95% CI)	*p*-Value
Promoter T-991C (rs5751129)				
TT	72 (78.2)	519 (89.5)	1.00 (ref)	
TC	**17 (18.5)**	**55 (9.5)**	**2.24 (1.25-4.08)**	**0.0157**
CC	3 (3.3)	6 (1.0)	3.61 (0.88-15.24)	0.0904
p-Value for trend			**2.91 (1.21-4.65)**	**0.0065**
(TC+CC) *vs.* TT			**2.38 (1.34-4.22)**	**0.0050**
CC *vs.* (TT+TC)			3.27 (0.76-13.28)	0.1127
Promoter G-57C (rs2267437)				
CC	61 (66.3)	394 (67.9)	1.00 (ref)	
CG	27 (29.3)	174 (30.0)	1.02 (0.60-1.68)	1.0000
GG	4 (4.4)	12 (2.1)	2.16 (0.65-6.91)	0.2556
p-Value for trend			1.43 (0.62-4.49)	0.4118
(CG+GG) *vs.* CC			1.06 (0.65-1.72)	0.8104
GG *vs.* (CC+CG)			2.02 (0.68-6.97)	0.2561
Promoter A-31G (rs132770)				
GG	74 (80.4)	475 (81.9)	1.00 (ref)	
GA	14 (15.2)	75 (12.9)	1.16 (0.64-2.17)	0.6186
AA	4 (4.4)	30 (5.2)	0.85 (0.31-2.54)	0.7952
p-Value for trend			1.07 (0.58-2.26)	0.8022
(GA+AA) *vs.* GG			1.10 (0.61-1.89)	0.7717
AA *vs.* (GG+GA)			0.84 (0.30-2.42)	1.0000
Intron 3 (rs132774)				
GG	72 (78.3)	466 (80.3)	1.00 (ref)	
GC	20 (21.7)	114 (19.7)	1.11 (0.63-1.92)	0.6736
CC	0 (0.0)	0 (0.0)		

[a]Adjusted for age, gender, smoking and alcohol drinking status; the line with ORs that significantly differ from 1.00 are in bold.

the mRNA level of *XRCC6* were measured and evaluated by real-time quantitative RT-PCR (Figure 1). The two samples with CC genotype were added to the samples of TC genotype for effective statistical analysis, and a statistically significantly lower level of *XRCC6* mRNA expression was identified in samples from patients with TC/CC genotypes than from those with the TT genotype (p=0.0039).

Discussion

In this study, the association of the *XRCC6* polymorphism and RCC risk was investigated in Taiwan, where the prevalence of end-stage renal disease is the second-highest, worldwide after Japan. From the genotyping analyses, we found that individuals carrying the TC genotype were at higher risk of RCC compared with those carrying the TT genotype of *XRCC6* T-991C. We have also investigated the effects of the *XRCC6* T-991C genotype on its mRNA expression level, finding that renal tissues from individuals with TC or CC genotypes had lower mRNA expression of *XRCC6* than those with the TT genotype. To the best of our knowledge, this is the first study on the role of *XRCC6* in RCC with conclusive findings.

XRCC6 may work together with XRCC5 as a heterodimer, or independently of it (32). *XRCC6*-knockout mice have less mature T-lymphocytes, higher incidence of thymic lymphomas, and a higher rate of fibroblast transformation, but *XRCC5*-knockout mice do not. The mechanisms causing the differences remain unclear (33). Proteomic defects in XRCC6 may cause not only lower DSB repair capacity, but also growth retardation, ionizing radiation hypersensitivity, and severe combination immune deficiency due to severely impaired variable division joining recombination capacity (9). From the genomic viewpoint, small genomic variations in *XRCC6*, such as polymorphisms, might escape the cell cycle checking point, and also lead to suboptimal DNA repair capacity, which would allow DNA damage to accumulate step by step triggering tumorigenesis (13, 14, 34).

In different types of cancer, there are some epidemiological studies investigating the association between *XRCC6* T-991C polymorphism and its risk for gastric (19), oral (16) and breast cancer (20), and cancer-like pterygium (35). The above evidence could be interpreted as suggesting that DNA repair genes may play a common role in the initiation of carcinogenesis. Interestingly, Wang *et al.* reported that the *XRCC6* A-31G and C-1310G polymorphisms were both associated with RCC risk in a Chinese population (36, 37). The genetic backgrounds of

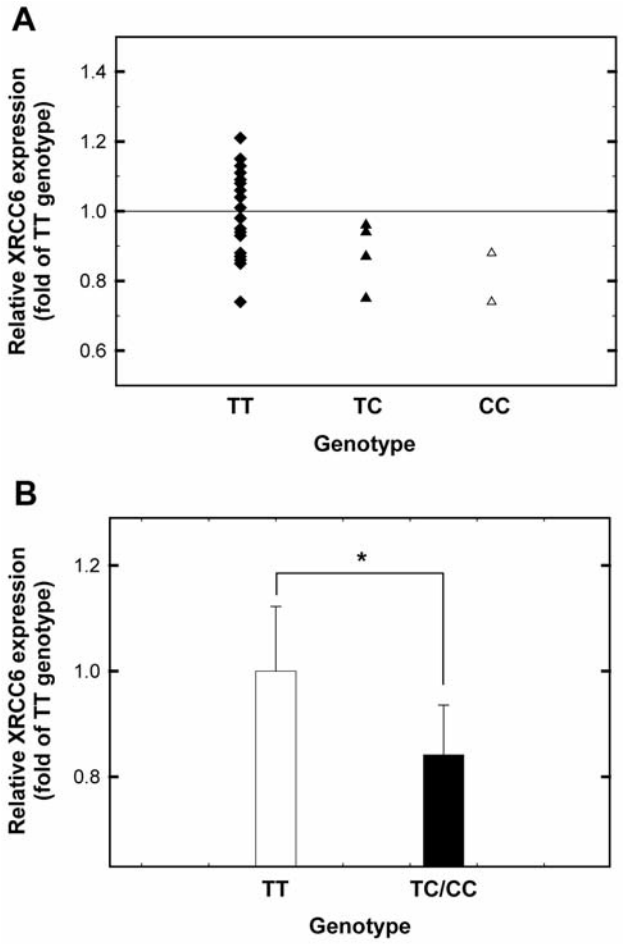

Figure 1. *Analysis of XRCC6 mRNA expression levels. A: Quantitative real-time quantitative PCR (RT-PCR) of three genotypes for XRCC6 from renal tissue samples was performed and GAPDH was used as an internal quantitative control. Fold changes were normalized by the levels of GAPDH expression, and each assay was carried out in, at least, triplicate. B: The groups of TC and CC in (A) were pooled and compared with the TT group. *p<0.05 compared with the TT genotype by the unpaired Student's t-test.*

the Taiwanese and Chinese populations are very similar, and T-991C is located between A-31G and C-1310G.

The *XRCC6* T-991C variation mapped in the promoter region of *XRCC6* does not directly result in amino acid coding alteration; it is possible to suspect that alternative spicing, intervention, modification, determination or involvement of this SNP influences the expression level or stability of the XRCC6 protein (16, 38). Therefore, we designed a functional experiment to investigate whether the T-991C SNP influences the expression levels of *XRCC6* mRNA *in vivo*. We found that normal renal tissues with the C allele had a lower expression level of *XRCC6* mRNA by real-time quantitative RT-PCR. This finding fully supports the hypothesis described above. The T allele might increase the expression level of *XRCC6* mRNA, which may lead to increased expression of the XRCC6 protein and elevated DSB repair capacity.

The present study has some limitations to be improved in future investigations. Firstly, our sample size is moderate, which may restrict the reliability and feasibility of stratification and interaction analyses. Secondly, the insufficient clinical and behavioral information, such as occupational exposure, daily diet and physical exercise habits, limited our capacity for performing risk factor analysis. Finally, the small sample size of the mRNA association study, especially tissues from individuals with the CC genotype of *XRCC6* T-991C, suggests that our findings should be further validated in both tumor tissues and normal adjacent tissues in future studies.

In conclusion, our present study indicates that the functional *XRCC6* T-991C polymorphism is associated with RCC susceptibility in Taiwanese patients, and this novel functional *XRCC6* polymorphism may lead to different expression levels of *XRCC6* mRNA. Further functional studies are required to reveal the role of *XRCC6* in RCC carcinogenesis.

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