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,
catalytic subunit (DNA-PKcs) (10). Genetic variations in NHEJ genes influence DNA repair capacity and confer predisposition to many 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 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 carried out in at least triplicate.

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'-AAGGACCACTTGGACAGA-3', and reverse 5'-GAG CCC CAG CCT TCT CCA TG-3'; for promoter G-57C were: forward 5'-AACTCATGGACCCAGTGGCTGGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-3'; for promoter G-991C were: forward 5'-AACTCATGGACCCAGTGGCTGGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-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'-GTA GTG ATG GCT TGC GAT GAG-3' (reverse), and the primers for GAPDH were 5'-GAA ATC CCA TCA CCA CCT TGC AG-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 DpnII. 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 HaeII. The restriction site was located at -57 with a G/C polymorphism, and the G-form PCR products could be further digested, while the C-form could not. Two fragments of 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 MspI. 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 MscI. 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...
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 \( (66.3\% \text{ versus } 52.1\%) \), 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 \( (95\% \text{ CI}=1.25-4.08) \) and 3.61 \( (95\% \text{ CI}=0.88-15.24) \) 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 \( (p = 0.0130) \). 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
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

<table>
<thead>
<tr>
<th>Promoter T-991C (rs5751129)</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Adjusted OR* (95% CI)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>72 (78.2)</td>
<td>519 (89.5)</td>
<td>1.00 (ref)</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>17 (18.5)</td>
<td>55 (9.5)</td>
<td>2.24 (1.25-4.08)</td>
<td>0.0157</td>
</tr>
<tr>
<td>CC</td>
<td>3 (3.3)</td>
<td>6 (1.0)</td>
<td>3.61 (0.88-15.24)</td>
<td>0.0904</td>
</tr>
<tr>
<td>$p$-Value for trend (TC+CC) vs. TT</td>
<td></td>
<td></td>
<td>2.91 (1.21-4.65)</td>
<td>0.0065</td>
</tr>
<tr>
<td>CC vs. (TT+TC)</td>
<td></td>
<td></td>
<td>2.38 (1.34-4.22)</td>
<td>0.0050</td>
</tr>
<tr>
<td>$p$-Value for trend (TC+CC) vs. TT</td>
<td></td>
<td></td>
<td>3.27 (0.76-13.28)</td>
<td>0.1127</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Promoter G-57C (rs2267437)</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Adjusted OR* (95% CI)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>61 (66.3)</td>
<td>394 (67.9)</td>
<td>1.00 (ref)</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>27 (29.3)</td>
<td>174 (30.0)</td>
<td>1.02 (0.60-1.68)</td>
<td>1.0000</td>
</tr>
<tr>
<td>GG</td>
<td>4 (4.4)</td>
<td>12 (2.1)</td>
<td>2.16 (0.65-6.91)</td>
<td>0.2556</td>
</tr>
<tr>
<td>$p$-Value for trend (CG+GG) vs. CC</td>
<td></td>
<td></td>
<td>1.43 (0.62-2.49)</td>
<td>0.4118</td>
</tr>
<tr>
<td>GG vs. (CC+CG)</td>
<td></td>
<td></td>
<td>1.06 (0.65-1.72)</td>
<td>0.8104</td>
</tr>
<tr>
<td>$p$-Value for trend (TC+CC) vs. TT</td>
<td></td>
<td></td>
<td>2.02 (0.68-6.97)</td>
<td>0.2561</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Promoter A-31G (rs132770)</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Adjusted OR* (95% CI)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>74 (80.4)</td>
<td>475 (81.9)</td>
<td>1.00 (ref)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>14 (15.2)</td>
<td>75 (12.9)</td>
<td>1.16 (0.64-2.17)</td>
<td>0.6186</td>
</tr>
<tr>
<td>AA</td>
<td>4 (4.4)</td>
<td>30 (5.2)</td>
<td>0.85 (0.31-2.54)</td>
<td>0.7952</td>
</tr>
<tr>
<td>$p$-Value for trend (GA+AA) vs. GG</td>
<td></td>
<td></td>
<td>1.07 (0.58-2.26)</td>
<td>0.8022</td>
</tr>
<tr>
<td>AA vs. (GG+GA)</td>
<td></td>
<td></td>
<td>1.10 (0.61-1.89)</td>
<td>0.7717</td>
</tr>
<tr>
<td>$p$-Value for trend (TC+CC) vs. TT</td>
<td></td>
<td></td>
<td>0.84 (0.30-2.42)</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intron 3 (rs132774)</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>Adjusted OR* (95% CI)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>72 (78.3)</td>
<td>466 (80.3)</td>
<td>1.00 (ref)</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>20 (21.7)</td>
<td>114 (19.7)</td>
<td>1.11 (0.63-1.92)</td>
<td>0.6736</td>
</tr>
<tr>
<td>CC</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aAdjusted for age, gender, smoking and alcohol drinking status; the line with ORs that significantly differ from 1.00 are in bold.
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 splicing, 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.

Acknowledgements

This study was supported by research grants from the Terry Fox Cancer Research Foundation and the National Science Council (NSC 101-2320-B-039-045). The assistance from Ping-Fang Wang in data collection, and that from Huang-Ting Chiang, Yi-Ting Chang, Hong-Xue Ji, Sue-Fung Chen in genotyping were highly appreciated by the authors.

References


Received June 11, 2012
Revised July 26, 2012
Accepted July 31, 2012