Association of DNA Double–strand Break Gene *XRCC6* Genotypes and Lung Cancer in Taiwan

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Abstract. *Aim: The DNA repair gene X-ray repair complementing defective repair in Chinese hamster cells 6 (XRCC6) is thought to play an important role in the repair of DNA double–strand breaks. It is known that defects in double–strand break repair capacity can lead to irreversible genomic instability. However, the association of polymorphic variants of XRCC6 with lung cancer susceptibility has never been reported. In this hospital-based case–control study, the association of XRCC6 promoter T-991C (rs5751129), promoter G-57C (rs2267437), promoter G-31A (rs132770), and intron 3 (rs132774) polymorphisms with lung cancer risk in a Taiwanese population, was studied. Materials and Methods: In total, 358 patients with lung cancer and 716 healthy controls recruited from the China Medical Hospital in Taiwan were genotyped. Results: The results showed that there were significant differences between lung cancer and control groups in the distribution of their genotypic ($p=3.7\times10^{-4}$) and allelic frequency ($p=2.7\times10^{-5}$) in the XRCC6 promoter T-991C polymorphism. Individuals who carried at least one C allele (TC or CC) had a 2.03-fold increased odds ratio of developing lung cancer compared to those who carried the wild-type TT genotype (95% conference internal=1.42-2.91, p=0.0001). For the other three polymorphisms, there was no difference between the case and control groups in the distribution of either genotypic or allelic frequency. Conclusion: In conclusion, the XRCC6 promoter T-991C, but not the promoter C-57G, promoter G-31A or intron 3, is associated with lung cancer susceptibility.*

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Worldwide, lung cancer is the most common cause of cancer-related death in both males and females, responsible for more than one million deaths annually in recent years (1-3). In Taiwan, lung cancer is characterized for its high incidence, high mortality, and low 5-year survival rate, especially in female adenocarcinoma cases (4), while smoking and polluted air are considered to be the two major enviromentol factors, closely related to lung cancer (4-6). The human genome is insulted tens to hundreds of thousand times per day and DNA repair mechanisms protect the genome from these insults both from endogenous and environmental agents. Mutations or defects in DNA repair genes and a lower DNA repair capacity are thought to be essential for tumorigenesis (7, 8). Therefore, it is logical to suspect that genetic variants of DNA repair genes, such as polymorphisms, might contribute to lung cancer susceptibility.

DNA double–strand breaks (DSBs) are repaired by two important repair subpathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ) (9). In humans, NHEJ is the predominant repair system in all the cell cycle phases. In recent years, several proteins involved in the NHEJ pathway have been identified, including ligase IV, X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4), XRCC6 (Ku70), XRCC5 (Ku80), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), artemis and XRCC4–liked factor (XLF) (10, 11). Genetic variation in DNA repair genes has been postulated as an important contributor to the etiology of cancer (12). Inappropriate NHEJ can also lead to translocations and telomere fusion, hallmarks of tumor cells (13). However, there is scant information regarding lung cancer and NHEJ–related gene polymorphisms. As for NHEJ, some

Table I. *Characteristics of lung cancer patients and controls.*

Characteristic	Controls (n=716)			Patients (n=358)			*p*-value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			64.8 (6.8)			64.0 (6.9)	0.58
Gender							0.36
Male	488	68.1%		254	70.9%		
Female	228	31.9%		104	29.1%		
Habit							
Cigarette smokers	563	78.6%		293	81.8%		0.23
Non-smokers	153	21.4%		65	18.2%		

[a]Based on chi-square test.

genetic polymorphisms were reported to influence DNA repair capacity and confer predisposition to several types of cancer, including skin (14), breast (15-17), bladder (18, 19), and oral cancer (20, 21).

In previous study of our group, we found that one polymorphism of the *XRCC4* NHEJ repair gene is associated with lung cancer susceptibility in Taiwan (22). In this study, we assumed that polymorphisms of the upstream gene *XRCC6* in NHEJ, like *XRCC4*, may also contribute to lung cancer susceptibility. To test this hypothesis, we determined the genotypic frequency of four polymorphisms of the *XRCC6* gene at promoter T-991C (rs5751129), promoter G-57C (rs2267437), promoter G-31A (rs132770), and intron3 (rs132774), using a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method. To the best of our knowledge, this is the first study carried out to evaluate the contribution of *XRCC6* genotypes to lung cancer risk.

Materials and Methods

Study population and sample collection. Three hundred and fifty-eight cancer patients diagnosed with lung cancer were recruited at the outpatient clinics of general surgery between 2005-2008 at the China Medical University Hospital, Taichung, Taiwan. The clinical characteristics of patients including histological details were all graded and defined by expert surgeons. All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. Twice as many non-lung cancer, healthy volunteers, were selected as controls by matching of 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 questions related to smoking habits. The study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all participants.

Genotyping conditions. Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and stored as previously published (19, 23-27). The primers used for *XRCC6* promoter C-991T were: forward 5'-AACTCATGGACCCACGGTTGTGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-3'; for promoter G-57C were: forward 5'-AACTCATGGACCCACGGTTGTGA-3', and reverse 5'-CAACTTAAATACAGGAATGTCTTG-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'-CATAAGTGCTCAGTACCTAT-3'. The following cycling conditions were used: one cycle at 94˚C for 5 min; 35 cycles at 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.

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

Table II. *Distribution of XRCC6 promoter T-991C genetic and allelic frequencies among lung cancer patient and control groups.*

XRCC6 T-991C	Controls		Patients		OR (95% CI)	*p*-value[a]
	n	%	n	%		
Genetic frequency						
TT	642	89.7%	290	81.0%	1.00 (ref)	3.7×10^{-4}
TC	66	9.2%	59	16.5%	1.98 (1.36-2.89)	
CC	8	1.1%	9	2.5%	2.49 (0.95-6.52)	
Carrier comparison						
TT+TC	708	98.9%	349	97.5%	1.00 (Reference)	NS
CC	8	1.1%	9	2.5%	2.28 (0.87-5.97)	
TT	642	89.7%	290	81.0%	1.00 (Reference)	0.0001
TC+CC	74	10.3%	68	19.0%	2.03 (1.42-2.91)	
Allelic frequency						
Allele T	1350	94.3%	639	89.3%	1.00 (Reference)	2.7×10^{-5}
Allele C	82	5.7%	77	10.7%	1.98 (1.43-2.75)	

OR: Odds ratio, CI: confidence interval; abased on Chi-square test, NS: non-significant.

Table III. *Distribution of XRCC6 promoter C-57G genetic and allelic frequencies among lung cancer patient and control groups.*

XRCC6 C-57G	Controls		Patients		OR (95% CI)	*p*-value[a]
	n	%	n	%		
Genetic frequency						
CC	490	68.4%	250	69.8%	1.00 (ref)	NS
CG	213	29.7%	101	28.2%	0.93 (0.70-1.23)	
GG	13	1.8%	7	2.0%	1.05 (0.42-2.68)	
Carrier comparison						
CC+CG	703	98.2%	351	98.0%	1.00 (Reference)	NS
GG	13	1.8%	7	2.0%	1.08 (0.43-2.73)	
CC	490	68.4%	250	69.8%	1.00 (Reference)	NS
CG+GG	226	31.6%	108	30.2%	0.94 (0.71-1.23)	
Allelic frequency						
Allele C	1193	83.3%	601	83.9%	1.00 (Reference)	NS
Allele G	239	16.7%	115	16.1%	0.95 (0.75-1.22)	

OR: Odds ratio, CI: confidence interval; [a]based on Chi-square test, NS: non-significant.

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, 46 and 114 bp, were present if the product was the digestible CCA form. The reaction was incubated for 2 h at 37˚C, and then 10 μl of product was loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was categorized as either (a) CCA/CCA homozygote (digested), (b) TGG/TGG homozygote (undigested), or (c) CCA/TGG heterozygote.

Statistical analyses. 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 *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) was used to compare the distribution of the *XRCC6* genotypes between cancer cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) and 95% confidence intervals (CIs) using unconditional logistic regression. Data was recognized as significant when the statistical *p*-value was less than 0.05.

Results

The characteristics of the lung cancer patients and the healthy controls are listed in Table I. There was no significant difference between groups with regards to their age, gender, and smoking habits (Table I). The frequencies of the genotypes and alleles of the *XRCC6* promoter T-991C

Table IV. *Distribution of XRCC6 promoter G-31A genetic and allelic frequencies among lung cancer patient and control groups.*

XRCC6 G-31A	Controls		Patients		OR (95% CI)	*p*-value[a]
	n	%	n	%		
Genetic frequency						
GG	574	80.2%	294	82.1%	1.00 (ref)	NS
GA	100	14.0%	46	12.9%	0.90 (0.62-1.31)	
AA	42	5.8%	18	5.0%	0.84 (0.47-1.48)	
Carrier comparison						
GG+GA	674	94.2%	340	95.0%	1.00 (Reference)	NS
AA	42	5.8%	18	5.0%	0.85 (0.48-1.50)	
GG	574	80.2%	294	82.1%	1.00 (Reference)	NS
GA+AA	142	19.8%	64	17.9%	0.88 (0.63-1.22)	
Allelic frequency						
Allele G	1248	87.2%	634	88.6%	1.00 (Reference)	NS
Allele A	184	12.8%	82	11.4%	0.88 (0.66-1.16)	

OR: Odds ratio, CI: confidence interval; [a]based on Chi-square test, NS: non-significant.

Table V. *Distribution of XRCC6 intron 3 genetic and allelic frequencies among lung cancer patient and control groups.*

XRCC6 intron 3	Controls		Patients%		OR (95% CI)	*p*-value[a]
	n	%	n			
Genetic frequency						
TGG/TGG	592	82.7%	299	83.5%	1.00 (ref)	NS
TGG/CCA	124	17.3%	59	16.5%	0.94 (0.67-1.32)	
CCA/CCA	0	0%	0	0%		
Allele frequency						
TGG	1308	91.3%	657	91.8%	1.00 (Reference)	NS
CCA	124	8.7%	59	8.2%	0.95 (0.69-1.31)	

OR: Odds ratio, CI: confidence interval; [a]based on Chi-square test, NS: non-significant.

polymorphism in the lung cancer and control groups are summarized in Table II. There were significant differences between both groups in the distribution of genotypic ($p=3.7\times10^{-4}$) and allelic frequency ($p=2.7\times10^{-5}$). The OR for those carrying TT and TC genotypes were 1.98 (95% CI=1.36-2.89) and 2.49 (95% CI=0.95-6.52), respectively, compared to those carrying the TT wild-type genotype. The former is significant while the latter is not significant. The lack of significance may be due to the limited sample size. Hence, individuals who carried at least one C–allele (TC and CC) had a 1.98-fold increased OR of developing lung cancer compared to those who carried the wild-type T–allele (95% CI=1.43-2.75) (Table II). On the contrary, for the *XRCC6* promoter C-57G (Table III), promoter G-31A (Table IV), and intron 3 polymorphisms (Table V), the distributions of these polymorphisms were in Hardy-Weinberg equilibrium but there was no difference between lung cancer and control groups in the distribution of either genotype or allelic frequency at these SNP sites (Tables III-V). The genotyping results by PCR-RFLP were presented in Figure 1.

Discussion

The present study is the first to investigate the role of *XRCC6* gene polymorphisms, which have never been reported to be associated with lung cancer risk. Our study revealed that the *XRCC6* promoter T-991C genotype (Table II), but not those of C-57G (Table III), G-31A (Table IV) or intron 3 (Table V) genotypes, was associated with the risk of lung cancer. The *XRCC6* promoter T-991C genetic variation may not directly result in amino acid coding change, but may possibly influence the expression level of the XRCC6 protein. In previous studies, the *XRCC6* promoter T-991C genotype was found to be associated with oral (20) and gastric cancer (28). In addition, this genotype was also found to be associated with two-side pterygium pathology, caused by uncontrolled cell proliferation like that of a tumor (29).

In this study, an effort was made to overcome some limitations in study design. For instance, in order to lower the possibility of false-positive or false-negative findings, we enlarged the sample size of the control group and avoided

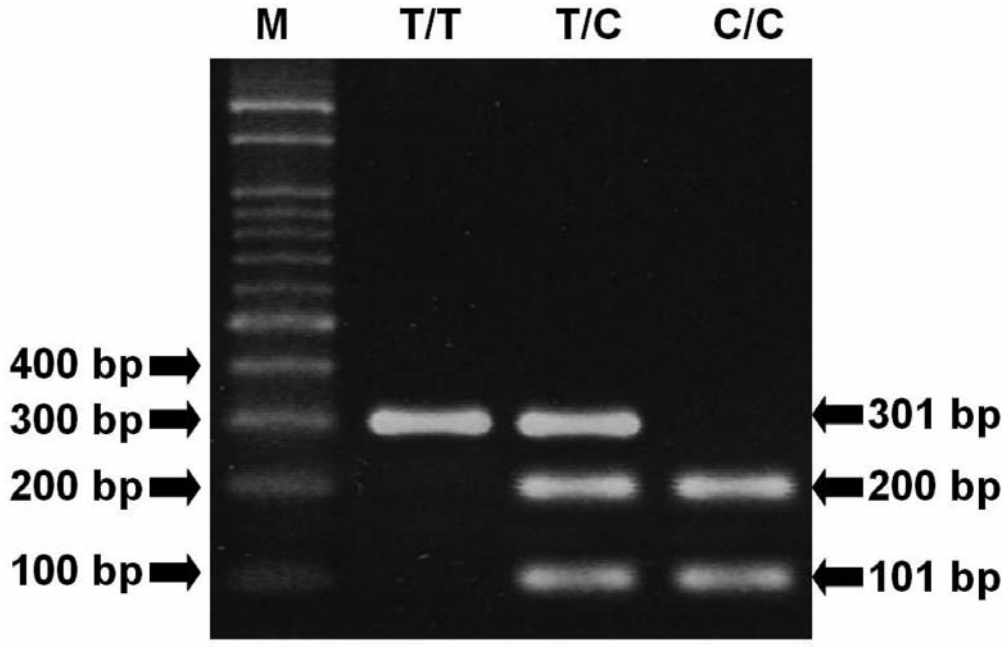

Figure 1. *PCR-based restriction analysis of the promoter T-991C polymorphism of XRCC6 gene shown on 3% agarose electrophoresis. Marker: 100 bp DNA size ladder marker, T/T: indivisible homozygote, T/C: heterozygote, and C/C: divisible homozygote.*

any sub-grouping and adjusting of the data. In Tables II to V, we provide only the main findings with these 358 lung cancer patients and 716 (twice the number of the patients) non-cancer controls, which is very convincing and reliable with multiple-checked significances ($p<0.05$ both in genotype and allelic frequencies, and the obvious ORs and 95% CIs). All the cases and the controls recruited in this study were also drawn from the same Taiwanese ethnic group and the Taiwanese population has a relatively homogenous genetic background (30), while little population bias can be produced in the sampling process. Therefore, the potential confounding effect of population stratification for genotyping data is not a major concern. Furthermore, possible selection bias was taken into consideration and reduced to the lowest level possible by frequency matching on age and gender between the cases and controls. Lastly, the frequencies of *XRCC6* polymorphisms variant alleles were similar to those reported in the NCBI website in the Asian population studies, for example the C allelic frequency of *XRCC6* promoter T-991C is 5.7% in our control group and 4.2-8.9% for Asian populations in NCBI, which also implies that there was no selection bias for participant enrolment in terms of various genotypes.

In this study, the allelic frequency of the C allele at *XRCC6* promoter T-991C was significantly higher in the lung cancer group (10.7%) than in the control group (5.7%) (Table II). It was also found that patients heterozygous (TC) for *XRCC6* promoter T-991C had a 1.98-fold higher risk of lung cancer (Table II). Although the CC genotype did not produce any significance, lung cancer risk for the combination of heterozygous and homozygous (TC or CC) groups was almost at the same level (OR=2.03) (Table II). All these data suggest that the C allele at *XRCC6* promoter T-991C is indeed a novel and important biomarker for lung carcinogenesis. In the future, C allele carriers, more susceptible to lung cancer, should prevent their exposure to environmental or other risky factors, such as smoking habit. If the sample size could be enlarged in the future, further stratification analysis regarding gene–gene or –environment interaction may add more information to the understanding of lung carcinogenesis and etiology.

In conclusion, this is the first report to investigate the association between *XRCC6* gene polymorphisms and lung cancer. Our findings suggested that *XRCC6* promoter T-991C, but not *XRCC6* promoter C-57G, promoter G-31A or intron 3 polymorphisms, are associated with higher susceptibility to lung cancer. *XRCC6* promoter T-991C polymorphism might become a potential biomarker for the prediction of lung oncology.

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