Association of XRCC4 Codon 247 Polymorphism with Oral Cancer Susceptibility in Taiwan

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Abstract. *Background: The DNA repair gene XRCC4, an important caretaker of overall genome stability, is thought to play a major role in the development of human carcinogenesis. However, the association of the polymorphic variants of XRCC4 with oral cancer susceptibility has never been reported. Materials and Methods: In this hospital-based case-control study, the association of XRCC4 codon 247 (rs3734091), G-1394T (rs6869366), intron 7 (rs28360317) and intron 7 (rs1805377) polymorphisms with oral cancer risk in a Central Taiwanese population was investigated. In total, 318 patients with oral cancer and 318 age- and gender-matched healthy controls recruited from the China Medical Hospital in Central Taiwan were genotyped. Results: A significantly different distribution was found in the frequency of the XRCC4 codon 247 genotype, but not the XRCC4 G-1394T or intron 7 genotypes, between the oral cancer and control groups. A/C heterozygosity at XRCC4 codon 247 conferred a significant (2.04-fold) increased risk of oral cancer. As for XRCC4 G-1394T and intron 7 polymorphisms, there was no difference in distribution between the oral cancer and control groups. Gene-environment interactions with smoking, but not with betel quid chewing or alcohol consumption, were significant for XRCC4 codon 247 polymorphism. The XRCC4 codon 247 A/C genotype in association with smoking conferred an increased risk of 3.44 (95% confidence interval = 1.24-9.60) for oral cancer. Conclusion: Our results provide the first evidence that the heterozygous A allele of the XRCC4 codon 247 may be associated with the development of oral cancer and may be a novel useful marker for primary prevention and anticancer intervention.*

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Oral cancer is one of the most commonly diagnosed cancers all over the world (1-4), and with continuously increasing incidence and mortality for the past two decades, oral cancer has become the fourth most common cause of male cancer death in Taiwan (5). The genomic etiology of oral cancer is of great interest but largely unknown. Human DNA repair mechanisms protect the genome from DNA damage caused by endogenous and environmental agents (6). Mutations or defects in the DNA repair system are essential for tumorigenesis (7). It is therefore logical to suspect that some genetic variants of DNA repair genes might contribute to oral cancer pathogenesis.

The responses of the cell to genetic injury and its ability to maintain genomic stability by means of a variety of DNA repair mechanisms is essential in preventing tumor initiation and progression which can be brought about by mutations or defects in the repair system (8). Sequence variants in DNA repair genes are also thought to modulate DNA repair capacity and consequently may be associated with altered cancer risk (9). The X-ray cross-complementing group 4 (*XRCC4*) gene, which is important in the non-homologous end-joining repair pathway, is found to restore DNA double-strand break repair and the ability to support V(D)J recombination of transiently introduced substrates in the XR-1 CHO cell line (10). The XRCC4 protein interacts directly with Ku70/Ku80 (11), and it is hypothesized that XRCC4 serves as a flexible tether between Ku70/Ku80 and its associated protein Ligase4 (11). XRCC4 is required for precise end-joining of blunt DNA double-strand breaks in mammalian fibroblasts (12). In the gene-targeting mutation mouse model, *XRCC4* gene inactivation leads to late

embryonic lethality accompanied by defective lymphogenesis and defective neurogenesis, manifested by extensive apoptotic death of newly generated postmitotic neuronal cells (13). These findings demonstrated that differentiating lymphocytes and neurons strictly require the XRCC4 end-joining proteins. Thus, it is reasonable that only polymorphisms of *XRCC4* gene, not mutations, can be sustained in the genome for such a long period of carcinogenesis. There are, however, few papers studying the single-gene role of the *XRCC4* gene in cancer, and no findings reporting the role of these *XRCC4* polymorphisms in oral cancer either.

Since DNA repair gene alterations have been shown to cause a reduction in DNA repair capacity, we hypothesized that DNA repair gene polymorphisms may be risk factors for oral cancer. To test this hypothesis, we determined the genotypic frequency of four polymorphisms of the *XRCC4* gene at codon 247 (rs3734091), G-1394T (rs6869366), Intron 7 (rs28360317) and Intron 7 (rs1805377), which will hereafter been referred to as XRCC4-1, XRCC4-2, XRCC4-3 and XRCC4-4, respectively, using a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method. To the best of our knowledge, this is the first study carried out to evaluate the contribution of *XRCC4* polymorphisms to oral oncology.

Materials and Methods

Study population and sample collection. Three hundred and eighteen patients diagnosed with oral cancer were recruited at the outpatient clinics of general surgery between 1998-2007 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. The mean age of the oral cancer patients and the controls were 66.45 (standard deviation, SD=10.43) and 63.32 (SD=8.64) years, respectively. All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. The same number of non-oral cancer healthy people as controls were selected by matching for age and gender after initial random sampling from the Health Examination Cohort of the hospital.

A questionnaire administered to the participants included questions on history and frequency of alcohol consumption, betel quid chewing and smoking habits. Self-reported alcohol consumption, betel quid chewing and smoking habits were evaluated and classified as categorical variables. Information on these factors when reported as more than twice a week for years was recorded as "ever", and all other cases were recorded as "never". 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 leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to PCR-RFLP methods. Briefly, the following primers (Perkin-Elmer, Taipei, Taiwan) were used: XRCC4-1: 5'-GCTAATGAGTTGCTGCATTTTA-3' and 5'-TTTCTAGGGAAACTGCAATCTGT-3'; XRCC4-2: 5'-GATGCGAACTCAAAGATACTGA-3' and 5'-TGTAAAGCCAGTACTCAAACTT-3'; XRCC4-3: 5'-ATACTGTGTTTGGAACTCCT-3' for CCT-positive forward primer, 5'-ATACTGTGTTTGGAACTAGA-3' for CCT-negative forward primer, and 5'-TATCCTATCATCTCTGGATA-3' as reverse common primer; XRCC4-4: 5'-TTCACTTATGTGTCTCTTCA-3' and 5'-AACATAGTCTAGTGAACATC-3'.

The following cycling conditions were performed: 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. The PCR products were studied after digestion with BbsI, Hinc II, and Tsp509I restriction enzymes (New England Biolabs, Beverly, MA) for XRCC4-1 (cut from 308 bp C type into 204+104 bp A type), XRCC4-2 (cut from 300 bp T type into 200+100 bp G type), and XRCC4-4 (cut from 237 bp G type into 79+158 bp A type) respectively. The PCR products of XRCC4-3 were 239 bp for the CCT positive form and no product for the CCT negative form. Ten percent of DNA samples were genotyped for a second time and the concordance rate was 100% .

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 *XRCC4* single nucleotide polymorphisms in the control group from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's χ^2 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 *XRCC4* 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. Data was recognized as significant when the statistical *p*-value was less than 0.05.

Results

The frequency of the alleles for XRCC4-1, XRCC4-2, XRCC4-3 and XRCC4-4 between oral cancer and control groups is shown in Table I. The distributions of all these polymorphisms were in Hardy-Weinberg equilibrium and were similar between oral patients and controls. It is apparent from Table I that the proportion of individuals with the A allele at XRCC4-1 was significantly greater in the patient group (p=0.026). In contrast, the G and T alleles at XRCC4-2, I and D alleles at XRCC4-3, and the A and G alleles at XRCC4-4, were not differently distributed in the oral cancer patient and control groups (p>0.05). The representative PCR-based restriction analyses for the XRCC4-1 polymorphisms are shown in Figure 1.

The frequencies of the genotypes of XRCC4-1, XRCC4-2, XRCC4-3 and XRCC4-4 polymorphisms in the oral cancer and control groups are shown in Table II. In XRCC4-1, using 247C as the reference group, there was an obvious association between the heterozygote of 247A at XRCC4-1 and oral cancer risk (Table II). There was no individual homozygous for the A allele in either control or oral cancer patients, and the data showed that the presence of the A allele at XRCC4-1 conferred a 2.04-fold risk factor for oral cancer (Table II). Neither heterozygotes of G at XRCC4-2, hetero- or homozygotes of D at XRCC4-3, nor hetero- or homozygotes of G at XRCC4-4 appear to be risky genotypes

Table I. *Allele frequencies for XRCC4 polymorphisms in the control and oral cancer groups.*

Allele	Controls (%) N=636	Cases (%) N=636	*p*-value[a]
XRCC4-1			
Allele A (Ser)	**16 (2.5)**	**31 (4.9)**	**0.0258**
Allele C (Ala)	**620 (97.5)**	**605 (95.1)**	
XRCC4-2			
Allele G	19 (3.0)	27 (4.2)	0.2296
Allele T	617 (97.0)	609 (95.8)	
XRCC4-3			
Allele I	478 (75.2)	492 (77.4)	0.3563
Allele D	158 (24.8)	144 (22.6)	
XRCC4-4			
Allele A	464 (73.0)	473 (74.4)	0.5667
Allele G	172 (27.0)	163 (25.6)	

[a]*p*-value based on χ^2 test.

Table II. *Association of XRCC4 polymorphisms and oral cancer risk.*

Genotype	Controls (%)	Cases (%)	Odds ratio (95% CI)[a]
XRCC4-1			
C/C	302 (95.0)	287 (90.3)	1.00 (ref)
A/C	16 (5.0)	31 (9.7)	**2.04 (1.09-3.81)[b]**
A/A	0 (0)	0 (0)	1.05 (0.02-53.21)
XRCC4-2			
T/T	299 (94.0)	291 (91.5)	1.00 (ref)
G/T	19 (6.0)	27 (8.5)	1.46 (0.79-2.68)
G/G	0 (0)	0 (0)	1.03 (0.02-51.96)
XRCC4-3			
I/I	195 (61.3)	196 (61.6)	1.00 (ref)
I/D	88 (27.7)	100 (31.4)	1.13 (0.80-1.60)
D/D	35 (11.0)	22 (6.9)	0.63 (0.35-1.11)
with D	123 (38.7)	122 (38.4)	0.99 (0.72-1.36)
XRCC4-4			
A/A	167 (52.5)	173 (54.4)	1.00 (ref)
A/G	130 (40.9)	127 (39.9)	0.94 (0.68-1.30)
G/G	21 (6.6)	18 (5.7)	0.83 (0.43-1.61)
with G	151 (47.5)	145 (45.6)	0.93 (0.68-1.27)

[a]CI, confidence interval; [b]statistically identified as significant.

for oral cancer (Table II). Thus, among the four XRCC4 polymorphisms investigated, only 247A at XRCC4-1 seems to contribute to increased oral cancer risk.

The joint effects of XRCC4-1 genotype and three environmental factors, namely smoking, betel quid chewing and alcohol consumption, on estimates of oral cancer risk are shown in Table III. Participants were classified into two groups in regard to smoking, alcohol consumption and betel quid chewing: ever (users) and never (non-users). Among the three well-known oral cancer-related habits evaluated, the effect of XRCC4-1 was shown to interact with that of smoking, but not of chewing of betel quid or the use of alcohol. The A/C variant conferred an increased risk compared to the C/C wild-type genotype in the smoking stratification, with an increased risk of 3.44 (95% CI=1.24-9.60) (Table III).

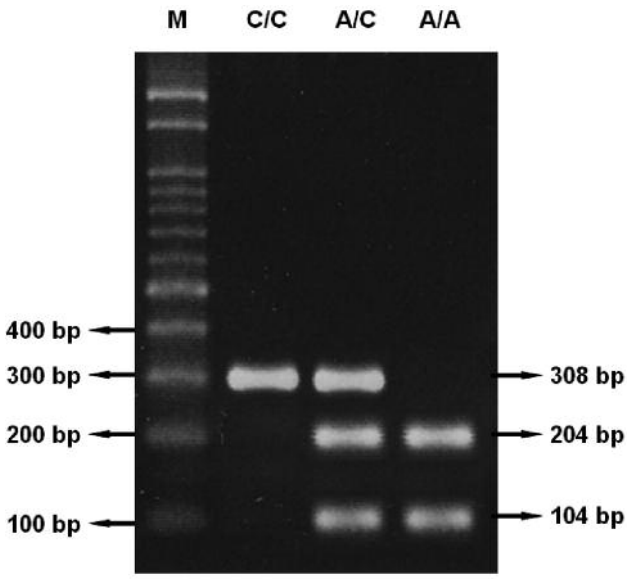

Figure 1. *PCR-based restriction analysis of the XRCC4-1 polymorphism of XRCC4 gene shown on 3% agarose electrophoresis. M: 100 bp DNA size marker, C/C: indivisible homozygote, A/C: heterozygote, and A/A: divisible homozygote.*

Table III. *Joint effects of XRCC4-1 polymorphisms and environmental factors on oral cancer risk.*

Habits	Genotype	Controls (n)	Cases (n)	Odds ratio (95% CI)[a]
Smoking				
Never	C/C	192	172	1.00 (ref)
	A/C	11	13	1.32 (0.58-3.02)
Ever	C/C	110	115	1.00 (ref)
	A/C	5	18	3.44 (1.24-9.60)[b]
Alcohol				
Never	C/C	137	128	1.00 (ref)
	A/C	8	14	1.87 (0.76-4.61)
Ever	C/C	165	159	1.00 (ref)
	A/C	8	17	2.21 (0.93-5.25)
Betel quid				
Never	C/C	231	212	1.00 (ref)
	C/C	13	22	1.84 (0.91-3.75)
Ever	A/C	71	75	1.00 (ref)
	C/C	3	9	2.72 (0.74-10.92)

[a]CI, confidence interval; [b]statistically identified as significant.

Discussion

The present study investigated the role of *XRCC4* gene polymorphisms, which has never been reported to be associated with oral cancer risk. At the time of writing, there were very few papers studying the *XRCC4* gene polymorphisms and their associations with carcinogenesis, not to mention their joint effects with environmental factors. To the best of our knowledge, there are no reports concerning any *XRCC4* polymorphism in oral cancer risk.

Our larger sample size and concise data analysis strengthen the accuracy and reliability of our finding. Moreover, the frequencies of *XRCC4* polymorphism variant alleles were similar to those reported on the NCBI website (http://www.ncbi.nlm.nih.gov) in the Asian population studies: for example A allele frequencies of XRCC-1 are 2.5~4.9 here and 4.2~15.9 in NCBI, which suggest no selection bias for enrolment of participants in terms of genotypes.

In this study, the genotype distribution of the A allele at *XRCC4* codon 247 (4.9%) was significantly higher in the oral cancer group than in the control group (2.5%) (Table I). It was also found that participants heterozygous for *XRCC4* codon 247A had a 2.04-fold higher risk of oral cancer (Table II). The lack of A/A *XRCC4* codon 247 homozygotes in our investigated populations and those in the NCBI may indicate that such individuals bear fetal defects related to this SNP which lead to apoptosis of the cells or early lethality, thus no cancer incident arises in this case. All these data suggest that 247A may be a novel and important biomarker for oral carcinogenesis, and when detected, the carriers may be more susceptible to oral cancer. As for other *XRCC4* polymorphisms, our results indicate that their genetic differences were not associated with oral cancer risk.

Three potential gene-environment interactions in oral carcinogenesis, cigarette smoking, alcohol consumption and betel quid chewing, were also investigated and further evaluated in our study. Smoking is associated with free radical-induced DNA damage and strand breaks (14), and tobacco smoke contains potential carcinogens, including polycyclic aromatic hydrocarbons, aromatic amines, tobacco nitroamines and 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(α)pyrene (BPDE), which may all form bulky DNA adduct (15). Our study provides some evidence for these gene-environment interactions. There were positive associations of variant XRCC4 codon 247 genotypes with smoking, but not betel quid chewing or alcohol use. Our results showed that *XRCC4* codon 247, A/C "ever" user groups exhibited an increased risk of 3.44 (95% CI=1.24-9.60) for smoking (Table III).

Together with our previous findings (16-18), these results suggest that genetic variants involved in DNA repair pathways are indeed involved in oral cancer etiology. Risk factors, such as consumption of betel quid and alcohol, may also modulate oral cancer risk in combination with genetic susceptibility in these repair pathways (19, 20). Therefore, replication in larger studies and other functional repair assays may be performed in the future to preclude chance findings, particularly those among subgroups, and clarify the mechanisms involved.

It is agreed that complex environmental carcinogens may generate various types of DNA damage, activate their responsible DNA proteins in different DNA repair pathways to remove them. In this case, further investigations of other SNPs in DNA repair genes and the repair capacity of cells from oral cancer patient can contribute to a better understanding of oral carcinogenesis.

In conclusion, this is the first report to investigate the association between *XRCC4* gene polymorphisms and oral cancer. Our findings suggest that the presence of the A allele of *XRCC4* codon 247 was associated with a higher susceptibility to oral cancer, and the A allele of *XRCC4* codon 247 may be a useful novel marker in oral oncology for primary prevention and intervention.

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