Significant Association of an XRCC4 Single Nucleotide Polymorphism with Bladder Cancer Susceptibility in Taiwan

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Abstract. *Background: The DNA repair gene XRCC4, a member of NHEJ for double strand breaks, is very important in maintaining the overall genome stability, and may play an important role in carcinogenesis. To reveal the relationship between XRCC4 and bladder cancer, seven polymorphic variants of XRCC4, including C-1622T (rs7727691), G-1394T (rs6869366), G-652T (rs2075685), C-571T (rs2075686), intron3 DIP (rs28360071), S247A (rs3734091) and intron7 DIP (rs28360317) were investigated and analyzed for their association with bladder cancer susceptibility. Patients and Methods: In this case-control study, the association of these variants of XRCC4 with bladder carcinogenesis in Taiwan was investigated. Bladder cancer patients (158) and 158 age- and gender-matched healthy controls were recruited and their genotypes were analyzed by PCR-based restriction fragment length polymorphism method. Results: It was found that XRCC4 G-1394T is a significant SNP in bladder carcinogenesis after analyzing the frequencies of each variant in both bladder cancer and control groups. The data indicated that the heterogeneous G of G-1394T is an obvious risk factor of bladder cancer susceptibility (p=0.005) and the data of G allele also showed a similar situation (p=0.0099). As for the other six polymorphisms, there was no difference between the bladder cancer and control groups. Conclusion: These findings suggest that the G allele of XRCC4 G-1394T may be involved in bladder carcinogenesis and useful in early detection of bladder cancer.*

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Bladder cancer is one of the most common urological cancers in Taiwan, with an increasing incidence and death rate during the past decades (1). Bladder cancer is strongly affected by environmental carcinogens and some risk factors have been confirmed, such as cigarette smoking, exposure of aromatic amines and intake of drugs like phenacetine, chlornaphrazine and cyclophosphamide (2, 3). These environmental carcinogens may induce bladder cancer by causing DNA damages which are often thought of as important origins of various carcinogenesis. Thus, the cellular capacities of repairing these DNA damages are closely correlated with the probabilities of cancer development. In previous studies, some genomic variants of DNA repair genes have been proved as risk factors or biomarkers of bladder cancer, such as the *XPD, XPG* and *XRCC3* (4, 5), supporting the idea that DNA adducts that are repaired by nucleotide excision repair and that homologous recombination may also involved. In addition to the BPDE-induced bulky adducts which should be removed by the nucleotide excision repair system (6), cigarette smoking contains may also induce a lot of oxidative damage causing DNA oxidative adducts together with single- and double-strand breaks (7, 8), which should be removed by base excision repair and double strand break (DSB) repair systems, respectively (9, 10). Among the various DNA injuries, the most severe is DSB. Only one fail of repairing this kind of DNA damage may cause a dramatic instability of the overall genome, leading to various types of carcinogenesis (11-17). In the cell, DSB is often repaired by two pathways, homologous recombination and non-homologous end-joining (NHEJ). Genes responsible of removing these DSBs are critical in various carcinogenesis, and the association between their genomic variants and cancer susceptibility has been confirmed (13-21).

The gene *XRCC4*, which is a specific member of NHEJ system, will cooperate with other proteins such as DNA ligase4 to reverse the broken DNA strands after DSB occurs. Therefore, this gene is quite important in maintaining the

genome stability, and its products play a critical role in prevention of carcinogenesis. Mounting evidences have indicated that some genomic polymorphisms of *XRCC4* are indeed significant in numerous diseases, including some cancers (13, 14). Thus, it is logical to propose that the subtle variants of *XRCC4* may also serve as a factor of bladder cancer susceptibility. The possible mechanisms are that the deficiencies of high-penetrate genes will lead to some lethal embryonic changes, and some serious mutations of high-penetrate genes will also cause early onset of lethal human diseases. Therefore, these kind of genetic variants are considered too severe to develop bladder cancers, since the ages of bladder cancer patients are usually more than fifty. In this study, it was thought reasonable to choose the polymorphisms of low-penetrate gene *XRCC4* to be the candidate targets. Seven *XRCC4* single nucleotide polymorphisms, four variants in promoter region (C-1622T, rs7727691; G-1394T, rs6869366; G-652T, rs2075685; C-571T, rs2075686), one amino acid substitution variant (S247A, rs3734091), and two variants in intron regions (Intron3 DIP, rs28360071; Intron7 DIP, rs28360317), were chosen to find some specific characteristics of Taiwanese bladder cancer. Furthermore, since cigarette smoking may induce DNA damage and individuals with a reduced DNA repair capacity are associated with increased cancer susceptibility, it was hypothesized that the genetic association might be modulated by exposure to cigarette smoke. Therefore, the aim was also to investigate the gene-environment interaction between the *XRCC4* genotypes and individual smoking habits in bladder cancer risk.

Patients and Methods

Study population and sample collection. One hundred and fifty eight patients diagnosed with bladder cancer were recruited at the outpatient clinics of general surgery between 2001-2007 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. The clinical characteristics were all defined by expert surgeons (Dr. Chang and Wu). All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. An equal number of non-bladder cancer healthy volunteers as controls were selected by matching for age, gender and some indulgences after initial random sampling from the Health Examination Cohort of the hospital. The study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all participants.

Questionnaire. At recruitment, a trained research nurse was assigned to obtain informed consent for the collection of a blood sample and to administer a structured questionnaire. The questionnaire collected information about demographic characteristics, lifestyle factors (such as number of cigarettes smoked), medical history and family history of cancer. For smoking status, a person who smoked at least once a day and had been doing so for more than 6 months was regarded as a smoker.

Genotyping assays. Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to previous papers (13, 14, 22-24). Briefly, the following primers were used for *XRCC4* C-1622T rs7727691: 5'-AAGATACTGAGACACTAATC-3' and 5'-CACAACATAACTAAGGATGA-3'; for *XRCC4* G-1394T rs6869366: 5'-GATGCGAACTCAAAGATACTGA-3' and 5'-TGTAAAGCCAGTACTCAAACTT-3'; for *XRCC4* G-652T rs2075685: 5'-GCTAGACACCACTCCAATAA-3' and 5'-GGCTACGTAGATTATGTGTG-3'; for *XRCC4* C-571T rs2075686: 5'-GGCTACTGACTAAACAGATG-3' and 5'-TAACACGTTGGC TACGTAGA-3'; for *XRCC4* intron3 DIP rs28360071: 5'-TCCTGTTACCATTTCAGTGTTAT-3' and 5'-CACCTGTGTTC AATTCCAGCTT -3'; for *XRCC4* S247A rs3734091: 5'-GCTAAT GAGTTGCTGCATTTTA-3' and 5'-TTTCTAGGGAAACTGCA ATCTGT-3'; and for *XRCC4* intron7 DIP rs28360317: 5'-ATACTGTGTTTGGAACTCCT-3' for CCT-positive forward primer, 5'-ATACTGTGTTTGGAACTAGA-3' for CCT-negative forward primer, and 5'-TATCCTATCATCTCTGGATA-3' as reverse common primer. The following cycling conditions were performed: one cycle at 95˚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 Fnu4H I, Hinc II, Mbo II, Mnl I and Bbs I, restriction enzymes for *XRCC4* C-1622T rs7727691 (cut from 218 bp T type into 32+186 bp C type), *XRCC4* G-1394T rs6869366 (cut from 300 bp T type into 200+100 bp G type), *XRCC4* G-652T rs2075685 (cut from 326 bp T type into 127+199 bp G type), *XRCC4* C-571T rs2075686 (cut from 197 bp C type into 69+128 bp T type) and *XRCC4* S247A rs3734091 (cut from 308 bp C type into 204+104 bp A type), respectively.

Statistical analyses. Only those matches with all DNA polymorphism data (control/case=158/158) were selected into final analyzing. 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* SNPs in the control subjects 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. Data was recognized as significant when the statistical p was less than 0.05. The bladder cancer risk associated with the genotypes was estimated as a crude odds ratio (ORs) and 95% confidence intervals (95% CIs) by unconditional logistic regression, then adjusted for the effect of possible confounders such as age, gender and individual habit of smoking. To evaluate effect modification by smoking, stratified analyses were conducted for *XRCC4* G-1394T to compare the association across exposure categories of smoking status (on-smokers *vs*. smokers), and assessed by logistic regression models. All statistical tests were performed using SAS, Version 9.1.3 (SAS Institute Inc., Cary, NC, USA) on two-sided probabilities.

Results

In this study, 158 bladder cancer patients and 158 age- and gender-matched controls were recruited. The frequencies of the genotypes for the *XRCC4* C-1622T, G-1394T, G-652T, C-571T, intron3 DIP, S247A and intron7 DIP between controls and bladder cancer patients are shown in Table I.

Table I. *Distribution of XRCC4 genotypes among bladder cancer patients and controls.*

Genotype	Controls	%	Patients	%	p^a
C-1622T rs7727691					0.41202
CC	139	88.0%	134	84.8%	
CT	19	12.0%	24	15.2%	
TT	0	0.0%	0	0.0%	
G-1394T rs6869366					0.00509
GG	0	0.0%	0	0.0%	
GT	31	19.6%	53	33.5%	
TT	127	80.4%	105	66.5%	
G-652T rs2075685					0.70194
GG	98	62.0%	93	58.9%	
GT	57	36.1%	60	38.0%	
TT	3	1.9%	5	3.2%	
C-571T rs2075686					0.79735
CC	96	60.8%	92	58.2%	
CT	57	36.1%	59	37.3%	
TT	5	3.2%	7	4.4%	
Intron3 DIP rs28360071					0.82604
II	98	62.0%	95	60.1%	
ID	57	36.1%	61	38.6%	
DD	3	1.9%	2	1.3%	
S247A rs3734091					0.59709
AA Ser/Ser	0	0.0%	1	0.6%	
AC Ser/Ala	32	20.3%	33	20.9%	
CC Ala/Ala	126	79.7%	124	78.5%	
Intron7 DIP rs28360317					0.66084
II	79	50.0%	72	45.6%	
ID	69	43.7%	73	46.2%	
DD	10	6.3%	13	8.2%	

[a]*p* based on Pearson's χ^2 test or Fisher's exact test (when the number in any cell was less than five).

Genotype distribution of various genetic polymorphisms of *XRCC4* G-1394T was significantly different between bladder cancer and control groups ($p<0.05$), while those for C-1622T, G-652T, C-571T, intron3 DIP, S247A and intron7 DIP were not significant ($p>0.05$) (Table I). Obviously, among the seven polymorphisms investigated, only the G-1394T was associated with bladder cancer risk. It is interesting to note that no homologous TT in C-1622T or GG in G-13894T were found. It may be explained by the importance of these two polymorphisms in suppressing the expression of *XRCC4* gene, and those people carrying homologous TT in C-1622T or GG in G-13894T have died at early age and could not be recruited in this study. To sum up, the *XRCC4* promoter G-1394T heterozygous is associated with higher susceptibility for bladder cancer risk. The representative PCR-based restriction analyses for the *XRCC4* promoter G-1394T polymorphisms are shown in Figure 1.

The frequencies of the alleles for the *XRCC4* C-1622T, G-1394T, G-652T, C-571T, intron3 DIP, S247A and intron7 DIP between controls and bladder cancer patients are shown in Table II. The distributions of all these polymorphisms were in Hardy-Weinberg equilibrium and were similar between controls and bladder cancer patients (data not shown). Allele frequency distributions of the *XRCC4* G-1394T *G are associated with higher susceptibility for bladder cancer. In detail, distributions of *XRCC4* C-1622T C/T allele in controls and bladder cancer patients were 94.0/6.0 and 92.4/7.6%, respectively (p=0.42962, Table II). Distributions of *XRCC4* G-1394T G/T allele in controls and bladder cancer patients were 9.8/90.2 and 16.8/83.2%, respectively (p=0.00994, Table II). Distributions of *XRCC4* G-652T G/T allele in controls and bladder cancer patients were 80.1/19.9 and 77.8/22.2%, respectively (p=0.49455, Table II). Distributions of *XRCC4* C-571T C/T allele in controls and bladder cancer patients were 78.8/21.2 and 76.9/23.1%, respectively (p=0.56547, Table II). Distributions of *XRCC4* intron3 DIP insertion/deletion allele in controls and bladder cancer patients were 80.1/19.9 and 79.4/20.6%, respectively (p=0.84308, Table II). Distributions of *XRCC4* S247A A/C allele in controls and bladder cancer patients were 10.1/89.9 and 11.1/88.9%, respectively (p=0.69829, Table II). Distributions of *XRCC4* intron7 DIP insertion/deletion allele in controls and bladder cancer patients were 71.8/28.2 and 68.7/31.3%, respectively (p=0.38423, Table II). Consistent with the findings in Table I, the *XRCC4* promoter G-1394T, and not the others, is associated with higher susceptibility for bladder cancer risk.

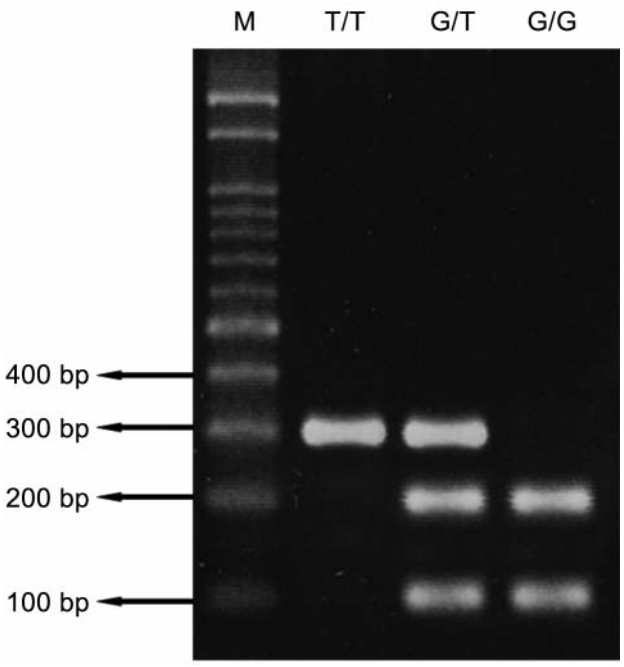

Figure 1. *PCR-based restriction analysis of the G-1394T polymorphism of XRCC4 gene shown on 3% agarose electrophoresis. M: 100 bp DNA size marker, T/T: indivisible homozygote, G/T: heterozygote, and G/G: divisible homozygote.*

Table II. *Distribution of XRCC4 alleles among bladder cancer patients and controls.*

Allele	Controls	%	Patients	%	p[a]	Odds Ratio (95% CI)[b]	Adjusted Odds Ratio (95% CI)[c]
C-1622T rs7727691					0.42962		
Allele C	297	94.0%	292	92.4%		1.00 (Reference)	1.00 (Reference)
Allele T	19	6.0%	24	7.6%		1.28 (0.69-2.40)	1.32 (0.71-2.28)
G-1394T rs6869366					**0.00994**		
Allele T	285	90.2%	263	83.2%		1.00 (Reference)	1.00 (Reference)
Allele G	31	9.8%	53	16.8%		**1.85 (1.15-2.98)[d]**	**1.96 (1.37-3.03)[d]**
G-652T rs2075685					0.49455		
Allele G	253	80.1%	246	77.8%		1.00 (Reference)	1.00 (Reference)
Allele T	63	19.9%	70	22.2%		1.14 (0.78-1.68)	1.18 (0.69-1.44)
C-571T rs2075686					0.56547		
Allele C	249	78.8%	243	76.9%		1.00 (Reference)	1.00 (Reference)
Allele T	67	21.2%	73	23.1%		1.12 (0.77-1.63)	1.09 (0.79-1.46)
Intron3 DIP rs28360071					0.84308		
Insertion	253	80.1%	251	79.4%		1.00 (Reference)	1.00 (Reference)
Deletion	63	19.9%	65	20.6%		1.04 (0.71-1.53)	1.10 (0.70-1.65)
S247A rs3734091					0.69829		
Allele C Ala	284	89.9%	281	88.9%		1.00 (Reference)	1.00 (Reference)
Allele A Ser	32	10.1%	35	11.1%		1.11 (0.67-1.84)	1.14 (0.88-1.73)
Intron7 DIP rs28360317					0.38423		
Insertion	227	71.8%	217	68.7%		1.00 (Reference)	1.00 (Reference)
Deletion	89	28.2%	99	31.3%		1.16 (0.83-1.64)	1.25 (0.87-1.93)

[a]p based on χ^2 test. [b]95% CI, 95% confidence interval. [c]95% CI, 95% confidence interval, and Date were calculated by unconditioned logistic regression and adjusted for age, gender, and smoking status. [d]Statistically significant.

Table III. *Association between XRCC4 G-1394T polymorphism and bladder cancer risk stratified by smoking habit.*

	Non-smokers			Smokers†		
Genotype	Cases/controls	OR (95% CI)	aOR (95% CI)*	Cases/controls	OR (95% CI)	aOR (95% CI)*
TT	74/85	1.00 (reference)	1.00 (reference)	31/42	1.00 (reference)	1.00 (reference)
TG	36/24	1.72 (0.94-3.15)	1.79 (0.96-3.42)	17/7	**3.29 (1.21-8.90)**	**3.35 (1.41-8.49)**

*Data were calculated by unconditional logistic regression and adjusted for age, gender and habit of smoking; aOR, adjusted odds ratio; 95% CI, 95% confidence interval. †Smokers were defined according to individual questionnaire.

Considering potential gene-environment interactions between the *XRCC4* gene and smoking status, the risk of bladder cancer related to *XRCC4* genotypes G-1394T was further examined with stratification by smoking status (Table III). A significant increased risk of G-1394T variants among smokers carrying G allele at their *XRCC4* G-1394T was observed (crude OR=3.29, 95% CI=1.21-8.90, adjusted OR=3.35, 95% CI=1.41-8.49) with an obvious gene-smoking interaction.

Discussion

The *XRCC4* G-1394T has significantly different distributions among controls and bladder cancer patients. The data showed that the *XRCC4* G-1394T is a positive genetic risk factor of bladder cancer (Table I and Table II). Former studies about the genetic factors of Taiwanese bladder cancer had indicated several polymorphisms correlated with bladder carcinogenesis. These evidences proved the importance of genetic variants in bladder cancer (25).

The *XRCC4* G-1394T is located on the promoter region of this gene, and the promoter is thought to play a major role in regulating gene expression. Thus, the polymorphism of the promoter region may influence gene expression and gene related functions. In this study, were chosen four variants which are located on the promoter region of *XRCC4*, with significance being found only in the G-1394T. This interesting data may be due to the fact that different parts of the promoter region are often responsible for different modifications and regulations, and the G-1394T may be locate on the part which is in charge of bladder cancer susceptibility. All the other six polymorphisms, including

three polymorphisms of promoter region, two polymorphisms of intron and one polymorphism of exon, had not observable contribution to bladder cancer in this study. The other polymorphisms may be of importance in regulating *XRCC4*'s other endpoints, but not in bladder cancer susceptibility.

The GT genotype of G-1394T is a significant risk factor in this study; its distribution is more frequently in bladder cancer patients than in the controls, and the data of allele frequencies indicated a similar situation (Table I and Table II). These data suggested people who with GT genotype have higher susceptibility to bladder cancer, and it is very possible that the products of GT type act as reducers of anticancer capacity. Interestingly in this polymorphism, there were no people of GG genotype found in both patients and controls. It is supposed that the GG genotype may cause some serious syndromes in early age and people who have this genotype may die young, and thus they cannot be found in the target population. According to this hypothesis, it is thought that suppose that the G allele will lower the expression or function of *XRCC4*, and one T allele will hold an overall normal capacity for *XRCC4*. If someone were of GT genotype, his total DSB repair capacity can still remove the DSBs, but to a lower or slower degree than people with TT genotype. When these people with GT genotype age, the chance of exposure to carcinogens will increase, and the DSBs remaining in their genome will also significantly rise. People with GT genotype may not have enough capacity to remove all DSBs on time, and they will have higher susceptibility to bladder cancer. In addition, it was observed that the *AXRCC4* variant genotypes were significantly associated with the risk of bladder cancer for smokers, but not for non-smokers, which reflects a gene-environment interaction (Table III). The data not only support the studies that smoking is indeed associated with Taiwanese bladder cancer (26, 27), but also provide evidence that NHEJ system may contribute to bladder carcinogenesis.

There are already plenty of approaches aimed at the genetic risk factors of bladder cancer. In addition to this report, the polymorphisms of DNA repair system have been thought to be related with various cancers (13-20). All of these findings strengthened the linkage of DNA repair systems, genome instability and carcinogenesis. This study, further corroborates this point, firstly indicating that *XRCC4* polymorphism is significantly related to bladder cancer susceptibility. This finding will be useful in revealing the genetic characteristics of bladder cancer, and finding the more efficient strategies to prevent it.

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