Interaction between Polymorphisms of DNA Repair Genes Significantly Modulated Bladder Cancer Risk

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Abstract

DNA repair is a primary defense mechanism against damage caused by exogenous and endogenous sources. We examined the associations between bladder cancer and 7 polymorphisms from 5 genes involved in the maintenance of genetic stability (MMR: MLH1-93G>A; BER: XRCC1-777T>C and Arg399Gln; NER: XPC Lys939Gln and PAT +/-; DSBR: ATM G555T and XRCC7 G6721T) in 302 incident bladder cancer cases and 311 hospital controls. Genotyping was done using a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique. The homozygous variant of XRCC7 G6721T (Odds Ratio [OR]: 2.36; 95% Confidence Interval [CI]: 1.13-4.92) was associated with increased bladder cancer risk. In an analysis of combined genotypes, the combination of XRCC1 Arg399Gln (Gln allele) with XRCC7 T allele (double strand break repair [DSBR]), the bladder cancer risk dramatically increased (OR: 4.42; 95% CI: 1.23-15.87). Our results suggest that there are multigenic variations in the DNA repair pathway involved in bladder cancer susceptibility, despite the existence of ethnic group differences.

Key words: Polymorphism, DNA repair, Ataxia telangiectasia mutated, MutL homolog 1, Transitional cell carcinoma, Multigenic variations.

Introduction

Bladder cancer is the fourth most common cancer in men and the tenth most common cancer in women in the United States[1]. Cancer of the urinary system is the most common cancer in men in China[2]. The established risk factors for bladder cancer include cigarette smoking, occupational exposure to certain chemical carcinogens such as aromatic amines and uptake of drugs such as phenacetin and cyclophosphamide. These carcinogens can cause DNA damage, introducing bulky adducts, crosslinks and single or double strand breaks[3].

DNA damage repair is the primary defense mechanism against mutagenic exposures. There are four major DNA-repair pathways in human cells: nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and double strand break repair (DSBR) [4]. The NER pathway mainly removes bulky DNA adducts typically generated by
exposure to polycyclic aromatic hydrocarbons in tobacco smoke. The MMR pathway corrects incorrectly paired bases during DNA replication errors. The BER pathway is responsible for removal of oxidized DNA bases that may arise from endogenous or exogenous agents. The DSBR pathway is responsible for repairing double strand breaks caused by a variety of exposures, including ionizing radiation, free radicals, etc. There are two distinct and complementary pathways for DSBR: homologous recombination (HR) and non-homologous end joining (NHEJ).

There are currently over 100 known DNA repair genes, and most are known to display variation in humans[5]. These genetic variations are involved in the maintenance of genome integrity and have been identified and reported in public databases. The NER pathway plays an important role in the repair of bulky lesions, such as pyrimidine dimers, photoproducts, larger chemical adducts and cross-links, and in the maintenance of genomic stability. Xeroderma pigmentosum complementation group C (XPC) is a component of the NER pathway[6, 7]. The two most common polymorphisms, Lys939Gln (rs2228001) in exon 15 and a poly (AT) insertion/deletion polymorphism in intron 9, have been associated with an increased risk of many human malignancies [8]. X-ray repair cross-complementing group 1 (XRCC1) is involved in the DNA BER pathway and plays a critical role in recruiting a complex of DNA repair proteins[9]. Codons 399 contain polymorphisms that result in amino acid substitutions within evolutionarily conserved regions [10]. MutL homolog 1 (MLH1) is the key component of the MMR system, which participates in the recognition of nucleotide mismatches occurring during DNA replication and in the recruitment of additional repair proteins to the site to correct the replication error[11]. Protein kinase DNA-activated catalytic polypeptide (XRCC7) encodes the catalytic subunit of a nuclear DNA-dependent serine/threonine protein kinase (DNA-PK). The protein mainly participates in the recognition and repair of double strand breaks via the non-homologous end joining mechanism[12]. Genetic variants of the XRCC7 6721G>T, which is located in intron 8, might regulate splicing and cause mRNA instability [13]. Ataxia telangiectasia mutated (ATM) belongs to the PI3/PI4-kinase family. This protein functions as a regulator of a wide variety of downstream proteins, including tumor suppressor proteins p53 and BRCA1, checkpoint kinase CHK2 and DNA repair protein NBS1[14]. Master controllers of cell cycle checkpoint signaling pathways are thought to be required for both cell response to DNA damage and genome stability[15]. Some studies have proposed a phenotypic effect for the common ATM missense variation 5557G>A that was associated with breast cancer risk[16,17].

Recent molecular epidemiological studies in different populations have shown inconsistent associations between the mentioned polymorphisms and an increased risk for bladder cancer[18-21]. To our knowledge, polymorphisms of MLH1 and ATM have not been reported to be associated with bladder cancer risk in the population of Southwest China. In addition to environmental factors, differences in susceptibility to cancer are also dependent on the genetic variation within different ethnic groups. Therefore, this study evaluated the association between bladder cancer risk and 7 critical single nucleotide polymorphisms (SNPs) in 4 DNA repair pathways: 1) MMR: MLH1-93G>A (rs1800734); 2) BER: XRCC1-77T>C (rs3213245) and Arg399Gln (rs25487); 3) NER: XPC Lys939Gln (rs2228001) and PAT +/- and (4) DSBR: ATM G5557A (rs1801516) and XRCC7 G6721T (rs7003908). These polymorphisms displayed to be associated with different human solid malignancy tumors[17, 22-24]. Based on multiple steps in carcinogenesis, above mentioned variants interaction would be evaluated in modulating bladder cancer risk in current population.

Materials and Methods

Study subjects

A total of 302 patients with pathologically confirmed transitional cell carcinoma of the bladder from the southwest hospital and 311 healthy control subjects were recruited in Grand Chongqing region without any gender or age restrictions between January 2007 and February 2010. The study subjects were southwestern Han Chinese and were permanently residing in Chongqing, China. Written informed consent was obtained from each participant for personal interviews and blood samples. Age, gender and smoking status were registered for all patients and controls. The studies were approved by the local ethics committees.

Genotyping

Each subject blood was collected in tubes containing ethylenediaminetetra-acetic acid (EDTA) and was stored at 4°C for genomic DNA extraction with TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). Genotyping for polymorphisms in XPC Lys939Gln, XRCC1-77T/C and Arg399Gln, XRCC7 6721G/T (rs7003908), hMLH1-93 G/A and ATM (G5557A) was performed using polymerase chain reaction restriction fragment length polymorphism
(PCR-RFLP) technique. PCR was used to amplify the fragments that contained intron 9 polymorphism of the XPC (an 83 bp poly [AT] insertion with a 5 bp deletion of GTAAC). The PCR products were electrophoresed in a 3% agarose gel. PAT -/- (the wild-type) has a 266 bp fragment. PAT+/- (the polymorphic type) has a 344 bp fragment. PAT +/- (the heterozygous type) has both fragments. One hundred nanograms of the extracted blood DNA were amplified in a PCR reaction containing 10xPCR buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl) 5 μl, 25 mM MgCl2 3 μl, dNTP Mixture (each 2.5 mM) 4μl, 200 nM of each primer (Table 1) and 5 μl TaKaRa Taq 0.25 ul (TaKaRa Taq Code: DR001A) in a final volume of 25 ul. PCR products were digested with the appropriate restriction endonucleases (New England Biolabs, Beverly, MA) that recognized and cut either wild type or variant sequences. The digested PCR products were separated by electrophoresis in 2-4% agarose gel and stained with ethidium bromide for visualization under ultraviolet (UV) light. To confirm the genotype ascribed by PCR-RFLP, 15% of the PCR-amplified DNA samples were examined by direct DNA sequencing (Invitrogen, Shanghai, China), and the results displayed 100% concordance (data not shown).

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software (version 13.0, SPSS Inc., Chicago, Illinois) was used for data analysis. Differences between cases and controls in selected demographic characteristics including gender and smoking status were evaluated by the Chi-square (χ2) test. The rank sum test was performed to evaluate the difference in age between cases and controls. Before analysis of disease risk associations was performed, we used the Hardy-Weinberg equilibrium (HWE) to test the genotype and allele frequencies by a goodness-of-fit χ2 test, with one degree of freedom to compare observed and expected genotype frequencies among cases and controls. Univariate and multivariate Logistic regression analyses with adjustment for age, gender and smoking status were used to estimate the associations between each genotype or genotype combinations and risk of bladder cancer by computing the crude and adjusted odds ratios and 95% confidence intervals. The genotype data were further stratified by smoking status. For all the genes, the homozygous and heterozygous carriers of the polymorphisms were classified as polymorphic genotypes and combined in statistical analysis for small numbers of homozygous polymorphisms. Two-sided tests of statistical significance were conducted, and a p-value of less than 0.05 was regarded as statistically significant.

Results

Characteristics of subjects

Characteristics of the study population are summarized in Table 2. Distributions of age and gender were comparable among cases and controls. The cases had a significantly higher percentage of smokers (52.3%) than the controls (34.7%) (p<0.001).

Almost all the genotype distributions among controls were consistent with the HWE (p>0.05) except for the intron 9 polymorphism of the XPC (PAT +/-) (controls: PAT -/- 200; PAT +/- 79; PAT +/- 32; χ^2=24.84, p<0.05; cases: PAT -/- 161; PAT +/- 93; PAT +/- 48; χ^2=24.33, p<0.05). HWE deviation for PAT +/- may have been related to limited sample size. In the current study, the ATM G5557A (rs1801516) polymorphism was not found.

Analyses of single genotypes

For each SNP, the distribution of gene variants in cases and controls and the crude and adjusted ORs and 95% CIs for bladder cancer risk are provided in Table 3. In univariate regression analysis, the homozygous for the XRCC7 T/T and XRCC7 T/T combined with G/T were significantly associated with bladder cancer risk (OR: 2.53; 95% CI: 1.23−4.92; χ^2=24.84, p<0.05; cases: PAT -/- 161; PAT +/- 93; PAT +/- 48; χ^2=24.33, p<0.05). HWE deviation for PAT +/- may have been related to limited sample size. In the current study, the ATM G5557A (rs1801516) polymorphism was not found.

Analyses of combined genotypes from different repair pathways

To determine whether the combined effect of two polymorphisms in DNA repair genes might modify the risk of developing bladder cancer, we analyzed combinations of multilocus genotypes (Table 4). Our results demonstrated that some of the combined variants displayed a trend to be more susceptible to bladder cancer than the two wild-type genotypes after adjustment of ORs for age, gender and smoking. Interestingly, the combination of the XRCC7 G 6721T (T allele)/XPC Lys939Gln (Gln allele) was associated with a more than 4-fold increase in bladder cancer risk (OR: 4.42; 95% CI: 1.23−15.87; p=0.023). The XRCC7 T allele combined with the XPC Lys939Gln...
had a significantly increased bladder cancer risk (OR: 3.78; 95% CI: 1.05−13.68; p=0.043). The combination of XRCC1Arg399Gln (Gln allele) with the XRCC1-77 T/T led to an increase in risk with ORs of 1.61 (95% CI: 1.10−2.36; p=0.014).

The DNA repair genes’ polymorphisms were further analyzed with the subjects stratified by smoking status. However, no association was observed between study polymorphisms in DNA repair genes and increased risk for bladder cancer in smokers (data not shown).

### Table 1 Details of RFLPs studied.

| Gene     | Primers sequence | Product size | Restriction enzyme |
|----------|------------------|--------------|--------------------|
| hMLH1    | F: 5’ -AGTAGCGGCTTCAGGGA-3’ | 259 bp | Pvu II |
| -93G/A   | R: 5’ -CTCGTCCAGCCGGAATAA-3’ |       |        |
| XRCC1    | F: 5’ -CGAATTTCTTCCAGACACCA-3’ | 287 bp | BsrB I |
| -77C/T   | R: 5’ -ATTCCCTGTGCCTCTC-3’ |       |        |
| XRCC1    | F: 5’ -TCTCCTTGTCCTCACAATT-3’ | 402 bp | Msp I |
| Arg399Gln| R: 5’ -AGTAGTCTGCTGGCTCTGG-3’ |       |        |
| XPC      | F: 5’ -GGAGGTGGACTCTCTTCTG-3’ | 765 bp | Pvu II |
| Lys939Gln| R: 5’ -TAGATCCCAGCAGATGACC-3’ |       |        |
| PAT+/-   | R: 5’ -GTGAAATAGCTTATAAGCT-3’ |       |        |
| XRCC7    | F: 5’ -GGCTGCAAAGGTCTTTC-3’ | 368 bp | Pvu II |
| G6721T   | R: 5’ -TGCCCTTAGTGGTTCCCTGG-3’ |       |        |
| ATM      | F: 5’ -GAT TCA TGA TAT TTT ACT CT-3’ | 88 bp | Dde I |
| G5557 A  | R: 5’ -AAG ACA GCT GGT GAA AAA TC-3’ |       |        |

* A mismatch to create the Dde I digestion site.

### Table 2 Demographic characteristics of bladder cancer cases and controls.

| Characteristic          | Controls (n=311) | Cases (n=302) | P<sup>a</sup> |
|-------------------------|------------------|---------------|---------------|
| Age, years Median (P25,P75) | 61(48.5,73) | 63(53,72) | 0.224 |
| 20-39                   | 5(1.6)          | 21(7.0)      |               |
| 40-59                   | 140(45.0)       | 97(32.1)     |               |
| >60                     | 166(53.4)       | 184(60.9)    |               |
| Gender (%)              |                 |               | 0.988         |
| Men                     | 247(79.4)       | 240(79.5)    |               |
| Women                   | 64(20.6)        | 62(20.5)     |               |
| Smoking status<sup>b</sup> |               | <0.001<sup>c</sup> |   |
| Never                   | 203(65.3)       | 144(47.7)    |               |
| Ever                    | 108(34.7)       | 158(52.3)    |               |

* Rank sum test was performed to evaluate the difference in age between cases and controls. Chi-square (χ2) test for gender and smoking status between cases and controls. <sup>b</sup> Those subjects who had smoked more than 100 cigarettes in their lifetime were considered ever smokers, and the others were never smokers.  
<sup>c</sup> P<0.05.

### Table 3 Distribution of gene polymorphisms among controls and cases.

| Genotype     | Controls (n=311) | Cases (n=302) | χ² | P<sup>-value</sup> | Crude OR | 95% CI | Adjusted<sup>a</sup> OR | 95% CI | P |
|--------------|------------------|---------------|----|---------------------|-----------|-------|-------------------------|-------|---|
| MLH1-93G>A   |                  |               |    |                     |           |       |                         |       |   |
| G/ G         | 43(13.8)         | 41(13.6)      | 1.00 | 1.00 | 1.00 | 1.00 |                         |       |   |
| G/ A         | 163(52.4)        | 161(53.3)     | 1.03 | (0.64,1.67) | 0.885 | 0.93 | (0.57,1.53) | 0.782 |
| A/ A         | 105(33.8)        | 100(33.1)     | 0.975 | 1.00 | (0.60,1.66) | 0.996 | 0.97 | (0.58,1.63) | 0.898 |
| G/ A+ A/ A   | 268(86.2)        | 261(86.4)     | 0.928 | 1.02 | (0.64,1.62) | 0.928 | 0.95 | (0.59,1.52) | 0.818 |

<sup>a</sup> P<0.05.
### Table 4 Interaction between genotypes and modulation of bladder cancer risk.

| Genotype                  | Controls (n=311) (%) | Cases (n=302) (%) | $\chi^2$ P-value | Crude OR 95%CI | Adjusted* OR 95%CI | P-value |
|---------------------------|----------------------|-------------------|------------------|----------------|---------------------|---------|
| **XRCC1-77T>C**           |                      |                   |                  |                |                     |         |
| T/ T                      | 229(73.6)            | 232(76.8)         | 1.00             | 1.00           | 1.00                |         |
| T/ C                      | 76(24.4)             | 61(20.2)          | 0.79 (0.54,1.16) | 0.234          | 0.279 (0.54,1.17)   | 0.239   |
| C/ C                      | 6(1.9)               | 9(3.0)            | 0.347            | 1.48 (0.52,2.43) | 0.463 (0.45,3.82)   | 0.628   |
| T/ C + C/ C              | 82(26.3)             | 70(23.2)          | 0.361            | 0.84 (0.58,1.22) | 0.361 (0.57,1.21)   | 0.332   |
| **XRCC1 Arg399Gln**       |                      |                   |                  |                |                     |         |
| Arg/ Arg                  | 148(47.6)            | 121(40.0)         | 1.00             | 1.00           | 1.00                |         |
| Arg/ Gln                  | 143(46.0)            | 151(50.0)         | 1.29 (0.93,1.80) | 0.131          | 1.26 (0.90,1.77)    | 0.186   |
| Gln/ Gln                  | 20(6.4)              | 30(10.0)          | 0.093            | 1.84 (0.99,3.39) | 0.053 (0.98,3.45)   | 0.057   |
| Arg/ Gln+ Gln/ Gln        | 163(52.4)            | 181(60.0)         | 0.061            | 1.36 (0.99,1.87) | 0.061 (0.96,1.84)   | 0.090   |
| **XPC Lys939Gln**         |                      |                   |                  |                |                     |         |
| Lys/ Lys                  | 138(44.4)            | 118(39.1)         | 1.00             | 1.00           | 1.00                |         |
| Lys/ Gln                  | 138(44.4)            | 136(45.0)         | 1.15 (0.82,1.62) | 0.415          | 1.14 (0.80,1.62)    | 0.464   |
| Gln/ Gln                  | 35(11.3)             | 48(15.9)          | 0.178            | 1.60 (0.97,2.65) | 0.064 (0.98,2.73)   | 0.059   |
| Lys/ Gln+ Gln/ Gln        | 173(55.7)            | 184(60.9)         | 0.184            | 1.24 (0.90,1.72) | 0.184 (0.89,1.72)   | 0.202   |
| **XRCC7 G 6721T**         |                      |                   |                  |                |                     |         |
| G/ G                      | 25(8.0)              | 12(4.0)           | 1.00             | 1.00           | 1.00                |         |
| G/ T                      | 134(43.1)            | 105(34.8)         | 1.63 (0.78,3.40) | 0.191          | 1.55 (0.73,3.28)    | 0.252   |
| T/ T                      | 152(48.9)            | 185(61.3)         | 0.004            | 2.54 (1.23,5.21) | 0.011 2.36 (1.13,4.92) | 0.022   |
| G/ T + T/ T              | 286(92.0)            | 290(96.0)         | 0.038            | 2.11 (1.04,4.29) | 0.038 1.98 (0.96,4.07) | 0.064   |

* Adjusted for age, gender, and smoking.
restingly, emerging evidence suggests that genetic polymorphisms in DNA repair genes may account for this diversity as the allele frequencies of the XRCC7 G6721T polymorphism have been reported to vary dramatically among different ethnic groups. For example, GG, GT and TT genotype frequencies were 8.0%, 43.1% and 48.9%, respectively, in our southwestern Han Chinese sample, compared with 21.6%, 46.4% and 32.0%, respectively, in the North Indian study population. These observations suggest that ethnic variations may modify the susceptibility of different ethnic groups.

**Discussion**

In this investigation, we showed that combinations of genetic variation in four DNA repair pathways, which are responsible for protecting against DNA damage caused by chemical carcinogens, significantly contributed to increased bladder cancer risk in a population in southwest China. Of the 7 SNPs in 5 repair-related genes evaluated, most were not found to be associated with bladder cancer risk, with the exception of XRCC7 G6721T. However, combined polymorphisms for XRCC7 G 6721T (T allele)/XPC Lys939Gln (Gln allele) dramatically increased bladder cancer risk.

In the individual genotype analysis, individuals who were both homozygous for the XRCC7+ 6721 T/T genotype and heterozygous for the XRCC7 G6721T genotype had an increased risk of developing bladder cancer. The association between polymorphisms in the XRCC7 gene and bladder cancer risk has been investigated in previous studies. Our finding is consistent with the results of a previous study of XRCC7 polymorphisms G 6721T in bladder cancer in a Chinese population[25]. In contrast, Gangwar et al. found that the XRCC7 + 6721 CG genotype was associated with increased susceptibility to urothelial bladder cancer in an Indian population[26]. Ethnic variations may account for this diversity as the allele frequencies of the XRCC7 G6721T polymorphism have been reported to vary dramatically among different ethnic groups. For example, GG, GT and TT genotype frequencies were 8.0%, 43.1% and 48.9%, respectively, in our southwestern Han Chinese sample, compared with 21.6%, 46.4% and 32.0%, respectively, in the North Indian study population. These observations suggest that ethnic variations may modify the susceptibility of different ethnic groups.

**XPC polymorphisms play a role in tissue-specific carcinogenesis.** Most reports indicate that XPC polymorphisms modulate the risk for lung, neck and head, breast, and bladder cancer. The XPC protein plays a key role in global NER by recognizing the distortion of damaged DNA. This pathway repairs bulky adducts, such as those induced by tobacco chemical carcinogens. Interestingly, emerging evidence suggests an additional role for XPC in the removal of oxidative damage[27] and its involvement in cell cycle regulation in the DNA damage response[28]. We didn’t observe that XPC Lys939Gln was associated with increased bladder cancer risk. This finding is consistent with the results of a previous study of XPC Lys939Gln polymorphisms in bladder cancer[18]. Although some studies showed the XPC Lys939Gln polymorphism significantly elevated the human bladder cancer risk, a recent pooled data meta-analysis indicated that the XPC Lys939Gln polymorphism was not related to bladder cancer risk except for XPC A499V[29].

One study has reported that the ATM G5557A polymorphism was associated with increased susceptibility to urothelial bladder cancer in nonsmokers[30]. The allele ratio for ATM 5557A was 6% in a South American population; ATM 5557A
was also significantly associated with breast cancer risk in a European population[16]. In our study, there was no ATM G5557A polymorphism found among southwestern Han Chinese. The results suggest that gene polymorphisms vary dramatically among different ethnic groups. Although the polymorphism of MLH1 was reported to convey some solid tumor risk[22,31,32], we know no evidence that the polymorphism of MLH1-93G>A increases bladder cancer risk. Our study indicated that the MLH1-93G>A polymorphism was not associated with bladder cancer risk in this population in southwest China.

Given that carcinogenesis is usually a multistep, multigenic process, it is unlikely that one individual genetic polymorphism would have a significant effect on cancer risk. Therefore, single-gene studies are likely to provide limited value in evaluating bladder cancer risk. Evaluation of the combined effects of a panel of polymorphisms that interact in different DNA repair pathways may amplify the effects of individual variation and enhance predictive power. In our analysis of combined genotypes, we found a significant trend of increased risk with increasing numbers of adverse alleles in the same genes and different DNA repair genes combined. For example, single XRCC1-Arg399Gln or XRCC1-77 C/T polymorphisms were not found to be associated with bladder cancer in the current population. However, the combinations of the XRCC1-Arg399Gln (Gln allele) with the XRCC1-77 T/T led to an increase in risk with an OR of 1.61 (95% CI: 1.10−2.36; p=0.014). Although we didn’t observe that XPC Lys939Gln was associated with increased bladder cancer risk, the XPC Lys939Gln increased the effect of other gene variation on bladder cancer risk. When the XPCArg399Gln (Gln allele) (NER) was present together with XRCC7 T (allele) (DSBR), the bladder cancer risk dramatically increased (OR: 4.42; 95% CI: 1.23-15.87). In contrast, regardless of whether it was single or in combination with other study genes, the MLH1-93G>A polymorphism did not increase the bladder cancer risk in the study population.

In summary, the current study demonstrated that the XRCC7+ 6721 T/T polymorphism independently increased the risk of bladder cancer in a population of southwest Han Chinese. More importantly, the combined influence of multiple SNPs in the XRCC7, XRCC1, and XPC genes of different DNA repair pathways led to dramatically increased bladder cancer risk. These results are based on a limited number of SNPs in one center and need replication by multiple study centers in the future.

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Competing Interests

The authors have declared that no competing interest exists.

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