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Association of Nijmegen Breakage Syndrome 1 Genotypes With Bladder Cancer Risk

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Abstract. *Background/Aim: We aimed to examine the association of the genotypes of Nijmegen breakage syndrome 1 (NBS1), a critical gene in DNA double strand break repair machinery, with bladder cancer risk in Taiwan. Materials and Methods: NBS1 rs1805794 genotypes among 375 bladder cancer patients and 375 non-cancer healthy controls were determined via the polymerase chain reaction-restriction fragment length polymorphism methodology and their association with bladder cancer risk were evaluated. Results: The results showed that the percentages of GG, CG and CC of NBS1 rs1805794 genotypes were 45.4%, 43.7% and 10.9% in the bladder cancer patient group and 47.2%, 43.2% and 9.6% in the non-cancer control group, respectively (p for trend=0.7873). The analysis of allelic frequency distributions showed that the variant C allele of NBS1 rs1805794 does not contribute to an increased bladder cancer susceptibility (p=0.5066). Conclusion: The genotypes of NBS1 rs1805794 are not closely associated with personal susceptibility to bladder cancer.*

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Statistically, bladder cancer (BLACA) is worldwide the sixth and seventeenth most prevalent cancer in men and women, respectively, with nearly 500,000 newly diagnosed cases in 2018 (1). In Taiwan, the incidence of BLACA is the ninth and fourteenth most common cancer in men and women, respectively, as recorded in the most updated government annual reports (2). From the viewpoints of epidemiology, the high incidence of BLACA is due to both environmental and genetic factors. As for the environmental factors, it is believed that smoking, exposure to chemicals and PM2.5, prior radiation therapy, and frequent bladder infections contribute to BLACA risk (3, 4). Although family history of the disease has supported the idea that genetic factors also play a role in BLACA risk determination, interestingly, the contribution of genetic factors and the underlying etiology of BLACA remains largely unclear. In the past decades, translational studies were focused into examining useful biomarkers for the prediction of BLACA (5-10), while genetic markers are urgently needed for various populations all over the world.

Among the various kinds of DNA repair proteins, the DNA repair and telomere maintenance protein nbs1, named for its underlying role in the Nijmegen breakage syndrome, plays a critical role in the DNA double strand break (DSB) repair machinery. As an early response to DNA DSBs, histone H2AX in the vicinity of DSBs is phosphorylated by Ataxia telangiectasia mutated (ATM) (11). Then, NBS1 targets the MRE11/RAD50 complex to the sites of DSBs *via* binding with the FHA/BRCT domain of the phosphorylated gamma-H2AX

(12). After that, the NBS1 complex directly binds to the damaged-DNA, initiating the DSB repair (13). In addition, NBS1 also teams up with ATM to control the cell cycle through the phosphorylation of SMC, CHK2 and FANCD2 (14-16). Thus, NBS1 plays a critical role not only in the maintenance of DNA integrity, but also in cell cycle regulation. Furthermore, NBS1 has also been reported to be involved in maintaining the length of telomeres, which have DSB-like structures and in which defects are closely associated with increased genomic instability and aging dysregulation (17, 18).

The *NBS1* gene, also called nibrin and NBN, is located in the human chromosome 8q21 (19, 20). In heterozygous NBS1 (+/−) mice, the tumor formation rates and ionizing radiation sensitivity are both much higher compared to wild-type mice, indicating that *NBS1* plays a critical role in DSB repair and carcinogenesis (21). In the *NBS1* gene, the most commonly investigated polymorphism is rs1805794 (Glu185Gln, E185Q), which has been widely studied regarding its association with susceptibility to several types of cancer including nasopharyngeal cancer (22), lung cancer (23-26), breast cancer (27), colorectal cancer (28), prostate cancer (29), and leukemia (30). Up to now, there is limited literature regarding the contribution of *NBS1* genotypes to bladder cancer risk (31-33). However, the results reported by these studies remained inconclusive, and none of them investigated the *NBS1* genotypes in Eastern countries. In this study, we aimed at examining the association between single nucleotide polymorphisms (SNPs) at the *NBS1* rs1805794 with the risk of bladder cancer in Taiwan.

Materials and Methods

Bladder cancer patients and non-cancer controls. This hospital-based case-control study was approved by the Institutional Review Board of China Medical University Hospital (DMR104-IRB-158) and all participants have provided written-informed consents. All clinical and pathological records were restrictively reviewed according to the principles expressed in the Declaration of Helsinki. Briefly, three hundred and seventy-five cases diagnosed with bladder cancer were recruited in this study, after completing a comprehensive questionnaire and providing 3 to 5 ml of their peripheral blood. An equal number (375) of non-cancer healthy individuals were obtained from the Health Examination Cohort of our hospital from an original pool of 15,000 subjects by matching for age, gender and smoking status. The exclusion criteria of the control subjects were defined as previously published (5-7). In brief, subjects with previous malignancy, metastasized cancer from another site or a tumor of unknown origin, or with any familial or genetic disease were excluded from the control group. As mentioned above, all participants completed a short questionnaire regarding personal characteristics, especially regarding their individual environmental exposures and life styles, such as smoking and alcohol drinking habits. Ever smokers were defined as daily or almost daily smokers who had smoked at least five packs of cigarettes per year in their lifetime for at least one year. The ever alcohol drinkers were defined as those who were twice drunken or had more than three cups per week for at least one year. The drunken status is defined as the loss of control in straight walking. Overall, the selective demographic characteristics of all the individuals investigated are summarized in Table I.

NBS1 rs1805794 genotyping conditions. Genomic DNA from the peripheral blood leucocytes of each patient and control was extracted using the QIAamp Blood Mini Kit, stored and processed as reported in our previous articles (34-36). The specific primer sequences of the forward and reverse primers of *NBS1* rs1805794, were 5'-TGTGCTCTTCTGACCATGAG-3' and 5'-CAGTGA CCAAAGACCGACTT-3', respectively. The specific polymerase chain reaction (PCR) cycling conditions for *NBS1* rs1805794 genotyping were set as one cycle at 94˚C for 5 min; followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec; and finally an extension step at 72˚C for 10 min. The PCR products were cut by the restriction enzyme *Hinf* I (New England BioLabs), overnight. The DNA adducts carrying the digestible C allele were cut into 321- and 255-base pair contigs, while those carrying the indigestible G allele remained intact with 576-base pair long contigs. The genotypic process was performed by at least two researchers independently and blindly at least twice. Also, PCR samples from 20 cases and 20 controls were directly sequenced and the results obtained along with those from the PCR–restriction fragment length polymorphism were 100% concordant.

Statistical analysis. The Student's *t*-test was adopted in the age (continuous variable) comparison between the case and control groups. The Pearson's chi-square was used for comparing the distributions of age, gender, personal habits, *NBS1* SNP genotypes and alleles among the subgroups. The associations between *NBS1* genotypes and bladder cancer risk were estimated with individual odds ratios (ORs) and 95% confidence intervals (CIs). A *p*-value less than 0.05 was identified as statistically significant.

Results

The demographic characteristics including age, gender, personal habits of all participants and the stage and grade of the 375 bladder cancer patients are summarized in Table I. First, the average age of the controls and bladder cancer patients were 62.9 and 61.4 years, respectively. The ratio of male *versus* female bladder cancer patients was about 3:1 (Table I). We adopted the matching strategy about their age, gender, smoking and alcohol drinking habits to recruit the same number of non-cancer healthy controls, and there was no difference in age, gender, cigarette smoking and alcohol drinking status between the two groups (p=0.7315, 0.5525, 0.3063 and 0.3807, respectively). As for the stage and grade of the bladder cancer patients, the percentages of non-muscle-invasive and muscle-invasive types were 62.7% and 37.3%, respectively; while those with low and high grades were 40.3% and 59.7%, respectively (Table I).

The distributions of the *NBS1* genotypes at rs1805794 among the non-cancer controls and the bladder cancer patients are presented in Table II. The results showed that the genotypes of *NBS1* rs1805794 were not -differentially

Table I. *Basic characteristics of the 375 bladder cancer patients and 375 non-cancer controls.*

Character	Controls (n=375)			Cases (n=375)			*p*-Value
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			62.9 (9.8)			61.4 (10.3)	0.7315[a]
Age group (years)							0.7108[b]
≤55	152	40.5%		158	42.1%		
>55	223	59.5%		217	57.9%		
Gender							0.5525[b]
Male	287	76.5%		279	74.4%		
Female	88	23.5%		96	25.6%		
Personal habits							
Cigarette smoking	186	49.6%		201	53.6%		0.3063[b]
Alcohol drinking	176	46.9%		189	50.4%		0.3807[b]
Stage							
Non-muscle-invasive				235	62.7%		
Muscle-invasive				140	37.3%		
Grade							
Low				151	40.3%		
High				224	59.7%		

SD: Standard deviation; [a]based on Student's *t*-test; [b]based on Chi-square test.

Table II. *Distribution of NBS1 rs1805794 genotypes among bladder cancer patients and healthy controls.*

rs1805794	Controls		Patients		OR (95%CI)	*p*-Value[a]
	n	%	n	%		
Genotype						
GG	177	47.2%	170	45.4%	1.00 (reference)	
CG	162	43.2%	164	43.7%	1.05 (0.78-1.43)	0.7330
CC	36	9.6%	41	10.9%	1.19 (0.72-1.94)	0.4993
P_{trend}						0.7873
Carrier analysis						
GG+CG	339	90.4%	334	89.1%	1.00 (reference)	
CC	36	9.6%	41	10.9%	1.16 (0.72-1.85)	0.5475
GG	177	47.2%	170	45.3%	1.00 (reference)	
CG+CC	198	52.8%	205	54.7%	1.08 (0.81-1.44)	0.6082

[a]Based on chi-square test without Yates' correction; *p<0.05; OR: odds ratio; CI: confidence interval.

distributed between bladder cancer and non-cancer control groups (p for trend=0.7873) (Table II). In detail, *NBS1* rs1805794 heterozygous CG and homozygous CC are not associated with increased bladder cancer risk (OR=1.05 and 1.19, 95%CI=0.78-1.434 and 0.72-1.94, p=0.7330 and 0.4993, respectively) (Table II). We further performed a carrier analysis, and the results showed that in both dominant and recessive models, the distributions of NBS1 rs1805794 genotypes were not significantly different between the bladder cancer and control groups (Table II).

In order to further validate the findings in Table II, we also conducted an analysis of allelic frequency distribution for the *NBS1* rs1805794 among the investigated population, and the results are summarized in Table III. Supporting the findings that neither the heterozygous variant CG nor the homozygous variant CC genotype at *NBS1* rs1805794 is responsible for altered risk of bladder cancer, the C allele was not present at a significantly higher rate in the cases compared to controls (p=0.5066) (Table III).

Table III. *Distribution of NBS1 rs1805794 allelic frequencies among bladder cancer patients and healthy controls.*

rs1805794	Controls	%	Patients	%	OR (95%CI)	*p*-Value[a]
Allele G	516	68.8%	504	67.2%	1.00 (reference)	
Allele C	234	31.2%	246	32.8%	1.07 (0.87-1.34)	0.5066

[a]Based on chi-square test; *$p<0.05$. *Statistically significant; OR: odds ratio; CI: confidence interval.

Discussion

The DNA DSB repair protein NBS1 encompasses lots of DNA damage sensors, signal transducers, and effectors, which enable our cells to maintain DNA integrity and genomic stability. One of its exonic polymorphisms, rs1805794, has been widely studied in case–control association studies for various types of cancer, however, the results were inconclusive. In the current hospital-based case–control association investigation, we focused on evaluating the contribution of *NBS1* rs1805794 genotype to bladder cancer risk. After examining the bladder cancer cases and non-cancer healthy controls, their genotypic results showed that neither heterozygous CG nor homozygous CC genotype of *NBS1* rs1805794 was significantly associated with risk of bladder cancer (Table II). In addition, the allelic frequency analysis also supported the findings of the genotypic frequency analysis indicating that the variant C allele at *NBS1* rs1805794 was not associated with bladder cancer risk (Table III). This negative finding is not consistent with the previous findings showing that *NBS1* rs1805794 was associated with bladder cancer risk, and the association was limited to ever smokers along was dependent on smoking dose and smoking duration (37). The inconsistency may come from the fact that a different population was investigated, and further validations in larger sample sizes and different populations are needed.

Gender difference is reported to be a risk factor for bladder cancer (38-40), especially for the non-invasive subtype (41-43). Although the underlining mechanism(s) for this gender-specific difference in bladder cancer risk has not been revealed, it is thought that sex steroids play a critical role in the etiology of bladder cancer (42). We are also interested in whether the genotype of *NBS1* rs1805794 contributes to the gender difference in bladder cancer susceptibility. After stratification by gender, it was found that the genotypes of *NBS1* rs1805794 were neither differently distributed among the males, nor among the females (data not shown). Interesting, after considering age and estrogen exposure status, women had a 1.58-fold higher risk of getting bladder cancer than men in the Taiwanese population (data not shown).

The contribution of smoking to bladder cancer risk is not so obvious as in other types of cancer, such as upper urinary tract urothelial carcinoma (44). The joint effects of genetic variation and smoking on bladder cancer is seldom examined. In 2017, Fu and his colleagues have investigated the gene-smoking interaction on bladder cancer risk in a Chinese population (45). They proposed that a haplotype containing the rs2010963-C and rs833052-A alleles of the *vascular endothelial growth factor* (*VEGF*) gene is associated with increased bladder cancer risk, and the genotypes of *VEGF* rs2010963 have a joint effect with smoking status on determining bladder cancer risk (45). In literature, it has been reported that tobacco smoking can induce lots of DNA lesions in the cells and defects in repair of tobacco carcinogen-induced DNA adducts may contribute to carcinogenesis (46). Therefore, in this study, the joint effects of *NBS1* rs1805794 and smoking status were also examined and the results showed that ever smokers who carried the homologous CC genotypes at *NBS1* rs1805794 were of increased risk of bladder cancer after adjusted for age, gender, and alcohol drinking status. On the contrary, there was no significantly elevated bladder cancer risk for those non-smokers with CG or CC genotypes at *NBS1* rs1805794 (data not shown). A phenotypic assay showed that the rs1805794 C allele will attenuate the ability of the NBS1 protein to repair DNA damage as the cells transfected with a plasmid carrying the rs1805794 C allele had a significantly higher number of DNA breaks than those transfected with a plasmid carrying the rs1805794 G allele after X-ray irradiation (47). To sum up, smoking behavior appears to be more strongly associated with bladder cancer risk in women than in men, which could be related to differences in metabolism, smoking behavior, exposure patterns, and DNA repair mechanisms (48).

In conclusion, our study provides evidence that the C allele of *NBS1* rs1805794 is not associated with an increased lung cancer risk among Taiwanese. Further investigations using the cells of patients with different gender, smoking status and genotypes can help to reveal the phenotypic role of NBS1 in bladder carcinogenesis.

Conflicts of Interest

All Authors declare no conflict of interest regarding this study.

Authors' Contributions

Research Design: Chen M, Tsai CW and Chang WS; Patient and Questionnaire Summarize: Wu HC and Shen TC; Experiment Performance: Wang YC, Chen CH and Chang WS; Statistical Analysis: Wang CH, Lin ML and Gong CL; Manuscript Writing: Chen M, Tsai CW and Bau DT; Reviewing and Revising: Bau DT, Chang WS and Tsai CW.

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