ANTICANCER RESEARCH *37*: 2417-2423 (2017)
doi:10.21873/anticanres.11581

Contribution of Double-strand Break Repair Gene Nijmegen Breakage Syndrome 1 Genotypes, Gender Difference and Smoking Status to Taiwanese Lung Cancer

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Abstract. *Background/Aim: Nijmegen breakage syndrome 1 (NBS1) is a component of MRE11/RAD50/NBS1 complex (MRN) that plays a critical role in the cellular response to DNA damage and maintenance of chromosomal integrity. Failure in DNA damage response affects the level of cell survival, increases the frequency of gene mutation or chromosomal instability and other cellular phenotypic abnormalities, which are the important mechanisms of carcinogenesis. However, the contribution of variant NBS1 genotypes to lung cancer is not known. The current study aimed to evaluate the contribution of the common variant NBS1 Glu185Gln (rs1805794, E185Q) genotypes to the risk of lung cancer. Materials and Methods: The contributions of the NBS1 Glu185Gln genotypes to lung cancer risk were investigated among 358 patients with lung cancer and 716 age- and gender-matched healthy controls by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Results: GG, CG and CC NBS1 Glu185Gln genotype percentages were 45.2%, 43.9% and 10.9% in the patient group and 46.1%, 45.1% and 8.8% in the non-cancer control group, respectively (p for trend=0.5423). Analysis of allelic frequency distributions showed that the C allele of NBS1 Glu185Gln did not increase lung cancer susceptibility (p=0.4916). Interestingly, the CC genotypes at NBS1 Glu185Gln enhanced the risk of lung cancer among the males adjusted odds ratio (aOR)=1.85, 95% confidence interval (CI)=1.12-2.83 and among the smokers (aOR=1.76, 95% CI=1.09-2.64) but not among the females and non-smokers. Conclusion: The CC genotype of NBS1 Glu185Gln may increase lung cancer risk only for males and smokers and may serve as a practical marker for early detective and predictive purposes of lung cancer.*

For many years, lung cancer has been the leading cause of cancer-related mortality all over the world (1, 2). Even though many antitumor therapeutic strategies are being developed, the prognosis of patients with lung cancer remains poor, as the 5-year survival rate is still less than 20% (3). From the epidemiologic point of view, the most well-known factor implicated in lung carcinogenesis is the individual long-term habit of tobacco consumption, which is also useful for prediction of prognosis (4, 5). Although cigarettes contain various kinds of carcinogens that may increase reactive oxygen species, DNA adducts and strand breaks in cells of lung and other organs, according to epidemiological reports, show that only 10-15% of all smokers actually develop lung cancer during their lifetime, indicating that individual susceptibility to carcinogens in cigarette smoke is unpredictable (6, 7). In this decade, lots of case-control studies have reported that specific genotypes

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Key Words: Double-strand break repair, gender, genotype, lung cancer, *NBS1*, polymorphism, smoking.

are associated with higher lung cancer risk for cigarette smokers than non-smokers (8-15) and *vice versa* (16-19). These population studies elucidating the gene-lifestyle interactions on lung cancer risk, especially for smokers and non-smokers, may provide predictive systems for revealing the personalized etiology of lung cancer, personalized therapy and genomic pharmacology.

Our cells are exposed continuously to exogenous and endogenous DNA damage mechanisms, while the DNA repair system is critically responsible for removing all types of damage as soon as possible from our genome. Among the various kinds of DNA repair proteins, the DNA repair and telomere maintenance protein NBS1, named after the Nijmegen breakage syndrome gene (*NBS1*), contains three functional regions: the forkhead-associated (FHA) domain and BRCA1 C-terminus (BRCT) domain at the N-terminus, several SQ motifs (consensus phosphorylation sites by ATM and ATR kinases) at a central region and MRE11-binding region at the C-terminus. In response to DNA damage, histone H2AX, in the vicinity of double-strand breaks (DSBs), is phosphorylated by ATM (20). NBS1 then targets the MRE11/RAD50 complex to the sites of DSBs through interaction of the FHA/BRCT domain with γ-H2AX (21). After that, NBS1 complex binds directly to the damaged DNA and the repair of DSB is initiated (22). In addition to fulfill the proper DSB repair processes, ATM also regulates cell-cycle checkpoints at G_1, G_2 and intra-S phases *via* phosphorylation of SMC, CHK2 and FANCD2. The phosphorylation of these proteins also requires the involvement of NBS1 (23-25). Thus, NBS1 has at least two important roles in genome maintenance; as a DNA repair protein in the homologous recombination (HR) pathway and as a signal modifier in intra-S phase checkpoints. NBS1 is also known to be involved in maintenance of telomeres, which have DSB-like structures and defects here can cause telomeric fusion, genomic instability and aging deregulation (26, 27).

The *NBS1* gene, located on human chromosome 8q21 (28), encodes the 754-amino acid protein NBS1 (29). In a previous animal study, higher susceptibility of cancer was detected among heterozygous *Nbs1*(+/−) mice (30), which suggested that mutation in the Nijmegen breakage syndrome gene might influence cancer development. One of the most commonly investigated polymorphisms was a G to C polymorphism leading to a substitution of glutamate (Glu) with glutamine (Gln). Known as Glu185Gln (rs1805794, E185Q), this polymorphism was widely studied on the association with susceptibility of cancers, such as breast (31), bladder (32), prostate (33), colorectal (34), leukemia (35) and nasopharyngeal cancer (36). As for lung cancer, there were also several reports that investigated the contribution of *NBS1* genotypes to lung cancer susceptibility (37-39). However, the results reported by these studies remained inconsistent. In the current study, we aimed to investigate the contribution of *NBS1* genotypes at Glu185Gln (rs1805794, E185Q) single-nucleotide polymorphic (SNP) site to the risk of lung cancer and, then, examine the joint effect of smoking habit and *NBS1* Glu185Gln genotypes on lung cancer risk in Taiwan.

Materials and Methods

Investigated population. Three hundred and fifty-eight patients diagnosed with lung cancer were recruited by the surgery team at the Outpatient Clinics of General Surgery at the China Medical University Hospital during 2005-2008. The clinical characteristics of patients, including histological details, were all graded and defined by expert surgeons. The patients with history of any other cancer and pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), pneumothorax and asthma, were excluded from the databank. All participants voluntarily completed a self-administered questionnaire and provided a 3- to 5-ml sample of peripheral blood. At the same time, twice as many non-lung cancer healthy volunteers as controls were selected by matching for age, gender and smoking behavior after initial random sampling from the Health Examination Cohort of China Medical University Hospital. The exclusion criteria of the controls included previous malignancy, metastasized cancer from other or unknown origin and any genetic or familial diseases. The study was approved by the Institutional Review Board of the China Medical University Hospital with the document coded DMR100-IRB-284 and written informed consent was obtained from all participants. Several selected characteristics of the subjects collected in this study are summarized in Table I.

Genotyping conditions. Genomic DNA from peripheral blood leucocytes of each patient and controls was prepared using the QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further stored and processed as previously described (40-42). The polymerase chain reaction (PCR) cycling programs for *NBS1* genotypes were: 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 sequences of forward and reverse primers for *NBS1* Glu185Gln genotyping work were 5'-TGTGCTCTTCTG ACCATGAG-3' and 5'-CAGTGACCAAAGACCGACTT-3', respectively. The PCR products were subject to restriction enzyme *Hinf* I (New England BioLabs, Ipswich, MA, USA). The digestible C allele was cut into 321- and 255-base pair contigs, while indigestible G allele was of intact 576-base pair long contig. The genotypic process was performed by two researchers independently and blindly. Five percent of the samples for *NBS1* Glu185Gln were randomly selected for direct sequencing and the results from PCR-restriction fragment length polymorphism analysis and direct sequencing were 100% concordant.

Statistical analyses. Seven hundred and sixteen of the controls and 358 cases with genotypic and clinical data were analyzed. 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 *NBS1* SNP in the controls from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test was used to compare the distribution of the *NBS1* genotypes between the cases and the controls. The associations between the *NBS1* genotypes and

Table I. *Distribution of selected demographic data of the 358 patients with lung cancer and the 716 matched controls.*

Characteristics	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)	0.5871
Gender							0.3642
Male	488	68.1%		254	70.9%		
Female	228	31.9%		104	29.1%		0.3642
Smoking status							
Ever smokers	563	78.6%		293	81.8%		
Non-smokers	153	21.4%		65	18.2%		0.2282
Histology							
Adenocarcinoma				218	60.9%		
SCC				106	29.6%		
Other				34	9.5%		

[a]Based on Chi-square test, SCC, Squamous cell carcinoma; SD, standard deviation.

Table II. *Distribution of NBS1 Glu185Gln genotypes among the 358 patients with lung cancer and the 716 matched controls.*

Glu185Gln	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
Genotype						
GG	330	46.1%	162	45.2%	1.00 (reference)	
CG	323	45.1%	157	43.9%	0.99 (0.76-1.29)	0.9422
CC	63	8.8%	39	10.9%	1.26 (0.81-1.96)	0.3024
P_{trend}						0.5423
Carrier analysis						
GG+CG	653	91.2%	319	89.1%	1.00 (reference)	
CC	63	8.8%	39	10.9%	1.27 (0.83-1.93)	0.3307
GG	330	46.1%	162	45.2%	1.00 (reference)	
CG+CC	386	53.9%	196	54.8%	1.03 (0.80-1.33)	0.7950

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

lung cancer risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression analysis. A value of $p<0.05$ was considered statistically significant.

Results

The frequency distributions of demographic characteristics, such as age, gender and smoking status, for the 358 patients with lung cancer and 716 non-cancer controls are summarized in Table I. The average age of the patients was 64.0 years, non-significantly different from 64.8 years of the controls, while the ratio of male versus female lung cancer patients was about 7:3 (Table I). Since we applied frequency matching to recruit the non-cancer healthy controls, there was no difference in the distributions of age and gender between the control and case groups (Table I). From the histological point of view, the percentages of adenocarcinoma, squamous cell carcinoma and patients of other histology were 60.9%, 29.6% and 9.5%, respectively (Table I).

The distributions of the *NBS1* genotypes at Glu185Gln among the non-cancer controls and the patients with lung cancer are presented and statistically analyzed in Table II. The genotypes of *NBS1* Glu185Gln were not differently distributed between lung cancer and non-cancer control groups (p for trend=0.5423) (Table II). In detail, *NBS1* Glu185Gln heterozygous CG was not associated with lung cancer risk (OR=0.99, 95% CI=0.76-1.29, p=0.9422), while the homozygous CC genotype seemed to be only slightly associated with increased lung cancer risk (OR=1.26, 95% CI=0.81-1.96, p=0.3024) but not statistically significant

Table III. *Distribution of NBS1 Glu185Gln allelic frequencies among the 358 patients with lung cancer and the 716 matched controls.*

	Controls	%	Patients	%	OR (95% CI)	*p*-Value[a]
Glu185Gln						
Allele G	983	68.6%	481	67.2%	1.00 (reference)	
Allele C	449	31.4%	235	32.8%	1.07 (0.88-1.30)	0.4916

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

Table IV. *Odds ratios for NBS1 Glu185Gln genotype and lung cancer after stratified by gender.*

Genotypes	Males		aOR (95% CI)[a]	Females		aOR (95% CI)[a]
	Controls	Cases		Controls	Cases	
GG	235	116	1.00 (Reference)	95	46	1.00 (Reference)
CG	214	105	1.04 (0.73-1.46)	109	52	0.97 (0.63-1.62)
CC	39	33	1.85 (1.12-2.83)*	24	6	0.64 (0.26-1.34)
Total	488	254		228	104	

[a]The aORs were estimated with multivariate logistic regression analysis after adjusted for age, smoking, alcohol drinking and areca chewing habits. *Statistical significant. aOR, Adjusted odds ratio; CI, confidence interval.

Table V. *Odds ratios for NBS1 Glu185Gln genotype and lung cancer after stratified by smoking status.*

Genotypes	Non-smokers		aOR (95% CI)[a]	Smokers		aOR (95% CI)[a]
	Controls	Cases		Controls	Cases	
GG	68	28	1.00 (Reference)	262	134	1.00 (Reference)
CG	64	32	1.19 (0.64-2.13)	259	125	1.06 (0.73-1.35)
CC	21	5	0.82 (0.36-1.86)	42	34	1.76 (1.09-2.64)*
Total	153	65		563	293	

[a]The aORs (odds ratios) were estimated with multivariate logistic regression analysis after adjusted for age, gender, alcohol drinking and areca chewing habits. *Statistical significant. aOR, Adjusted odds ratio; CI, confidence interval.

(Table III). The results of carrier analysis indicated that, in both dominant and recessive models, the differential distribution of genotypes were not so obvious between the lung cancer and control groups (Table III).

To confirm the findings of Table III, analysis of allelic frequency distribution for the *NBS1* Glu185Gln was also conducted, with the results being summarized in Table IV. Supporting the findings that neither heterozygous CG nor homozygous CC genotype of *NBS1* Glu185Gln was associated with increased lung cancer risk, the C allele was not significantly more frequently higher in cases than in controls ($p=0.4916$) (Table III).

The joint effects of *NBS1* Glu185Gln with gender and smoking status were examined (Tables IV and V). First, lung cancer patients and controls were stratified according to their gender and ORs were analyzed. The results showed that males carrying the homologous CC genotypes at *NBS1* Glu185Gln were of increased risk of lung cancer after adjusted for age, smoking, alcohol drinking and areca chewing habits (adjusted OR[aOR]=1.85, 95% CI=1.12-2.83) (Table IV). On the contrary, there was no significantly elevated lung cancer risk for females with CG or CC genotypes at *NBS1* Glu185Gln (Table IV). Second, lung cancer patients and controls were stratified according to their smoking status and ORs were analyzed. The results showed that ever-smokers carrying the homologous CC genotypes at *NBS1* Glu185Gln were of increased risk of lung cancer after adjusted for age, gender, alcohol drinking and areca chewing habits (aOR=1.76, 95%

CI=1.09-2.64) (Table V). On the contrary, there was no significantly elevated lung cancer risk for non-smokers with CG or CC genotypes at *NBS1* Glu185Gln (Table V).

Discussion

NBS1 plays pivotal roles in maintaining genomic stability and the initiation and progression of carcinogenesis. The exon variantrs1805794 (Glu185Gln) of *NBS1* has been studied in case-control association studies. However, the results were conflicting. In the current case-control association study in Taiwan, the contribution of *NBS1* Glu185Gln genotype, gender and smoking status to lung cancer risk was evaluated. Among lung cancer patients and non-cancer healthy control subjects, the genotyping results showed that neither heterozygous CG nor homozygous CC genotypes of *NBS1* Glu185Gln was significantly associated with an increased risk of lung cancer (Table II). The allelic frequency analysis supports the findings from genotypic frequency analysis that the C allele of *NBS1* Glu185Gln was not associated with an increased lung cancer risk (Table III). This negative association has also been evident from several studies (37, 43, 44). On the contrary, there were two reports with positive association. In 2010, Fan and co-workers found that, compared to the GG genotype, the C genotypes (CG/CC) at *NBS1* Glu185Gln conferred a significantly 1- to 46-fold increased OR of lung cancer (45). Similar positive association was proposed by Fang and colleagues reporting that, compared to the GG genotype, the C genotypes (CG/CC) at *NBS1* Glu185Gln conferred a significantly increased risk of lung cancer in a Chinese cohort (OR=1.40). They have also investigated the contribution of this polymorphism with phenotypic assays and shown that X-ray radiation induced more chromatid breaks in lymphocyte cells from C genotype individuals than those from the GG genotype carriers (46). Noticeably, the current findings, as well as others, all showed that the C allele of *NBS1* Glu185Gln is slightly positively associated with an increased lung cancer risk; however, further validations in larger and different populations are needed.

Gender is a risk factor for lung cancer. Although the mechanism(s) explaining this gender-dependent difference in lung cancer risk is not known, it is thought that endocrine factors may play a critical role (47). In the National Health Insurance Research Database of Taiwan, investigating 33,919 patients with lung cancer recorded from 2002 to 2008 in Taiwan, about two-thirds of patients were males (48), with the ratio being very similar to the gender ratio of this study. During recent years, there has been an increasing trend for female patients with lung cancer in Taiwan as the prevalence and mortality rates of females with non-small cell lung cancer adenocarcinoma are very high in Taiwan. Therefore, we were interested whether the genotype of *NBS1* Glu185Gln contributes to the gender difference in lung cancer susceptibility. After stratification by gender, it was found that the genotypes of *NBS1* Glu185Gln were differently distributed among males but not among females (Table IV). One explanation for the trend for female patients with lung cancer is the increase of cigarette consumption. The results of epidemiological studies suggest that, after one can control the number of cigarettes smoked, women have a three-time higher risk of suffering lung cancer than men (47).

As mentioned above, smoking is another well-known risk factor for lung cancer. Cigarette smoke may enhance remodeling in the developing human airway smooth muscle through hyperplasia and alteration of extracellular microenvironment, thus contributing to the development of neonatal and pediatric airway disease (49). In addition, tobacco smoking can induce DNA lesions and defects in repair of tobacco carcinogen-induced DNA adducts may contribute to carcinogenesis (50). Therefore, the interaction of the genotype of *NBS1* Glu185Gln and cigarette smoking status of the participants was also analyzed. As expected, the results showed that the genotypic distribution of the variant genotypes of *NBS1* Glu185Gln was significantly different between lung cancer and control sub-groups who were ever smokers (Table V). On the contrary, no differential distribution was observed among non-smokers (Table V). There was a phenotypic assay showing that the rs1805794C allele attenuated NBS1's capacity to remove DNA damage as cells transfected with a plasmid carrying the rs1805794C allele had significantly higher DNA breaks than those transfected with a plasmid carrying the rs1805794G allele after X-ray irradiation (46). Further investigations using cells from patients rather than cell lines should be conducted to explore the joint effects of tobacco smoking, other risk factors and this polymorphism on cancer risk. Likewise, future investigations should be designed based on cells from different gender, drinking status and other lung cancer risk factors.

In conclusion, our study provides evidence that the C allele of *NBS1* Glu185Gln is associated with an increased lung cancer risk among males, especially smokers.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

The Authors are grateful to Tissue-bank of China Medical University Hospital and doctors/nurses under Professor Hsia's leadership for their excellent sample collection and technical assistance. This study was supported partially by research grants from Taichung Armed Forces General Hospital (106A06) to Chuang, from Asia University (ASIA-105-CMUH-11) to Hsu and from Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW106-TDU-B-212-113004).

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Received March 10, 2017
Revised March 18, 2017
Accepted March 20 2017