Screening of Homologous Recombination Gene Polymorphisms in Lung Cancer Patients Reveals an Association of the NBS1-185Gln Variant and p53 Gene Mutations

Abstract
Carcinogens present in tobacco smoke lead to several types of DNA damage in bronchial cells. In lung cancer, karyotype, allelotype, and fluorescence in situ hybridization analyses have demonstrated the common presence of aneuploidy, although its severity varies considerably among tumors. Deficiencies in the DNA-double strand break (DSB) repair system may be critical in the generation and persistence of chromosomal gains or losses during lung tumorigenesis. Therefore, we examined whether specific DSB repair gene polymorphisms were associated with an increase in tobacco-induced DNA damage, including gene mutations (p53 and KRAS) and chromosomal alterations. Nonsynonymous polymorphisms with a frequency higher that 0.1 at the XRCC3, NBS1, and BRCA2 genes were selected for the study. A PCR-RFLP analysis was performed to identify the Met241Thr, Glu185Gln, and Asn372His polymorphisms in the XRCC3, NBS1, and BRCA2 genes, respectively, in 109 lung cancer patients. Interestingly, the prevalence of p53 mutations was significantly greater among individual homozygous for the NBS1-185Gln allele (8 of 8, 100%) than among individuals for the wild-type allele (24 of 52, 46%). This increase in p53 mutation frequency was largely attributable to an increased prevalence of G→T or C→A transversions among these patients (P < 0.001). In addition, the association between this type of mutation and the NBS1-185Gln allele remained statistically significant after adjusting for age, smoking, and histological cell-type (odds ratio = 3.42 for heterozygous and odds ratio = 38.3 for NBS1-185Gln homozygous). Germ-line variants in the NBS1 gene may play a role in the lung carcinogenesis in cigarette smokers.

Introduction
The contribution of DNA repair deficiencies to sporadic cancer is not completely elucidated. Interestingly, individuals with homozygous germ-line mutations in several genes implicated in the repair of DSBs such as the Ataxia Telangiectasia, BRCA1, BRCA2, and NBS1 genes develop syndromes that share, among other characteristics, a predisposition to several types of cancer and high levels of aneuploidy (Ref. 1 for review). These genetic syndromes reveal a clear cause/effect association between defects in the HR repair system and the development of cancer.

In lung cancer, cigarette smoking has been identified as the main causative agent in smokers (2, 3). The activated form of the tobacco compound benz(a)pyrene, BPDE, can irreversibly damage DNA (4, 5) and selective BPDE adduct formation occur at guanine positions of codons 157, 248, and 273 of the p53 gene and at codons 12 and 13 of the KRAS gene (6, 7), strongly supporting an active role of this chemical agent in lung carcinogenesis. In addition, chromosomal aberrations have been induced in lymphoblastoid cell lines by BPDE treatment, highlighting the role of tobacco carcinogens as the main cause of the widespread chromosomal abnormalities present in lung tumors (5). Stable covalent DNA adducts can induce single-strand breaks and one-strand DNA damage can generate DSBs in cells that are replicating their DNA, thus, contributing to several forms of chromosomal damage (5). In agreement with this hypothesis, several molecular alterations such as chromosomal losses or gene promoter hypermethylation have been observed in apparent normal bronchial epithelium and in sputum from smokers or ex-smokers compared with never-smokers (8–11). Moreover, we previously observed that lung tumors from smokers have a significantly increased number of chromosomal alterations compared with lung tumors from non-smokers (12), additionally supporting that tobacco carcinogens are among the causes for chromosomal abnormalities arising in lung tumors.

To define whether some gene variants at the HR repair pathway contribute to lung carcinogenesis and genetic damage...
in lung tumors, we studied the \textit{XRCC3}-Thr241Met, \textit{NBS1}-Glu185Gln and \textit{BRCA2}-Asn372His gene polymorphisms and their correlation with clinical, pathological, and genetic characteristics of NSCLC.

\textbf{Patients and Methods}

\textbf{Patient Samples, DNA Extraction, and Genetic Characterization of NSCLC.} Primary tumors and blood were obtained from 109 patients with NSCLC at The Johns Hopkins University School of Medicine. Informed consent was obtained from each patient. The characteristics of the patients included in the study for which we had data available were as follows: median age, 65 years (range, 31–84 years); gender, 38 males and 58 females; race, 29 blacks and 68 whites; smoking history, 95 smokers and 13 nonsmokers; tumor histology, 36 SCCs, 49 ACs, and 18 bACs; and stage, 39 stage I, 38 stage II, 18 stage III, and 5 stage IV. Smokers and nonsmokers were as defined previously (14, 15). Mutations and FALs for these tumors have been determined and reported previously (12–15). DNA was extracted from normal and normal lymphocytes as described previously (14, 15). 

\textbf{Gene Polymorphism Analysis.} We searched databases and previously published data (16–18) and selected gene polymorphisms at \textit{XRCC3}, \textit{NBS1}, and \textit{BRCA2} genes (\textit{XRCC3}-Thr241Met, \textit{NBS1}-Glu185Gln and \textit{BRCA2}-Asn372His).4,5 These genes were chosen among those that participate in DSB repair and all of the variants led to amino acid changes and had a high prevalence (allele frequency higher than 0.1). In addition, \textit{NBS1} and \textit{BRCA2} are implicated in genetic syndromes leading to high predisposition to cancer. These polymorphisms were identified in lymphocyte DNA using PCR/RFLP assays. PCR reactions were performed in a total reaction volume of 25 \(\mu\)l containing: 30 ng of DNA; 6 ng/\(\mu\)l of each primer; 200 \(\mu\)M of each deoxynucleotide triphosphate; 0.75 units of DNA polymerase (Biotools); and 2 mM MgCl\(_2\). The \textit{XRCC3}-Thr241Met gene polymorphism was determined using the following primers: forward, 5'-CTACAAAAAGTTAA-GAACTAGCAAGACTG-3' and reverse, 5'-GTCACCTTC-CACCTCTAAAGGGCTCTCAT-3' (the underlined base introduces a restriction site for the \textit{Hsp92 II} enzyme). The PCR cycling conditions consisted of initial denaturation at 94°C for 2 min followed by 10 cycles of denaturation (30 s at 94°C), annealing (30 s at 50°C), and elongation (30 s at 72°C) and 25 cycles of denaturation (30 s at 94°C), annealing (30 s at 45°C), and elongation (30 s at 72°C). After the last cycle, a final extension (5 min at 72°C) was added. The product of 190 bp was digested with \textit{Hsp92 II} (Promega) and resolved on 3% agarose gels. The three possible genotypes are identified by distinct banding patterns: (a) Asn/Asn (147 and 43 bp); (b) Asn/His (147, 119, 43, and 28 bp); and (c) His/His (119, 43, and 28 bp).

As quality control, the three assays were repeated on 30% of the samples, and the replicates were 100% concordant. PCR products from the \textit{BRCA2} and \textit{XRCC3} genes have an additional restriction site that was used as internal control for complete restriction reaction. As control for the complete restriction digestion of the \textit{NBS1} gene, we used a sample with a known homozygous 185Glu genotype.

\textbf{Statistical Analysis.} Hardy-Weinberg equilibrium in the prevalence of \textit{XRCC3}, \textit{NBS1}, and \textit{BRCA2} gene polymorphisms among lung cancer patients was tested using the likelihood ratio test. Association between genotype frequencies of these polymorphisms with demographic and tumor characteristics was assessed by \(\chi^2\) test and Fisher’s exact test. For quantitative variables, the nonparametric Wilcoxon test was used. The relationship between the \textit{NBS1} genotype and \textit{C}→\textit{T} or \textit{C}→\textit{A} mutations at p53 was additionally explored using logistic regression taking into account age, histological type, and smoking habit. Finally, differences in survival according to the studied genotypes were analyzed using Kaplan-Meier and the log-rank test.

\textbf{Results}

\textbf{Prevalence of \textit{XRCC3}, \textit{NBS1}, and \textit{BRCA2} Gene Variants.} One hundred nine NSCLC patients were genotyped for these variants in the \textit{XRCC3}, \textit{NBS1}, and \textit{BRCA2} genes. Frequencies for each gene polymorphism and patient clinical characteristics are presented in Table 1. None of the genotype distributions differed significantly from the HWE, although \(P\)s for \textit{XRCC3} and \textit{BRCA2} were rather low because of the distinct frequencies among blacks and whites. No statistically significant differences in the distribution of the polymorphisms according to smoking history or lifetime cigarette consumption were observed (data not shown). Representative examples of PCR and restriction analysis are depicted in Fig. 1.

\textbf{Gene Variants at the \textit{XRCC3}, \textit{NBS1}, and \textit{BRCA2} Genes and Their Association with the Pathological and Genetic Characteristics of Lung Tumors.} We also examined the distribution of gene polymorphisms among different tumor characteristics such as tumor histology (SCC, AC, and bAC) and tumor genetic background (\textit{KRAS} and p53 gene mutations and FAL). Patients with the \textit{NBS1}-185Gln gene variant had an increased frequency of lung tumors carrying p53 gene mutations (Table 2). The prevalence of p53 mutations was significantly higher among individuals homozygous for the \textit{NBS1}-185Gln allele (8 of 8, 100%) than among individuals homozygous for the wild-type allele (24 of 52, 46%, \(P = 0.018\)). In addition, \textit{G}→\textit{T} or \textit{C}→\textit{A} transversions were clustered in individuals with the Gln/Gln genotype (\(P < 0.001\)), whereas the frequency of p53 mutations other than \textit{G}→\textit{T} or \textit{C}→\textit{A} was not related with this

\footnote{Internet address: http://www.genome.utah.edu.}
\footnote{Internet address: http://www.ncbi.nlm.nih.gov.}
polymorphism ($P = 0.2$; Table 2). The association between the Gln variant at NBS1 with G$\rightarrow$T or C$\rightarrow$A transversions at p53 gene was additionally examined using logistic regression (Table 3). The risk of G$\rightarrow$T or C$\rightarrow$A changes at p53 was significantly higher among heterozygous and homozygous carriers of the 185Gln allele, after adjusting for age, smoking history, and histological cell type. These differences were not attributable to the unequal distribution of the NBS1 genotypes among tumors with and without p53 data available ($P = 0.40$; Fisher's exact test). No correlation was found between KRAS mutations considering only lung AC and BC and the XRCC3, NBS1, and BRCA2 gene polymorphisms. The presence of DSB repair gene variants was also compared with the degree of chromosomal abnormalities in the tumors. The percentage of chromosomal alterations was calculated as the frequency of chromosomal gains/losses per chromosomal arms (FALs/gains, FAL; Ref. 12). Data on the FAL status was available in only 26 tumors from smokers. Tumors arising in individuals with the Gln/Glu and Gln/Gln genotypes for the NBS1 gene had higher levels of FAL (mean, 24.3), compared with tumors from Glu/Glu individuals (mean, 21.2), but differences did not reach statistical significance ($P = 0.182$). This preliminary observation was not because of differences in the distribution of the polymorphisms because it was comparable among patients with and without FAL data available: (XRCC3, $P = 0.60$; NBS1, $P = 0.10$, and BRCA2, $P = 0.84$; Fisher’s exact test).

### Discussion

Although the implication of DNA repair genes in cancer predisposition syndromes has been well established, their relevance in sporadic tumors is still under intense investigation. The XRCC3 protein participates in the repair of DSBs throughout the HR system, contributing to the maintenance of genomic stability. However, the role of XRCC3 gene in cancer development is still uncertain, and no cancer predisposition syndrome has been linked to germ-line mutations in the XRCC3 gene. The XRCC3-241Met gene variant has been associated with predisposition to melanoma, bladder cancer, and in head
### Table 2  Distribution of gene polymorphisms according to the histology and the genetic background of the lung tumors

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Tumor histology*</th>
<th>( p )</th>
<th>KRAS*</th>
<th>( p )</th>
<th>p53</th>
<th>( p )</th>
<th>p53</th>
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<tbody>
<tr>
<td></td>
<td>AC</td>
<td>bAC</td>
<td>SCC</td>
<td>Mutant</td>
<td>WT</td>
<td>Mutant</td>
<td>WT</td>
</tr>
<tr>
<td>XRC3-(Thr241Met)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Thr/Thr</td>
<td>24 (49%)</td>
<td>8 (44%)</td>
<td>15 (42%)</td>
<td>13 (59%)</td>
<td>18 (41%)</td>
<td>23 (44%)</td>
<td>23 (49%)</td>
</tr>
<tr>
<td>Thr/Met</td>
<td>18 (37%)</td>
<td>8 (44%)</td>
<td>14 (39%)</td>
<td>3 (14%)</td>
<td>6 (27%)</td>
<td>6 (12%)</td>
<td>10 (21%)</td>
</tr>
<tr>
<td>Met/Met</td>
<td>7 (14%)</td>
<td>2 (11%)</td>
<td>7 (19%)</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
<td>6 (12%)</td>
<td>6 (14%)</td>
</tr>
<tr>
<td>Thr/Met and Met/Met</td>
<td>25 (51%)</td>
<td>10 (56%)</td>
<td>21 (58%)</td>
<td>0.79</td>
<td>9 (41%)</td>
<td>26 (59%)</td>
<td>0.20</td>
</tr>
<tr>
<td>NBS1-(Glu185Gln)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Glu/Glu</td>
<td>24 (49%)</td>
<td>13 (72%)</td>
<td>19 (53%)</td>
<td>13 (59%)</td>
<td>23 (51%)</td>
<td>24 (46%)</td>
<td>28 (61%)</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>20 (41%)</td>
<td>5 (28%)</td>
<td>13 (36%)</td>
<td>0.43</td>
<td>8 (36%)</td>
<td>17 (38%)</td>
<td>0.76</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>5 (10%)</td>
<td>0 (0%)</td>
<td>4 (11%)</td>
<td>1 (5%)</td>
<td>5 (11%)</td>
<td>8 (15%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Glu/Gln and Gln/Gln</td>
<td>25 (51%)</td>
<td>5 (28%)</td>
<td>17 (47%)</td>
<td>0.23</td>
<td>9 (41%)</td>
<td>22 (49%)</td>
<td>0.79</td>
</tr>
<tr>
<td>BRCA2-(Asn372His)</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Asn/Asn</td>
<td>26 (53%)</td>
<td>10 (56%)</td>
<td>21 (58%)</td>
<td>10 (45%)</td>
<td>25 (57%)</td>
<td>28 (54%)</td>
<td>25 (54%)</td>
</tr>
<tr>
<td>Asn/His</td>
<td>19 (39%)</td>
<td>5 (28%)</td>
<td>11 (31%)</td>
<td>0.80</td>
<td>10 (45%)</td>
<td>14 (32%)</td>
<td>0.55</td>
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<td>His/His</td>
<td>4 (8%)</td>
<td>3 (17%)</td>
<td>4 (11%)</td>
<td>2 (9%)</td>
<td>5 (11%)</td>
<td>4 (8%)</td>
<td>7 (15%)</td>
</tr>
<tr>
<td>Asn/His and His/His</td>
<td>23 (47%)</td>
<td>8 (44%)</td>
<td>15 (42%)</td>
<td>0.39</td>
<td>12 (55%)</td>
<td>19 (43%)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

*Calculated for those patients with available information.

*Calculated only in the ACs and bACs histological types for which we had KRAS data available.

*Calculated for individuals with p53 mutational status available.

* Corresponding to the comparisons between p53-wild type tumors versus tumors with mutations G→T/C→A.

* Corresponding to the comparisons between p53-wild type tumors versus non-G→T/C→A mutations.
and neck SCCs from previous smokers but not to lung cancer (19–22). The XRCC3-241Met gene polymorphism has been associated with higher DNA adduct levels in WBCs from peripheral blood of healthy individuals (23), suggesting that the XRCC3 protein may participate in the repair of bulky DNA adducts and that the XRCC3-241Met gene variant could generate a protein with less efficient DNA repair ability. However, recent in vitro studies have demonstrated no difference in the homology-directed repair of DSBs between the variant XRCC3-241Met protein and the wild-type 241Thr protein (24). In our study, the XRCC3 gene polymorphism did not cluster with any of the tumor parameters studied, suggesting that such gene variant does not significantly contribute to the genetic or pathological characteristics of NSCLC.

On the other hand, germ-line mutations at BRCA2 and NBS1 genes clearly predispose to cancer (1). Most publications on the BRCA2-Asn372His and the NBS1-Glu185Gln gene variants have focused on breast cancer risk assessment and not lung cancer (17, 25). Homozygous germ-line mutations at the NBS1 gene lead to the NBS, a rare disease characterized by microcephaly, growth and mental retardation, radiosensitivity, immunodeficiency, high incidence of malignancies at an early age, and elevated rates of chromosomal abnormalities (26, 27).

Interestingly, our observations show that virtually all individuals homozygous for the Gln variant have tumors with p53 mutations in contrast with only 46% of p53 mutations in tumors from individuals homozygous for the Glu variant. Even more intriguing was the strong association between the NBS1-185Gln gene polymorphism and G→T or C→A transversions at p53 but not with other types of p53 mutations. However, these observations are still preliminary and additional studies, including larger number of patients are needed to confirm our findings. Compared with other tumor types, point mutations at KRAS and p53 genes in lung tumors have an increased rate of G→T or C→A transversions. In tumors other than lung, the total frequency of G→T or C→A mutations at the p53 gene is 15% compared with 33% in lung tumors (7, 28). This specific mutational spectrum of p53 mutations in lung cancer is likely to occur as a consequence of bulky adducts originated by the exposure to tobacco carcinogens such as BDPE and others being repaired through the NER pathway (1). Although most of the lung tumors included in our study were from smokers, it is unlikely that our observations apply also to tumors from non-smokers because they harbor a significant lower proportion of p53 mutations and G→T or C→A transversions (28).

The NBS1 protein is a member of the hMre11/hRad50 complex that actively participates in DSB repair but not in the NER pathway (27). However, it has been shown that the slow growth phenotype of rad50 mutants can be suppressed by mutations in RAD1, which encodes a nuclease that mediates in the recognition and incision of DNA damage during NER (30).

Such results may link the NER system with the Mre11/Rad50 complex and could help explain our observations. Moreover, recent studies have uncovered that the hMre11/hRad50 complex is also implicated in the checkpoint signaling and in DNA replication, additionally suggesting that the hMre11/hRad50 complex has a broader biological function outside the DSBs repair system (29, 30). To our knowledge, there is no information regarding changes in the activity of the NBS1-185Gln isoform. The region between amino acid 108–196 of the NBS1 protein constitutes a BRCA1 COOH-terminus domain that is presumably involved in cell-cycle checkpoints or in DNA repair. Most of the NBS patients (80%) harbor a 5-bp deletion, a mutation of Slavic origin, at nucleotide 657 (31). Other six types of mutations have been found in these families, restricted to the central region of the protein (between codons 233 and 385). All mutations lead to protein truncations. Thus, the mutational pattern of NBS1 gene in NBS patients does not gives us clues about the functional relevance of the NBS1-185Gln gene polymorphism.

Previous studies have reported that p53 mutations accumulate in lung tumors with high levels of chromosomal abnormalities (12, 32), suggesting that p53 mutations may prevent the efficient repair of the chromosomal alterations caused by tobacco carcinogens. We failed to detect a significant correlation between the presence of the gene polymorphisms analyzed and higher levels of FAL, although there was a trend toward a higher levels of FAL in tumors from individuals carrying either the NBS1-185Gln or XRCC3-241Met variant allelic alleles. However, the number of samples for which we had FAL status available was too low to reach definitive conclusions.

Overall, our observations suggest that the NBS1-185Gln protein variant could affect repair of DNA adducts and would, thereby, allow the accumulation of G→T or C→A transversions in p53. Examples of the association between deficiencies in DNA repair genes and specific mutational profiles in important cancer genes have already been described. In colorectal and lung tumors, gene promoter hypermethylation at the DNA repair protein O6-methylguanine-DNA methyltransferase, in charge of removing mutagenic adducts from the O6 position of guanine, correlates with G→A transitions at KRAS and p53, demonstrating how DNA repair deficiencies affect the status of important cancer genes (33, 34).

In conclusion, our results show that NBS1-185Gln gene polymorphism associates with p53 mutations in NSCLC, suggesting that it may contribute to human lung carcinogenesis in smokers.

### References
Homologous Recombination Repair Genes in Lung Tumors


Screening of Homologous Recombination Gene Polymorphisms in Lung Cancer Patients Reveals an Association of the NBS1-185Gln Variant and p53 Gene Mutations

Pedro P. Medina, Steven A. Ahrendt, Marina Pollan, et al.