Differential Association of the Codon 72 p53 and GSTM1 Polymorphisms on Histological Subtype of Non-Small Cell Lung Carcinoma

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ABSTRACT

Traditionally, non-small cell lung cancer (NSCLC) has been evaluated as a unique entity in genotyping studies. However, recent biological data suggest that different NSCLC subtypes, specifically adenocarcinomas (AC) and squamous cell carcinomas (SCC), differentially alter cancer behavior. Several studies have associated a p53 polymorphism at codon 72 with NSCLC susceptibility. This study investigated whether different p53 genotypes altered the overall risk of developing AC versus SCC. Polymorphisms in metabolizing enzymes, together with prolonged exposure to tobacco carcinogens, can result in accumulation of DNA damage; these effects may potentiate the effects of subtle differences in p53 function. Thus, interactions between polymorphisms of p53 and either GSTM1 or GSTT1 were also evaluated. We analyzed 1168 incident lung cancer cases and 1256 control subjects using multiple logistic regression. Histological data were available for 1144 cases (98%): 585 with AC, 284 with SCC, and 275 with other histological subtypes (large cell, small cell, mixed, and other). An increase in the NSCLC risk posed by the p53 Pro allele (versus Arg/Arg) was seen in AC compared with controls [adjusted odds ratio (OR), 1.36; 95% confidence interval (CI), 1.1–1.7] but not in SCC (adjusted OR, 1.04; 95% CI, 0.8–1.4). Among AC and SCC cancer patients, individuals with the GSTM1-null genotype had an OR of 1.80 (95% CI, 1.1–2.8; case-only analysis) of having AC versus SCC if they also carried a p53 Pro allele. We conclude that different genotype combinations of p53 and GSTM1 increase the risk of developing specific histological subtypes of NSCLC.

INTRODUCTION

Studies evaluating the role of genetic polymorphisms in lung cancer risk have generally grouped all NSCLCs together. However, NSCLC encompasses several histological subtypes: AC, SCC, and large cell carcinoma (1). There is growing biological and epidemiological data to suggest that different NSCLC histological subtypes, particularly the two most common, AC and SCC, are distinct etiological entities that should be analyzed separately (2–4). For example, in active smokers, AC comprises a smaller proportion (30–50%) of all NSCLC than in never-smokers (60–80%; Refs. 5 through 12). In former smokers, the proportion of ACs is higher in individuals with a longer period since quitting smoking, with the differences most marked ≥20 years after smoking cessation (13). Controversy exists as to whether the different histological subtypes are different manifestations of the same disease process or different processes shared by some distinct risk factors (4).

p53 is a tumor suppressor gene involved in multiple pathways including apoptosis, cellular transcriptional control, and cell cycle regulation (14, 15). Somatic p53 mutations are found more frequently in SCC than in AC, although this may be a function of higher exposures to tobacco in individuals with SCC (16). Individuals with AC and p53 somatic mutations have a shorter survival compared with SCC (17). p53 somatic mutations have been associated with specific loss of heterozygosity patterns, and both loss of heterozygosity and somatic mutation patterns among AC and SCC patients are distinctly different (2, 3, 18). We reported recently an association between a germline p53 functional polymorphism at codon 72 and lung cancer susceptibility (19). Combinations of polymorphisms of different xenobiotic metabolizing genes have also been evaluated in lung cancer (20–23).

In this study, we sought to confirm our original preliminary findings of an overall lung cancer susceptibility of the Pro allele of the p53 codon 72 polymorphism, using a greatly expanded sample of >2400 cases and controls. Furthermore, we examined whether different p53 polymorphisms altered the risk of developing AC versus SCC, the two most common subtypes of NSCLC. Finally, we evaluated the interactions of p53 polymorphisms and the metabolizing genes, GSTM1 and GSTT1, by histological subtype.

MATERIALS AND METHODS

Sample Collection. Patient recruitment and sample collection methods have been described elsewhere (19). Briefly, histologically confirmed incident adult lung cancer patients assessed at Massachusetts General Hospital were recruited, and controls were blood-unrelated family or friends of patients with either cancer or cardiothoracic problems. An interviewer-administered questionnaire obtained relevant demographic and epidemiological information, including detailed diet and smoking information. Peripheral blood samples were obtained from all subjects, and DNA was extracted using a Puregene DNA isolation kit (Genta, Minneapolis, MN). All histological classification was reviewed by a lung pathologist at Massachusetts General Hospital, and data abstraction of histological, staging, and grade data was supervised by a thoracic oncologist. This study was approved by the human subjects committee of all involved institutions.

Genotyping of p53, GSTM1, and GSTT1. PCR-RFLP analysis of codon 72 of the p53 gene, modified from a technique described by Ara et al. (24), was used to identify p53 genotypes. The forward primer used was 5′-TCGCTCTTCTGCCGGTCTC-3′ whereas the reverse primer was 5′-TCTGGGAAGGACAGAGATGAC′-3′. Each PCR reaction mixture (50 μl) contained 10 pmol of each primer, 2.0 mM of MgCl2, 200 mM each deoxynucleotide triphosphate, 1 unit of Taq polymerase, and 100–300 ng of genomic DNA. Reaction mixtures were preincubated for 5 min at 95°C. PCR conditions were 95°C for 30 s and 65°C for 1 min, followed by 72°C for 1 min, for 35 rounds. The final extension was at 72°C for 8 min. After confirmation of an amplified fragment of the expected size (199 bp) on an agarose gel, 10 μl of PCR product was digested with 6 units of restriction enzyme BstUI (New England Biolabs, ME) at 60°C for at least 3 h. DNA fragments were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The Arg allele is cleaved by BstUI and yields two small fragments (112 and 86 bp). The Pro allele is not cleaved by BstUI and has a single 199-bp band. The heterozygote contains three bands (199, 113, and 86 bp).

For quality control purposes, the PCR-RFLP genotyping technique...
scribed by Ara et al. (24) was compared with a PCR-pyrosequencing technique for genotyping. This pyrosequencing technique used the same PCR primers as the PCR-RFLP technique, except that the reverse primer strand was biotinylated. Twenty-five μl of PCR product was mixed with magnetic streptavidin beads that bind the biotinylated primer to obtain single-stranded DNA. Then 15 pmol of sequencing primer (5'-CAGAGGGCTGTCCCCC-3') was added. The single-stranded biotinylated PCR product is sequenced by adding nucleotides sequentially to a solution containing the single-stranded PCR product and a mix of enzymes involved in the reaction. Nucleotides were dispensed in the following order: TGGCTG, where bold characters G and C represent the polymorphic site and the italicized Ts indicate negative control nucleotides used to determine the amount of background signal generated by reaction. Pyrophosphate (PPi), released when a specific nucleotide was incorporated, reacts with sulfurylase to create ATP. The ATP drives a luciferase-mediated reaction releasing visible light, whereas asparagine continuously degrades unincorporated nucleotides and excess ATP before the next nucleotide is dispensed. The amount of light released is measured and is proportional to the number of nucleotides incorporated (Pyrosequencing AB, Uppsala, Sweden).

The GSTM1 and GSTT1 genetic polymorphisms were evaluated using multiplex PCR techniques, modified from a GSTM1 technique by Zhong et al. (25). Five primers were used: (a) 5'-CGCAATCTTGTGGCTATTG-3'; (b) 5'-ATCCCTCTCCCTCTG-3'; (c) 5'-TCTGAGTTAGGACAGTACA-3'; (d) 5'-TTCTCCATCTGTCCTCACT-3'; and (e) 5'-TCACCGGATCATGCAGCAGA-3'. Each 25 μl of the PCR reaction mixture contained 3.0 mm MgCl2, 200 μM each deoxynucleotide triphosphate, 1.25 units of Taq polymerase, and -100 ng of genomic DNA. The reaction mixtures were preincubated for 5 min at 94°C. Initial amplification involved the following PCR conditions: 94°C for 15 s, 67°C for 15 s, and 72°C for 22 s for two cycles. Then the main amplification involved the following PCR conditions: 94°C for 30 s and 62°C for 30 s, followed by 72°C for 45 s for 37 rounds, and followed by a final extension at 72°C for 5 min. The primers listed in a and c above amplified a 230-bp fragment in the presence of the GSTM1 allele; the primers listed in a and b amplified a 157-bp fragment as an internal control; and the primers listed in d and e amplified a 480-bp fragment in the presence of the GSTT1 allele. DNA fragments were electrophoresed through a 2% agarose gel and stained with ethidium bromide.

**Statistical Analysis.** Age, gender, smoking status, pack-years (defined as the product of years smoked and the average number of cigarettes smoked per day), and p53 genotypes were tabulated for all cases: AC, SCC, and controls. Differences were tested by using the χ² and t tests, all two-sided, where appropriate. HWE was evaluated by using the χ² goodness-of-fit test (26). For the purpose of modeling the association between the p53 variant gene frequency and lung cancer, multiple logistic regression was performed to evaluate lung cancer risk for the entire sample and for the risk of SCC and AC separately. In the main effects analyses, we compared both the Pro/Pro and Arg/Pro genotypes to Arg/Arg respectively in the same model by allowing a different lung cancer risk for each genotype. Generalized additive models (27), which allow the relationship between the outcome and each covariate to be an unspecified smooth function, were used initially to examine whether pack-years was linearly related to the log odds of lung cancer. The relationship between pack-years and the log odds of cancer was nonlinear, whereas the relationship was approximately linear when the square root transformation of pack-years was used instead. Multiple logistic regression was used to model the relationship between the log odds of lung cancer and p53 genotype, adjusting for age, gender, smoking status (current, former, and never smokers separately), the square root of pack-years, and the number of years since quitting smoking for ex-smokers. Under the hypothesis that p53 genotype may be differentially associated with histological subtype, we tested for gene-gene interactions between p53 and either of the GST polymorphism (GSTM1 or GSTT1) genotypes in models for SCC and separately for AC. In addition, the association between these genotypes and histology was compared directly in case-only analyses, because control subjects could not be classified by histology. These case-only logistic regressions were performed, using AC and SCC as the outcomes, to evaluate: (a) the association between p53 polymorphisms (Pro allele versus Arg allele) and tumor histology (AC versus SCC); (b) the gene-gene interaction of the p53 polymorphism with GSTM1 in determining tumor histology; and (c) the gene-gene interaction of p53 with GSTT1 in determining tumor histology.

**RESULTS**

The distribution of demographic variables for cases and controls, and separately for AC and SCC, is summarized in Table 1. Controls were significantly younger and were more likely to be female, have lower cumulative smoking exposures (measured by lower pack-years and a higher proportion of nonsmokers), and have a lower frequency of variant p53 alleles (P < 0.05 for all variables). In 24 cases, the histological subtype was not available. In 16 cases, the histological subtype was not identified by the pathologist (the majority of these were small needle aspirate samples), 51% of the known cases were AC, 25% were SCC, and the other 24% were of other histological subtypes (n = 97 small cell, 89 large cell, and 73 with mixed or other histological subtypes). When comparing AC to SCC, AC patients were significantly younger, more likely to be female and to have lower cumulative smoking exposures, to have quit smoking for a longer period (for ex-smokers), and to have a higher frequency of variant p53 alleles (P < 0.05 for all variables).

Initially, we evaluated p53 genotypes using the technique adapted from Ara et al. (24). We found that these results were not in HWE (χ² goodness-of-fit test; P = 0.01). Concerned that these results were affected by incomplete BstUI digestion, we developed a PCR-based pyrosequencing technique to verify our PCR-RFLP technique. We found that 2–3 units of BstUI, initially used in our modified technique, was inadequate and led to incomplete digestion of up to 30% of the homozygote variants and 10% of the heterozygotes. We optimized the PCR conditions and repeated all potentially affected samples (65% of the total sample) using 6 units of BstUI. Using 6 units of BstUI, there was 99% concordance with pyrosequencing results in 285 samples that included randomly selected samples and samples selected on the basis that they were potentially affected heterozygote or homozygote variants. A random 50% of the agarose gels was interpreted by two independent authors and 50% of the data entry was checked by at least two individuals, with 99.9% and 99.8% concordance, respectively. Data analysis was checked by at least two authors, independently. The results from using 6 units of BstUI form the basis of this report and were in HWE.

**Table 1 Baseline characteristics of all lung cancer cases and controls and of AC and SCC cases**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>Total</th>
<th>AC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>58 ± 12</td>
<td>65 ± 11</td>
<td>64 ± 11</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>576 (46%)</td>
<td>629 (54%)</td>
<td>203 (48%)</td>
<td>140 (65%)</td>
</tr>
<tr>
<td>Female</td>
<td>680 (54%)</td>
<td>539 (46%)</td>
<td>216 (52%)</td>
<td>77 (35%)</td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>442 (35%)</td>
<td>73 (6%)</td>
<td>51 (9%)</td>
<td>7 (2%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>571 (46%)</td>
<td>618 (53%)</td>
<td>326 (56%)</td>
<td>156 (55%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>243 (19%)</td>
<td>477 (41%)</td>
<td>208 (36%)</td>
<td>121 (43%)</td>
</tr>
<tr>
<td>Pack-yrs (ever-smokers only)</td>
<td>32 ± 27</td>
<td>60 ± 36</td>
<td>52 ± 34</td>
<td>73 ± 41</td>
</tr>
<tr>
<td>Yrs since smoking cessation (ex-smokers only)</td>
<td>19 ± 12</td>
<td>14 ± 11</td>
<td>15 ± 11</td>
<td>13 ± 10</td>
</tr>
</tbody>
</table>

For age and pack-years, data is reported as mean ± standard deviation. Pack-years is the product of the number of years of smoking and the average number of cigarettes per day. Age, gender, smoking status, pack-years, years since smoking cessation, and p53 genotype were significantly different between cases and controls and between AC and SCC at α = 0.05 for each variable. No differences were noted for GSTM1 or GSTT1 genotypes (P > 0.05 for cases vs controls and for AC vs SCC).

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For the entire sample, when compared with the wildtype Arg/Arg genotype the crude OR of lung cancer for the Pro/Pro genotype was 1.37 (95% CI, 1.0–1.9), and for the Arg/Pro genotype, the crude OR was 1.27 (95% CI, 1.1–1.5). The corresponding adjusted ORs were 1.29 (95% CI, 0.9–1.8) and 1.32 (95% CI, 1.1–1.6), respectively. The crude and adjusted ORs of AC for the Pro/Pro genotype were 1.53 (95% CI, 1.1–2.2) and 1.33 (95% CI, 0.9–2.0), respectively. The corresponding crude and adjusted ORs of the Arg/Pro genotype were 1.31 (95% CI, 1.1–1.6) and 1.36 (95% CI, 1.1–1.7), respectively. In contrast, for SCC the crude and adjusted ORs for the Pro/Pro genotype were 0.94 (95% CI, 0.5–1.6) and 0.75 (95% CI, 0.4–1.5), whereas the corresponding ORs for the Arg/Pro genotype were 1.04 (95% CI, 0.8–1.4) and 1.11 (95% CI, 0.8–1.6), respectively. In the case-only analysis, the p53 Pro/Pro variant also increased the risk of AC compared with SCC (P = 0.05), compared with the wildtype Arg/Arg genotype. No association was seen between p53 polymorphism and tumor grade, disease stage, or gender (Table 2).

The association between the p53 Pro allele (p53 Pro/Pro and p53 Arg/Pro) versus Arg/Arg genotypes in individuals with specific genotypes of GSTM1 and GSTT1 in the model for AC, and separately for SCC, is shown in Fig. 1 (estimators obtained from logistic regression models individually comparing each of two histological subtypes with controls, using an interaction term for p53 and each of the two GST genes separately). In all interaction analyses, homozygous and heterozygous variant p53 genotypes were analyzed as a single group (labeled as the “Pro allele” category), for two reasons: the primary ORs of lung cancer risk for heterozygote and homozygote Pro variants were similar (both adjusted ORs were ~1.3), and the numbers of individuals carrying the homozygous variant genotype were too small for separate consideration in interaction analyses. When comparing the results of separate logistic regression models for AC versus controls and SCC versus controls, GSTM1-null individuals with the Pro allele versus Arg/Arg genotype had an adjusted OR of 1.18 (0.8–1.6) for developing AC and 0.63 (0.4–1.0) for developing SCC. In contrast, GSTM1-present individuals had an adjusted OR of 1.47 (1.0–2.1) for developing AC and an adjusted OR of 1.77 (1.0–3.1) for developing SCC. In the case of AC, GSTM1 did not modify the p53 polymorphism-lung cancer relationship; however, GSTM1 strongly modified this relationship in SCC (P < 0.01). Case-only analyses also found an interaction between GSTM1 and p53 genotypes. In the case-only analysis, the risk of developing SCC versus AC in GSTM1-null individuals was significantly lower for the Pro allele (adjusted OR, 0.56; 95% CI, 0.4–0.8). No differences were found in the GSTM1-present stratum between AC and SCC. In the case of GSTT1, no significant interaction between p53 and GSTT1 polymorphisms was observed for AC or SCC, and no associations were found in the corresponding case-only analyses.

<table>
<thead>
<tr>
<th>Category</th>
<th>AC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pro/Pro</td>
<td>Arg/Pro</td>
</tr>
<tr>
<td>GSTM1-null</td>
<td>29 (11%)</td>
<td>96 (37%)</td>
</tr>
<tr>
<td>GSTM1-present</td>
<td>16 (7%)</td>
<td>90 (42%)</td>
</tr>
<tr>
<td>GSTT1-null</td>
<td>13 (13%)</td>
<td>34 (34%)</td>
</tr>
<tr>
<td>GSTT1-present</td>
<td>34 (8%)</td>
<td>168 (41%)</td>
</tr>
<tr>
<td>Stage I or II</td>
<td>34 (10%)</td>
<td>136 (39%)</td>
</tr>
<tr>
<td>Stage III or IV</td>
<td>20 (9%)</td>
<td>99 (43%)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well- or moderately differentiated</td>
<td>27 (10%)</td>
<td>114 (40%)</td>
</tr>
<tr>
<td>Poorly or undifferentiated</td>
<td>19 (11%)</td>
<td>74 (43%)</td>
</tr>
<tr>
<td>Male</td>
<td>29 (10%)</td>
<td>117 (41%)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (8%)</td>
<td>118 (39%)</td>
</tr>
</tbody>
</table>

Fig. 1. The fitted ORs and 95% CIs for the risk of AC or SCC with the p53 Pro allele (i.e., Pro/Pro + Arg/Pro) versus Arg/Arg genotype for all individuals and individuals with different GSTM1 and GSTT1 genotypes as calculated from different interaction models, each adjusting for age, gender, smoking status, square root of pack-years, and number of years since smoking cessation.

DISCUSSION

Although some genetic polymorphism studies have evaluated small cell lung cancer and NSCLC separately, only a few have analyzed specific NSCLC histological subtypes (28–30). This is the first study examining the differential effects of codon 72 p53 polymorphisms on the development of AC versus SCC. We investigated these associations in a large Caucasian sample of lung cancer patients with a wide

Table 2. Frequency of p53 genotypes by GSTM1 genotype, GSTT1 genotype, stage, grade, or gender, separately for AC and SCC

* Percentages reflect the proportion of the specific p53 genotype among all p53 genotypes in each row (category) for either AC or SCC.

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age distribution and roughly equal numbers of men and women, as histological frequencies vary with these variables (4, 31). Even in our large lung cancer sample, the low prevalence of the large cell subtype prevents its subset analysis. However, all lung histological subtypes were included in the main association analysis of overall lung cancer risk, and there were reasonable numbers of samples to compare specifically AC versus SCC. We confirm that there is an overall elevation in lung cancer risk resulting from the Pro allele; however, this overall relationship is attributable largely to the subset of patients with AC.

Epidemiological and functional studies of the p53 polymorphism suggest that the Arg and Pro alleles confer increased susceptibilities to different types of cancers, implying an interplay of different pathogenetic mechanisms by each allele. Several mechanisms have been proposed to explain the role of the Arg allele in cancer development. The Arg allele has in vitro weaker affinity for several transcription-activating factors (32). In addition, the Arg allele is found to be more susceptible to degradation by the human papillomavirus E6 protein than the Pro allele in vivo (33), although the epidemiological evidence is somewhat contradictory (33–36). The Arg allele may enhance mutant p53 binding to p73, thus neutralizing p73-induced apoptosis independently of human papillomavirus-related mechanisms in squamous cell cancers (37, 38), with supportive epidemiological data (38, 39).

In contrast, the Pro allele of the codon 72 p53 polymorphism has been linked epidemiologically to smoking-related lung and bladder cancers in some but not all studies (40–45). We reported recently that the Pro allele was associated with lung cancer (19); in this study of >2400 cases and controls we confirm this overall association. The exact biological mechanisms for an increased baseline risk of AC by the Pro allele are not fully understood. This polymorphism is located in a proline-rich domain of p53 that has been shown to be required for activation of cyclin-dependent kinase activities in human fibroblast during radiation-induced G1 arrest. Cell, 1023, 1994.


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