Glutathione-S-Transferase M1 and Codon 72 p53 Polymorphisms in a Northwestern Mediterranean Population and Their Relation to Lung Cancer Susceptibility

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Abstract

Several polymorphic genes have been reported to be possibly involved in modifying lung cancer risk in smokers. The gene GSTM1 is frequently deleted in human populations, and the null genotype has been reported to be a risk factor for developing lung carcinoma. A germline polymorphism of p53 with a single-base change at codon 72 that causes an amino acid replacement of arginine (Arg; CGC) by proline (Pro; CTC) is also associated with susceptibility in a Japanese population. Both polymorphisms were genotyped by PCR in a northwestern Mediterranean healthy population (n = 147) and in a group of lung cancer patients (n = 139). The results showed that the frequency of the GSTM1 null genotype was higher in the lung cancer patients compared to the controls (odds ratio (OR), 1.57; 95% confidence interval (CI), 0.99–2.51). The historical subtypes most clearly modified were small cell carcinoma (OR, 1.89; CI, 0.97–3.65) and adenocarcinoma (OR, 1.93; CI, 1.40–2.64). The null GSTM1 genotype was more frequent among those cancer patients who were medium/light smokers (≤50 pack-years) and in those who showed an onset of the disease at a more advanced age. The study of the p53 polymorphism in the healthy population showed allele frequencies of 0.79 (Arg) and 0.21 (Pro). The frequencies found in the lung cancer patients were statistically similar. Both polymorphisms were studied together, and the relative risk of the combination null GSTM1 and Pro/Pro or Arg/Pro genotypes was calculated taking the combination of GSTM1+ together with Arg/Arg as a baseline. The OR found (1.97; CI, 1.83–3.73) suggests that the Pro allele of the p53 germline polymorphism may slightly increase the risk of the GSTM1 null genotype among smokers.

Introduction

Lung cancer is unequivocally associated with tobacco consumption, but many cigarette smokers do not develop this disease and consequently some host factors are suspected of playing a role in determining individual susceptibility to the smoke chemical mixture. Several polymorphic genes (CYP1A1 and CYP2D6) have been reported to be possibly involved in modifying lung cancer risk in smokers. These genes encode for enzymes such as aryl hydrocarbon hydroxylase and debrisoquine hydroxylase involved in the Phase I bioactivation of smoke procarcinogens. Other genes encode for Phase II enzymes that participate in the detoxication reactions and favor elimination of the reactive electrophiles formed in Phase I. This prevents the possibility of significant amounts of the intermediate reaching nuclear DNA and causing mutation and cancer. One of these genes (GSTM1) encodes for a specific isoenzyme (GST Mu) of the cytosolic GST family (EC 2.5.1.18). Substrates of GST Mu include several epoxides of polycyclic aromatic hydrocarbons, which are present in tobacco smoke and that may lead to the formation of stable DNA adducts and trigger the early steps of carcinogenesis. This enzyme frequently lacks activity in Caucasians due to an homozygous GSTM1 gene deletion (3, 4). The lack of activity and/or the null genotype have been reported to be a risk factor for developing smoking-related lung cancer (5–10). Other authors, however, did not find this type of association (11, 12) or found only a slightly elevated risk for light smokers (13).

Germline polymorphisms of other genes involved in different steps of tumorigenesis may also determine individual susceptibility to lung cancer. p53 is a tumor suppressor gene that encodes for a protein which probably plays a critical role in regulating the cell cycle through interaction with proteins required for DNA replication or by regulating the expression of different cell cycle-related genes. A large number of human tumors, including smoke-induced lung tumors, show mutations and deletions of the p53 gene that result in loss of tumor suppression function and cell cycle deregulation (14).

The wild-type p53 gene shows a polymorphism at codon 72 with a single-base change that causes an amino acid replacement in the transactivation domain of the protein of Arg (CGC) by Pro (CCC) (15–18). The functional differences of these two variants of p53 protein are unknown, but the genotype Pro/Pro was found to be overrepresented in a Japanese lung cancer cohort and was reported to be associated with increased cancer risk.
susceptibility (19). A similar study with African-Americans and Caucasians in the United States reported no significant differences between lung cancer patients and controls. The Pro variant at codon 72, however, was found to be in excess in patients with adenocarcinoma in an initial report (20), although it could not be confirmed in a follow-up (21). An enhanced risk was found, more recently, for African-Americans with the Pro/Pro genotype and a relatively early onset of the disease (22), whereas a Swedish study suggested that the codon 72 alleles need not be functionally involved in lung cancer but may rather be markers in linkage disequilibrium with other cancer susceptibility sites on p53 (23).

In the present study, we used PCR-based genotyping methods to examine both the GSTM1 polymorphism and the codon 72 germline p53 polymorphism in a northwestern Mediterranean population (Barcelona, Catalonia, northeastern Spain). We studied a control group of healthy blood donors and a group of lung cancer patients to know the genotype frequencies of these polymorphisms in the general population and to evaluate their possible importance as lung cancer risk modifiers.

Materials and Methods

Subjects. The present study involved 139 lung cancer patients and 147 healthy individuals from the general population. The present study involved 139 lung cancer patients and 147 healthy individuals from the general population. The criteria for the definitive inclusion in the case group were: (a) northwestern Mediterranean Caucasians (Catalonia); (b) residence in the area of Barcelona (minimum, 10 years); (c) available and complete clinical history, including unequivocal histological diagnostic of lung cancer, according to the WHO criteria; (d) recent diagnostic tests (up to 1 year from inclusion date); (e) written consent for inclusion in the protocol and blood extraction. Lung cancer patients who presented, in addition, other cancers or other major pathologies were excluded.

The patients were interviewed by specifically trained members of the Oncology Unit medical staff for a detailed occupational and smoking history, asbestos exposure, drinking habits, and cancer in family members. Questions about smoking habits included whether they smoked or not, the age they started smoking, the average amount smoked per day, and the age at which they eventually quit smoking. PYs were calculated from daily cigarette consumption and the number of years of smoking (1 PY = daily consumption of 20 cigarettes for 1 year).

The patients had histological diagnoses of squamous cell carcinoma (n = 46), small cell carcinoma (n = 49), adenocarcinoma (n = 34), and large cell carcinoma (n = 10). Some patients with less common types, such as mesothelioma (1 patient) or with a mixed histology (4 patients), were not included, although blood and DNA was kept for an additional study.

A summary of the patients’ characteristics is presented in Table 1. No cases were found with a referred exposure to carcinogens other than tobacco smoke, except the patient with mesothelioma, who presented a history of occupational exposure to asbestos.

The controls were selected from a large DNA bank (>2000 samples) from healthy and unrelated volunteers (blood donors, employees, and staff members) and northwestern Mediterranean Caucasians with a minimum 10-year residence in the metropolitan area of Barcelona, who were participating in an ongoing research of genetic polymorphisms in the general population of Catalonia (24, 25). Since all of the polymorphisms studied to date fitted Hardy-Weinberg equilibrium, the DNA bank appears to be representative of the general population under study.

The DNAs for inclusion in the control group (n = 147) were randomly selected from the bank with the only restriction being the matching of a gender distribution (136 males and 11 females), similar to that of the patient group. Mean age was 48 ± 22 years (range, 18–75). Smoking habits were not known for these subjects. The study design was approved by the local ethical committees. DNA was extracted from fresh peripheral leukocytes using the protocol described by Auxbel et al. (26) for the analysis of the genetic polymorphisms.

p53-AccII Polymorphism. The polymorphic site in exon 4 (codon 72) of the p53 gene was achieved by RFLP of PCR-amplified fragments. The amplification reaction was carried out in a 50-μl volume containing 300–400 ng of genomic DNA template, 20 pmol of each oligonucleotide, 200 μM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris (pH 9), 1 mM MgCl₂, 0.1% Triton X-100, and 2 units of Taq polymerase. The primers used to amplify the target DNA were: p53-1 primer, 5’- ATCTACAATCTCCCTGGCGC-3’; and p53-2 primer, 5’- GCAACTGATCCGACCTGAAAAT-3’. The samples were processed through 30 temperature cycles of 1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C. The last elongation step was extended to 8 min. The PCR product, 296-bp size, was purified by Wizard PCR Prep (Promega). Fourteen μl (± 200 to 500 ng) of the PCR purified product was digested with 6 units AccII (5'-CG/GC-3'). The detection of the different alleles was carried out routinely by horizontal submarine ethidium bromide 2% agarose gel electrophoresis, along with a 100-bp ladder (Fig. 1).

In some randomly selected samples, the genotype was confirmed by PCR direct sequencing using the M13 oligonucleotide and the Automated Laser Fluorescent ALF DNA sequencer (Pharmacia). The two methods were in complete agreement in their identification of the genotypes.

GSTM1 Polymorphism. The polymorphic deletion of the GSTM1 gene was determined by PCR (27). The amplification reaction was carried out in a 25-μl volume containing 100–200 ng of genomic DNA as template, 10 pmol of each oligonucleotide, 250 μM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, and 2 units of Taq polymerase. The primers used to amplify the target DNA were: GSTM1-1 primer, 5’- GCTGCCTCTCGTCTGTTCTC-3’; and GSTM1-2 primer, 5’- CAGACAGCTCTTCGTCC-3’. The samples were processed through 30 temperature cycles of 1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C. The last elongation step was extended to 8 min. The PCR product, 296-bp size, was purified by Wizard PCR Prep (Promega). Fourteen μl (± 200 to 500 ng) of the PCR purified product was digested with 6 units AccII (5'-CG/GC-3'). The detection of the different alleles was carried out routinely by horizontal submarine ethidium bromide 2% agarose gel electrophoresis, along with a 100-bp ladder (Fig. 1).

In some randomly selected samples, the genotype was confirmed by PCR direct sequencing using the M13 oligonucleotide and the Automated Laser Fluorescent ALF DNA sequencer (Pharmacia). The two methods were in complete agreement in their identification of the genotypes.

### Table 1: Characteristics of lung cancer patients by histological subtype

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Age (± SD)</th>
<th>PYs (range)</th>
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<tbody>
<tr>
<td>All cancers</td>
<td></td>
<td>58 ± 10 (32–82)</td>
<td>53 ± 23 (0–120)</td>
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<tr>
<td>Small cell</td>
<td></td>
<td>60 ± 10 (45–82)</td>
<td>54 ± 23 (10–100)</td>
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<td>(n = 49)</td>
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<tr>
<td>Adenocarcinoma</td>
<td></td>
<td>57 ± 13 (38–81)</td>
<td>48 ± 18 (0–88)</td>
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<td>(n = 34)</td>
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<td></td>
<td></td>
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<tr>
<td>Squamous cell</td>
<td></td>
<td>59 ± 10 (36–82)</td>
<td>59 ± 22 (20–120)</td>
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<tr>
<td>(n = 42)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Large-cell</td>
<td></td>
<td>56 ± 10 (40–73)</td>
<td>50 ± 24 (20–110)</td>
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<td>(n = 10)</td>
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</tr>
<tr>
<td>Control</td>
<td></td>
<td>48 ± 22 (18–75)</td>
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<tr>
<td>population</td>
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<td>(n = 147)</td>
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Fig. 1. Detection of germline polymorphism of the p53 gene by PCR and AccII digestion. Lanes 1 and 8, Arg/Arg homozygotes; Lanes 2, 4, and 7, Arg/Pro heterozygotes; Lanes 3 and 6. Pro/Pro homozygotes; Lane 5 is a size marker. The fragment of 296 bp is the nondigested PCR product from the Pro allele. Fragments of 169 and 127 bp result from AccII digestion of the Arg allele.

Fig. 2. Detection of GSTM1 polymorphism by PCR. Lanes 2 and 4, null GSTM1 genotypes (absence of 1650-bp fragment). Lanes 3 and 5, GSTM1+ genotypes. As an amplification control, a 312-bp coamplified fragment (CYP1A1) appears in the sample lanes. Lanes 1 and 6 are size marker ladders.

10 mM Tris (pH 9), 1.5 mM MgCl₂, 0.1% Triton X-100, and 0.75 unit Taq polymerase. The oligonucleotides used to amplify the target DNA were: GSTM1–1 primer, 5'–GAAGGTGGCCTCCTCTTGG-3'; and GSTM1–2 primer, 5'–AATTCTGGATTGTAGCAGAT-3'. As a control, exon 7 of the CYP1A1 gene was coamplified. Primers for the coamplified fragment were 5'–GAACTGCCACTTCAGCTGTCT and 5'–CAGCTGCATTTGGAAGTGCTC, yielding a 312-bp product. Thirty temperature cycles were used: 1 min at 95°C, 1 min at 55°C, and 1 min 30 s at 72°C. The last elongation step was extended to 5 min. Negative (without DNA) and positive (cell line K562) control samples were included in each amplification series.

The presence of at least one GSTM1 allele [GSTM1 (+)], identified by a 165-bp PCR product, or its complete deletion [GSTM1 (–), null genotype] were analyzed by 1% ethidium bromide agarose gel electrophoresis (Fig. 2).

Results

The frequency of the GSTM1 null genotype in the general population was 45.6%, which is consistent with other genotyping studies in Caucasian and Japanese populations (9, 10, 12). The samples were stratified according to age intervals (<30; 30–50; and >50 years), but the genotype frequencies were similar in all of the groups (data not shown), thus demonstrating no association between the frequency of the GST null genotype and age within the general population.

Table 2 shows the distribution of the GSTM1 genotypes in the lung cancer patients. The proportion of the GSTM1 null genotype was significantly greater in the lung cancer patients compared to the controls (56.8%; OR, 1.57; 95% CI, 0.99–2.51). When the patients were divided according to tumor histology, the null genotype was clearly increased in the group of patients with small cell carcinoma (61.2%; OR, 1.89; CI, 0.97–3.65) and with adenocarcinoma (58.3%; OR, 1.93; CI, 0.90–4.14), whereas the group of small cell carcinoma showed a less significant increase.

The lung cancer patients were divided into two groups according to smoking habits, using the median number of PYs as a cutoff (<50 PY and >50 PY) and in two age groups using the median age (<50 years and >50 years) in search for possible associations between the GST null genotype and smoking history or age at the onset of the disease. The genotype distribution was then compared with that of the control population. The results (Table 3) showed that there was a tendency of the GST null genotype to be overrepresented more clearly in the medium or light smokers (<50 PY), especially within the small cell carcinoma subgroup, and in the patients who showed an onset of the disease at a more advanced age.

The frequencies of the three p53 genotypes Arg/Arg, Arg/Pro, and Pro/Pro found in the healthy population were 92 (62; 6%), 47 (32; 0%) and 8 (5; 4%), respectively, and fitted the Hardy-Weinberg equilibrium (χ² = 0.345; df = 1; P > 0.80) with allele frequencies of 0.79 (Arg) and 0.21 (Pro). These results show slight but significant differences with other reports in Japanese and Caucasians (16–18), which found a somewhat higher frequency of the Pro allele. Table 4 shows the distribution of the p53 genotypes in the lung cancer patients grouped according to the tumor histology.

The distribution showed no significant differences in any cancer group when compared to the healthy controls; however, the Pro allele showed a tendency
to be overrepresented in the adenocarcinoma and small cell carcinoma groups. No significant variations in the genotype distribution among the adenocarcinoma patients were found to be those who most frequently showed the enzyme lack of activity. On the other hand, other authors did not find any positive correlation between adenocarcinoma of the lung and the GSTM1 null genotype (9), and there is even a study that reported a significant negative correlation (11). Furthermore, some studies found a particularly increased susceptibility to squamous cell carcinoma but resulted in inconclusive results, due to the small number of cases concerning the other major type of lung cancer, small cell carcinoma (9, 5).

The results reported here for the whole group of patients suggest that the GSTM1 null genotype may increase the susceptibility to lung cancer. The null genotype showed a tendency to be overrepresented in the three major histological subtypes, with the small cell carcinoma and the adenocarcinoma groups presenting higher ORs than the squamous cell carcinoma group. The GSTM1 distribution among the adenocarcinoma patients tends to confirm particularly the original observations of Seidegard et al. (5), in spite of this type of tumor being less clearly related to smoking than the two other major subtypes. The patients with large cell carcinoma presented a contrary tendency but due to small numbers, the results were not significant and require an additional study with a larger number of patients.

Sources of bias in the selection of cases for the GSTM1 studies have been reviewed by London et al. (13) as a possible
explanation for the discordant results reported in the literature. In the present study, we selected, for a period of time, all of the patients who attended the Hospital Oncology Unit for treatment or surgery follow-up and fitted the inclusion criteria described above. This resulted in a distribution of histological types (small cell carcinoma > squamous cell carcinoma > adenocarcinoma > large cell carcinoma) somewhat different to that found in the general Spanish cancer registries (squamous cell carcinoma > adenocarcinoma > small cell carcinoma > large cell carcinoma). We found, however, a tendency for the GST null genotype to be overrepresented in the three major subtypes; therefore, a major bias in the calculated risk for the whole lung cancer group seems unlikely. We also limited inclusion to recently diagnosed patients to avoid a possible bias due to the inclusion of a number of long-term survival patients. Imbalance in age and sex between the patients and the general population must be considered. In this study, the gender distribution was similar in both groups, and although mean age was considerably lower in the general population studied, we did not find any difference in the frequency of the GST null genotype among the different age strata within this group, discarding significant genotype variations between patients and controls due to age imbalance. An association between smoking habits and GST null distribution in the general population would also be a confounding factor. This could not be assessed in our study due to a lack of sufficient information about the smoking status of the control group. London et al. (13), however, did not find such an association in a control population stratified according to smoking status.

The analysis of the possible association between the GSTM1 genotype and the patients’ lifetime cigarette consumption revealed that the null genotype may be more clearly associated with an increased risk of small cell carcinoma in medium and light smokers. This would require confirmation with a much larger study. It is in accordance with some reports (13, 28) and is discordant with others (10) and suggests that genetic susceptibility may play a role in certain medium/low exposure conditions but be overpowered by heavy exposure to high doses of smoke carcinogens.

The distribution of the p53 genotypes among the lung cancer patients do not permit the confirmation of the significant differences that were found in a former Japanese study (19). If the p53 genotype is combined, however, with GSTM1, a significant increased OR is found for patients with both GSTM1 null and the “rare” Pro allele, suggesting that the risk associated with the GST null genotype may be slightly increased by a Pro/Arg or a Pro/Pro genotype. Both polymorphisms probably work independently, but unlike the GSTM1 polymorphism, the functional differences eventually associated with the germline codon 72 p53 polymorphism are still not elucidated. According to our data, the smokers with both the GSTM1 null genotype and a Pro/Pro or Pro/Arg genotype may have a nearly 2-fold higher risk of developing lung carcinoma compared with those with a GST+ and a Arg/Arg genotype. Confirmation of this finding would require further studies with a larger number of samples.

In conclusion, the results reported here tend to reinforce the view that GSTM1 null genotype may increase susceptibility to lung cancer among smokers. The ORs found to date, in this and other similar studies, suggest only a weak association, but the combination with other polymorphic genes such as p53 or CYP1A1 (29) may increase the calculated risks.

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References

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