SHORT COMMUNICATION

Glutathione S-transferase μ1 null genotype is associated with K-ras gene mutation in lung adenocarcinoma among smokers

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Tobacco smoke contains several carcinogens, including benzo[a]pyrene (BP) and other polycyclic aromatic hydrocarbons (PAHs) that initiate cancer. Many of these carcinogens are oxidized by phase I enzymes into reactive metabolites that are detoxified by phase II enzymes. Glutathione S-transferases (GSTs), important phase II enzymes, play a major role in the detoxification of carcinogens and protect DNA against cellular damage. The glutathione S-transferase μ1 (GSTM1) gene locus is polymorphic owing to a gene deletion, which results in the virtual absence of GSTμ enzyme activity in individuals with both alleles deleted [GSTM1(−)]; approximately half of the population in various ethnic groups are of both alleles deleted [GSTM1(−)] (1). The GSTM1 gene product is suggested to be particularly important for detoxifying BP diol epoxide (2); individuals genetically lacking GSTM1 activity can potentially be at an enhanced risk for smoking-related lung cancer. Many studies show that lack of GSTM1 activity or of its gene is responsible for the genetic susceptibility to tobacco-related lung cancer, however, little attention has been paid to whether the gene mutations are indeed increased in cases with GSTM1(−).

The ras genes have been shown to be mutationally activated in a number of cancers in humans (3) and in a carcinogen-specific manner in experimental animals (4). There are many studies showing an association between K-ras gene mutation and smoking in lung cancer cases (3,5–7). It has been demonstrated that when compared with non-smokers, smokers have higher frequencies of K-ras gene mutations (8). Various studies have shown that chemical carcinogens can selectively induce specific base pair changes found in codons 12, 13 and 61 of the K-ras gene (9–11). It has, therefore, been speculated that there may be a correlation among GSTM1 genotype, K-ras gene mutation and smoking. The purpose of this study was to investigate the possible relation between the GSTM1 null genotype and K-ras gene mutation in human non-small cell lung cancer.

We studied 312 lung tumors from 312 patients who underwent surgical resection for primary non-small cell lung carcinoma between 1986 and 1996 at the Second Department of Surgery of Fukuoka University Hospital. The tumors included 193 adenocarcinomas and 119 squamous cell carcinomas. In adenocarcinoma patients the mean age was 63.8 years and the group was made up of 115 men and 78 women. In squamous cell carcinoma patients the mean age was 68.4 years and the group was made up of 101 men and 18 women. Information about smoking habits was obtained from the hospital patient records. The patients were classified as smokers and non-smokers. Smokers included patients who were former smokers. Non-smokers were patients who had never smoked. DNA was isolated from frozen tissue and blood using standard procedures with proteinase K and phenol/chloroform extraction.

The GSTM1 genotypes were at first determined using DNAs from peripheral granulocytes and carcinoma tissues in 100 of the 312 lung carcinoma patients. We could obtain neither a blood sample nor normal tissue from the rest of the 212 patients. The GSTM1 genotype of peripheral granulocytes was in complete agreement with that of the carcinoma tissue in each subject and somatic mutation of the GSTM1 gene is not known to occur in lung carcinoma. Therefore, the GSTM1 genotyping study was performed using tumor tissue in the remaining 212 carcinoma patients. Furthermore, we determined the GSTM1 genotype in 138 healthy Japanese controls using DNA from peripheral granulocytes. The GSTM1 genotyping was carried out by PCR according to the method of Comstock et al. (12). The GSTM1 genotype was determined by the presence or absence of PCR product. Primers MD65-L (GCACACACACATGAAATGCA) and MD65-R (CTTTCCTTTCGCGTACAG), which amplify a 154–170 bp CA repeat marker on chromosome arm 16q, were used as internal controls (D16S267; 13) (1). If PCR products were faint assays were repeated with a sample diluted 5-fold with deionized pyrene; GSTs, glutathione S-transferases; GSTM1, glutathione S-transferase μ1; PAHs, polycyclic aromatic hydrocarbons; SSOPs, sequence-specific oligonucleotide probes.

Abbreviations: BP, benzo[a]pyrene; GSTs, glutathione S-transferases; GSTM1, glutathione S-transferase μ1; PAHs, polycyclic aromatic hydrocarbons; SSOPs, sequence-specific oligonucleotide probes.
Codons 12 and 13 of the K-ras gene were examined for mutations by PCR dot-blot hybridization analysis, because almost all of the K-ras mutations occur in codons 12 and 13 in lung carcinoma. PCR amplifications were done in a 50 μl total reaction volume using 0.1μg genomic DNA. The PCR reaction conditions were 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 μM MgCl2, 50 μM each dNTP, 0.3 μM each primer and 1.25 U Taq polymerase (Takara, Osaka, Japan). The PCR primers used for amplification of the 102 bp region including codons 12 and 13 of the K-ras gene were 5'-GCCTGCTGAA-AATGACTGTA-3' (sense) and 5'-CGTCCACAACTTGA-TTCTGAAAT-3' (antisense). Amplification conditions were an initial cycle at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min. Human ras Muta-Lyzer Probe Panels (Clontech, Palo Alto, CA) were used as sequence-specific oligonucleotide probes (SSOPs). The SSOPs were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Toyobo, Osaka, Japan). The amplified DNAs were spotted onto a nylon membrane (Hybond N+; Amersham, Tokyo, Japan) and immobilized by alkaline denaturation in 0.4 N NaOH. The membranes were prehybridized for 1 h at 54°C in 10 ml of hybridization buffer composed of 50 mM Tris–HCl, pH 8.0, 3.0 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, 5× Denhart’s solution, 0.1% SDS and 100 μg/ml heat-denatured herring sperm DNA. After hybridization with labeled SSOPs at 54°C for 1 h the filters were washed twice for 5 min at room temperature in 2× SSPE and 0.1% SDS with constant gentle agitation, once for 10 min at room temperature in TMAC solution (50 mM Tris–HCl, pH 8.0, 3.0 M tetramethylammonium chloride, 2 mM EDTA, pH 8.0, and 0.1% SDS), twice for 15 min at 59°C in the TMAC solution and once for 5 min at room temperature in 2× SSPE. After removing excess fluid on Wattmann 3MM filters the filters were exposed to Kodak XAR5 film for 2 h at room temperature to obtain hybridization signals.

The statistical significance of each finding was determined using Fisher’s direct probability test or the Kruskal–Wallis test. The odds ratio and 95% confidence interval were estimated by logistic regression analysis. Differences with a P value <0.05 were considered significant.

The GSTM1 genotype was determined by detection of at least one GSTM1 gene [GSTM1(+) or its homozygous deletion [GSTM1(−)]. Although loss of heterozygosity at chromosome 1p has since been described in ~15–18% of non-small cell lung cancers (14,15) there is little possibility of mistyping the GSTM1 genotype in tumor tissue due to loss of heterozygosity at 1p32, because the GSTM1 genotype of peripheral granulocytes was in complete agreement with that of the carcinoma tissue in our 100 patients and somatic mutation of the GSTM1 gene is not known to occur in lung carcinoma. However, the possibility of GSTM1 mistyping in 212 patients cannot be completely disproved. Among the 138 healthy Japanese controls 66 (47.8%) and 72 (52.2%) were identified as GSTM1(+) and GSTM1(−), respectively. Among the 312 patients with lung carcinoma 152 (48.7%) and 160 (51.3%) were GSTM1(+) and GSTM1(−), respectively. The frequency of the GSTM1 null genotype, GSTM1(−), did not differ significantly between the healthy controls and patients. There was no significant difference in the GSTM1 genotype among the patients classified by histological tumor type or sex. In addition, there was no significant correlation between the GSTM1 genotype and smoking in the patients; GSTM1(−) was found in 51.3 and 51.2% of habitual smokers and non-smokers, respectively.

Using the PCR SSOP hybridization assay K-ras gene mutations were detected in 29 of 312 (9.3%) lung cancer specimens (Table I). Mutations were found in 21 of 193 (10.9%) adenocarcinomas and in 8 of 119 (6.7%) squamous cell carcinomas. All patients having tumors with a K-ras mutation were smokers. K-ras mutations were significantly more frequent in male patients, 26 of 216 (12%), than in female patients, 3 of 96 (3.1%) (P = 0.010). However, when we compared the K-ras mutation rates in the patients with smoking history there was no significant gender difference; 26 of 205 (12.7%) smoking male patients and 3 of 25 (12%) smoking female patients.

We first analyzed the association between GSTM1 genotype and K-ras gene mutation in all patients. Mutations of the K-ras gene were found in 9 of 152 (5.9%) patients with the GSTM1(+) genotype and in 20 of 160 (12.5%) with the GSTM1(−) genotype. K-ras gene mutation was significantly more frequent in GSTM1(−) than in GSTM1(+) carriers (P = 0.045). When we investigated this association by taking into account the histological type of lung tumors, GSTM1 genotype and K-ras gene mutation were significantly associated in adenocarcinoma (P = 0.024) but not in squamous cell carcinoma (Table II).

The K-ras gene is mutated in ~20–30% of non-small cell lung cancers in Europeans and Americans (5,16), while the frequency in Japanese is 6.9–12% (17,18); our results are consistent with the reported frequency in Japanese. K-ras gene mutations were found more frequently in adenocarcinoma than in squamous cell carcinoma in this study, as has been reported (3.16–19). K-ras mutations are more frequently detected in adenocarcinomas obtained from patients who were habitual smokers than in those from patients who never smoked. The association between smoking and the development of mutations

### Table I. Mutations of codons 12 and 13 of the K-ras gene

<table>
<thead>
<tr>
<th>Codon</th>
<th>Mutation</th>
<th>Nucleotide substitution</th>
<th>Amino acid change</th>
<th>No. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>12a</td>
<td>TGT</td>
<td>G→T</td>
<td>Cys</td>
<td>15</td>
</tr>
<tr>
<td>GTT</td>
<td></td>
<td></td>
<td>Val</td>
<td>5</td>
</tr>
<tr>
<td>GAT</td>
<td></td>
<td>G→A</td>
<td>Asp</td>
<td>4</td>
</tr>
<tr>
<td>GCT</td>
<td></td>
<td>G→C</td>
<td>Ala</td>
<td>1</td>
</tr>
<tr>
<td>TGC</td>
<td>G→T</td>
<td></td>
<td>Cys</td>
<td>3</td>
</tr>
<tr>
<td>GAC</td>
<td>G→A</td>
<td></td>
<td>Asp</td>
<td>1</td>
</tr>
</tbody>
</table>

*aWild-type GGT (Gly).  
*bWild-type GGC (Gly).

### Table II. Association of GSTM1(−) genotype and K-ras mutation rate in lung cancers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>K-ras mutation (rate)</td>
</tr>
<tr>
<td>GSTM1(−)</td>
<td>93</td>
<td>15 (16.1%)</td>
</tr>
<tr>
<td>GSTM1(+)</td>
<td>100</td>
<td>6 (6.0%)</td>
</tr>
</tbody>
</table>

*Versus GSTM(+), odds ratio 3.011 (95% confidence interval 1.116–8.129), P = 0.024.
in the K-ras gene suggests that carcinogenic agents in tobacco smoke may induce these mutations.

Tobacco smoke contains several carcinogens, including PAHs. Slebos et al. (20) noted that exposure to BP in tobacco smoke was an important factor in the induction of point mutations in the K-ras gene in human lung adenocarcinomas. The most commonly identified K-ras gene mutations in lung cancers from smokers were G→T transversions followed by the G→A transitions (3,21). You et al. (9) reported that 9 of 13 (69.2%) mutations of the K-ras gene found in BP-induced lung adenocarcinoma of mice were G→T transversions and 4 of 13 (30.8%) mutations were G→A transitions. In contrast to lung cancer, K-ras gene mutations in gastrointestinal malignancies, especially in colon and pancreatic adenocarcinomas, were not preferentially G→T transversions (22,23). Despite a similar histological appearance to adenocarcinoma, the pattern of K-ras mutations in lung cancer differs from that in gastrointestinal malignancies. This difference might relate to the degree of association between smoking and K-ras gene mutations, because gastrointestinal cancers are not as strongly correlated with smoking as is lung cancer. Experimental studies have indicated that PAH compounds like BP primarily induce G→T transversions (24,25). GSTM1 genotype was found to be associated with transition mutations at G:C base pairs in the p53 and K-ras genes in lung cancer (26).

BP is metabolized into a carcinogenic diol epoxide that is able to bind covalently to DNA. Adducts of BP diol epoxide have been found in the lung DNA of smokers (27). BP diol epoxides are detoxified by GSTM1, a phase II enzyme, protecting the DNA against damage. Our study has shown that K-ras mutations occurred more frequently in lung adenocarcinomas from smokers with the GSTM1(−) genotype than in those from smokers with the GSTM1(+) genotype or in those from nonsmokers. These observations suggest that one mechanism causing K-ras gene mutation in smokers might be accumulation of BP diol epoxide. In clinical practice lung squamous cell carcinoma is related to smoking, however, there was no relation between the GSTM1(−) genotype and K-ras gene mutation in squamous cell carcinomas in this study. As regards gene mutations in squamous cell carcinomas of the lung, another gene, probably p53, might be a specific target of the mutagenic activity of tobacco smoke, because the frequency of p53 gene mutation is higher in lung squamous cell carcinomas than in lung adenocarcinomas (5). Ryberg et al. (28) reported that individuals with the GSTM1(−) genotype were at a higher risk of p53 gene mutations caused by PAH compounds, at least when heavily exposed. Kawajiri et al. (29) reported that CYP1A1 germline polymorphisms were related to cigarette smoking-associated p53 gene mutations. They also examined K-ras gene mutations in 187 lung cancer patients genotyped for GSTM1, however, K-ras gene mutations were not associated with either smoking or GSTM1 genotype in their study. We have here shown a correlation between the GSTM1(−) genotype and K-ras gene mutations in lung adenocarcinoma, especially in smokers. Needless to say, there is no simple relation between smoking, the development of gene mutations and carcinogenesis, however, our results suggest that one of the carcinogenetic processes involves smoking, the GSTM1 null genotype and K-ras gene mutations in lung adenocarcinomas. However, the frequency of K-ras gene mutations in Japanese is low and the number of cases with K-ras gene mutations was still small in our study. Thus, further study is required.

References


