Impact of Genetic Polymorphisms in Cytochrome P450 2E1 and Glutathione S-Transferases M1, T1, and P1 on Susceptibility to Esophageal Cancer among High-Risk Individuals in China

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Abstract

Esophageal cancer, which is prevalent in China, is believed to be induced by environmental carcinogens such as nitrosamines and other agents. The disproportionate geographical distribution of this cancer among individuals suggests a role for gene-environment interactions in developing the disease. We have shown in our preliminary study that a genetic polymorphism in cytochrome P450 2E1 (CYP2E1) that is known to activate nitrosamines may be a susceptibility factor involved in the early events leading to the development of esophageal cancer (Lin et al., Cancer Epidemiol. Biomark. Prev., 7: 1013–1018, 1998). This relatively larger study was conducted to compare the results with our previous findings. One hundred and fifty cases with esophageal cancer, 146 cases with esophageal dysplasia, and 150 normal controls were residents of Linxian, China, a high-risk area. Genomic DNA samples were assayed for restriction fragment length polymorphisms in the CYP2E1 and GSTP1 loci by PCR amplification followed by digestion with Rsal and Alw261, respectively. Deletion of the GSTM1 and GSTT1 genes was detected by multiplex PCR. The distribution of CYP2E1 c1/c1 allele frequency was found to be significantly different between controls (44.0%) and cases with cancer (71.3%) or cases with dysplasia (70.6%; P < 0.0001). Individuals having the c1/c1 genotype were at a 3.1-fold [95% confidence interval (CI), 2.4–3.9] increased risk of developing squamous cell carcinoma of the esophagus. Although polymorphisms in the GSTT1 and GSTP1 were not significantly different between cases with cancer or cases with dysplasia and controls, the frequency of the GSTM1 non-null (+/+) and +/0 genotypes appeared to be overrepresented in cases with cancer compared with controls (odds ratio, 2.3; 95% CI, 1.8–3.0). Furthermore, a joint effect of the CYP2E1 c1/c1 genotype and GSTM1 non-null genotype on the cancer risk was observed, showing an odds ratio of 8.5 (95% CI, 3.7–19.9). These results demonstrate that CYP2E1 and perhaps GSTM1 are genetic determinants in the development of squamous cell carcinoma of the esophagus.

Introduction

SCC3 of the esophagus is one of the most common cancers in China, with ~250,000 cases diagnosed every year. The distribution of the cancer is markedly different over the country. Linxian County in Henan Province is an example of high-risk areas, where the mortality rate of the disease is over 150 per 100,000 (1). Esophageal carcinogenesis follows a multistage progression, from normal epithelium to basal cell hyperplasia, dysplasia, or carcinoma in situ, and finally to invasive SCC (2–4). Many environmental agents, particularly nitrosamines, have been suggested to be involved in the etiology of SCC of the esophagus in this area (5–8). However, even in a high-risk area, only a small portion of people develops this disease. The disproportionate occurrence of this cancer among individuals suggests a role of host susceptibility factors in the development of the disease. In recent years, evidence has accumulated to support the hypothesis that genetic polymorphisms in carcinogen-metabolizing enzymes may be of importance in determining individual susceptibility to cancer (9, 10).

CYP2E1 is an enzyme responsible for the metabolic activation of many carcinogens, including nitrosamines (11, 12). This enzyme is also believed to participate in the oxidation of other compounds, such as ethanol, to produce reactive free radicals that may initiate lipid peroxidation and consequently carcinogenesis (13, 14). CYP2E1 represents a major CYP isoenzyme in the liver and is also expressed at significant levels in human esophagus and other extrahepatic tissues (15–17). Although certain chemicals and pathophysiological status can induce the activity of CYP2E1, considerable interindividual variations have been observed before and after induction (18–20), suggesting that the variation may be determined by genetic factors in the locus. Several restriction fragment length polymorphisms of the human CYP2E1 have been identified (21–23), and the variant c2 allele recognized by Rsal digestion in the

1 The abbreviations used are: SCC, squamous cell carcinoma; CYP, cytochrome P450; GST, glutathione S-transferase; OR, odds ratio; CI, confidence interval.
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The GSTs, a family of multifunctional enzymes, metabolize a variety of xenobiotics with a large overlap in substrate specificity. Individuals who are homozygous for the null GSTM1 or null GSTT1 alleles lack the respective enzyme functions (29, 30). The null GSTM1 genotype appears to be common in both Asians and Caucasians, whereas the null GSTT1 genotype exhibits population frequencies that depend on ethnicity (31–33). The GSTM1 and GSTT1 defects seem to be associated with increased risk of certain cancers (34, 35); however, conflicting data have been observed (36–38). This may reflect the fact that certain GSTs may be more able to detoxify certain carcinogens, but others may result in a higher risk for some types of exposure, which has been especially demonstrated with GSTT1 (34, 39). GSTP1 is a major GST isoform expressed in human esophagus (17, 40) and has been shown to be genetically polymorphic. Two single-base mutations within exons 5 and 6 have been identified that result in Ala105→Val and Ala140→Val changes in the amino acid sequence of the protein, which alter affinity and activity for some substrates of the enzyme (41–43). Therefore, the GSTP1 polymorphism may also have potential effects on cancer susceptibility (44, 45).

Although several studies have been undertaken to examine the association between susceptibility to some types of cancer and genetic polymorphisms in CYP2E1 (22, 46–51) and GSTs (see above), there are limited data on their association with SCC of the esophagus (52–54). A case-control study based on the population in a high-risk area such as Linxian provides an excellent opportunity for assessment of the impact of a genetic susceptibility factor on esophageal carcinogenesis because the population is relatively stable and has similar life-styles and environmental exposures. Recently, we reported in a pilot study an association between genetic polymorphisms in CYP2E1, but not GSTs, and the risk of developing squamous cell dysplasia, a precancerous lesion, and SCC of the esophagus in Linxian, China (32). This independent case-control study was carried out as a comparison with our initial findings. We also assessed the joint effect of genetic polymorphisms and tobacco smoke, a known risk factor for many cancers, on the risk of SCC of the esophagus in this study population.

Materials and Methods

Study Population. This case-control study contained 150 patients with SCC of the esophagus, 146 patients with squamous cell dysplasia of the esophagus, and 150 controls. All subjects were residents in Linxian County, Henan Province, China. The cases were recruited over a period from January 1997 to November 1998 at two local hospitals. All cases diagnosed at these two hospitals during the study period were recruited, yielding a 100% response rate. Case subjects were newly diagnosed as having SCC of the esophagus and were previously untreated. The diagnoses of cancer were confirmed histologically. Cases with squamous cell dysplasia and normal controls were accrued from a cancer-screening program for early detection of esophageal cancer and its precancerous lesions in the same area. The diagnoses of dysplasia were determined by esophageal biopsy. Controls randomly selected on the basis of cytological examination or biopsy were frequency-matched to the cancer cases for age and gender. All subjects were required to respond to a personal interview and provide information on sociodemographic characteristics, recent and prior tobacco use, and family history of cancer.

Polymorphism Analysis. Genomic DNA was isolated from surgically removed “normal” tissues adjacent to tumors of patients with SCC of the esophagus or from biopsy samples of cases with dysplasia and controls, using standard methods as described previously (32). Genotypes were analyzed using PCR-based methods as described below. Genotyping was conducted with blinding to case/control status.

CYP2E1. Because a Ras-loss but not Draf1-recognized polymorphism in the CYP2E1 locus was found to be associated with increased risk of esophageal cancer in our pilot study (32), only the Ras site polymorphism in the transcription regulation region was analyzed in this study. The PCR primers used were 5’-CCAGTCGAGTCTACATGTC-3’ and 5’-AGACCTCCACATTGAC-3’. All PCR amplifications were performed in 25-μl reaction mixtures containing 0.1 μg of template DNA, 1.0 μM each primer, 0.2 mM deoxynucleotide triphosphates (Promega, Madison, WI), 2.5 mM MgCl2, and 1.5 units of Taq polymerase in buffer [10 mM Tris-HCl (pH 9), 1% Triton X-100, 2% DMSO, Promega]. After an initial denaturation at 95°C for 2 min, the DNA was amplified by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C, followed by a final extension step of 10 min at 72°C in a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, CT). Ten μl of amplified 552-bp product were then digested with 10 units of Ras restriction enzyme (New England Biolabs, Inc., Beverly, MA) at 37°C for 4 h, and the restricted product was analyzed by electrophoresis in 2.5% agarose gel containing ethidium bromide. Ras digestion produced three CYP2E1 genotypes, i.e., the predominant homozygote c1/c1, the heterozygote c1/c2, and the rare homozygote c2/c2. The predominant allele (c1) was sensitive to Ras digestion and resulted in two fragments at 352 and 200 bp, whereas the c2 allele was resistant to Ras digestion.

GSTM1 and GSTT1. The GSTM1 and GSTT1 genes were identified by a multiplex PCR procedure (32), based on that described by Arand et al. (55). In brief, this PCR method had both GST-specific primers in the same reaction mixture together with primers for amplification of the albumin gene as an internal control. The absence of a GSTM1-specific 219-bp product or GSTT1-specific 459-bp product indicated the corresponding null genotype when the albumin-specific 350-bp product was present in the reaction. PCR was carried out in a 25-μl mixture consisting of reaction buffer, template DNA (0.1 μg), deoxynucleotide triphosphates (0.2 mM), MgCl2 (2.5 mM), each primer (1.0, 0.3, and 0.1 μM for GSTM1, GSTT1, and albumin, respectively), and Taq polymerase (1.25 units). Amplification was achieved by 35 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. Amplified products were resolved on a 1.5% agarose gel.

GSTP1. The A→G mutation within exon 5 of the GSTP1 gene was detected by a PCR-restriction fragment length polymorphism method using the primers 5’-AGCCACATCCTCTCTCCCTC-3’ and 5’-TACTTGGTCGTTGATGTCC-3’. The conditions for PCR amplification and AluI digestion of the amplified product were as described previously (32). A sample of the resulting fragment was separated by electrophoresis to detect the mutation with polymorphic bands of 213 and 227 bp (GSTP1*B) or 440 bp (GSTP1*A).

Statistical Analysis. Pearson’s χ2 test was used to examine differences in distributions of genotypes studied between cases and controls. ORs with 95% CIs calculated using unconditional logistic regression and adjusted for age, gender, smoking status, and pack-years of smoking were used to estimate the association between certain genotypes or tobacco smoking and diseases. Gene-gene interactions and gene-smoking interactions, after adjusting for age, gender, and smoking, were also ana-

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were homozygous for the c1 allele of GSTM1 in Table 2, 71.3% of cancer cases and 70.6% of dysplasia cases compared with 0.82 and 0.18 among cancer cases (P < 0.05). Subjects with the c1/c1 genotype had a 3-fold increased risk of developing SCC (adjusted OR, 3.1; 95% CI, 2.4 –3.9) and 3-fold increased risk of developing squamous cell dysplasia (adjusted OR, 3.2; 95% CI, 2.5–4.1), compared with subjects with variant c1/c2 and c2/c2 genotypes.

Although the GSTM1 gene deletion was common in both controls and cases, a significant difference in the distribution of GSTM1 genotypes was also observed between cancer cases and controls (Table 2). Seventy percent of cancer cases were GSTM1 non-null (+/+ and +/0) genotypes, which was significantly higher (P < 0.001) than that of control subjects (44.0%). The adjusted OR of esophageal cancer for the GSTM1 non-null genotype compared with the GSTM1-null genotype was 2.3 (95% CI, 1.8–3.0). The GSTM1 genotype was not significantly associated with squamous cell dysplasia. Because the distributions of genotypes of both GSTT1 and GSTP1 did not differ significantly between the controls and either group of cases, further analysis was focused on CYP2E1 and GSTM1.

Results of the analysis of interaction between the CYP2E1 and GSTM1 genotypes and the GSTM1 genotypes on risk of SCC of the esophagus are shown in Table 3. Although no significant interaction between the two genes was observed (P = 0.34), cases with the CYP2E1 c1/c1 genotype were more likely to carry GSTM1 null alleles than their corresponding controls. Among individuals who carried the CYP2E1 c1/c1 and GSTM1-null genotypes, the OR of SCC of the esophagus was 4.4 (95% CI, 1.8–10.8). However, the OR was increased to 8.5 (95% CI, 3.7–19.9) among individuals who had both CYP2E1 c1/c1 and GSTM1 null-genotypes (P < 0.05, test for homogeneity).

The potential interaction between tobacco smoking and CYP2E1 or GSTM1 polymorphisms on the risk of the cancer was also examined. Table 4 shows the ORs of SCC of the esophagus related to CYP2E1 and GSTM1 genotypes by exposure to tobacco smoking. An excessive risk with borderline significance (OR, 2.3; 95% CI, 1.0–5.2) was found to be related to smoking only among those with at least one copy of the CYP2E1 c2 allele. This pattern, although not statistically significant, was also seen among those with the GSTM1-null genotype compared with nonsmokers. The interactions between both genes and the levels of smoking (pack-years) were not significant.

### Discussion

The results obtained from the current study confirm our previous finding that CYP2E1 genetic polymorphism is a susceptibility factor involved in the early events leading to SCC of the esophagus (32). On the basis of this study of 146 cases with dysplasia, 150 cases with carcinoma, and 150 controls, a 3-fold increased risk of both dysplasia and cancer of the esophagus was observed among subjects with the c1/c1 genotype of CYP2E1. This observation is in agreement with recent studies showing that the c1/c1 genotype of CYP2E1 was associated with increased risk of lung cancer (48, 57, 58) and liver cancer.

### Table 1: Demographic variables of the study subjects

<table>
<thead>
<tr>
<th>Gender, n (%)</th>
<th>Control subjects (n = 150)</th>
<th>Dysplasia cases (n = 146)</th>
<th>Cancer cases (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>99 (66.0)</td>
<td>74 (50.7)*</td>
<td>99 (66.0)</td>
</tr>
<tr>
<td>Female</td>
<td>51 (34.0)</td>
<td>72 (49.3)*</td>
<td>51 (34.0)</td>
</tr>
<tr>
<td>Mean age ± SD (years)</td>
<td>53.6 ± 8.2</td>
<td>51.3 ± 7.1</td>
<td>54.5 ± 8.2</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>96 (64.0)</td>
<td>110 (75.3)*</td>
<td>90 (60.0)</td>
</tr>
<tr>
<td>Current</td>
<td>54 (36.0)</td>
<td>36 (24.7)*</td>
<td>60 (40.0)</td>
</tr>
<tr>
<td>≤20 pack-years smoked</td>
<td>23 (42.6)</td>
<td>21 (58.3)</td>
<td>26 (43.3)</td>
</tr>
<tr>
<td>&gt;20 pack-years smoked</td>
<td>31 (57.4)</td>
<td>15 (41.7)</td>
<td>34 (56.7)</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with controls.

### Table 2: Genotype frequencies of CYP2E1 and GSTM1, GSTT1, and GSTP1 among cases with dysplasia, cases with carcinoma, and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control subjects (n = 150)</th>
<th>Dysplasia cases (n = 146)</th>
<th>Carcinoma cases (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c2/c2</td>
<td>7 (4.7)</td>
<td>1 (0.7)</td>
<td>12 (8.0)</td>
</tr>
<tr>
<td>c1/c2</td>
<td>77 (51.3)</td>
<td>42 (28.8)</td>
<td>31 (20.7)</td>
</tr>
<tr>
<td>c1/c1</td>
<td>66 (44.0)</td>
<td>103 (70.6)</td>
<td>107 (71.3)</td>
</tr>
<tr>
<td>OR (95% CI)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTM1, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/0</td>
<td>76 (50.7)</td>
<td>86 (58.9)</td>
<td>46 (30.7)</td>
</tr>
<tr>
<td>+/- and +/-</td>
<td>74 (49.3)</td>
<td>60 (41.1)</td>
<td>104 (69.3)</td>
</tr>
<tr>
<td>OR (95% CI)*</td>
<td>0.7 (0.5–1.2)</td>
<td>2.3 (1.8–3.0)</td>
<td></td>
</tr>
<tr>
<td>GSTT1, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/0</td>
<td>59 (39.3)</td>
<td>53 (36.3)</td>
<td>60 (40.0)</td>
</tr>
<tr>
<td>+/- and +/-</td>
<td>91 (60.7)</td>
<td>93 (63.7)</td>
<td>90 (60.0)</td>
</tr>
<tr>
<td>OR (95% CI)*</td>
<td>1.1 (0.7–1.9)</td>
<td>0.9 (0.7–1.2)</td>
<td></td>
</tr>
<tr>
<td>GSTP1, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*A/*A</td>
<td>91 (60.7)</td>
<td>83 (56.8)</td>
<td>93 (62.0)</td>
</tr>
<tr>
<td>*A/*B</td>
<td>53 (35.3)</td>
<td>55 (37.7)</td>
<td>48 (32.0)</td>
</tr>
<tr>
<td>*B/*B</td>
<td>6 (4.0)</td>
<td>8 (5.5)</td>
<td>9 (6.0)</td>
</tr>
<tr>
<td>OR (95% CI)*</td>
<td>0.8 (0.6–1.0)</td>
<td>1.0 (0.8–1.3)</td>
<td></td>
</tr>
</tbody>
</table>

*ORs and 95% CIs were calculated by logistic regression, with the CYP2E1 c1/c2 + c2/c2, GSTM1 null, GSTT1 null, and GSTP1*A/*A genotypes as the reference groups. GSTP1*A/*B and GSTP1*B/*B were combined for analysis. Adjusted for age, gender, and tobacco smoking.
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In addition, no interactions between smoking and genetic polymorphisms in the three loci known to detoxify tobacco smoke-related carcinogens such as polycyclic aromatic hydrocarbon epoxides were significantly correlated with the risk of the cancer. These results are essentially in agreement with previous reports (69, 70) suggesting that tobacco smoking is not a major etiological factor for esophageal cancer in this high-risk area. In addition to nitrosamines, however, other carcinogens present in the environment should not be overlooked because CYP2E1 may also catalyze the oxidation and DNA adduct formation of many low-molecular weight carcinogens (11) and catalyzes the production of reactive oxygen species (13, 14) that may cause DNA damage and consequently initiate carcinogenesis.

Another provocative but unexpected observation from the present study was that increased risk of SCC of the esophagus was associated with the GSTM1 non-null genotypes. Moreover, the GSTM1 non-null genotype showed a synergistic effect with CYP2E1 c1/c1 genotype on the risk of esophageal cancer. Because there are few reports (38), to our knowledge, that show that the risk of cancer is associated with GSTM1 non-null genotypes, caution should be made in interpreting this finding and further confirmation may be warranted. However, several possibilities exist for explanation of the observation. One possibility is that carcinogen(s) involved in the etiology of esophageal cancer in this high-incidence region or their intermediates derived from CYP2E1-mediated metabolism are further activated by GSTM1-catalyzed conjugation. It is generally believed that the great majority of GST-catalyzed reactions of electrophiles with reduced glutathione are detoxification processes. However, enhanced genotoxicity and carcinogenicity of some carcinogens after conjugation with glutathione have also been documented (71–73). At present, there is little information on the detoxification or activation of nitrosamines by GSTM1, GSTT1, or GSTP1. Another possibility could be related to dietary anticarcinogens that inhibit CYP2E1 and are inactivated by GSTM1. An inverse relationship between high vegetable consumption and low risk of developing esophageal cancer has been well established (74). Of the mechanisms by which such protection could arise, inhibition of activation and/or induction of detoxification of carcinogens by certain components such as isothiocyanates found in cruciferous vegetables were suggested to play an important role (75). It has been shown that isothiocyanate-containing cruciferous vegetables, including Chinese cabbage, broccoli, watercress, and their active constituents inhibit CYP2E1 activity (27, 76–79). Accordingly, it is reasonable to assume that protection of esophageal carcinogenesis by vege-

### Table 3  Risk of esophageal carcinoma associated with the CYP2E1 genotypes by GSTM1 genotypes

<table>
<thead>
<tr>
<th>CYP2E1 genotype</th>
<th>GSTM1 0/0</th>
<th>GSTM1 +/+ and +/0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases/controls</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>c1/c2 and c2/c2</td>
<td>11/44</td>
<td>1.0</td>
</tr>
<tr>
<td>c1/c1</td>
<td>35/32</td>
<td>4.4 (1.8–10.8)</td>
</tr>
</tbody>
</table>

* ORs were adjusted for age, gender, and smoking, with the CYP2E1 c1/c2 + c2/c2 and GSTM1 null genotypes as the reference groups. P < 0.05, test for homogeneity.

### Table 4  Interaction of CYP2E1 and GSTM1 genotypes and tobacco smoking on risk of esophageal carcinoma

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>CYP2E1 genotype</th>
<th>GSTM1 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c1/c2 and c2/c2</td>
<td>c1/c1</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>19/54</td>
<td>71/42</td>
</tr>
<tr>
<td>Smokers</td>
<td>23/30 (1.0–5.2)</td>
<td>36/24</td>
</tr>
<tr>
<td>≤20 pack-years</td>
<td>11/13</td>
<td>24/30</td>
</tr>
<tr>
<td>&gt;20 pack-years</td>
<td>13/17</td>
<td>24/30</td>
</tr>
</tbody>
</table>

a No. of cases/no. of controls.


In conclusion, this study demonstrates a significant association between CYP2E1 genetic polymorphisms and SCC of the esophagus in Linxian, China, confirming our previous pilot study conducted in the same area. In addition, we found that the presence of GSTM1 gene is also a risk factor for esophageal cancer in this population, probably because of specific exposure or via a mechanism of gene-environment interaction relative to dietary anticarcinogens. Genetic susceptibility factors for SCC of the esophagus identified in our study could serve as useful biomarkers for targeting prevention of the cancer.
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