Susceptibility to Esophageal Cancer and Genetic Polymorphisms in Glutathione S-Transferases T1, P1, and M1 and Cytochrome P450 2E1

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Introduction
Cancer of the esophagus is one of the most common fatal diseases in certain regions of China such as Linxian County, where the mortality rate is as high as 150/100,000 (1). The risk for esophageal cancer in these areas has been associated with exposure to environmental carcinogens, particularly nitrosamines (2–4). However, even in high-risk regions, only certain individuals develop esophageal cancer. Most people live a normal life span, suggesting that host susceptibility factors may play an important role in cancer development. Most chemical carcinogens require metabolic activation for DNA-damaging capabilities, a step widely believed to be essential in carcinogenesis (5, 6). On the other hand, carcinogens may also be detoxified before damaging DNA by in vivo metabolic detoxifying systems (7, 8). Thus, it has been suggested that individual differences in carcinogen metabolism, which may be heritable or due to sustained environmental exposure to agents that affect the expression of enzymes involved in carcinogen activation or detoxification, may determine the susceptibility to chemically induced cancers. The major enzymes involved in the metabolic activation of chemical carcinogens are CYPs (4), a multigene superfamily of enzymes. Of the 25 or more CYP enzymes known to be expressed in human tissues, CYP2E1 is believed to be involved in the activation of most carcinogenic nitrosamines (9–12). Furthermore, this enzyme has the ability to metabolically activate many low molecular weight carcinogens (9) and to produce reactive free radicals from ethanol (13, 14), which also might be of importance in cancer etiology.

CYP2E1 represents a major CYP isomorph in the human liver and is also expressed in extrathoracic tissues (15–17). Although the enzyme can be induced by certain chemicals such as ethanol, large interindividual variations have been observed before and after induction (18–20), suggesting that the variation may be due to genetic polymorphisms. RFLPs of the human CYP2E1 gene have been identified, and a polymorphism in the upstream region of the gene detected by Rsal has been shown to be associated with the transcriptional regulation of gene expression (21). Another genetic polymorphism that is detectable with DraI is located in intron 6 (22), and this allele has been found to contain a number of functional mutations affecting protein expression and catalytic activity (23). Associations between these genetic polymorphisms in CYP2E1 and the susceptibility to some types of cancer have been reported in case-control studies (22, 24–29), although some negative results have been reported (30–40). To date, one study in Japan has evaluated the role of the Rsal polymorphism in relation to
esophageal cancer and found no association (36). However, an increased risk of both oral (41) and nasopharyngeal (42) cancer has been associated with the prevalence of this variant allele in Taiwan.

The GSTs are a family of multifunctional enzymes that play a central role in the detoxification of toxic and carcinogenic electrophiles (43). Individuals who are homozygous for the null GSTM1 or GSTT1 alleles lack the respective enzyme function (44, 45), and these genotypes have been associated with an increased risk of cancer at many sites (44, 46–49). GSTP1, which is a major GST isozyme expressed in the human esophagus (17, 50), has also been shown to be genetically polymorphic (51, 52). A mutation of A to G within exon 5 results in an I to V change at position 105 in the amino acid sequence of the protein, which alters the specific activity and affinity for the electrophilic substrates of the enzyme (52, 53). Therefore, the GSTP1 polymorphism may also have potential effects on cancer susceptibility (51). Whereas numerous studies have been undertaken to examine the association between genetic polymorphisms in CYPs and/or GSTs and cancer susceptibility (vide supra), there is limited information on their association with esophageal cancer (36, 54). In this study, we analyzed genetic polymorphisms in GSTM1, GSTT1, GSTP1, and CYP2E1 in subjects with esophageal cancer and severe epithelial hyperplasia, which is a precancerous lesion, and frequency-matched controls from Linxian County, China, an area where dietary nitrosamine exposures are known to be high (3).

**Materials and Methods**

**Study Subjects.** A pilot case-control study was designed to evaluate the possible role of genetic polymorphisms in carcinogen-metabolizing enzymes in the susceptibility to esophageal cancer. All subjects were residents of Linxian County, Henan Province, China. Smoking status was not available; however, recent studies with a similar population indicate that smoking prevalence is comparable between cases and controls. The diagnoses of esophageal cancer and epithelial hyperplasia were confirmed either histologically or cytologically. Cases were grouped as follows: (a) those with advanced dysplasia or severe hyperplasia (n = 45); (b) those with squamous cell carcinoma or adenocarcinoma (n = 45); and (c) those with squamous cell carcinoma, adenocarcinoma, or advanced dysplasia (n = 62).

DNA samples were isolated from surgically removed esophageal tissues using standard methods (55) or from epithelial cells scraped from the esophagus. The yields of DNA varied with the amount of tissue available, and the analysis of each sample for all genotypes was not possible.

**GSTM1 and GSTT1 Genotyping.** GSTM1 and GSTT1 genotyping for gene deletions was carried out by a multiplex PCR using primer pairs 5′-GAATCTCCCTGAAAAGCTAA-3′ and 5′-GTTGGCTCATAATACGGTG-3′ for GSTM1, which produced a 219-bp product, and 5′-GTTGGC-TCAAATATACGGTG-3′ and 5′-GTTGGCTCAAATATACGGTG-3′ for GSTT1, which produced a 350-bp product. PCR was performed in a 50-µl reaction buffer and contained each primer (1.0, 0.3, and 0.1 µM), dNTP (0.2 mM), MgCl2 (2.5 mM), and Taq polymerase (1.25 units). Amplicons were detected on a 2% agarose gel.

**GSTM1 RFLP Analysis.** The primers 5′-ACCCACATCT-CTTCCCTCCCTC-3′ and 5′-TACCTGGCTGGTTAGTGC-C-3′ was used to amplify exon 5 in the GSTP1 gene that includes the Alw261 enzyme recognition site (cf. Ref. 49). PCR reactions were carried out in a 50-µl mixture containing sample DNA (0.1 µg), reaction buffer, dNTP (0.2 mM), MgCl2 (2.5 mM), each primer (1.0 µM), and Taq polymerase (1.25 units). Amplification, which resulted in a 440-bp fragment, was achieved by 35 cycles of 30 s at 94°C, 30 s at 66°C, and 2 min at 72°C. Ten µl of the PCR reaction products were subjected to digestion with 20 units of the restriction enzyme in the buffer, as recommended by the manufacturer (Fermentas, Vilnius, Lithuania), for 2 h at 37°C. The samples were then analyzed by electrophoresis in 2% agarose gels.

**CYP2E1 RFLP Analysis.** The RFLPs in the 5′-flanking region and in intron 6 of the CYP2E1 gene were determined by PCR amplification followed by digestion with RsaI or DraI, using the methods described previously (21, 22). The PCR primers used for the 5′-flanking region (the RsaI site) were 5′-CTCTCTGTGTTAGGAGG-3′ and 5′-AGACCTC- TACCTGCTGATG-3′ and produced a 459-bp product. The predominant allele (c1) was sensitive to RsaI digestion and yielded two products at 201 and 258 bp. The c2 allele was resistant to RsaI digestion. The PCR primers for intron 6 (the DraI site) were 5′-GGAGTTGAAAGGACCCGCTC-3′ and 5′-GGAGTTGAAAGGACCCGCTC-3′ and produced a 686-bp product. Only the D allele was sensitive to DraI cleavage, resulting in fragments of 335 and 351 bp. All PCR amplifications were performed in a total volume of 50 µl. The mixture used reaction buffer and contained each primer (1.0 µM), dNTP (0.2 mM), MgCl2 (2.5 mM), and Taq polymerase (1.25 units). Amplification was carried out in a 50-µl mixture containing sample DNA (0.1 µg), reaction buffer, dNTP (0.2 mM), MgCl2 (2.5 mM), each primer (1.0 µM), and Taq polymerase (1.25 units). Amplification, which resulted in a 440-bp fragment, was achieved by 35 cycles of 30 s at 94°C, 30 s at 66°C, and 2 min at 72°C. Ten µl of the PCR reaction products were subjected to digestion with 20 units of the restriction enzyme in the buffer, as recommended by the manufacturer (Fermentas, Vilnius, Lithuania), for 2 h at 37°C. The samples were then analyzed by electrophoresis in 2% agarose gels.

The presence of the polymorphic Alw261 restriction site yielded two fragments of 213 and 227 bp (GSTM1*), whereas the absence of the polymorphic site was determined by the presence of a 440-bp fragment (GSTM1*A).

**Fig. 1.** An ethidium bromide-stained gel of electrophoresed products of the amplification of GSTM1, GSTT1, and albumin genes. Lanes 1–7 correspond to individual subjects. The absence of the PCR product indicates the null genotype. M, the molecular weight markers.
jected to agarose gel electrophoresis. Lanes 1-4, and 9. ii. 

Fig. 2. The RFLPs of PCR-amplified fragments obtained using RsaI and DRAI (n = 62) were digested with 10 units of RsaI or 10 units of DraI (New England Biolabs, Inc., Beverly, MA), respectively, at 37°C for 4 h, and the products were analyzed by electrophoresis in a 2.5% agarose gel.

Statistical Analysis. The \( \chi^2 \) test was used to examine the differences in the distribution of genotypes between cases and controls. ORs with 95% CIs were computed using unconditional logistic regression (SPSS Inc., Chicago, IL) and adjusted for age and gender (57). DNA analyses of all of the genotypes for all study participants were not possible due to sample limitations; thus, the number of participants varies for each polymorphism.

Results

The characteristics of study subjects are presented in Table 1. Age and gender distributions among cases with cancer, cases with hyperplasia, and controls were very similar, reflecting the matching criteria used in this case-control study. DNA samples subjected to PCR and enzymatic digestion with RsaI revealed the expected fragment lengths, resulting in three genotypes of CYP2E1 (Fig. 2). The three genotypes of CYP2E1 recognized by PCR and DRAI restriction fragment length analyses were readily discerned (Fig. 3). Distributions of the CYP2E1 genotypes identified by these two restriction endonuclease analyses were similar to those described previously (21, 22), as shown in Tables 2 and 3 for controls, cancer cases, and hyperplasia cases. The frequencies of the homozygous RsaI-sensitive allele (c1/c1) and the combined genotypes (c1/c2 + c2/c2) detected by RsaI were found to be 44 and 56%, respectively, in the control subjects, and those for the CC genotype and the CD + DD genotypes recognized by DRAI were found to be 42 and 58%, respectively. As shown in Table 2, the frequency of variant genotypes of CYP2E1 detected by RsaI was significantly higher (\( \chi^2 = 20.8; P < 0.001 \)) in controls (56%) than in epithelial hyperplasia and dysplasia cases (17%) or in cancer cases (20%). Subjects with the c1/c1 genotype had a 6-fold increased risk of developing epithelial hyperplasia and dysplasia (OR, 6.0; 95% CI, 2.3–16) and an almost 5-fold increased risk for cancer (OR, 4.8; 95% CI, 1.8–12.4) as compared to subjects with variant genotypes. The frequencies of the CD and DD genotypes detected by DRAI were also slightly higher in control subjects (58%) than in cases with epithelial hyperplasia and dysplasia (41%) or in cases with cancer (45%), although the differences were not statistically significant. When cases with cancer and advanced dysplasia (n = 62) were analyzed together, the results for the RsaI (OR, 6.0; 95% CI, 1.8–2.4) and DRAI (OR, 1.5; 95% CI, 0.7–3.6) polymorphisms were similar to that described for the cancer cases only.

The distributions of the GSTM1, GSTT1, and GSTP1 genotypes in cases and controls are shown in Table 4. GSTM1 and GSTT1 gene deletion was common in both controls and cases. The distribution of the genotypes of the three GST genes did not differ significantly between the controls and either group of cases, although the frequencies of the GSTT1 gene deletion and the frequencies of the GSTP1*B minor alleles were slightly lower in the cancer and hyperplasia cases than in the controls. No interactions were observed for combined contributions of GST genetic polymorphisms, together and with CYP2E1, on the risk of developing epithelial hyperplasia and esophageal cancer.

Discussion

Because the etiology of esophageal cancer has been hypothesized to be associated with environmental nitrosamine exposure, we investigated whether genetic factors that might modulate the activation and/or detoxification of these carcinogens could have an impact on the risk of developing this malignant disease. We analyzed genetic polymorphisms at the CYP2E1, GSTM1, GSTT1, and GSTP1 loci and found that the CYP2E1 genotype was associated with susceptibility to esophageal cancer. Subjects who were homozygous c1/c1 for CYP2E1 were at a more than 4-fold excess risk of developing epithelial hyp-
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The mechanisms involved in CYP2E1 expression and the resulting amount and activity of the enzyme are not well characterized. Hu et al. (23) recently identified several mutations in the flanking regions in the Drai C allele using single-strand conformational polymorphism and found determinants of mRNA stability in some motifs. The PsrI or RsaI site is located in the 5'-flanking region and may affect gene transcription. Based on experiments with an in vitro expression system, Hayashi et al. (21) and Watanabe et al. (58) suggested that the c2/c2 genotype detected by RsaI produced higher enzyme activity than the c1/c1 genotype. However, this suggestion could not be confirmed in several in vivo and in vitro studies using chlorozoxazone and other specific substrates as probes for enzyme activity (59–61). Moreover, in view of the role of CYP2E1 in the metabolic activation of carcinogens and our results showing a protecting effect of the c2/c2 genotype against esophageal cancer, we suggest that this genotype may result in less CYP2E1 activity toward esophageal carcinogens in Linxian County than the corresponding c1/c1 genotype.

The basal expression of CYP2E1 seems to be relatively low, but it can be highly induced. The polymorphism in the 5'-flanking region might be expected to modify the inducibility of the enzyme. Although the mechanism of CYP2E1 induction remains controversial and seems to be mediated by different mechanisms, transcriptional activation of the gene is likely to be involved (62, 63). It is interesting that Lucas et al. (20) observed that chlorozoxazone metabolism did not differ for either polymorphism across the genotypes for the basal rates present in controls or in withdrawn alcoholics. However, after alcohol induction, chlorozoxazone metabolism was significantly lower in heterozygous genotypes than it was in homozygous c1 genotypes detected by analysis using either RsaI or DraI. These results indicate that the c2 genotypes might be associated with a lack of inducibility in CYP2E1 activity (20). To date, there has been no evidence showing that enhanced CYP2E1 activity in vivo is related to the c2 genotypes.

Several case-control studies have been conducted to examine the associations between CYP2E1 polymorphisms and the susceptibility to a number of cancers, although the results are inconsistent. In a Swedish study, Persson et al. (26) found that the frequency of the c2 genotype was significantly lower in lung cancer cases than it was in controls and suggested that this could constitute a protective factor against the disease. These data were also supported by recent data from a study by Wu et al. (64). Yu et al. (29) recently showed that the risk of developing hepatocellular carcinoma in cigarette smokers in Taiwan was significantly associated with the genotypes of CYP2E1 detected by Pstl or RsaI. They found that the frequency of the c1/c1 genotype detected by Pstl or RsaI was higher in cases than it was in controls, and this difference was more pronounced among smokers. The data reported herein are consistent with these previous studies. Another case-control study conducted in Taiwan on CYP2E1 genetic polymorphisms and the risk of nasopharyngeal cancer, which is also hypothesized to be associated with exposure to nitrosamines, showed an increased risk of the c1/c1 genotype (42). However, in contrast to our results and those reported by Yu et al. (29) and Persson et al. (26), a lower risk of the cancer was associated with variant genotypes detected by both RsaI and DraI (28).

In another recent study in Taiwan (41), the c2 allele was found to increase the risk of oral cancer among those who did not chew betel quid. In a case-control study of breast cancer, Shields et al. (27) also found the DraI polymorphism C alleles to be associated with increased risk among premenopausal smokers.

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### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
<th>Cancer (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n = 45)</td>
<td>(n = 32)</td>
<td>(n = 45)</td>
<td>(n = 58)</td>
</tr>
<tr>
<td>c1/c1</td>
<td>20 (44)</td>
<td>25 (83)</td>
<td>36 (80)</td>
<td>48 (80)</td>
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<tr>
<td>c1/c2</td>
<td>22 (49)</td>
<td>7 (17)</td>
<td>6 (13)</td>
<td>4 (7)</td>
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<tr>
<td>c2/c2</td>
<td>3 (7)</td>
<td>0 (0)</td>
<td>3 (7)</td>
<td>4 (7)</td>
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<tr>
<td>OR (CI)*</td>
<td>1.0</td>
<td>6.0 (2.3–16.0)</td>
<td>4.8 (1.8–12.4)</td>
<td>4.0 (1.8–12.4)</td>
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</table>

* ORs and 95% CIs were calculated by logistic regression, with the c1/c1 genotype as the reference category, adjusted for age and sex.

### Table 3

<table>
<thead>
<tr>
<th>Genotype^a</th>
<th>Controls (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
<th>Cancer (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
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</thead>
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<td></td>
<td>(n = 45)</td>
<td>(n = 38)</td>
<td>(n = 45)</td>
<td>(n = 56)</td>
</tr>
<tr>
<td>CC</td>
<td>19 (42)</td>
<td>21 (59)</td>
<td>25 (55)</td>
<td>34 (57)</td>
</tr>
<tr>
<td>CD</td>
<td>23 (51)</td>
<td>16 (39)</td>
<td>16 (36)</td>
<td>16 (33)</td>
</tr>
<tr>
<td>DD</td>
<td>3 (7)</td>
<td>1 (2)</td>
<td>4 (9)</td>
<td>6 (10)</td>
</tr>
<tr>
<td>OR (CI)^b</td>
<td>1.0</td>
<td>2.0 (0.9–4.4)</td>
<td>1.5 (0.7–3.6)</td>
<td>1.5 (0.7–3.6)</td>
</tr>
</tbody>
</table>

^a CC, homozygous for the common allele; CD, heterozygous; DD, homozygous for the rare allele.
^b ORs and 95% CIs were calculated by logistic regression, with the CC genotype as the reference category and groups (CD + DD) combined, adjusted for age and sex.

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### Table 4

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Controls (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
<th>Cancer (%)</th>
<th>Hyperplasia and dysplasia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 45)</td>
<td>(n = 32)</td>
<td>(n = 45)</td>
<td>(n = 58)</td>
</tr>
<tr>
<td>GSTM1</td>
<td>n = 45</td>
<td>24 (53)</td>
<td>28 (62)</td>
<td>25 (56)</td>
</tr>
<tr>
<td>+</td>
<td>21 (47)</td>
<td>17 (38)</td>
<td>20 (44)</td>
<td>30 (48)</td>
</tr>
<tr>
<td>OR (CI)*</td>
<td>1.0</td>
<td>0.7 (0.3–1.8)</td>
<td>1.0 (0.4–2.3)</td>
<td>1.1 (0.5–2.4)</td>
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<tr>
<td>GSTT1</td>
<td>n = 45</td>
<td>22 (49)</td>
<td>27 (60)</td>
<td>26 (55)</td>
</tr>
<tr>
<td>+</td>
<td>23 (51)</td>
<td>18 (40)</td>
<td>19 (45)</td>
<td>28 (45)</td>
</tr>
<tr>
<td>OR (CI)^a</td>
<td>1.0</td>
<td>0.6 (0.3–1.5)</td>
<td>0.7 (0.3–1.5)</td>
<td>0.7 (0.3–1.6)</td>
</tr>
</tbody>
</table>

^* ORs and 95% CIs were calculated by logistic regression, with the 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 and sex.
A significant association of the variant genotype recognized by DraI with increased lung cancer risk was also reported in a Japanese case-control study (22). In addition, contrary results showing no association between CYP2E1 genotypes and cancer risk in Caucasians were reported by other investigators (30, 31, 33–35). The reasons for these discrepancies are not clear, and specific exposures may need to be further addressed. However, the very limited statistical power in Caucasian studies due to the extreme rarity of the variant genotypes of CYP2E1 determined by either Rsal or DraI in this population could make such studies difficult to evaluate. Interracial difference in CYP2E1 activity may also account for the discrepancy. It is worthy to note that CYP2E1 activity was lower in Japanese subjects than it was in Caucasian subjects (61). Again, it is possible that the risk associated with polymorphisms alone is not evident without relevant exposure data.

GSTs, as an enzyme class, show activity against a broad range of DNA-damaging chemical substrates. Although several polycyclic aromatic hydrocarbon epoxides, such as benzo(a)pyrene diol-epoxide, are known substrates for GSTM1 and GSTP1 (65), there is little information on the detoxification of nitrosamines by these GSTs. It seems that genetic polymorphisms of GSTM1 and GSTP1 are mainly associated with an increased susceptibility for tobacco smoke-related cancers (44, 46, 48, 51). GSTT1 has significant activity in human erythrocytes and is implicated in the conjugation of natural and synthetic haloalkanes. The association between GSTT1 genetic polymorphisms and cancer risk has not been studied as extensively. Our results in the present study did not indicate an association between polymorphisms at the three GST genes and susceptibility to esophageal cancer. On the basis of what is known about substrates for human GSTM1, GSTP1, and GSTT1 and given the present findings, we suggest that the carcinogen(s) involved in the etiology of esophageal cancer in this high-incidence region may not be substrates for these GSTs. For example, previous epidemiological data already show that tobacco smoke plays a negligible role in the etiology of esophageal cancer in this area (66, 67).

In summary, we report a significant role of CYP2E1 but not GST genotypes in the development of esophageal cancer in Linxian County, China. Although the number of individuals with the CYP2E1 c2 alleles was small, the differences in allele frequencies between cases and controls were striking. Of particular note was the apparent similarity in the frequencies of alleles in cases with esophageal cancer and in those with severe epithelial hyperplasia, in contrast to those among controls. Because this is one of the few reports on the association between genetic polymorphisms in carcinogen-metabolism enzymes and susceptibility to cancer of the esophagus, studies in larger populations would be desirable to confirm the findings of this pilot study.

References


Susceptibility to esophageal cancer and genetic polymorphisms in glutathione S-transferases T1, P1, and M1 and cytochrome P450 2E1.

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