Genetic polymorphisms of cytochrome p4502E1 and susceptibility to alcoholic liver disease and hepatocellular carcinoma in a white population: a study and literature review, including meta-analysis

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Abstract

Aims—To investigate the associations between the Rsa I, Dra I, and Taq I genetic polymorphisms of cytochrome p4502E1 and susceptibility to alcoholic liver disease or to hepatocellular carcinoma.

Methods—DNA samples isolated from 61 patients with alcoholic liver disease, 46 patients with hepatocellular carcinoma, and 375 healthy controls were subjected to polymerase chain reaction amplification followed by digestion with the endonucleases Rsa I, Dra I, or Taq I. Meta-analysis was performed using data from previous studies of Rsa I polymorphism and the risk of alcoholic liver disease.

Results—No association was found between any of the three polymorphisms and susceptibility to hepatocellular carcinoma. The distributions of Rsa I and Dra I alleles among the patients with alcoholic liver disease were not significantly different from those among the control group. Meta-analysis of this data and previous data concerning Rsa I polymorphism and alcoholic liver disease risk failed to demonstrate any significant association between the two. However, the alcoholic liver disease group in this study showed a significantly lower frequency of the less common Taq I allele compared with the healthy control group (odds ratio, 0.33; 95% confidence interval, 0.12 to 0.78).

Conclusions—Possession of the less common Taq I cytochrome p4502E1 allele is associated with reduced susceptibility to alcoholic liver disease. There is no existing evidence that the Taq I polymorphism is directly associated with altered alcohol metabolism, but it might be in linkage disequilibrium with as yet unidentified protective factors.


Keywords: cytochrome p4502E1; alcoholic liver disease; hepatocellular carcinoma

Cytochrome p4502E1 (CYP2E1) is a key microsomal enzyme that metabolises alcohol in the non-alcohol dehydrogenase pathway. Because the gene encoding CYP2E1 shows several polymorphisms, it has been speculated that these polymorphisms might, at least in part, explain the inherited variability in susceptibility to alcoholic liver disease. To date, the groups that have searched for possible associations between CYP2E1 gene polymorphisms and alcoholic liver disease susceptibility have reported varied, often contradictory, results. Some of these discrepancies might relate to CYP2E1 having different putative roles in the pathogenesis of alcoholic liver disease in different ethnic groups. There have been nine published reports of the relation between CYP2E1 polymorphisms and alcoholic liver disease in white populations. Rsa I/Pst I polymorphism was investigated in all nine studies, most of which were unable to demonstrate any significant association between the polymorphism and the risk of developing alcoholic liver disease. This conclusion has, however, not gained universal acceptance, particularly because one UK study reported a strong link between carriage of the less common Rsa I allele (c2) and an increased risk of developing alcoholic liver disease. The Dra I polymorphism of the CYP2E1 gene is considerably more prevalent in white populations than the Rsa I/Pst I polymorphism and, therefore, could conceivably play a more important role in inherited susceptibility for alcoholic liver disease. However, the four studies that have investigated this hypothesis produced conflicting results: the earliest study found the less common Dra I allele (C) to be less frequent in patients with alcoholic cirrhosis than in healthy controls; two later studies reported the opposite, and the most recent study has shown no significant difference in C allele frequency between patients with alcoholic cirrhosis and healthy controls.

The use of Taq I to demonstrate polymorphism of intron 7 of the CYP2E1 gene was first reported in 1987. Taq I polymorphism is as frequent in white populations as Dra I polymorphism, with the less common Taq I allele (A1) having a reported population frequency of 0.08 to 0.10. Therefore, it is surprising that there have been no reported studies into the relation between CYP2E1 Taq I polymorphism and susceptibility to alcoholic liver disease.

Because hepatic CYP2E1 has been shown to activate various carcinogens, there has been interest in whether certain CYP2E1 polymorphisms might predispose to liver cancer. We are aware of only one previous study that has investigated this hypothesis in a white population. Although Ladero and colleagues...
demonstrated that possession of the less common Rsa I/Pst I allele (c2) was associated with increased susceptibility for hepatocellular carcinoma, the relations between Dra I and Taq I polymorphisms and hepatocellular carcinoma risk were not studied.

Our study aims to investigate the above unresolved issues regarding CYP2E1 genetic polymorphisms and their relation to susceptibility for alcoholic liver disease and hepatocellular carcinoma. Hence, in a white population, we have: (1) performed meta-analysis of our data and data from previous studies of Rsa I/Pst I polymorphism and alcoholic liver disease risk to help reach a definitive conclusion about their relation; (2) restated the hypothesis that possession of the less common Dra I allele is associated with an increased risk of developing alcoholic liver disease; (3) studied the relation between Taq I polymorphism and susceptibility to alcoholic liver disease; and (4) investigated whether Dra I and/or Taq I polymorphisms are associated with susceptibility to hepatocellular carcinoma.

Methods
Blood samples were obtained from 61 patients with alcoholic liver disease receiving care at the Centre of Liver and Digestive Diseases at the Royal Infirmary of Edinburgh. These patients had a median age of 56 years (range, 32 to 90) and showed a male to female patient ratio of 2:2. All 61 patients had alcohol consumptions of greater than 60 g/day for greater than 10 years (median intake, 150 g/day for 18 years); these alcohol consumption data were obtained directly from the patients with, where possible, corroboration from relatives and/or partners. All 61 patients had undergone liver biopsies, which demonstrated at least one of the following features: alcoholic hepatitis, precirrhotic pericellular/perivenular fibrosis, and/or cirrhosis. None of the patients showed clinical, histological, or serological evidence of an alternative cause of liver disease. Forty six consecutive hepatocellular carcinoma resection tissue sections (patient median age, 66 years; age range, 21 to 92; male to female patient ratio, 2:3) were obtained from the archives of the department of pathology, University of Edinburgh. The hepatocellular carcinoma group did not include any patients with clinical and/or histological evidence of alcoholic liver disease. Excluding such cases allowed us to study, in isolation, the relation between CYP2E1 polymorphisms and hepatocellular carcinoma risk, and to avoid the confounding effects of coexisting alcoholic liver disease. Three hundred and seventy five control blood samples were obtained from the Scottish National Blood Transfusion Service. The anonymous donors were aged between 18 and 65 years and consisted of roughly equal numbers of men and women. All 375 donors appeared healthy on routine questioning and examination, and met the usual criteria for blood donation. The use of healthy individuals as the control group as opposed to alcoholic patients with no clinical or biochemical evidence of alcoholic liver disease—as used by several previous studies—is explained below. All patients and control individuals were white.

Because the study of patients with hepatocellular carcinoma represented only a sideline of the main study, the number of patients with alcoholic liver disease required took precedence in the calculation of sample sizes. This calculation was performed using the computer software package Epi Info v6.04b (Centers for Disease Control and Prevention, Atlanta, USA) with the following variables: a power of 80%; a significance level of 95%; a control to patient ratio of 4:1; an expected proportion of control individuals possessing the less common allele of 7.0% for Rsa I, 19% for Dra I, and 25% for Taq I; and a significant difference defined as a threefold higher proportion of patients carrying the less common allele compared with control individuals. Based on the trends shown by previous studies on Rsa I and Dra I polymorphisms, we did not anticipate a decreased frequency of the less common alleles in the patient group. The expected proportions were derived from the genotyping of our control group, which had preceded our study. The largest sample sizes required were those required for Rsa I analysis, and consisted of 224 control individuals and 61 patients.

Our study design was approved by the Lothian Health Board ethical review committee, and informed consent was obtained from the patients attending the Centre of Liver and Digestive Diseases. DNA was obtained from sections of archival tissue samples without neoplastic changes or from fresh peripheral blood leucocytes, as has been described previously.7 21

The endonucleases Rsa I and Pst I each detect a different polymorphism in the 5' flanking region of the CYP2E1 gene: an allele possessing a positive restriction point for Rsa I is designated as a c1 allele and one with a Pst I restriction point, a c2 allele.1 2 With rare exceptions, the two polymorphisms show complete linkage disequilibrium in white populations.6 7 22 Therefore, for the purposes of our study, only Rsa I was used to demonstrate the polymorphisms, and absence of the Rsa I restriction point was taken to define a c2 allele.

Dra I digestion detects a polymorphism in intron 6 of the CYP2E1 gene and Taq I a polymorphism in intron 7.14 10 The more common and less common alleles demonstrated using Dra I are designated D and C, respectively, and those demonstrated using Taq I, A2 and A1, respectively.14 15

Polymerase chain reaction (PCR) for the Rsa I assay was carried out with an Omnigene thermocycler (Hybaid, Ashford, UK) with 200 μM of each dNTP, 1.25 units of DNA polymerase (Advanced Biotechnologies, Epsom, UK), 50 ng of each primer, and 1.5 mM magnesium chloride. The primer sequences were 5'-CCAGTGGAGTCTACATTGTCAG and 5'-TTCAATCTGTCCTTCTAATTGG, as used in previous studies.1 5 7 9 11 21 The amplification protocol comprised 94°C for 20 seconds, 54°C for 20 seconds, and 72°C for 20 seconds (36 cycles), followed by 54°C for 10 minutes, and 72°C for 10 minutes. PCR products were run
on a 2% agarose gel. The products were digested with Rsa I overnight at 37°C, and the fragments separated on a 10% acrylamide gel.

PCR for the Dra I assay was carried out with an Omnigene thermocycler (Hybaid), with 200 μM of each dNTP, 1.25 units of Taq polymerase (Life Technologies, Paisley, UK), TaqStart antibody (Clontech, Basingstoke, UK), 50 ng of each primer, and 1.5 mM magnesium chloride. The primer sequences were 5′-GACAGGGTTCATCATGTTGG and 5′-GACAGGGTTCATCTTCATTTCGA, and 5′-AGTCGACATGTGATGGATCCA, as used in previous studies.91 12 4 The amplification protocol comprised 94°C for 30 seconds, and 72°C for 30 seconds (36 cycles), followed by 62°C for two minutes, and 72°C for 10 minutes. PCR products were run on a 2% agarose gel. The products were digested with Dra I overnight at 37°C, and the fragments run on an 8% acrylamide gel.

PCR for the Taq I assay was carried out with an Omnigene thermocycler (Hybaid), with 200 μM of each dNTP, 1.25 units of Taq polymerase (Advanced Biotechnologies), 50 ng of each primer, and 1.5 mM magnesium chloride. The primer sequences were 5′-GGGCTTTCATCTTCATTTCGA and 5′-GGGCTTTCATCTTCATTTCGA, and were designed from the CYP2E1 gene sequence published in the Genbank database. By introducing a single base mismatch, the upstream primer contains an engineered Taq I site as a control for the restriction enzyme. The amplification protocol comprised 94°C for 20 seconds, 56°C for 20 seconds, and 72°C for 15 seconds (36 cycles), followed by 56°C for two minutes, and 72°C for 10 minutes. PCR products were run on a 2% agarose gel. The products were digested with Taq I overnight at 65°C, and the fragments run on an 8% acrylamide gel.

All gels were visualised by means of ethidium bromide staining and examined under ultraviolet irradiation.

Associations between disease groups and specific genotypes were analysed for significance by the χ² test using the Yates correction or, where more appropriate, by the two tailed Fisher’s exact test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to assess relative risk of disease conferred by a particular allele. The above statistical analyses were carried out using the computer software package Epi Info v6.04b.

Data were extracted from eight of the nine previous studies of Rsa I/Pst I polymorphism and alcoholic liver disease in white populations (all except the “random effects” model). The decision to compare alcoholic liver disease groups with healthy control groups in the meta-analysis is explained below. Five of the eight studies included in the meta-analysis only investigated patients with cirrhosis whereas three, like our own, also included patients with alcoholic hepatitis in their alcoholic liver disease study groups.6 8 11 Because neither of these two groups appeared more or less likely to demonstrate any association between c2 carriage rate and alcoholic liver disease risk, all eight studies were pooled and considered together in the meta-analysis. The meta-analysis was carried out using the computer software package Rev Man v3.1 (The Cochrane Collaboration, Copenhagen, Denmark).

Results

The PCR produced amplimers of the expected size, and differentiated between individual Rsa I (data not shown), Dra I (data not shown), and Taq I genotypes (fig 1) in the study groups. Among the hepatocellular carcinoma group,
reproducible diagnostic bands could not be produced in 18 samples for Rsa I, 15 samples for Dra I, and two samples for Taq I. The fact that the paraffin wax blocks for several of these samples were more than 10 years old suggests that the failure to yield diagnostic bands might be the result of age related deterioration of the DNA extracted from these samples.

Within the control group, the most even distribution of CYP2E1 genotypes was demonstrated using Taq I (less common allele frequency, 0.135), followed by Dra I (0.096), and then Rsa I (0.033). There were no significant differences in the frequencies of the less common Rsa I allele between the control and alcoholic liver disease groups (table 1). Similarly, there were no significant differences in the frequencies of the less common Dra I allele between the control and alcoholic liver disease groups (table 1). In contrast, the frequency of the less common Taq I allele in the alcoholic liver disease group was significantly less than that in the control group (χ² = 6.35; p = 0.012; table 1).

There were no significant differences in the frequencies of the less common Rsa I allele in the hepatocellular carcinoma and control groups (table 1). Comparisons of the observed distributions of Rsa I, Dra I, and Taq I genotypes and those predicted by allele frequencies by χ² analysis showed that the populations studied were in Hardy-Weinberg equilibrium, indicating that the control and study groups were sufficiently random and representative (data not shown).

Meta-analysis using the fixed effects model demonstrated heterogeneity among the nine studies of c2 allele carriage rate and alcoholic liver disease risk (data not shown), therefore suggesting that analysis using the random effects model would be preferable. The latter yielded an overall OR of 1.41 (95% CI, 0.78 to 2.55) and therefore failed to demonstrate any significant association between c2 carriage and susceptibility for alcoholic liver disease (table 2).

### Discussion

Our study investigated the relation between different polymorphisms of the CYP2E1 gene and susceptibility to both alcoholic liver disease and hepatocellular carcinoma. Of the eight previous studies included in our meta-analysis, four compared the c2 carriage rate of patients with alcoholic liver disease with that of healthy individuals and also with that of alcoholic patients with no evidence of liver disease. Using the latter group as controls is theoretically preferable in permitting the study, in isolation, of the direct association between CYP2E1 genetic polymorphisms and risk of liver disease on a background of alcoholism. In the four mentioned studies, the “no liver disease alcoholic” groups were defined as alcoholic patients with either no clinical or no biochemical evidence of liver disease. However, in these groups, liver disease was not excluded historically, even though almost a fifth of alcoholic patients without clinical or biochemical abnormalities may show advanced disease on liver biopsy. The subsequent potential for significant error in defining these “no liver disease alcoholic” groups discouraged us from performing meta-analysis to compare these groups with patients with alcoholic liver disease. For similar reasons, we had initially planned to use only alcoholic patients with no histological evidence of liver disease as our control group. However (as other investigators presumably found), we were unable to recruit large enough numbers of such individuals. The alcoholic liver disease/cirrhosis patient groups used by previous studies were defined by documented alcohol consumption (range of median individual intake, 148–257 g/day for 10–17 years) and, in almost all cases, confirmatory histological features on liver biopsy. The “healthy” control groups used in these studies showed more heterogeneity, ranging from university staff to blood donors. Because alcohol intake was not recorded and liver biopsies were not performed in most of these control groups, it is theoretically possible that some of the groups may have inadvertently included a small number of patients with alcoholic liver disease. Another weakness of using healthy control groups is the possibility of recruiting individuals who have the genetic predisposition to develop alcoholic liver disease, but who have not drunk sufficient alcohol to develop the disease. Finally, although our control group was selected because of its similar ethnic background to our patient groups, like other case control association studies, our study may suffer from the “founder effect” or population admixture. These issues of selecting an appropriate control group can be circumvented through the use of family studies, although the recruitment of relatives for such analyses does itself introduce new logistical problems.

Accepting the aforementioned limitations, in keeping with the individual findings of most previous studies on the subject, we found no significant association between Rsa I/Pst I polymorphism and susceptibility to alcoholic liver disease in our own study population or from our meta-analysis (tables 2 and 3). It is difficult to reconcile this absence of association with the recent findings of Pirmohamed and colleagues, who used similar methodologies to our study and previous studies, but found possession of the less common allele (c2) to be strongly associated with an increased risk of developing alcoholic liver disease. However,
regardless of the reasons for this discrepancy, the low frequencies at which the c2 allele exists in white populations suggests that, if present, it only makes a minor contribution to inherited susceptibility to alcoholic liver disease.

The less common Dra I allele (C) is found two to four times more frequently in healthy white control groups than the c2 allele. To date, two research groups have demonstrated the frequency of the C allele to be higher among patients with alcoholic liver disease than among healthy controls, or alcohol drinkers without liver disease. Although an earlier study reported the opposite, the association was not significant. In agreement with the findings of Parsian and colleagues’ recent study, our data failed to show a significant association between possession of the less common Dra I allele and susceptibility to alcoholic liver disease. It is possible, however, that our findings and those of Parsian and colleagues only found a significant association among patients with hepatocellular carcinoma who had high daily alcohol intakes, a group excluded from our analysis. The arguments raised earlier in the discussion point against this association being caused by c2 allele carriage increasing susceptibility to alcoholic cirrhosis and, thus, indirectly increasing susceptibility to hepatocellular carcinoma. Indeed, the same workers failed to relate Rsa I/Pst I polymorphism to alcoholic liver disease risk in a later study. It is not certain whether alcohol or its metabolites have a direct hepatocarcinogenic effect or, as discussed above, whether CYP2E1 activity and inducibility varies with different CYP2E1 genotypes. Thus, confirming and explaining the discrepant results of our study and the Spanish study will require further analysis of c2 allele carriage among alcoholic and non-alcoholic patients with hepatocellular carcinoma in other white populations.

In conclusion, we were unable to demonstrate any association between the two, it is possible that our findings regarding the A1 allele are attributable to its linkage to some as yet unidentified protective factor(s).

In contrast to the findings of a recent Spanish study, we were unable to demonstrate any association between possession of the c2 allele and increased risk of developing hepatocellular carcinoma. Interestingly, Ladero and colleagues only found a significant association among patients with hepatocellular carcinoma who had high daily alcohol intakes, a group excluded from our analysis. The arguments raised earlier in the discussion point against this association being caused by c2 allele carriage increasing susceptibility to alcoholic cirrhosis and, thus, indirectly increasing susceptibility to hepatocellular carcinoma. Indeed, the same workers failed to relate Rsa I/Pst I polymorphism to alcoholic liver disease risk in a later study. It is not certain whether alcohol or its metabolites have a direct hepatocarcinogenic effect or, as discussed above, whether CYP2E1 activity and inducibility varies with different CYP2E1 genotypes. Thus, confirming and explaining the discrepant results of our study and the Spanish study will require further analysis of c2 allele carriage among alcoholic and non-alcoholic patients with hepatocellular carcinoma in other white populations.

Table 3 Summary of the findings of previous studies on the association between CYP2E1 genetic polymorphisms and susceptibility to alcoholic liver disease (ALD) in white populations

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population studied</th>
<th>Study group (n)</th>
<th>c2 allele frequency</th>
<th>p Value</th>
<th>C allele frequency</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingelman-Sundberg et al (1993)</td>
<td>Italian</td>
<td>Control# (114)</td>
<td>0.044</td>
<td>0.565</td>
<td>0.083</td>
<td>0.22*</td>
</tr>
<tr>
<td>Carr et al (1995)</td>
<td>North American</td>
<td>Control# (32)</td>
<td>0.027</td>
<td>0.016</td>
<td>0.415</td>
<td>–</td>
</tr>
<tr>
<td>Ball et al (1995)</td>
<td>British</td>
<td>Control# (114)</td>
<td>0.047</td>
<td>0.028</td>
<td>0.705</td>
<td>–</td>
</tr>
<tr>
<td>Pirmohamed et al (1995)</td>
<td>British</td>
<td>Control# (100)</td>
<td>0.041</td>
<td>0.015</td>
<td>0.0006*</td>
<td>–</td>
</tr>
<tr>
<td>Lucas et al (1996)</td>
<td>French</td>
<td>Control# (260)</td>
<td>0.018</td>
<td>0.025</td>
<td>0.35*</td>
<td>0.079</td>
</tr>
<tr>
<td>Agündez et al (1996)</td>
<td>Spanish</td>
<td>Control# (110)</td>
<td>0.041</td>
<td>0.026</td>
<td>1.005</td>
<td>–</td>
</tr>
<tr>
<td>Savolainen (1997)</td>
<td>Finnish</td>
<td>No ALD (33)</td>
<td>0.017</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Parsian et al (1998)</td>
<td>North American</td>
<td>Control# (89)</td>
<td>0.011</td>
<td>0.010</td>
<td>1.005</td>
<td>0.130</td>
</tr>
<tr>
<td>Grove et al (1998)</td>
<td>British</td>
<td>Control# (121)</td>
<td>0.041</td>
<td>0.17</td>
<td>0.000</td>
<td>0.90*</td>
</tr>
<tr>
<td>Wong et al (current study)</td>
<td>British</td>
<td>Control# (375)</td>
<td>0.016</td>
<td>0.033</td>
<td>0.415</td>
<td>0.096</td>
</tr>
</tbody>
</table>

*p Chi squared test with Yates correction; §two tailed Fisher’s exact test; #healthy controls; ¶alcohol related cirrhosis; †no raw data given.
characterise the mechanisms that underlie the association.

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