RsaI polymorphism at the cytochrome P4502E1 locus and risk of hepatocellular carcinoma

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Abstract
Background—CYP2E1, the coding gene for the ethanol inducible cytochrome P4502E1, is polymorphic at the RsaI restriction site in the 5' flanking region. The mutant allele c2 has a higher transcriptional activity than the wild-type gene c1. P4502E1 catalyses the activation of several environmental carcinogens at a rate that is increased, if only moderately, by longterm ethanol intake.

Aims—To establish the distribution of CYP2E1 RsaI polymorphism in patients with hepatocellular carcinoma and to evaluate its possible role in the multifactorial pathogenesis of this tumour.

Subjects—101 (84 males) patients with hepatocellular carcinoma and 178 (128 males) healthy controls of the same ethnic (white) and Spanish origin.

Methods—After extraction of DNA from white blood cells, alleles c1 and c2 of CYP2E1 were identified by restriction fragment length polymorphism (RFLP) with endonuclease RsaI.

Results—Homozogous c1c1: 90 patients and 169 controls; heterozygous c1c2: 11 and 9; homozogous c2c2: none (non-significant difference). C2 allele frequencies: 0.055 in patients, 0.025 in controls (non-significant difference) and 0.108 in the 37 patients who had drunk more than 50 g of ethanol/day (p=0.0035, odds ratio versus controls: 4.67; 95% confidence limits 1.57 to 13.81).

Conclusion—The carrier state of one copy of the c2 CYP2E1 gene increases the risk of hepatoma in previously regular ethanol users with chronic liver disease.

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Keywords: hepatocellular carcinoma, ethanol, CYP2E1. P4502E1, polymorphism.

The ethanol inducible cytochrome P-450 isozyme CYP2E1 catalyses the oxidation of more than 75 xenobiotic substrates, including ethanol itself, drugs (paracetamol, chloroxazone), and several potential carcinogens (some nitrosamines, benzene, aniline) that are transformed to their ultimate reactive forms. High ethanol intake is a well known carcinogenic factor for the upper alimentary tract, specially if associated with tobacco use. Primary liver cancer usually complicates several chronic liver diseases, mainly those induced by hepatitis B and C virus. Although liver cancer is a frequent terminal event in alcoholic cirrhosis, the high incidence of coexisting HCV chronic infection in these patients makes doubtful the role of alcohol itself as a definite risk factor for hepatoma. Nevertheless, tobacco use may be related to the incidence of hepatoma in cirrhotic patients, although current data are controversial.

Theoretically, the induction of CYP2E1 would result in an increase of the rate of activation of carcinogens that reach the liver in subjects who misuse alcohol. Many carcinogens are present in tobacco smoke and indeed in many foods, and the broad spectrum of substrates for CYP2E1 includes many of them.

CYP2E1 gene is polymorphic at several levels. One of these polymorphisms involves Pst I and Rsa I sites in the 5' flanking region; the rare allele c2 is associated with increased gene expression but, up to now, its real effect on the enzymic activity in vivo has not been fully investigated, partly because of its low incidence. Nevertheless, the liver is a better candidate for these type of studies because its high rate of specific CYP2E1 activity after alcohol induction and because it must cope with all the CYP2E1 substrates absorbed in the gut, including tobacco specific carcinogens dissolved in swallowed saliva.

Therefore, CYP2E1 may modulate the risk of liver cancer through two different mechanisms that influence its rate of activity. One is genetic and lies upon functional differences between Rsa I polymorphic c1 and c2 alleles. The other depends on environmental factors, mainly ethanol or other inducers, which frequently show also a carcinogenic potential in the liver. This study aims to elucidate the role of the Rsa I genetic polymorphism in liver cancer risk in a large series of patients with liver cancer and different aetiological profiles.

Methods

Study population
One hundred and one patients (84 males, mean (SD) age 67 (8-5) years) diagnosed with primary liver cancer (hepatocarcinoma) were included in the study. The diagnosis of hepatoma was based upon positive imaging studies (ultrasonography, computed tomography or angiography, or all three) in all patients, and confirmed by fine needle aspirative cytology or histological analysis of the tumour (75 patients) or by an a fetoprotein concentration of at least 700 ng/ml in absence of other known primary malignancy in the remaining 26 cases.
Seventy seven patients had a previous diagnosis of chronic liver disease: 67 were cirrhotic (by clinical, analytical, and imaging criteria), seven had a definite histological diagnosis of chronic active hepatitis, and there were two other patients with porphyria cutanea tarda without evidence of cirrhosis and one with haemochromatosis. There were no data of previous liver disease in 24 patients, but the histology of the non-tumoral liver was known only in one (normal liver). Viral hepatitis B (VHB) and C (VHC) markers were known in all but two patients. Six were positive for HBsAg, 27 for anti-HBs, 46 for anti-HBc, and 71 for anti-HCV (ELISA second generation or western blot assays, or both). Thirteen patients were negative for all serum VHB and VHC markers. Data on tobacco and alcohol consumption were collected; alcohol use was categorised in two levels: teetottlers or drinkers less than 50 g/day and drinkers of more than 50 g/day. This limit is somewhat lower than that fixed by most authors for the cirrhogenic effect of ethanol,\textsuperscript{19} 20 but it was chosen because it is probably closer to the threshold for the ethanol inducing effect on CYP2E1 activity.\textsuperscript{12}

The control group was composed of 178 subjects (128 males) of the same ethnic (white Spanish) and geographical (the central area of Spain) origin. Ages in the control group are bimodally distributed: 137 subjects were healthy volunteers (116 males, mean (SD) age 32 (18-4) years), whereas 41 subjects were older than 90 years (12 males, mean (SD) age 92-8 (2-5) years); these elderly controls were in comparatively good health and none suffered any malignant or neurodegenerative diseases.

The study was approved by the ethics committees of the San Carlos University (Madrid) and Infanta Cristina (Badajoz) hospitals, under the guidelines of the 1975 Declaration of Helsinki. Patients and controls gave their previous informed consent to be included in the study.

Study procedure

Venous blood samples (about 20 ml) were obtained by venipuncture and anticoagulated in sterile glass tubes containing sodium citrate or ethylenediaminetetraacetic acid. The blood was then transferred to sterile plastic vials and kept at -80°C until use. Genomic DNA samples were isolated from leucocytes as described elsewhere.\textsuperscript{21}

The analysis for the RsaI mutation at the 5' flanking region of CYP2E1 was carried out by an amplification restriction procedure\textsuperscript{13} with minor modifications. Briefly, a 565 bp fragment of the 5' flanking region of CYP2E1 was amplified by polymerase chain reaction by the use of primers (forward: 5' GTC CCT GCC ACC TCA CAC T; reverse: 5' CCC TCT TCC ACC TTC TAT G). The amplification mixture contained, in a final volume of 25 μl: 200-400 ng of genomic DNA of proband, 50 nM of each primer, 200 μM of each nucleotide (dATP, dGTP, dCTP, and dTTP), 10 ml TRIS-HCl, pH 8-3, 1-5 mM MgCl2, 50 mM KCl, 0-1 mg/ml gelatine, and 2-5 units of Taq DNA polymerase. The reactions were assembled at room temperature. The amplification conditions were: one cycle at 94°C for 10 seconds, 62°C for 10 seconds, and 72°C for 50 seconds. In the following four cycles the annealing temperature was decreased one °C every cycle. Then, 30 cycles at 94°C for 15 seconds, 57°C for 20 seconds, and 72°C for 20 seconds were performed. A final extension of 74°C for seven minutes was carried out. The PCR products were ethanol precipitated and resuspended in 10 μl water. The samples were then digested during three hours with three units RsaI and electrophoresed in 1-2% agarose gels.

The RsaI genotype was estimated by the presence of digests of 421 and 144 bp (allele c1) or 565 bp (allele c2).\textsuperscript{15} Adequate controls of subjects with genotypes cl-cl, c1-c2, and c2-c2 were included and analysed in parallel with every set of reactions.

Statistical analysis

It was made through non-parametrical (U Mann-Whitney) and parametrical (Mantel-Haenszel or two tailed Fisher exact) tests, each when adequate. The odds ratio was 95% confidence intervals was calculated to compare the distribution of the CYP2E1 genotype between cases, subgroups of cases, and controls. We also performed a multivariate analysis that included previously suggested (age, HBV and HCV markers, chronic liver disease, and alcohol and tobacco use) and newly proposed (CYP2D6 and NAT2 genotypes) risk factors to control for confounding variables. All calculations were made with the Epi-Info6 software. The null hypothesis was rejected when \( p<0.05 \).

Results

Eleven (10-9%) patients, seven controls in the younger age group (5-4%) and two subjects in the elderly control group (4-9%) were classified as heterozygote c1c2. The frequency of the c1c2 genotype in the whole control group is 5-1% (Table). No homozygous c2c2

<table>
<thead>
<tr>
<th>Genotype and allele frequencies</th>
<th>P4502E1 genotype frequencies</th>
<th>Allele frequencies</th>
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<tr>
<td></td>
<td>clcl</td>
<td>clc2</td>
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<tr>
<td>Normal controls (n=178)</td>
<td>0.949</td>
<td>0.051</td>
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<tr>
<td>All patients (n=101)</td>
<td>0.981</td>
<td>0.019</td>
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<td>Ethanol (+) patients (n=37)</td>
<td>0.784</td>
<td>0.216</td>
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<tr>
<td>Ethanol (-) patients (n=64)</td>
<td>0.953</td>
<td>0.047</td>
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NS: non-significant difference. *: comparison with controls.
Bilateral comparisons were non-significant between the distribution of CYP2E1 Rs1 genotype and age, sex, previous liver state, HBV and HCV markers, and tobacco use. Twelve of 13 patients HBV and HCV negative were c1c1. Nine of 11 patients classified as c1c2 were cirrhotic and the remaining two had chronic active hepatitis, HBV and HBV-HCV related, respectively.

Thirty seven patients (36 men) drank more than 50 g ethanol/day. There was a pronounced excess of c1c2 genotypes in this subgroup when compared with non-excessive drinkers (two tailed Fisher exact test p=0.016, odds ratio 5.61, 95% confidence intervals 1.21 to 24.6) and with controls (p=0.0028 odds ratio 5.18, 95% confidence intervals 1.65 to 16.22) (Table). Multivariate analysis, which included VHB and VHC serum markers, age, previous liver state, and tobacco use, showed that this relation between c1c2 genotype and ethanol intake in patients was independent from the other analysed risk factors for hepatoma (p=0.022). When comparing the distribution of gene frequencies, a significant excess of the c2 gene among patients with previous alcohol intake was found both when compared with controls (p=0.035, odds ratio=4.67, 95% confidence intervals 1.57 to 13.81) and with non-drinking patients (p=0.02, odds ratio 5.05, 95% confidence intervals 1.15 to 30.28).

CYP2D6 genotype was known in 100 patients (published in part22) and N-acetyl transferase-2 genotype in 96 patients (unpublished data). The three genotypes were known in 94 patients. When these two polymorphic traits were included in the multivariate analysis, they had no influence on the existing relation between ethanol use and c1c2 CYP2E1 Rs1 genotype (p=0.03), even when only ethanol and the three genotypes were analysed (p=0.027).

Discussion
This study shows the distribution of the CYP2E1 Rs1 polymorphism in patients with hepatocellular carcinoma. The pronounced excess of the rare c2 gene, which we found only in patients with previous high ethanol intake, suggests a synergism between these two factors in the pathogenesis of liver cancer. Other known risk factors for hepatoma, as chronic HBV and HCV infection, liver cirrhosis, tobacco use or CYP2D6 genotype,22 were excluded as confounding factors through the adequate multivariate analysis. Nevertheless, the c2 allele should be a secondary factor in the aetiopathogenic spectrum of hepatoma in alcohol consuming patients, because most of them are homozygote for the c1 allele. Moreover, 12 of 13 patients negative for all viral markers carried the c1c1 genotype, indicating that the c2 allele is not especially linked to the risk of HCC of non-viral origin.

We have not found any relation between tobacco use, Rs1 CYP2E1 genotype, and risk of hepatoma, a possible link detected among alcoholic consuming patients of Taiwanese origin. In that study23 the 13 patients with hepatoma who smoked carried the c1c1 genotype. It must be stated that the prevalence of the c2 allele in populations of Oriental origin is much higher than in white people. Thus, the absolute prevalence of the c1 allele in this small subgroup reached statistical significance.

There are various possibilities to explain the link between the CYP2E1 c2 allele carrier state and ethanol intake. Cytochrome 2E1 enzyme is inducible by ethanol at comparatively low doses,12 which increases its rate of activation of specific carcinogens. Aflatoxin, a well known liver carcinogen, does not follow the CYP2E1 route for its activation24; however, it should be kept in mind that aflatoxin B1 is not a relevant factor in the etiology of liver cancer in Western countries. More relevant may be the role of N-nitrosodimethylamine (NDMA), an environmental procarcinogen. Human beings are exposed to very low concentrations of NDMA, the same that are metabolised exclusively by CYP2E1.25 Rats exposed to such low doses of NDMA develop hepatocarcinoma only when they are simultaneously treated with ethanol.26

The CYP2E1 c2 gene has a higher transcriptional activity than its c1 counterpart.13 Induction of cytochrome P-4502E1 in human liver by ethanol is probably secondary to an increase in encoding mRNA,27 but that study only included five ‘induced’ patients with unknown genotype and the chance of having selected at least one carrier of one c2 gene is quite low. In fact, the rate of increase of enzymic activity after treatment with ethanol was very similar in these five subjects. If the c2 gene had a higher rate of ethanol inducibility, carriers of one copy of it (homozygote c2c2 are very uncommon) should be exposed to greater doses of activated carcinogens if they were drinkers, even moderate, of ethanol. The increased transcriptional activity of the C2 gene can also cause, even without ethanol related induction, an increased rate of activation of carcinogens. At present, this hypothesis is merely speculative and should be confirmed through studies in humans. Lucas et al.28 have reported that patients with the genotype c1c2 seem to be less inducible than those with the genotype c1c1 after ethanol induction. This could be explained if the increases in the enzymic activity caused by the C2 allele increased transcriptional rate and by the ethanol inductive properties were not additive. However, it seems that we still do not have adequate non-toxic substrates to confirm these type of studies, because the only one proposed by Kim et al.14 and used in the study by Lucas et al.28 patients has been isolated in the metabolic metabolite by CYP2E1 mediate catalysis, does not fulfil the minimum requirements to be used as a reliable probe for these studies29 nor is it specific for CYP2E1.30

It could be argued that the c2 gene is related to the risk of alcoholic cirrhosis instead of...
influencing the subsequent malignant transformation. Nevertheless, ethanol is probably not the main aetiological factor of the liver disease developed by these patients, because only a few were actually heavy drinkers and 25 were anti-HCV reactive. Moreover, we have found that the distribution of this polymorphism in 58 patients with severe chronic alcoholic liver disease (cirrhosis) is very similar to that found in normal controls (unpublished data), confirming the findings made in other white populations.31 32

In conclusion, we have found an over-representation of carriers of the CYP2E1 c2 gene among patients with hepatocarcinoma complicating chronic liver disease and with a history of regular significant ethanol intake. Studies are needed to elucidate the basis of the interaction between this infrequent allele and ethanol, which may increase the risk of hepatocarcinoma in otherwise predisposed patients.

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