The H63D mutation in the HFE gene is related to the risk of hepatocellular carcinoma


ABSTRACT

Aim: to disclose whether mutations in the HFE gene inducing liver iron overload are related to the risk of hepatocellular carcinoma (HCC) in otherwise predisposed patients.

Patients and methods: one hundred and ninety-six patients (161 males) diagnosed with HCC and 181 healthy controls were included in the study. All subjects were white Spaniards.

C282Y and H63D mutations in the HFE gene were identified in leucocyte genomic DNA using a polymerase chain reaction (PCR) and specific restriction enzymes.

Results (cases/controls): 1. Genotype distribution: a) C282Y mutation: homozygotes 1/0, heterozygotes 12/23, wild type 183/158 (p = 0.07, non significant); b) H63D mutation: homozygotes 9/5, heterozygotes 85/52, wild type 102/124 (odds ratio 2.00, 95% C.I. 1.29-3.12, p = 0.002). Four cases and 6 controls were carriers of heterozygous mixed genotypes. 2. Allele frequencies: a) C282Y mutation: wild type allele 378/339, mutated allele 14/23 (p = 0.11, n.s.); b) H63D mutation: wild type allele 289/300, mutated allele 103/62 (odds ratio 1.72, 95% C.I. 1.19-2.50, p = 0.004). Age at diagnosis, gender and etiology of the underlying liver disease do not influence these findings.

Conclusion: the C282Y mutation in the HFE gene is not related to the risk of HCC in non-hemochromatosis patients. The H63D mutation is associated with a higher risk of HCC in cirrhotic patients irrespective of their underlying liver disease.


INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most frequent cancer in the world, with an incidence rate that varies from 3 to 80 cases per 100,000 inhabitants/year (1),
and that is increasing (2), though with wide geographical variations (3). HCC occupies the third place in cancer-related deaths (4). More than 80% of cases develop in cirrhotic livers (5), a proportion that in Spain rises up to 90% (6). The risk depends on the etiology of cirrhosis, and is maximum for hemochromatosis (7,8), though probably less than previously thought (9). In Europe, the most important risk factor is cirrhosis from hepatitis C virus (HCV), alone or in association with ethanol abuse (10,11). In our hospital 68% of HCC complicates HCV-induced cirrhosis (12).

The identification by Feder et al. (13) of the hemochromatosis gene (HFE), and of mutation Cys282Tyr, has allowed to establish a genetic basis for the majority of cases of hereditary hemochromatosis in white subjects of anglo-celtic descent (14), although in European countries by the Mediterranean basin the prevalence of homozygosity for mutation C282Y in patients with genetic hemochromatosis is only 64% (15). In addition, only 50% of homozygotes for mutation C282Y develop phenotypical hemochromatosis, and an additional 25% has iron overload without clinical repercussion (16). Hence, it might exist other factors that modulate the patogenicity of this mutation. One of them is a second mutation of the HFE gene, designated H63D, which when coincident in double heterozygosis with mutation C282Y can induce a syndrome clinically undistinguishable from that common in C282Y homozygotes (17).

Mutations in the HFE gene can be a cofactor aggravating other chronic diseases of the liver (18). Studies exist on the possible relation of mutations in the HFE gene with the risk and clinical behavior of different liver diseases, including HCV chronic infection (19-22), alcoholic liver disease (23,24), porphyria cutanea tarda (25), and non-alcoholic steatohepatitis (26-28), with controversial results. The item warranting these studies is suspected iron overload as induced by some mutation of the HFE gene capable of inducing liver damage by increasing oxidative stress (29). In the specific case of HCC, which is the subject of this study, liver iron overload might be carcinogenic through the direct stimulus of cellular proliferation (30) and induction of DNA damage, specially by inducing mutations in the p53 gene (31,32), but also indirectly through the already-mentioned increased oxidative stress, which would cause a peroxidation of membrane lipids, DNA damage via the formation of adducts, and a stimulus of stellate cells, with a resulting acceleration of fibrogenesis (29,33).

It is well known that genotypes C282Y/C282Y and C282Y/H63D are frequently associated with a certain degree of iron overload, though with a minor frequency to the hemochromatosis phenotype (16,17). A recent study of a wide sample has verified that the possession of any mutation in gene HFE increases hepatic iron in patients with chronic HCV infection (34), which confirms the findings previously reported by smaller series or based only on blood iron parameter measurements (35-41). Nevertheless, the repercussion of mutation H63D, both in homo- and in heterozygosis, on iron deposition in the body is controversial, and several groups have found no association between this mutation and liver iron content (42-45). Interestingly, mutation H67D in the mouse, which is equivalent to H63D in humans, favors iron deposition in the liver (46).

The HFE gene codes for a class I major histocompatibility complex (MHC) molecule whose peptide fixation fold is too narrow for antigen presentation (47); however, mutations in this gene are related to functional alterations in classic class-I MHC molecules, which indicates that the HFE gene can play a role in immune response (48), a preponderent element in the evolution of chronic liver disease, on which most HCCs develop.

This study was performed to investigate the potential relationship between C282Y and H63D mutations in the HFE gene, and the risk of developing HCC in Spanish patients diagnosed with this tumor, as well as its possible association with other etiological factors.

**PATIENTS AND METHODS**

In all, 196 white unrelated consecutive patients (161 males, mean age 66.1 years, SD 9.7, range 29-88) of Spanish ancestry were included, all of them diagnosed with HCC in the Gastroenterology Dept. at Hospital Clínico San Carlos (Madrid, Spain) from January, 1994 until September, 2003. HCC diagnosis was based on histopathological results or on a concentration of alpha fetoprotein > 399 ng/dl in the presence of a liver mass on imaging studies (ultrasonography or CT scanning). Table I shows the demographic and epidemiological characteristics of patients. All patients gave their informed consent for blood sampling after being briefed about the nature of the study.

The control group was constituted by 181 non-related healthy blood donors, who also gave their informed consent. Only those for whom donation had been rejected for any cause were excluded. Information on these subjects is absolutely confidential, and thus neither their sex nor their age is known, except for their white race and Spanish origin.

For the molecular analysis, genomic DNA was extracted from peripheral blood leukocytes by means of a standard extraction procedure (49). The study of the HFE gene was done by PCR (Polymerase Chain Reaction) using oligonucleotides as described previously (13,50).

For the C282Y mutation, the amplified product (387 bp) was digested with restriction enzyme Rsa I, with a target GT ↓ AC located on the mutated allele. For the H63D mutation, the amplified fragment of 208 bp was digested with the restriction enzyme Dpn II, whose target ( ↓ GATC) is located in the normal allele.

The statistical analysis was performed by applying tests for continuous independent variables (Student’s t or
Mann-Whitney test, each when adequate), and the Chi-square test (Mantel-Haenszel) for categorical variables, with odds ratios and 95% confidence interval being estimated for dichotomous variables. In addition, the Chi-square test was used for linear trends when pertinent (analyses of dose-gene effect), with Bonferroni’s correction for multiple comparisons. The possible influence of other risk factors (age, sex, etiology) was analyzed by means of contingency tables, as having no comparable control group for these variables made a multivariate analysis not feasible. The null hypothesis was rejected when p < 0.05 or when the confidence interval did not include value 1. We used the statistical software packages EpiInfo 2000, by Center for Disease Control and Prevention (Atlanta), and SPSS 11.5.

### RESULTS

The distribution of HFE genotypes is shown in table II, and allele frequencies for these mutations are shown in table III. A patient not previously diagnosed with hemochromatosis was homozygote for mutation C282Y. C282Y allele frequency and the proportion of single C282Y heterozygotes were low in both groups, and showed no significant differences. Four patients and six controls were compound heterozygotes C282Y/H63D. Of all 13 patients carrying mutation C282Y four were anti-HCV positive, three were excessive ethanol drinkers, and in six both factors were coincidental.

In the group of cases there is a significant excess of mutation H63D carriers (48.0 vs. 31.5%, table II) and in the allele frequency of this mutation (0.263 vs. 0.171, table III). This difference persists after having eliminated from the analysis all 13 cases and 23 controls carrying mutation C282Y, and a possible dose-gene effect is appreciated by means of the test for linear trend (Table II, right column).

Twelve patients (11 males) were HbsAg-positive. None of them carried the C282Y mutation, but 10 were carriers of the H63D mutation (9 heterozygotes and 1 homozygote). This excess reaches statistical significance (Chi square 6.597, p = 0.01). In 6 of these 12 patients there were other simultaneous risk factors (HCV infection in 2 cases, ethanol abuse in 2 cases, both in 2 cases).

We found no significant variation in the frequency of HFE gene mutations in relation with sex, age at diagnosis, positive or negative status for anti-HCV and anti-HBc, and excessive ethanol use.

### Table I. Characteristics of all 196 patients

| Sex (males/females): 161/35 |
| Mean age: 66.1 years, SD 9.7 (range: 29-88) |
| Diagnosis: |
| Presence of one or more liver masses and at least one of the following: |
| Confirmatory pathology: 122 cases |
| Alpha-feto protein > 399 ng/dl: 113 cases |
| Both criteria: 41 cases |
| Ethanol abuse (> 50 g/day): 93 cases (91 males). |
| HBSAg positive/negative/unknown: 12/171/13* |
| Anti-HBc positive/negative/unknown: 71/114/11** |
| Anti-HCV positive/negative/unknown: 128/61/7*** |

* In these 13 cases, anti-HBc was negative in 2 and unknown in 11. Anti-HCV was positive in 6 cases, negative in 3, and unknown in 4. **: in 6 of these 11 cases anti-HCV was positive; ***: two of these 7 cases showed positivity for one or more HBV markers. Fifteen patients were negative for HBSAg, antiHBc, and anti-HCV, and were not ethanol users.

### Table II. Distribution of HFE genotypes in cases and controls

<table>
<thead>
<tr>
<th>HFE mutation</th>
<th>Group</th>
<th>Without mutations (%)</th>
<th>Carriers Homozygotes/compound heterozygotes/single heterozygotes</th>
<th>Total serie</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Patients without C282Y (study of the H63D mutation)</td>
</tr>
<tr>
<td>C282Y</td>
<td>Cases</td>
<td>183 (93.4)</td>
<td>13 (1/4/8)</td>
<td>Odds ratio = 0.49</td>
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<tr>
<td></td>
<td>Controls</td>
<td>158 (87.3)</td>
<td>23 (0/6/17)</td>
<td>C95% = 0.23-1.05</td>
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<td></td>
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<td></td>
<td>p = 0.07</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Ch2 = 3.35</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td>p = 0.07</td>
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</tr>
<tr>
<td>H63D</td>
<td>Cases</td>
<td>102 (52)</td>
<td>94 (9/4/81)</td>
<td>Odds ratio = 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>124 (68.5)</td>
<td>57 (5/6/46)</td>
<td>C95% = 1.29-3.12</td>
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<tr>
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<td>p = 0.002</td>
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<td></td>
<td></td>
<td></td>
<td>Ch2 = 9.95</td>
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<td></td>
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<td></td>
<td>p = 0.002</td>
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</tbody>
</table>

### Table III. Allele frequencies in cases and in controls

<table>
<thead>
<tr>
<th>HFE Allele</th>
<th>Group</th>
<th>Normal (frequency)</th>
<th>Mutated (frequency)</th>
<th>Odds ratio</th>
<th>Statistical analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>C282Y</td>
<td>Cases</td>
<td>378 (0.964)</td>
<td>14 (0.036)</td>
<td>0.55</td>
<td>95% CI = 0.26-1.13</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>339 (0.937)</td>
<td>23 (0.063)</td>
<td>1.72</td>
<td>Ch2 = 8.63, p = 0.004</td>
</tr>
<tr>
<td>H63D</td>
<td>Cases</td>
<td>289 (0.737)</td>
<td>103 (0.263)</td>
<td>0.55</td>
<td>Ch2 = 1.19-2.50</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>300 (0.83)</td>
<td>62 (0.17)</td>
<td>1.72</td>
<td>p = 0.004</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study we detected an excess of gene HFE mutation H63D carriers among patients with HCC versus a group of healthy controls with the same nationality and ethnic origin, all of them recruited in the city of Madrid. The prevalence of the H63D mutation in the control group (allele frequency 0.171) is slightly lower than that detected in another group of controls with the same nationality and race, though from a different geographical zone (Catalonia). The authors of this study (51) indicate in their controls the finding of one of the highest frequencies of the H63D allele reported in the literature. In a study performed by our group in the blood from the umbilical cord of 298 neonates of Spanish origin and white race born in Madrid, allele frequencies were, respectively, 0.018 for mutation C282Y and 0.191 for mutation H63D (52). If we had used this group for control, the statistical significance we found for the identified differences in the respective proportions of H63D carriers would still persist (0.006), and that would also be the case regarding H63D allele frequencies (p = 0.017). Moreover, these data are very similar to those found by other groups in Western Mediterranean Europe (53).

The C282Y mutation of the HFE gene was present in 13 cases and 23 controls, with an allele frequency of 0.036 and 0.063, respectively (Table III). These data confirm the low prevalence of this mutation in the Spanish population as already reported by other authors (51). Although this difference is not significant, it points opposite to the hypothetical relationship of risk for this mutation with HCC, theoretically due to its ability to induce iron overload (54), something that no doubt occurs when in homozygosis (51) but that has also been verified, although to a much lesser extent, when in heterozygosis (34). We have not been able to measure liver iron content in our patients, since most of the pathological diagnoses were reached by means of a cytology obtained with fine-needle aspiration, and ferritinemia does not reflect body iron content in cirrhotic patients with HCC. In a study of 35 patients with HCC developed in non-cirrhotic livers, iron overload was detected in the liver parenchyma of 19 cases, seven of whom were C282Y carriers, whereas the remaining 16 patients, without iron overload, had no mutations in the HFE gene (55). In our literature review some reports detect an excess of C282Y mutations; special interest has the study by Lauret et al. (56), performed in Spain, in which the authors detected a significant excess of heterozygotes for the C282Y mutation in 43 patients with HCC developed in the setting of alcoholic cirrhosis (20.9%), versus 136 subjects with alcoholic cirrhosis and no superimposed tumor (4.4%), but not in 34 patients with HCC complicating HCV-related cirrhoses. Hallerbrand et al. (57), in a group of 137 patients with HCC, did not identify any C282Y homozygotes, but they detected a significantly greater proportion of heterozygotes (12.4%) in comparison with cirrhotic patients without HCC (3.7%, p = 0.020) and with healthy subjects (4.8%, p = 0.031). Fargion et al. (58) detected an excess of C282Y mutations in 81 Italian patients with HCC as compared with controls (8.6 vs. 1.6%, p < 0.03), suggesting that carrying this mutation can increase the inherent risk of ethanol- and/or HCV-induced cirrhosis, but the allele frequency for this mutation in their control group is lower than that found in another series (53) in the same country (3.2%). Franczazini et al. (59) and Shi et al. (60) found an unequivocal relation between risk of hepatocellular carcinoma and carrier status for C282Y only in those patients with chronic hepatitis B and male sex. Other studies confirm the relation of C282Y with HCC risk only in homozygosis (61,62), or report negative results (63-65). Beckman et al. (66) have identified a significant relation to risk when the presence of mutation C282Y in the HFE gene and of genotype Ser/Ser at position 142 of the transferrin receptor gene is seen together, but not if they appear separately. Although these authors do not comment on this fact, it should be noted that 22% of these patients were carriers of mutation C282Y.

In this study we detected a significant excess of carriers of mutation H63D among HCC patients that had not been reported before. This difference is very high in carriers of HBsAg, but affects all the etiological groups and is independent of variables like age and sex. Only two studies (64,66) have detected a quantitatively smaller excess of H63D mutation that never reached statistical significance due to the small size of samples in the studied series.

The risk associated with H63D mutation and a hypothetical iron overload are difficult to relate to each other; although non negligible, as previously seen, such overload is much smaller than that associated with the C282Y mutation. An alternative explanation would be based on the adscription of the HFE gene to class-I HLA complex molecules, and on the possible linkage disequilibrium between mutation H63D and the HLA-A29-B44 haplotype (48), but at present the possible interaction between HFE gene mutations and the immune function have only been explored for the C282Y mutation (67).

It can be conjectured that HFE gene mutations may facilitate the progression towards cirrhosis that usually precedes the development of HCC, instead of exerting a direct oncogenic effect. In a previous study we detected no differences in the histological severity of chronic hepatitis C as related to HFE genotype (22), although Geier et al. (68) found a greater involvement in patients carrying any one of these two mutations. With respect to alcoholic liver disease, we have detected a greater prevalence of the H63D mutation in subjects with cirrhosis of this origin when compared to the general population (23).

Our study, based on a wide series of cases, suggests that the possession of an H63D mutation is a risk factor for the development of HCC in subjects with predisposing factors, mainly liver cirrhosis due to chronic HCV infection and/or ethanol abuse and chronic HBV-infection.
More studies are needed on the function of the HFE gene and the repercussions that the H63D mutation can have on it to clarify the pathophysiological basis for this association.

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REFERENCES


