Genetic Polymorphisms in Antioxidant Enzymes Modulate Hepatic Iron Accumulation and Hepatocellular Carcinoma Development in Patients with Alcohol-Induced Cirrhosis

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Abstract

Manganese superoxide dismutase (MnSOD) converts the superoxide anion into H2O2 which, unless it is detoxified by glutathione peroxidase 1 (GPx1), can increase hepatic iron and can react with iron to form genotoxic compounds. We investigated the role of Ala/Val-MnSOD and Pro/Leu-GPx1 polymorphisms on hepatic iron accumulation and hepatocellular carcinoma development in patients with alcoholic cirrhosis. Genotypes were determined in 162 alcoholic patients with cirrhosis but without hepatocellular carcinoma initially, who were prospectively followed up for hepatocellular carcinoma development. Genotypes were determined in 162 alcoholic patients with cirrhosis but without hepatocellular carcinoma initially, who were prospectively followed up for hepatocellular carcinoma development. We found that patients with two Val-MnSOD alleles (slow H2O2 production) and two Pro-GPx1 alleles (presumably quick H2O2 detoxification) had a lower risk of hepatocellular carcinoma development than other patients (χ2 trend test, P = 0.001; log-rank, P = 0.0009). Indeed, hepatocellular carcinoma percentage was 0% in subjects with this “2Val-MnSOD/2Pro-GPx1” genotype versus 16%, 27%, and 32% in “2Val-MnSOD/1or2Leu-GPx1,” “1or2Ala-MnSOD/2Pro-GPx1,” and “1or2Ala-MnSOD/1or2Leu-GPx1” patients, respectively. The percentage of patients with stable iron accumulation progressively increased with these genotypic associations: 22%, 28%, 50%, and 53%, respectively (χ2 trend test, P = 0.005). Stable iron was a risk factor for hepatocellular carcinoma (log-rank, P = 0.0002; relative risk, 3.40). In conclusion, polymorphisms in antioxidant enzymes modulate hepatic iron accumulation and hepatocellular carcinoma development in French alcoholic patients with cirrhosis. (Cancer Res 2006; 66(5): 2844-52)

Introduction

Hepatocellular carcinoma is the fifth cause of cancer worldwide and the third cause of cancer-related deaths (1). Its incidence is rising in Western countries due to the recent hepatitis C epidemics (1). However, alcohol abuse still remains the prevailing cause of hepatocellular carcinoma in some countries, including France (1).

Alcohol intoxication increases reactive oxygen species (ROS) formation in several cell compartments, including mitochondria (2, 3), causing oxidative stress and mitochondrial damage (4, 5). ROS and ROS-induced cytokines (6) lead to the apoptosis of some hepatocytes (7–9) followed by inflammation, fibrogenesis (10), and carcinogenesis (11).

Mitochondrial ROS are detoxified by the successive action of manganese superoxide dismutase (MnSOD) and glutathione peroxidase 1 (GPx1; ref. 12). MnSOD dismutates the superoxide anion into H2O2, which GPx1 detoxifies into water (12). GPx1 is the main glutathione peroxidase in the mammalian liver (13, 14). It is encoded by nuclear DNA, with an internal, incomplete mitochondrial targeting sequence, whose optional use targets GPx1 to either mitochondria or cytosol (13, 14). The active site of GPx1 contains a selenocysteine encoded by an UGA codon (15). Albeit usually a stop codon, UGA encodes for selenocysteine in GPx1, thanks to the formation of a quaternary structure involving the ribosome, a stem-loop structure in the 3′-untranslated region of the GPx1 mRNA, an RNA-binding protein, and selenocysteinyl-tRNAsec (15). Insufficient selenium concentrations impair GPx1 mRNA stability, mRNA translation, and GPx1 activity (16). A genetic polymorphism encodes for either proline (Pro) or leucine (Leu) at codon 198 of human GPx1 (reference SNP cluster identifier number: 1050450; refs. 17, 18). Although basal GPx1 activities were similar in breast carcinoma cells transfected with either Leu-GPx1 or Pro-GPx1 variants, selenium at concentrations anticipated in the human serum increased the activity of the Pro-GPx1 variant more than the Leu-GPx1 variant (19). The less active Leu-GPx1 variant was associated with lung (17), prostate (18), and breast (19) cancer. Furthermore, loss of heterozygosity affecting GPx1 can occur in the tumors of patients with breast cancer (19) or cancer of head and neck (20).

MnSOD is encoded by nuclear DNA and is inducible by ROS, cytokines, and ethanol (21–23). MnSOD is synthesized with a mitochondrial targeting sequence, which drives its mitochondrial import (24). In the matrix, the targeting sequence is cleaved, and the mature protein assembles into the active tetramer (24). A genetic polymorphism incorporates either alanine (Ala) or valine (Val) in the targeting sequence of MnSOD (reference SNP cluster identifier number: 1799725; ref. 24). The Ala-MnSOD variant, whose presequence has an α-helix structure, is easily imported and achieves high mitochondrial activity, whereas the Val-MnSOD presequence has an α-helical conformation in several cell compartments, including mitochondria.
We previously reported that French alcoholics with two Val-MnSOD alleles were protected against the development of cirrhosis (26) and then hepatocellular carcinoma and death (27) compared with patients with one or two copies of the Ala-MnSOD–encoding allele.

A limitation of these previous studies was that a possible comodulatory role of the GPx1 polymorphism was not investigated, and the possible role of iron was not assessed (26, 27). Not only MnSOD activity but also GPx1 activity could modulate the intracellular levels of H$_2$O$_2$, which could itself modulate hepatic iron accumulation and hepatocellular carcinoma development. Indeed, hydrogen peroxide reacts with Fe$^{2+}$ to form the hydroxyl radical, which damages DNA (28). Furthermore, H$_2$O$_2$-mediated signaling activates cytosolic iron-responsive protein 1 (IRP1), which binds to an iron-responsive element in the promoter of the transferrin receptor gene, to increase its transcription and hepatic iron uptake (29, 30). Increased iron stores could then further increase hydroxyl radical formation in the presence of high H$_2$O$_2$ levels, thus further increasing DNA damage, and the probability of cancer. Indeed, hepatic iron is an important predictor of the risk of death in patients with alcoholic cirrhosis (31), who can develop mild hepatic iron overload. More severe iron overload can occur in hereditary hemochromatosis (31), which is due to cysteine (Cys) to tyrosine (Tyr), or histidine (His) to aspartate (Asp) mutations in the HFE gene (32, 33). About 80% of hereditary hemochromatosis patients are homozygous for the Cys$^{282}$Tyr HFE mutation (32, 33). Patients with both one Cys$^{282}$Tyr allele and one His$^{63}$Asp allele may also occasionally develop iron overload, although most have normal hepatic iron stores. The major Cys$^{282}$Tyr mutation prevents the formation of a disulfide bond in the HFE protein, thus decreasing its association with $\beta_2$-microglobulin and its cell surface expression (34). This may prevent the association of the HFE protein with the transferrin receptor in duodenal crypt cells, which may trigger a compensatory increase in iron absorption by villus cells (35).

In the present study, we looked for a combined role of the Ala/Val-MnSOD and Pro/Leu-GPx1 genetic polymorphisms on hepatic iron accumulation and hepatocellular carcinoma development in a large series of French patients with alcoholic cirrhosis. Results suggest that patients with two low-activity MnSOD alleles and two high-activity GPx1 alleles are protected from hepatocellular carcinoma development. Patients with two low-activity MnSOD alleles, but either none, or only one, high-activity GPx1 allele have an intermediate risk of hepatocellular carcinoma. Finally, patients with either one or two high-activity MnSOD allele(s) frequently develop hepatic iron accumulation and have a high risk of hepatocellular carcinoma, whatever their GPx1 status.

Patients and Methods

Patients. The present study was part of an ongoing prospective study, to prospectively assess the rates of hepatocellular carcinoma development in diverse liver diseases (27).

In the present study, we compiled all new patients who were consecutively admitted between January 1, 1981 and December 31, 2001 and who fulfilled the following inclusion criteria: (a) biopsy-proven hepatic cirrhosis; (b) daily alcohol intake of $>$80 g (c) no other cause of liver disease and no infection by the HIV, hepatitis C, or hepatitis B virus; (d) no evidence of hepatocellular carcinoma at the time of inclusion, as judged by negative ultrasonographic findings and serum $\alpha$-fetoprotein (AFP) $<$50 ng/mL; (e) residence in France and Caucasian origin; (f) availability of either a frozen liver sample or a blood sample to prepare DNA; and (g) acceptance of a regular follow-up for the detection of hepatocellular carcinoma. A total of 162 patients met all these inclusion criteria and had a known outcome as of August 31, 2004.

For each patient, the date of inclusion was the date of the first liver biopsy showing cirrhosis. Gender, age, previous daily alcohol intake, presence of ascites or hepatic encephalopathy, serum bilirubin, albumin and prothrombin levels, serum alanine aminotransferase (ALT) activity, platelet count, blood iron, and ferritin levels were recorded at inclusion.

Patients were prospectively evaluated every 6 months by physical examination, ultrasonography, and AFP measurements (27). When these investigations suggested possible hepatocellular carcinoma, computed tomodensitometry and/or magnetic resonance imaging and/or a guided liver biopsy were done. Hepatocellular carcinoma was diagnosed on either one of the following criteria: histologic evidence or convergent demonstration of a focal lesion $>$2 cm in size and with arterial hypervascularization by two different imaging techniques, or the combination of one imaging technique showing this morphologic aspect with an AFP level of $>$400 ng/mL (27).

The two main end points of the study were the occurrence of hepatocellular carcinoma and the occurrence of liver transplantation or death. Follow-up ended at the date of death or liver transplantation, or at the last recorded visit (or information) within the last 6 months before August 31, 2004, which was set as the final time limit for upgrading the patients' file. Alcohol consumption was again recorded at end point.

DNA extraction, amplification, and MnSOD/GPx1/HFE genotyping.

DNA samples were prepared from blood or frozen liver samples. All patients gave written consent for blood sampling and genotyping. The use of left over liver specimens (no longer used for diagnostic purposes) for research purposes had been approved by the Comité Consultatif d'Éthique Médicale du Centre Hospitalier Bichat-Beaujon.

To determine the MnSOD genotype, DNA was amplified with primers 5'-CAGCCCGACCTGCTAGACGG-3' and 5'-CTTG-GCCAAAGCCTTCGTACTT-3' and genotyped by restriction analysis, as described (26, 27). In each experiment, DNA samples from patients, together with three previously sequenced DNA samples serving as quality controls (one of each genotype), were concomitantly amplified and digested with BsaWI (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France; refs. 26, 27). This restriction enzyme only cleaves the MnSOD amplification product, when a thymine is present at position 1183 of the MnSOD gene (i.e., in the valine-encoding allele), thus giving a 183-bp fragment and an 84-bp fragment. Lack of digestion, half digestion, or complete digestion was detected after migration on 3% agarose gel (2% Nusieve, 1% Seakem; Tebu, Le-Perray-en-Yvelines, France), allowing clear distinction of Ala/Val, Ala/Val, and Val/Val patients (26, 27).

To determine the GPx1 genotype, DNA was amplified with primers 5'-TTGTCCTCAGCCAGTACA-3' and 5'-CCCCCGAGACACGACGA-3' (17). In each experiment, DNA samples from the patients, together with three previously sequenced DNA samples serving as quality controls (one of each genotype), were concomitantly amplified and digested with ApaI (Invitrogen, Cergy-Pontoise, France). This restriction enzyme only cleaves the GPx1 amplification product, when a cytosine is present at position 593 of the GPx1 gene (i.e., in the proline-encoding allele), thus giving an 81-bp fragment and a 66-bp fragment. Lack of digestion, half digestion, or complete digestion was detected after migration on 3% agarose gel (4% Nusieve), allowing clear distinction of Ala/Ala, Ala/Val, and Val/Val patients (26, 27).

To determine the HFE genotype, DNA was amplified with primers 5'-GCAAAGCCTTCGTACTT-3' and genotyped by restriction analysis, as described (26, 27). In each experiment, DNA samples from patients, together with three previously sequenced DNA samples serving as quality controls (one of each genotype), were concomitantly amplified and digested with RsaWI (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France; refs. 26, 27). This restriction enzyme only cleaves the MnSOD amplification product, when a thymine is present at position 1183 of the MnSOD gene (i.e., in the valine-encoding allele), thus giving a 183-bp fragment and an 84-bp fragment. Lack of digestion, half digestion, or complete digestion was detected after migration on 3% agarose gel (2% Nusieve, 1% Seakem; Tebu, Le-Perray-en-Yvelines, France), allowing clear distinction of Ala/Ala, Ala/Val, and Val/Val patients (26, 27).

Histologic assessment of hepatic iron. Hepatic iron was assessed on the initial liver biopsy showing cirrhosis. Liver specimens were fixed in Bouin’s fluid and routinely processed. Hepatic sections (4-μm thick) were

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stained with the Perls’ stain and examined by two observers unaware of genotypic and clinical data. To semiquantitatively evaluate hepatic iron deposits, we used the Deugnier’s histologic hepatic iron score (37), adapted to cirrhotic samples (31).

**Statistical analysis.** Qualitative variables were compared using the Fischer exact \( \chi^2 \) test or \( \chi^2 \) trend test with 1 degree of freedom, whereas quantitative variables were compared using the nonparametric Wilcoxon test. Multivariate analysis (ANOVA) was also conducted to compare more than two means. The Kaplan-Meier method was used to estimate death and the occurrence of hepatocellular carcinoma for each variable noted at enrollment, and the distribution of death and hepatocellular carcinoma were compared with the log-rank test (38). A significant level below 0.10 was used to select the variables in the Cox’s proportional hazards model (39), using a stepwise backward procedure with a threshold of \( \alpha = 0.05 \). Variables (iron score, age, and sex) associated with risks of death or hepatocellular carcinoma based on knowledge and findings from previous studies were also selected. Statistical analysis used the SAS System Package version 8.02 (SAS Institute, Cary, NC). All reported \( P \)s are two tailed. Associations were first considered statistically significant at a two-tailed \( \alpha = 0.05 \). Bonferroni adjustment was also applied to correct for the number of primary outcomes tested (i.e., for 10 primary outcomes, \( \alpha = 0.005 \)).

**Results**

**Characteristics of patients classified according to the Ala/Val-MnSOD genotype, the Pro/Leu-GPx1 genotype, or combined MnSOD/GPx1 genotypes.** The MnSOD and GPx1 genotypic distributions in these patients did not differ from the Hardy-Weinberg equilibrium expectations. Table 1 displays the initial presentation and the final outcome in 162 patients with alcoholic cirrhosis classified according to the MnSOD genotypes. Except for a lower prevalence of stainable hepatic iron in patients with two Val MnSOD alleles, the initial presentation and the persistence of alcohol consumption at end point did not differ among the three MnSOD genotypes (Table 1). However, as previously reported (27), hepatocellular carcinoma and death occurred less frequently in patients with two copies of the Val-MnSOD allele (Table 1). In patients with at least one Ala-MnSOD allele (compared with patients with two Val-MnSOD alleles), the hazard ratio (HR) of hepatocellular carcinoma development was 11.494, and the confidence interval (95% CI) was 2.659 to 50.000 (\( P = 0.001 \)). The HR for death in patients with at least one Ala-MnSOD allele was 11.235, and the 95% CI was 2.710 to 47.619 (\( P = 0.0009 \)). Table 2 displays the same entries in patients classified according to the GPx1 genotypes. Except for a higher plasma ferritin level in patients with two Pro-GPx1 alleles, the clinical presentation and outcome did not differ with the GPx1 genotypes. Because there were only 10 patients with two Leu-GPx1 alleles, and because the mortality rate and the prevalence of hepatocellular carcinoma at end point were similar in patients with either one or two Leu-GPx1 alleles (Table 2), these two GPx1 genotypes were combined into a single group for the combined MnSOD/GPx1 genotypic classification.

In keeping with the putative role of hydrogen peroxide in hepatocellular carcinoma development as suggested by our previous study (27), we finally classified the 162 patients into four groups according to their combined MnSOD and GPx1 genotypes (Table 3). The first group comprises patients with two alleles for the low-

| Table 1. Initial presentation at inclusion, persistence of alcohol consumption, and outcome in 162 patients with alcoholic cirrhosis classified according to the MnSOD genotypes |
|---------------------------------|---------------------------------|---------------------------------|--------|
| **Group 1: 2 Val-MnSOD** (n = 40; 25%) | **Group 2: 1 Val-MnSOD, 1 Ala-MnSOD** (n = 83; 51%) | **Group 3: 2 Ala-MnSOD** (n = 39; 24%) | **P** |
| Age (y)* | 52.2 ± 9.1 | 53.7 ± 9.4 | 53.7 ± 10.9 | 0.702 |
| Male gender † | 31 (77.5) | 56 (67.5) | 28 (71.8) | 0.571 |
| Alcohol consumption (g/d)* | 116 ± 31 | 114 ± 32 | 105 ± 28 | 0.212 |
| ALT (× upper limit of normal)* | 1.4 ± 0.9 | 1.4 ± 0.8 | 1.3 ± 0.9 | 0.859 |
| AST (× upper limit of normal)* | 2.1 ± 1.2 | 2.5 ± 1.6 | 2.4 ± 1.7 | 0.421 |
| GGT (× upper limit of normal)* | 7.2 ± 10.0 | 8.0 ± 10.8 | 5.5 ± 5.2 | 0.414 |
| Albumin (g/L)* | 35.2 ± 7.2 | 34.7 ± 6.8 | 34.9 ± 7.4 | 0.924 |
| Prothrombin level (% control)* | 63 ± 20 | 59 ± 19 | 58 ± 17 | 0.449 |
| Bilirubin (μmol/L)* | 60.5 ± 98.3 | 67.5 ± 78.3 | 82.2 ± 105.0 | 0.547 |
| Platelets (10³/mm³)* | 153 ± 79 | 139 ± 57 | 150 ± 93 | 0.522 |
| Blood iron (μmol/L)* | 21 ± 8 | 20 ± 9 | 19 ± 9 | 0.620 |
| Ferritinemia (mg/L)* | 693 ± 1036 | 463 ± 621 | 387 ± 364 | 0.176 |
| Ascites † | 16 (40.0) | 30 (61.3) | 18 (46.1) | 0.583 |
| Encephalopathy † | 2 (5.0) | 2 (4.2) | 1 (2.6) | 0.530 |
| Child Pugh score* | 8.0 ± 2.8 | 8.0 ± 2.6 | 8.2 ± 2.4 | 0.932 |
| Stainable hepatic iron in initial liver biopsy | 10 (25) | 42 (50.6) | 21 (53.8) | 0.018 |
| Persisting alcohol consumption at end point † | 18 (45.0) | 43 (51.8) | 16 (41.0) | 0.732 |
| HCC development † | 3 (7.5) | 25 (30.1) | 12 (30.8) | 0.016 |
| Death † | 2 (5) | 31 (37.3) | 16 (41.0) | 0.001 |

NOTE: All biological and clinical parameters were recorded at inclusion. Alcohol consumption was recorded at inclusion and again at end point. Abbreviations: AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma.

*Mean ± SD.
†Number (percentage) of patients.
activity Val-MnSOD variant (25) and two alleles for the Pro-GPx1 variant, which is associated with high GPx1 activity in the presence of physiologic concentrations of selenium (19). These "2Val-MnSOD/2Pro-GPx1" subjects may slowly form H2O2 and quickly detoxify it and should therefore have low intracellular H2O2 concentrations. In contrast, patients with one or two copies of the high-activity Ala-MnSOD-encoding allele were combined into a single subgroup (the "1or2Ala-MnSOD" subgroup), because the Ala/GPx1 genotype was a risk factor for lung (17), prostate (18), and breast (19) cancer, and should therefore have low intracellular H2O2 concentrations.

During follow-up, 40 of 162 (24.6%) patients developed hepatocellular carcinoma. The incidence of hepatocellular carcinoma increased with the genotypic group (Table 3). This incidence was 0% (0 of 22) in group 1 ("2Val-MnSOD/2Pro-GPx1" patients), 16.6% (3 of 18) in group 2 ("2Val-MnSOD/1or2Leu-GPx1" patients), 27.5% (16 of 58) in group 3 ("1or2Ala-MnSOD/2Pro-GPx1" patients), and 32.8% (21 of 64) in group 4 ("1or2Ala-MnSOD/1or2Leu-GPx1" patients; \( \chi^2 \) trend test, \( P = 0.001 \)).

Using the Kaplan-Meier method (Fig. 1), the "1or2Ala-MnSOD" genotypes and "1or2Leu-GPx1" genotypes were associated with a higher cumulative incidence of hepatocellular carcinoma compared with patients in group 1 who did not develop hepatocellular carcinoma. In the three groups of patients who developed hepatocellular carcinoma, first quartiles up to hepatocellular carcinoma occurrence were 180 months in group 2, 60 months in group 3, and 57 months in group 4 (log-rank test, \( P = 0.0009 \)).

In multivariate analysis according to the Cox model, old age \( (P < 0.0001) \), male gender \( (P = 0.01) \), and group 4 of combined MnSOD/GPx1 genotypes (Table 3) were independent risk factors for hepatocellular carcinoma. Indeed, the HR for hepatocellular carcinoma was 2.0 in group 4 compared with patients of the three other groups, and the 95% CI was 1.31 to 3.052 (\( P = 0.001 \)).

### MnSOD/GPx1 Polymorphisms and hepatocellular carcinoma percentage

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### MnSOD/GPx1 polymorphisms and death/transplantation

During follow-up, 49 of 162 (30.2%) patients died, all of them from liver-related complications (due to advanced hepatocellular
carcinoma in 19 cases, or due to variceal bleeding and/or liver failure in other patients. Four patients underwent liver transplantation. The incidence of death or transplantation differed markedly according to the MnSOD genotype (Table 1), with no influence of the GPx1 genotype (Table 2). This incidence was 4.5% (1 of 22) in group 1 (“2Val-MnSOD/2Pro-GPx1” patients) and 5.5% (1 of 18) in group 2 (“2Val-MnSOD/1or2Leu-GPx1” patients), contrasting with incidences of 37.9% (22 of 58) in group 3 (“1or2Ala-MnSOD/2Pro-GPx1” patients) and 39% (25 of 64) in group 4 (“1or2Ala-MnSOD/1or2Leu-GPx1” patients; \(\chi^2\) test, \(P = 0.001\); Table 3). Similar conclusions were reached when the cumulative incidence of death/transplantation was analyzed with the Kaplan-Meier method (log-rank test, \(P = 0.0005\); Fig. 2).

Multivariate analysis according to Cox model including age, sex, and MnSOD/GPx1 polymorphisms was done. The MnSOD/GPx1
polymorphism associations were the only independent risk factor for death/transplantation in this cohort (HR, 1.808 in group 4 patients compared with other groups; 95% CI, 1.271-2.573; \( P = 0.0006 \)).

MnSOD/GPx1 polymorphisms and liver iron accumulation. Of the 162 patients, 73 (45%) had stainable liver iron. The proportion of patients with stainable iron differed with the MnSOD genotype (Table 1), with no influence of the GPx1 genotype (Tables 2 and 3). Whereas this proportion was 22% (5 of 22) and 28% (5 of 18) in groups 1 and 2, it was 50% (29 of 58) and 53% (34 of 64) in groups 3 and 4 (\( \chi^2 \) trend test, \( P = 0.003 \)).

In patients with stainable liver iron, the mean iron score increased mainly according to the MnSOD genotype. This score was 1.22 ± 2.50 in group 1 (median, 0; 25th percentile, 0; 75th percentile, 1), 0.66 ± 1.23 in group 2 (median, 0; 25th percentile, 0; 75th percentile, 1), 1.98 ± 2.81 in group 3 (median, 0.5; 25th percentile, 0; 75th percentile, 3), and 2.84 ± 3.45 in group 4 (median, 2; 25th percentile, 0; 75th percentile, 5; Wilcoxon test, \( P = 0.003 \)).

In multivariate analysis, old age (\( P = 0.002 \)), male gender (\( P = 0.003 \)), and group 4 of the combined MnSOD/GPx1 genotypes (\( P = 0.01 \)) were independent risk factors for hepatic iron accumulation according to ANOVA.

Liver iron accumulation and risk of hepatocellular carcinoma. The incidence of hepatocellular carcinoma was higher in patients with stainable liver iron than in those without iron. Indeed, 40% (29 of 73) of patients with stainable iron developed hepatocellular carcinoma versus only 11% (10 of 89) of patients without stainable iron (\( \chi^2 \) test, \( P = 0.001 \)). Stainable hepatic iron was a major risk factor for hepatocellular carcinoma (log-rank test, \( P = 0.0002 \)). In patients who developed hepatocellular carcinoma, first quartiles were 54 months in patients with stainable iron versus 180 months in patients without iron. The relative risk of developing hepatocellular carcinoma in patients with stainable liver iron was 3.40 (95% CI, 1.70-6.81; Fig. 3).

When hepatic iron overload was considered as a continuous variable, the relative risk for hepatocellular carcinoma per unit of hepatic iron score was 1.23 (95% CI, 1.13-1.34; \( P < 0.0001 \)). However, stepwise backward multivariate analysis showed that the hepatic iron score was not an independent risk factor for hepatocellular carcinoma occurrence, possibly due to its dependence on age, sex, and the combined MnSOD/GPx1 genotypes. In patients without stainable hepatic iron, Cox model, including age, sex, and genotypes, selected only age as a risk factor for hepatocellular carcinoma (HR, 1.094; 95% CI, 1.022-1.171; \( P = 0.010 \)), whereas genotype associations were not significantly associated with hepatocellular carcinoma risk (HR, 1.723; 95% CI, 0.856-3.469; \( P = 0.128 \)). In patients with stainable hepatic iron, Cox model, including age, sex, and genotypes, selected both age (HR, 1.058; 95% CI, 1.013-1.105; \( P = 0.011 \)) and genotype associations (HR, 1.889; 95% CI, 1.086-3.284; \( P = 0.024 \)) as risk factors for hepatocellular carcinoma.

Influence of MnSOD/GPx1 polymorphisms on death and hepatocellular carcinoma in patients without stainable liver iron. We also studied the influence of MnSOD/GPx1 polymorphisms on death and hepatocellular carcinoma in the subgroup of 89 patients without stainable liver iron. In this subgroup, the MnSOD polymorphism was still a risk factor for death/transplantation, which occurred in 0% (0 of 17) of patients in group 1 and 0% (0 of 13) of patients in group 2, but 41.3% (12 of 29) of patients in group 3, and 33.3% (10 of 30) of patients in group 4 (\( P = 0.0001 \)).

In this subgroup of 89 patients without stainable hepatic iron, the incidence of hepatocellular carcinoma was 0% (0 of 17) in group 1, 15.3% (2 of 13) in group 2, 13.7% (4 of 29) in group 3, and 16.6% (5 of 30) in group 4. However, these differences did not reach statistical significance (\( P = 0.2 \)) in this small subgroup of patients with a low overall rate of hepatocellular carcinoma development.

Lack of influence of HFE gene mutations on hepatic iron, hepatocellular carcinoma, and death. None of the 162 patients was homozygous for the Cys282Tyr mutation. Two patients were compound (Cys282Tyr and His63Asp) heterozygotes, and three were homozygous for the His63Asp mutation. Ten patients had a heterozygous Cys282Tyr mutation, and 36 patients had a heterozygous His63Asp mutation.

In this cohort, carrying or not the Cys282Tyr mutation and carrying or not the His63Asp mutation did not significantly influence the percentage of patients with stainable iron (30% versus 44.3%, \( P = 0.7 \); 47.2% versus 43.3%, \( P = 0.4 \), respectively), the incidence of hepatocellular carcinoma (40% versus 20.4%, \( P = 0.1 \); 33.3% versus 24.2%, \( P = 0.2 \)), and the incidence of death or transplantation (10% versus 30.9%, \( P = 0.1 \); 40.4% versus 31.2%, \( P = 0.3 \)).

Discussion

Previous studies have shown that French alcoholic patients with one or two copies of the high activity Ala-MnSOD allele have a higher risk of developing cirrhosis than patients with two copies of the low activity Val-MnSOD allele (26, 27). At the time of the first liver biopsy showing cirrhosis, the percentage of alcoholic cirrhotic patients with two Val-MnSOD alleles (16%) was less than in a control population (31%; ref. 27). However, the
mortality rate was lower in patients with two Val-MnSOD alleles than in other patients (27). As the time of follow-up increased, the proportion of surviving patients with two Val-MnSOD alleles increased (27). This may explain why the genotypic reparation of alcoholic patients differed from control subjects when patients were studied at the time of the first liver biopsy showing cirrhosis (26, 27), but not when blood samples were drawn at various times after diagnosis (40, 41). In the present study, only patients with a documented HFE genotype were included. Because the HFE genotype was mostly determined on blood samples drawn at various times after the first diagnosis of cirrhosis, rather than from the initial liver tissue, this resulted in a relative enrichment in patients with two Val-MnSOD alleles, who represented 25% of patients in the present study compared with 16% in our previous study (27).

Our previous study had also shown that patients with at least one Ala-MnSOD allele have a higher risk of hepatocellular carcinoma development than patients with two Val-MnSOD alleles (27). The present study extends these data by showing that patients with the Ala-MnSOD allele have more hepatic iron accumulation, which may contribute to their increased risk of hepatocellular carcinoma. This study also shows that the hepatocellular carcinoma risk is further modulated by the Pro/Leu-GPx1 genetic polymorphism in patients with two copies of the low-activity Val-MnSOD allele (Table 3).

In a previous study, transfection of an Ala-MnSOD–encoding vector led to a 4-fold higher MnSOD activity than the Val-MnSOD–encoding vector (25). Thus, subjects having one or two copies of the Ala-MnSOD allele may have a high basal MnSOD activity, which may be further increased by alcohol abuse, because MnSOD is inducible by ROS, cytokines, and ethanol (21–23). Conversely, the activity of GPx1 may be decreased in alcoholics: first, by the ethanol-induced decrease in mitochondrial glutathione (42) and second, by ROS-mediated GPx1 inactivation (43). Therefore, patients with one or two copies of the Ala-MnSOD allele may have a major imbalance between a high formation rate of $\text{H}_2\text{O}_2$ by MnSOD and a low detoxification rate of $\text{H}_2\text{O}_2$ by GPx1 (26, 27). These patients may have high intracellular $\text{H}_2\text{O}_2$ concentrations, whatever the more or less active GPx1 variant(s) present in these subjects. Although the influence of hydrogen peroxide on iron metabolism has been poorly evaluated in humans, several in vitro studies have highlighted this regulation. High $\text{H}_2\text{O}_2$ concentrations may have three consequences (Fig. 4, groups 3 and 4). First, $\text{H}_2\text{O}_2$-induced signaling activates cytosolic IRP1, which binds to an iron-responsive element in the promoter of the transferrin receptor gene, to increase the transcription of this receptor and hepatocellular iron uptake (29, 30). Indeed, the expression of the transferrin receptor and hepatic iron stores are increased in patients with alcoholic liver disease (44). Although hepatic iron is mainly deposited as ferric iron, the metabolism of ethanol increases the NADH/NAD$^+$ and NAPDH/NADP$^+$ ratios, thus partly reducing ferric iron into ferrous iron (3). A second consequence of high $\text{H}_2\text{O}_2$ concentrations, particularly in the presence of ferrous iron, is to form the hydroxyl radical, which damages DNA directly, and also indirectly, by forming reactive, lipid peroxidation products (28). A last consequence of high $\text{H}_2\text{O}_2$ levels and $\text{H}_2\text{O}_2$-induced cytokine expression is to trigger mitochondrial permeability transition and cell death in some hepatocytes (6–9). This apoptotic rate (8, 9) must be compensated by an adaptive increase in progenitor cell proliferation, to maintain liver cell mass (45). As somatic mutations accumulate over the years, and as there is a constant apoptotic pressure, cells may be selected that resist apoptosis and/or escape the control of the cell cycle, to finally develop a malignant clone (45).

Whereas patients with either one or two Ala-MnSOD alleles had iron overload and a high risk of hepatocellular carcinoma, whatever their GPx1 genotypes (Fig. 4, groups 3 and 4), in contrast, patients with two copies of the low activity–associated Val-MnSOD allele exhibited either a low, or an intermediate, hepatocellular carcinoma risk, depending on their GPx1 genotype (Table 3). In patients with two copies of the Pro-GPx1 allele, no hepatocellular carcinoma occurred. In a previous study, transfection of a Pro-GPx1–encoding vector in cells exposed to selenium resulted in a higher GPx1 activity than with the Leu-GPx1 vector (19). Thus, patients with two Val-MnSOD alleles and two Pro-GPx1 alleles may slowly form $\text{H}_2\text{O}_2$ and quickly detoxify it and may have very low intracellular concentrations of $\text{H}_2\text{O}_2$. These low levels may avoid hydroxyl radical formation, DNA damage, and hepatocellular carcinoma development (Fig. 4, group 1).

In contrast, patients with two Val-MnSOD alleles but one or two copies of the low activity–associated Leu-GPx1 allele (group 2) had an intermediate hepatocellular carcinoma risk (Table 3). Although these patients may slowly form $\text{H}_2\text{O}_2$, they may also slowly detoxify it. Accordingly, they could have an intermediate concentration of $\text{H}_2\text{O}_2$, resulting in moderate hydroxyl radical formation, and an intermediate hepatocellular carcinoma risk (Fig. 4, group 2). Although low $\text{H}_2\text{O}_2$ concentrations do not trigger cell death and
thus will not trigger compensatory cell proliferation, they may directly increase cell proliferation (46), which might contribute to the higher hepatocellular carcinoma risk in group 2 compared with group 1 patients (Fig. 4).

Although iron has been previously identified as a risk factor for death in cirrhotic patients (31), the role of hepatic iron accumulation in hepatocarcinogenesis is difficult to assess, as iron overload is dependent on male gender (no menstruation) and age (progressive accumulation), which are by themselves the most determinant risk factors for hepatocellular carcinoma development. Our study indicates that not only old age and male gender but also group 4 of the combined MnSOD/GPx1 genotypes are independent risk factors for hepatic iron accumulation (histologic score) according to multiple regression analysis. As discussed above, patients of group 4 may have high intracellular H₂O₂ concentrations, which may induce the transferrin receptor and increase hepatic iron uptake (Fig. 4). However, there was no relationship between histologic iron accumulation, which tended to increase from genotypic groups 1 and 2 to genotypic groups 3 and 4, and ferritinemia, which was not significantly different in the four genotypic groups (Table 3). Further studies are needed to better understand how ferritinemia may be regulated under conditions associating both increased cell iron, which would tend to increase ferritin synthesis, and H₂O₂-mediated activation of cytosolic IRP1, which would tend to decrease ferritin synthesis.

It is difficult to discriminate the respective role of cellular iron accumulation versus a direct effect of anti-oxidant enzymes on hepatocellular carcinoma development. Stepwise backward multivariate analysis showed that the hepatic iron score was not an independent risk factor for hepatocellular carcinoma occurrence, possibly due to its strong dependence on old age, male sex, and the MnSOD/GPx1 genotype. However, although hepatic iron is dependent on other factors, it may still play an important pathogenic role in hepatocellular carcinoma development by increasing the formation of the DNA-damaging hydroxyl radical from H₂O₂ (Fig. 4). Indeed, the incidence of hepatocellular carcinoma was higher in patients with stainable liver iron (40%) than those without iron (11%), and the relative risk of developing hepatocellular carcinoma was 3.40 (95% CI, 1.70-6.81) in patients with stainable iron compared with patients without iron (Fig. 3). Nevertheless, a few patients without stainable iron still developed hepatocellular carcinoma. Taken together, these observations may suggest that the antioxidant genotypes may modulate hepatocellular carcinoma development both by modulating hepatic iron stores and also by directly modulating the formation of the hydroxyl radical as a direct consequence of changes in intracellular H₂O₂ concentrations (Fig. 4).

Because our findings could help predict the risk of hepatocellular carcinoma, which might modulate hepatocellular carcinoma screening programs, it will be important to repeat this study in large groups of patients in France and other countries. Although dietary antioxidant intake may modulate oxidative stress and the effects of antioxidant genotypes (47), this information was not available in the present cohort. In future studies, it will be useful to record information on diet, calorie intake, antioxidant intake, the type of alcohol beverages (wine, beer, or spirits), and the drinking pattern (steady drinking versus binges; ref. 27). Patients should be included at the time of first diagnosis, and blood samples for genotyping should be obtained at that time, before a prospective follow-up (27).

In summary, we suggest that genetic polymorphisms affecting MnSOD and GPx1 may modulate the risk of hepatic iron accumulation and hepatocellular carcinoma development in French alcoholic patients with cirrhosis. If confirmed by other studies, these findings could improve the assessment of hepatocellular carcinoma risk and clinical management in these patients.

Acknowledgments

Received 7/21/2005; revised 12/16/2005; accepted 12/30/2005.

Grant support: Institut de Recherche et d’Etude sur les Boissons, Société Nationale Française de Gastroentérologie, and Université Paris XIII.

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