Genetic Dimorphism in Superoxide Dismutase and Susceptibility to Alcoholic Cirrhosis, Hepatocellular Carcinoma, and Death

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Background & Aims: A genetic dimorphism encodes for either alanine (Ala) or valine (Val) in the mitochondrial targeting sequence of manganese superoxide dismutase (MnSOD), and modulates its mitochondrial import. However, the role of this dimorphism in the susceptibility of alcoholic patients to develop cirrhosis is controversial, and its influence on the occurrence of hepatocellular carcinoma (HCC) and death in patients with alcoholic cirrhosis is unknown. Methods: We compared MnSOD genotypes in 94 control subjects and 264 patients with alcoholic cirrhosis. Patients were included at the time of the first liver biopsy examination showing cirrhosis, and were followed-up prospectively. Results: Alcohol consumption was similar, whatever the patients’ genotype. At inclusion, the percentage of Val/Val homozygotes was lower in patients than in controls (16% vs. 31%), whereas the percentage of Ala/Ala homozygotes was higher in patients than in controls (30% vs. 21%) (P = .008). During follow-up evaluation, only 9% of Val/Val patients developed HCC, vs. 30% and 29% of Ala/Val and Ala/Ala patients, respectively (P = .02). Only 28% of Val/Val patients died or were transplanted, vs. 49% and 50% of Ala/Val and Ala/Ala patients, respectively (P = .03). Because of the progressive decrease in surviving Ala patients, the genotypic distribution in patients surviving for 5 or 10 years no longer differed from the genotypic distribution in controls. Conclusions: The presence of at least 1 Ala MnSOD allele increases the risk for developing cirrhosis in French alcoholics, and increases the rates of HCC development and death in cirrhotic patients.

Excessive alcohol consumption is a major cause of cirrhosis, hepatocellular carcinoma (HCC), and death. 1 However, there is wide variability in susceptibility to alcoholic cirrhosis and its outcome. Despite similar alcohol consumption, many alcohol abusers only have a fatty liver, whereas others develop cirrhosis. Of those who have progressed to cirrhosis, some patients survive for many years, whereas others soon develop HCC and/or die from various complications of cirrhosis.

Although twin concordance studies suggest a role of genetic predisposition in susceptibility to alcoholic cirrhosis, 2 we still barely know which genetic polymorphisms actually are involved. 3 Furthermore, we do not know which genetic traits, if any, might speed up HCC development and/or death in some patients.

Alcohol consumption increases reactive oxygen species (ROS) formation in mitochondria 4 and several other cell compartments, thus causing oxidative stress and mitochondrial damage. 5–7 The increase in ROS and cytokines leads to cell death, fibrogenesis, and carcinogenesis. 8,9 Mitochondrial ROS are detoxified by 2 mitochondrial enzymes, namely manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPX). 10 MnSOD is encoded by nuclear DNA and is synthesized with a mitochondrial targeting sequence, which allows its import into the mitochondrial matrix. 11 The targeting sequence then is cleaved, and the mature protein assembles into the active tetramer. 12

A genetic dimorphism affects the mitochondrial targeting sequence of MnSOD. 13 The presence of either a cytosine or a thymine at position 1183 of the MnSOD gene results in either a guanine-cytosine-thymine codon or a guanine-thymine-thymine codon, leading to the incorporation of either alanine (Ala) or valine (Val) at the mitochondrial targeting sequence. This results in the production of either the Ala or Val MnSOD allele. 14

Abbreviations used in this paper: Ala, alanine; CI, confidence interval; GPX, glutathione peroxidase; HCC, hepatocellular carcinoma; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; Val, valine.

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position −9 of the MnSOD mitochondrial targeting sequence. The genetic dimorphism therefore is referred to as the “Ala −9Val” MnSOD dimorphism. The Ala-encoding allele and the Val-encoding allele are about equally distributed in Caucasian populations. Because the human MnSOD gene is located on chromosome 6, and each of the 2 chromosomes carry either the Ala or the Val allele, subjects can be Ala/Ala homozygotes, Ala/Val heterozygotes, or Val/Val homozygotes. The Ala −9Val genetic dimorphism affects the secondary structure of the mitochondrial targeting sequence and modulates the mitochondrial import of MnSOD.

The role of the Ala −9Val MnSOD dimorphism in susceptibility to alcoholic liver disease has been studied in French and English alcoholics. In the French study, the frequencies of the Ala −9Val MnSOD genotypes were compared in 79 control subjects and 71 patients with diverse alcoholic liver lesions, including 13 patients with cirrhosis. The frequency of Val/Val homozygotes was found to be lower in the group of patients with alcoholic liver disease than in controls (17% vs. 28%), whereas the frequency of Ala/Ala homozygotes was higher (44% vs. 19%). The daily alcohol consumption was similar in Val/Val, Ala/Val, and Ala/Ala alcoholics, suggesting that the Ala allele increases the risk for developing severe liver disease in alcoholics. These findings, however, were not confirmed in 2 subsequent English studies, which, collectively, found no differences in the MnSOD genotypic distributions of control subjects and patients with fibrosis/cirrhosis of the liver.

The 3 aims of the present study were as follows: (1) to check the results of Degoul et al. by comparing the distribution of MnSOD genotypes in control subjects and an entirely new, and much larger, cohort of French alcoholic patients included at the time of first histologic diagnosis of cirrhosis; (2) to determine prospectively the influence of the MnSOD genotype on the occurrence of HCC and death in these patients; and (3) to compare again the genotypic distribution of control subjects with that of cirrhotic patients having survived for either 5 or 10 years.

**Patients and Methods**

**Patients**

The present study was part of a large prospective study at the Jean Verdier Hospital to assess prospectively the rates of HCC development in the course of diverse liver diseases.

We included all new patients who were admitted consecutively between January 1, 1981, and December 31, 1999, and who fulfilled the following inclusion criteria: (1) biopsy-proven hepatic cirrhosis; (2) daily alcohol intake of 80 g or more; (3) no other cause of liver disease and no infection by the human immunodeficiency virus, hepatitis C virus, or hepatitis B virus; (4) no evidence of HCC at the time of inclusion, as judged by negative ultrasonographic findings and serum α-fetoprotein level less than 50 ng/mL; (5) residence in France and Caucasian origin; (6) availability of either a frozen liver sample or a blood sample to prepare DNA for MnSOD genotyping; and (7) acceptance of a regular follow-up evaluation for the detection of HCC. A total of 264 patients met all these inclusion criteria with a known final outcome as of December 31, 2002.

At the date of inclusion, which was the date of the first liver biopsy examination showing cirrhosis, sex, age, previous daily alcohol intake, presence of ascites or hepatic encephalopathy, serum bilirubin, albumin, prothrombin, serum alanine transaminase levels, and platelet counts were recorded.

HCC screening was conducted every 6 months by physical examination, ultrasonography, and α-fetoprotein measurements. When these investigations suggested possible HCC, computed tomography and/or magnetic resonance imaging and/or a guided liver biopsy examination were performed. HCC was diagnosed on histologic evidence, or convergent demonstration of a focal lesion more than 2 cm in size and with arterial hypervascularization by 2 different imaging techniques, or the combination of 1 imaging technique showing focal lesions >2 cm with an α-fetoprotein level of 400 ng/mL or more.

The 2 main end points of the study were the occurrence of HCC, and the occurrence of liver transplantation or death, whatever the latter’s cause. However, we also looked for the cause of death to differentiate between liver-related deaths (caused by liver failure, bleeding, and/or HCC) and nonrelated deaths.

The follow-up period ended at the date of death or liver transplantation, or at the last recorded visit (or information) within the last 6 months before December 31, 2002, which was set as the final time limit for evaluating the patients’ outcome. Alcohol consumption again was recorded at end point.

**Control Subjects**

To assess the prevalence of the 3 MnSOD genotypes in the general population, we used a control group of 94 Caucasian blood donors living in the same geographic area.

**DNA Extraction, Amplification, and Manganese Superoxide Dismutase Genotyping**

DNA samples were prepared from blood or frozen liver samples. Patients gave written consent for blood sampling and MnSOD genotyping. The use of leftover 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. Polymerase chain amplification was performed using primers P1, 5’-CAGCCCGAGCCTTGGTACGACGG-3’, and P2, 5’-CTTGGCAACGGCCTCCTGGTACTTT-3’, and genotyping was performed by restriction analysis, as previously described. In each ex-
per experiment, DNA samples from the patients, together with previously sequenced DNA samples serving as quality controls, were concomitantly amplified and digested with BsaW1 (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France). This restriction enzyme only cleaves the MnSOD amplification product when a thymine is present at position 1183 of the MnSOD gene (ie, in the Val-encoding allele), thus giving a 183-base pair fragment lacking 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 heterozygotes and 29% of Ala/Ala homozygotes.18

Statistical Analysis

Qualitative variables were compared using the Fischer exact $\chi^2$ test, whereas quantitative variables were compared using the nonparametric Wilcoxon test.

The Kaplan–Meier method was used to estimate death and the occurrence of HCC for each parameter noted at enrollment, and the distribution of death and HCC were compared with the log-rank test. A significance level less than .10 was used to select the variables in the Cox proportional hazards model,22 using a stepwise backward procedure with a threshold of .05. Statistical analysis used the SAS System Package, version 8.02 (SAS Institute, Cary, NC). All reported $P$ values are 2-tailed.

Results

Characteristics of Patients According to Their Ala –9Val MnSOD Superoxide Dismutase Genotype

The 264 patients with alcoholic cirrhosis were classified according to their MnSOD genotype (Table 1). The demographic, biological, and clinical features at inclusion did not differ significantly between the 3 MnSOD genotypes (Table 1). Age, sex, daily alcohol intake at inclusion, as well as serum alanine transaminase, albumin, and prothrombin levels were similar in Val/Val, Ala/Val, and Ala/Ala patients (Table 1). Although slight trends were noticeable toward increasing bilirubin levels (49, 61, and 80 $\mu$mol/L), decreasing platelets counts (151, 141, and 133 × 10⁹/mm³), increasing prevalences of ascites (30.2%, 42.9%, and 40.5%), and increasing prevalences of encephalopathy (4.6%, 6.3%, and 8.8%) from Val/Val patients to Ala/Val and Ala/Ala patients, none of these trends was statistically significant (Table 1). Furthermore, there was no difference between the 3 groups when these criteria were combined according to Child–Pugh score (Table 1).

The prevalence of persisting alcohol consumption at end point (60.4%, 47.8%, and 51.9%) also did not differ significantly between Val/Val, Ala/Val, and Ala/Ala patients (Table 1).

Manganese Superoxide Dismutase Genotype and Hepatocellular Carcinoma Development

Although inclusion criteria required the absence of detectable HCC at the time of inclusion, 69 of the 264 cirrhotic patients (26%) developed HCC during the follow-up period. The cumulative incidence of HCC was lower in Val/Val homozygotes than in patients with other MnSOD genotypes ($\chi^2$ test, $P = .02$). Indeed, only 9% of Val/Val homozygotes developed HCC vs. 30% of Ala/Val heterozygotes and 29% of Ala/Ala homozygotes (Figure 1).

Using the Kaplan–Meier method, the Ala –9Val MnSOD genotype was predictive of HCC development,
with a first quartile of 180 months in Val/Val homozygotes, and a median time to occurrence of 153 months in Ala/Val heterozygotes and 139 months in Ala/Ala homozygotes (log-rank test, $P = .009$) (Figure 2). The predictive value of Ala $\rightarrow$Val MnSOD genotype for HCC development was confirmed by a Cox model ($P = .02$).

Because time to occurrence of HCC was not statistically different in Ala/Val and Ala/Ala patients, we combined these 2 groups to compare their risk for developing HCC with that of Val/Val homozygotes. The relative risk for developing HCC in patients with at least 1 Ala allele (ie, either Ala/Val or Ala/Ala patients) was 4.59, with a 95% confidence interval (CI) of 1.61–13.06 in Cox multivariate analysis including age and sex. The Ala $\rightarrow$Val MnSOD genotype was an independent predictive factor of HCC.

**Manganese Superoxide Dismutase Genotype and Death/Transplantation**

During the follow-up period, 122 of the 264 patients (46.2%) died or underwent liver transplantation (14 patients). Only 1 death was owing to an extrahepatic cause (suicide). In the other patients, death was attributable to the liver disease, to advanced HCC in 41 patients, or to variceal bleeding and/or liver failure in the other patients.

The cumulative incidence of death or transplantation during the follow-up period was lower in Val/Val homozygotes than in patients with other MnSOD genotypes ($\chi^2$ test, $P = .03$); only 28% of Val/Val homozygotes died or underwent transplantation, vs. 49% of Ala/Val heterozygotes and 50% of Ala/Ala homozygotes (Figure 3).

By using the Kaplan–Meier method, the Ala $\rightarrow$Val MnSOD genotype was predictive of death, with a median survival of 249 months in Val/Val homozygotes, 81 months in Ala/Val heterozygotes, and 82 months in Ala/Ala homozygotes (log-rank test, $P = .008$) (Figure 4). The predictive value of Ala $\rightarrow$Val MnSOD genotype on death/transplantation was confirmed by a Cox model ($P = .01$).

Because the survival distribution function was similar in Ala/Val and Ala/Ala patients, we combined these 2
groups to compare their survival with the survival of Val/Val homozygotes. The relative risk for death/transplantation in patients with at least 1 Ala allele was 2.49 (95% CI, 1.36–4.57) in Cox multivariate analysis including age and sex. The Ala−9Val MnSOD genotype was an independent predictive factor of death/transplantation.

**Comparison of the Distribution of Manganese Superoxide Dismutase Genotypes in Control Subjects and in Patients**

The distribution of Ala−9Val MnSOD genotypes in the control group of blood donors was similar to published data in Caucasians.14,15 The allelic distribution was 45% for the Ala allele and 55% for the Val allele, leading to a genotype distribution of 31% for Val/Val homozygotes, 48% for Ala/Val heterozygotes, and 21% for Ala/Ala homozygotes (Figure 5).

At inclusion, namely the date of the first liver biopsy examination showing cirrhosis, cirrhotic patients had a significantly different repartition of the MnSOD genotypes compared with control subjects ($\chi^2$ test, $P = .008$). In cirrhotic patients, the frequency of Val/Val homozygotes was 48% lower than in control subjects (16% in patients vs 31% in controls), the percentage of Ala/Val heterozygotes was 12.5% higher (54% in patients vs. 48% in controls), and the percentage of Ala/Ala homozygotes was 43% higher (30% in patients vs 21% in controls) (Figure 5). Compared with the risk for Val/Val patients, the relative risk for developing cirrhosis in patients with at least 1 Ala allele (either Ala/Val or Ala/Ala patients) was 4.59 (95% CI, 1.61–13.06), the relative risk for Ala/Val patients was 4.68 (95% CI, 1.62–13.58), and the relative risk for Ala/Ala patients was 4.45 (95% CI, 1.49–13.28) in Cox multivariate analysis including age and sex.

However, the lower survival of patients with 1 or 2 Ala alleles (Figure 4) depleted this allele in survivors. The distribution of MnSOD genotypes in patients surviving without transplantation for 5 or 10 years came back to the distribution of control subjects and no longer differed significantly from the control distribution (Figure 5).

**Discussion**

A role of the Ala−9Val MnSOD dimorphism in susceptibility to severe alcoholic liver disease has been reported in French alcoholics,18 but was not confirmed in 2 English studies.19,20 A first aim of the present study was to assess again the Ala−9Val MnSOD genotypes in a larger and entirely new cohort of French alcoholic patients who were followed-up prospectively every 6 months as part of the HCC screening program of the Jean Verdier Hospital. We found that the distribution of the MnSOD genotypes in 264 alcoholic patients included at the time of the first liver biopsy examination showing cirrhosis differed significantly ($P = .008$) from the genotypic distribution of control subjects. The frequency of Val/Val homozygotes was 48% lower in patients than in controls (16% vs. 31%), whereas the frequency of Ala/Ala homozygotes was 43% higher (30% vs. 21%) (Figure 5). Compared with the lower risk for Val/Val patients, the relative risk for developing cirrhosis in patients with at least 1 Ala allele (either Ala/Val or Ala/Ala patients) was 4.59 (95% CI, 1.61–13.06). Because alcohol consumption was similar in Val/Val, Ala/Val, and Ala/Ala alcoholics (Table 1), it can be concluded that the Ala−9Val MnSOD polymorphism does not act on alcohol intake, but modulates the susceptibility to develop cirrhosis in French alcoholics. Thus, the present study confirms that the presence of at least 1 Ala allele is a risk factor for developing cirrhosis in French alcoholics compared with the lower risk for Val/Val homozygotes.18 However, unlike the study of Degoul et al.,18 the present study does not indicate a greater risk for developing cirrhosis in Ala/Ala homozygotes than in Ala/Val heterozygotes.

A second aim of the present study was to test the hypothesis that the Ala MnSOD allele may not only increase the likelihood of alcoholic patients to progress to the stage of cirrhosis, but may also go on to speed up...
HCC development and other lethal complications of cirrhosis. Although the prevalence of continued alcohol consumption at end point was similar in Val/Val, Ala/Val, and Ala/Ala cirrhotic patients (Table 1), the presence of at least 1 Ala allele markedly hastened the occurrence of HCC (Figures 1 and 2), and markedly increased the percentage of patients who underwent transplantation and/or died (Figures 3 and 4). If these major prognostic differences are indeed confirmed by other studies, then the Ala−9Val MnSOD genotype might become used to genetically adapt HCC screening strategies and transplantation programs in patients with alcoholic cirrhosis.

Previous studies may shed light on the possible mechanisms whereby the Ala-encoding allele may favor, whereas the Val-encoding allele may prevent, severe alcoholic liver disease. Alcohol consumption increases ROS formation in several cell compartments, including mitochondria. The mitochondrial respiratory chain initially forms the superoxide anion, which is transformed by MnSOD into hydrogen peroxide, which is detoxified into water by mitochondrial GPX. Although the successive action of MnSOD and GPX ensures detoxification of the superoxide anion, a proper balance is required between the activities of these 2 enzymes because the intermediacy product, hydrogen peroxide, is itself toxic and furthermore forms the extremely reactive hydroxyl radical in the presence of iron. Alcohol consumption may cause a basal imbalance between MnSOD and GPX activities. On the one hand, MnSOD is inducible by cytokines and ethanol, on the other hand, GPX activity may be decreased, first by the low intramitochondrial glutathione levels caused by the ethanol-induced impairment of mitochondrial glutathione uptake, and second by ROS-mediated GPX inactivation. This basal imbalance between a high MnSOD activity and a low GPX activity may be aggravated further in alcoholic subjects having an Ala-encoding MnSOD allele, compared with alcoholic subjects having 2 Val-encoding alleles. Indeed, the presence of Val confers a β-sheet structure to the mitochondrial targeting sequence of MnSOD, thus hampering the mitochondrial import of the enzyme through the narrow translocase pore of the inner mitochondrial membrane. In contrast, the presence of Ala confers an α-helical structure to the MnSOD presequence, ensuring rapid and full MnSOD import across the inner membrane. Thus, the Ala MnSOD presequence may increase further the basal imbalance between a high MnSOD activity and a low GPX activity in alcoholics. This aggravated imbalance may cause high steady-state levels of hydrogen peroxide and thus high hydroxyl radical formation. ROS up-regulates both death receptor ligands and death receptors, causing chronic hepatocyte apoptosis. The engulfment of apoptotic bodies by both macrophages and stellate cells increases transforming growth factor β formation by both cell types, and increases collagen production by stellate cells. Finally, both ROS and lipid peroxidation products damage DNA, whereas chronic apoptosis is compensated for by increased cell proliferation rates. The combination of DNA damage with active DNA replication causes gene mutations. As these mutations accumulate over the years, some of them eventually may hamper the control of the cell cycle and/or apoptosis to produce a cancerous clone.

The last aim of the present study was to assess reasons behind the divergent results reported in French and English alcoholics. In the present study and the study by Degoul et al., French patients were included at the time of the first liver biopsy examination showing cirrhosis, whereas the time of inclusion was not specified (and biopsy examination was not required) in the English studies. Because the latter studies relied on blood samples to prepare DNA, it is possible that blood may have been drawn at diverse times, from the time of first referral in some patients to much later times for other patients. An important observation of the present study is that the lower survival of Ala patients during the follow-up period (Figures 3 and 4) brings back the MnSOD genotypic distribution of patients surviving for 5 or 10 years without transplantation, toward the genotypic distribution of control subjects (Figure 5).

In addition to the inclusion time, French and English patients have differences in diet and drinking patterns (steady drinking of red wine in French vs either steady or binge drinking of beer and spirits in English alcoholics) and may differ in the persistence of alcohol consumption after diagnosis. This study should be repeated in other, different countries.

In summary, the Ala-containing mitochondrial targeting sequence of MnSOD increases the susceptibility to develop cirrhosis, and the Ala-encoding allele predicts quick HCC development and death in French alcoholics. Finally, our study shows that care must be taken to include cirrhotic patients at the time of first diagnosis. Otherwise, the selective death of susceptible patients during the follow-up period can bring back genotypic distributions toward the genotypic distribution of controls.

References


