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Do Genetic Variations in Antioxidant Enzymes Influence the Course of Hereditary Hemochromatosis?

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Abstract

Iron-induced oxidative stress promotes hepatic injury in hereditary hemochromatosis, which can be influenced by genetic traits affecting antioxidant enzymes. We assessed the influence of Ala16Val-superoxide dismutase 2, Pro198Leu-glutathione peroxidase 1, and –463G/A-myeloperoxidase genotypes (high activity for the Ala, Pro, and G alleles, respectively) on the risks of cirrhosis and hepatocellular carcinoma (HCC) in patients homozygous for the C282Y-hemochromatosis (HFE) gene mutation. Both the 2G-myeloperoxidase genotype and carriage of one or two copies of the Ala-superoxide dismutase 2 allele were more frequent in patients with cirrhosis or HCC. Patients cumulating these two genetic traits had higher rates of cirrhosis and HCC than other patients. *Antioxid. Redox Signal.* 15, 31–38.

Hereditary Hemochromatosis and Oxidative Stress

Hereditary hemochromatosis (HH) is a genetic disorder characterized by excessive iron deposition in various organs, including the liver. In Western countries, 80%–100% of patients with HH are homozygous for the C282Y-hemochromatosis (HFE) mutation. However, the phenotypic expression of this mutation is variable, as only some of these patients develop cirrhosis or HCC. Besides well-identified factors such as gender and age, phenotypic expression of the C282Y-HFE mutation could be related to yet unidentified host factors (18).

Iron-induced reactive oxygen species (ROS) formation is considered as the main mechanism underlying the progression of liver disease in HH (1). Iron-induced ROS could activate stellate cells, thus favoring fibrogenesis (19), and could cause DNA damage and somatic mutations, with the subsequent development of HCC (5). Several mitochondrial enzymes act in concert as ROS-scavenging enzymes. Manganese superoxide dismutase (MnSOD) accelerates the dismutation of the superoxide anion ($O_2^{\cdot -}$) produced by the mitochondrial respiratory chain (27). MnSOD generates hydrogen peroxide (27), which, unless detoxified by glutathione peroxidase 1 (GPx1) into water, can form the hydroxyl radical in the presence of iron, or can form hypochlorous acid in the presence of myeloperoxidase (MPO) (10) a cytosolic enzyme present in neutrophils and Kupffer cells (3).

Antioxidant Enzymes, Genetic Polymorphisms, Cancer, and Liver Diseases

Genetic dimorphisms modulate the activities of MnSOD, GPx1, and MPO. The Ala16Val-SOD2 dimorphism leads to the incorporation of either alanine (Ala) or valine (Val) in the mitochondrial targeting sequence of MnSOD (25). In acute mitochondrial import and transfection experiments, the Ala-SOD2 variant achieved higher mitochondrial activity than the Val-SOD2 variant (24, 25) and has been shown to increase iron overload in hepatoma cell lines (12). A genetic dimorphism encodes for either proline (Pro) or leucine (Leu) at codon 198 of human GPx1 (20), leading to an increased activity of the Pro-GPx1 variant in the presence of selenium (7). Finally, a G→A base exchange at position −463 affects the MPO promoter (17), the 2G-MPO genotype being associated with higher MPO protein expression than the GA- or 2A-MPO genotypes (21).

The influence of this genetic heterogeneity on the course of human disease, including various cancers, has been widely studied. We previously reported that these functional genetic dimorphisms (and particularly their combination) modulate the risks of death and HCC occurrence through modification

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Table 1. Characteristics of 198 Patients with Hereditary Hemochromatosis

	All	*No cirrhosis*	*Cirrhosis: with or without HCC*	p[a]	*No HCC*	*HCC*	p[b]
Number	198	134	64		175	23	
Age (years)[c]	50.4 ± 0.7	48.2 ± 0.7	54.9 ± 0.6	<0.0001	48.8 ± 0.8	62.2 ± 0.9	<0.0001
Male gender[d]	152 (76.7%)	96 (71.6%)	56 (87.5%)	0.01	130 (74.2%)	22 (95.6%)	0.02
BMI (kg/m^2)[c]	26.0 ± 0.4	25.3 ± 0.7	27.3 ± 0.5	<0.0001	25.6 ± 0.5	29.0 ± 0.3	<0.0001
AST (UI)[c]	43.4 ± 2.2	36.4 ± 1.7	56.7 ± 1.5	<0.0001	41.0 ± 1.9	59.9 ± 1.5	0.0002
ALT (UI)[c]	56.1 ± 1.8	52.8 ± 1.6	63.1 ± 2.5	0.001	56.9 ± 1.4	51.0 ± 2.4	0.7
GGT (UI)[c]	53.5 ± 1.8	46.9 ± 2.6	66.7 ± 3.5	<0.0001	53.3 ± 2.8	55.0 ± 3.2	0.04
Albumin (g/l)[c]	44.1 ± 0.4	44.4 ± 0.4	43.6 ± 0.3	0.2	44.3 ± 0.6	43.1 ± 0.4	0.04
Prothrombin level (%)[c]	92.0 ± 1.2	94.3 ± 1.7	87.9 ± 1.8	0.005	92.0 ± 1.6	91.9 ± 1.5	0.4
Bilirubin (μM)[c]	12.2 ± 6.2	11.0 ± 4.2	14.0 ± 4.6	0.001	11.8 ± 4.1	14.2 ± 4.7	0.02
Blood iron (μM)[c]	34.8 ± 0.6	35.3 ± 0.6	32.6 ± 0.5	0.1	34.6 ± 0.6	38.5 ± 0.7	0.9
Serum Ferritin (mg/l)[c]	1938 ± 78	1542 ± 153	2768 ± 142	<0.0001	1926 ± 147	2032 ± 148	0.2
Transferrin saturation (%)[c]	81.6 ± 0.9	79.7 ± 1.7	83.1 ± 1.6	0.3	87.3 ± 1.7	81.4 ± 1.6	0.7
Liver iron content ($\mu mol/g$)[c]	308 ± 10	286 ± 11	406 ± 12	0.006	311 ± 12	284 ± 14	0.2
SOD2 genotype[d]							
2Val	44 (22.2%)	38 (28.3%)	6 (9.4%)	0.001	43 (24.6%)	1 (4.3%)	0.01
Ala/Val	99 (50.0%)	65 (48.5%)	34 (53.1%)		87 (49.7%)	12 (52.1%)	
2Ala	55 (27.8%)	31 (23.2%)	24 (37.5%)		45 (25.7%)	10 (43.5%)	
1- or 2Ala	154 (77.8%)	96 (71.6%)	58 (90.6%)		132 (75.4)	22 (95.6%)	
MPO genotype[d]							
2A	16 (7.6%)	14 (10.5%)	1 (1.6%)	0.01	15 (8.5%)	0	0.001
A/G	73 (36.8%)	52 (38.8%)	21 (32.8%)		70 (40.0%)	3 (13.1%)	
2G	110 (55.6%)	68 (50.7%)	42 (65.6%)		90 (51.5%)	20 (86.9%)	
Glutathione peroxidase 1 genotype[d]							
2Pro	98 (49.6%)	68 (50.7%)	30 (46.8%)	0.9	87 (49.7%)	11 (47.8%)	0.7
Pro/Leu	87 (43.9%)	56 (41.8%)	31 (48.5%)		77 (44.0%)	10 (43.5%)	
2Leu	13 (6.5%)	10 (7.5%)	3 (4.7%)		11 (6.3%)	2 (8.7%)	

[a]Cirrhosis *vs.* no cirrhosis.
[b]HCC *vs.* no HCC.
[c]Mean ± SEM.
[d]Number (percentage) of patients.
AST, aspartate adino transferase; ALT, alanine adino transferase; GGT, gamma glutamyl transpeptidase; BMI, body mass index; HCC, hepatocellular carcinoma; MPO, myeloperoxidase; SEM, standard error of the mean; SOD, superoxide dismutase.

of hepatic iron accumulation in patients with alcoholic cirrhosis (13, 14, 26).

The aim of the present work was to assess the influence of the Ala16Val-SOD2, Pro198Leu-GPx1, and −463G/A-MPO dimorphisms, alone or combined, on the risks of cirrhosis and/or HCC in patients with C282Y-HFE-related HH. Although either the Ala16Val-SOD2 dimorphism or the −463G/A-MPO dimorphism has been assessed independently in HH patients (15, 23); their role, if any, on HCC development has not been studied.

The HH Patients with Cirrhosis or HCC Differ by Biological and Clinical Criteria

One hundred and ninety-eight patients were enrolled in the present study (Hôpital Jean Verdier: 59, Hôpital Pontchaillou: 139). Their characteristics are displayed in Table 1.

Cirrhosis was present in 64/198 patients (32.3%). As expected, cirrhotic patients were older; they were more often men, and had a higher body mass index than patients without cirrhosis. They also had slightly worse liver tests and a much higher serum ferritin level and liver iron content than those without cirrhosis (Table 1). HCC was diagnosed in 23 of 198 (11.6%) patients, including 17 patients with cirrhosis and 6 patients with a noncirrhotic liver. Patients with HCC were older, and they were more often men and overweight than patients without HCC (Table 1).

The Ala16Val-SOD2 and −463G/A-MPO, But Not the Pro198Leu-GPx1, Genetic Dimorphisms Are Associated with the Presence of Cirrhosis or HCC

Both in control subjects and in patients, genotype distributions followed the Hardy-Weinberg equilibrium expectations. The distributions of the three genetic dimorphisms did not differ between the 187 control subjects and the whole group of 198 patients with HH (Fig. 1).

The distribution of the GPx1 genotypes was similar in control subjects and in HH patients with cirrhosis or in HH patients with HCC (Fig. 1). In contrast, HH patients with cirrhosis and those with HCC more frequently had one or two Ala-SOD2 allele(s) and more frequently had the 2G-MPO genotype than control subjects (Fig. 1). Grossly similar differences were observed when the HH patients with cirrhosis

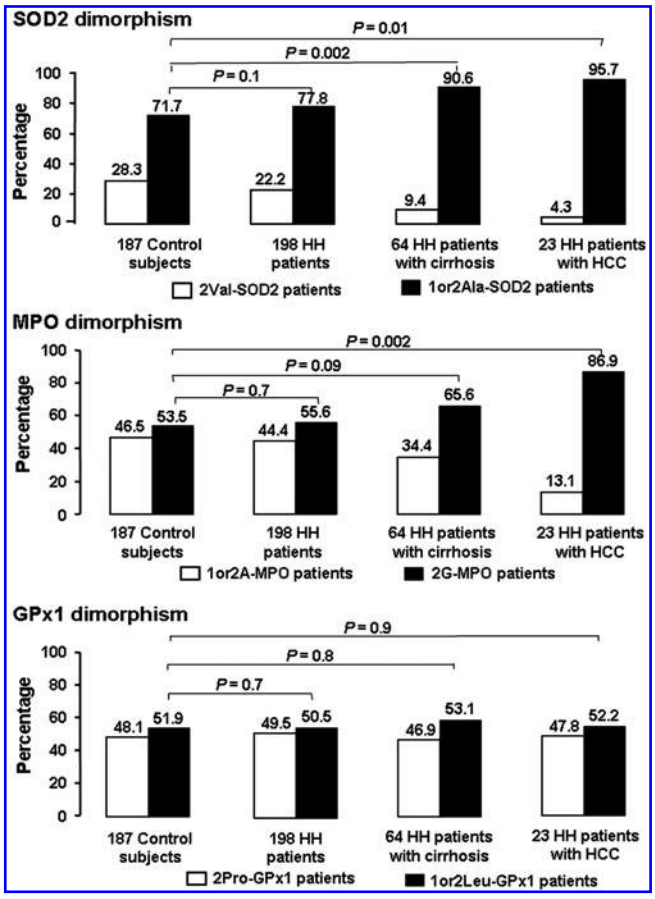

FIG. 1. Ala16Val-SOD2, −463G/A-MPO, and Pro198Leu-GPx1 genotype distributions in control subjects and patients with HH. The SOD2, MPO, and GPx1 genotype distributions did not differ between control subjects and the whole group of HH patients. Compared to control subjects, the subgroup of HH patients with cirrhosis exhibited an increased prevalence of (1- or 2Ala)-SOD2 allele(s), whereas the subgroup of patients with HCC exhibited an increased prevalence of (1- or 2Ala)-SOD2 allele(s) and an increased prevalence of the 2G-MPO genotype. The GPx1 genotype distributions were similar in all groups of patients. GPx1, glutathione peroxidase 1; HCC, hepatocellular carcinoma; HH, hereditary hemochromatosis; MPO, myeloperoxidase; SOD, superoxide dismutase.

were compared to the HH patients without cirrhosis, and even more pronounced when the HH patients with HCC were compared to the HH patients without HCC (Table 1).

When HH patients were classified according to their SOD2 genotypes (Table 2), age, male gender, serum ferritin, and liver iron content did not differ between 2Val-, Ala/Val-, or 2Ala-SOD2 patients. However, the prevalence of cirrhosis was 13.7%, 34.3%, and 43.6% in 2Val-, Ala/Val-, or 2Ala-SOD2 patients, respectively ($p = 0.002$), and the prevalence of HCC was 2.2%, 12.1%, and 18.1%, respectively ($p = 0.01$). When HH patients were classified according to their MPO genotypes (Table 3), age, male gender, serum ferritin and liver iron content did not differ between 2A-, A/G-, or 2G-MPO patients.

However, the prevalence of cirrhosis was 6.7%, 28.7%, and 38.1% in HH patients with the 2A-MPO, A/G-MPO, and 2G-MPO genotypes, respectively ($p = 0.01$), and the prevalence of HCC was 0%, 4.1%, and 18.2%, respectively ($p = 0.01$). In particular, the six patients who developed HCC in noncirrhotic liver were all 2G-MPO homozygotes.

In univariate analysis both the SOD2 and the MPO dimorphisms were associated with both cirrhosis and HCC (Table 4). However, in multivariate analysis, carriage of (1- or 2Ala)-SOD2 allele(s) was the only polymorphic genetic trait independently associated with the presence of cirrhosis in HH patients, along with old age and male gender. In contrast, the multiple logistic regression model only selected the 2G-MPO genotype as a genetic feature independently associated with HCC, along with old age and the presence of cirrhosis (Table 4).

The Combination of One or Two Ala-SOD2 and 2G-MPO Variants Increases the Risks of Cirrhosis and HCC

Having found protective and "at risk" genotypes for both the −463G/A-MPO and the Ala16Val-SOD2 dimorphisms (Tables 2 and 3), we then classified the 198 patients into four groups according to their combined MPO and SOD2 genotypes (Table 5).

Group 1 patients neither had the "at risk" 2G-MPO genotype nor the "at risk" (1- or 2Ala)-SOD2 genotypes. Group 2 patients had only the "at risk" 2G-MPO genotype without the (1- or 2Ala)-SOD2 genotypes. Group 3 patients had only the "at risk" (1- or 2Ala)-SOD2 genotypes, without the 2G-MPO genotype. Finally, group 4 patients had both the "at risk" 2G-MPO genotype and the "at risk" (1- or 2Ala)-SOD2 genotypes. Group 4 represented 45% of the whole study population. Demographic data, features estimating the severity of liver disease, serum iron, and liver iron content, did not differ between the four groups. In keeping with a role of carriage of (1- or 2Ala)-SOD2 alleles in cirrhosis development (Table 4), the prevalence of cirrhosis was similar in group 1 (12.5%) and group 2 (15%), but doubled between group 2 and group 3 (29.6%), with a moderate further increase in group 4 (43.3%) (Table 5). In keeping with the role of cirrhosis (and thus carriage of [1- or 2Ala]-SOD2 alleles)] and an independent role of the 2G-MPO genotype in HCC development (Table 4), the prevalence of HCC was 8.2-fold higher in group 4 (22.2%) than in the three other groups combined (2.7%) ($p = 0.0008$). Overall, patients cumulating both (1- or 2Ala-SOD2 allele(s) and a 2G-MPO genotype represented 86.9% (20/23) of the cases of HCC. In total, of the 90 patients in group 4, 45 patients (50%) had either cirrhosis alone (39 patients) or HCC alone (6 patients) and 14 had both cirrhosis and HCC.

Conclusions and Open Questions

The present study reports the influence of the Ala16Val-SOD2 and −463G/A-MPO genetic dimorphisms on the prevalence of cirrhosis and that of HCC in patients with HH. Results highlight a possible role of the Ala-SOD2 allele on the risk of cirrhosis, and a role of the 2G-MPO genotype on the risk of HCC development in HH patients. As a consequence of these two added effects, the subgroup of patients combining these two genetic traits, which represented 45% of the study population, had an eightfold higher prevalence of HCC than other patients. These findings offer new insights into genetic factors behind the variable phenotypic expression of the homozygous C282Y-HFE gene mutation, and the role of oxidative stress in the development of both cirrhosis and HCC in

Table 2. Comparison of 198 Hereditary Hemochromatosis Patients Classified According to Their Ala16Val-Superoxide Dismutase 2 Genotype

	Patients with two low activity-associated Val-SOD2 alleles (n = *44; 23%*)	*Patients with one Val-SOD2 and one Ala-SOD2 alleles* (n = *99; 50%*)	*Patients with two high activity-associated Ala-SOD2 alleles* (n = *55; 27%*)	p
Age (years)[a]	51.3 ± 0.7	45.8 ± 0.6	49.8 ± 0.7	0.4
Male gender[b]	33 (75.0%)	74 (74.5%)	45 (81.8%)	0.4
BMI (kg/m^2)[a]	26.5 ± 0.4	25.2 ± 0.5	27.1 ± 0.7	0.3
AST (UI)[a]	37.5 ± 2.2	39.4 ± 1.5	50.4 ± 1.7	0.006
ALT (UI)[a]	54.5 ± 1.8	53.4 ± 2.5	61.4 ± 1.6	0.2
GGT (UI)[a]	38.1 ± 1.8	53.5 ± 3.5	65.4 ± 2.6	0.007
Albumin (g/l)[a]	44.3 ± 0.4	44.6 ± 0.3	44.9 ± 0.4	0.6
Prothrombin level (% control)[a]	92.1 ± 1.2	90.7 ± 1.8	93.8 ± 1.7	0.8
Bilirubin (μM)[a]	10.8 ± 6.2	12.4 ± 4.6	13.4 ± 4.2	0.1
Blood iron (μM)[a]	36.5 ± 0.6	35.4 ± 0.5	38.7 ± 0.6	0.07
Ferritinemia (mg/l)[a]	1468 ± 78	2032 ± 142	2144 ± 153	0.1
Transferrin saturation (%)[a]	82 ± 0.9	80 ± 1.6	83 ± 1.7	0.5
Liver iron content (μmol/g)[a]	287 ± 10	330 ± 12	300 ± 11	0.6
Cirrhosis[b]	6 (13.7%)	34 (34.3%)	24 (43.6%)	0.002
HCC[b]	1 (2.2%)	12 (12.1%)	10 (18.1%)	0.01

[a]Mean ± SEM.
[b]Number (percentage) of patients.

these patients. If confirmed in large prospective cohort studies, these genetic predispositions could help adapt HCC screening strategies in HH patients.

Carriage of at least one copy of the Ala-SOD2 allele was associated with the presence of cirrhosis and, to a lesser extent, HCC in this population (Fig. 1; Tables 2 and 4). The Ala-SOD2 allele has been reported to be involved in the emergence or progression of various diseases in which mitochondrial oxidative stress plays a pivotal role (2). Regarding liver disease, the implication of the Ala-SOD2 allele on the constitution of alcoholic cirrhosis has been debated, possibly due to several methodological bias encountered in case–control studies (4, 13, 22). This could also be the case in HH, as a previous study conducted with a methodology similar to ours did not find an association between the Ala-SOD2 variant and cirrhosis in patients with HH (23). In this previous study, no HH patient was reported as having HCC, whereas 23 patients (including 17 patients with cirrhosis) had HCC in the present study. Since patients with HCC (with or without cirrhosis) had an extremely low prevalence of the two Val-SOD2 genotype (4.4%) and conversely an extremely high prevalence of (1- or 2Ala)-SOD2 allele(s) (95.6%) in the present study, differences in the inclusion/prevalence of patients with HCC might account at least in part for the different results of the

Table 3. Comparison of 198 Hereditary Hemochromatosis Patients Classified According to Their −463G/A-Myeloperoxidase Genotype

	Patients with two low activity-associated A-MPO alleles (n = *15; 7%*)	*Patients with both one A-MPO allele and one G-MPO allele* (n = *73; 37%*)	*Patients with two high activity-associated G-MPO alleles* (n = *110; 56%*)	p
Age (years)[a]	49.4 ± 0.5	48.7 ± 0.6	51.7 ± 0.8	0.1
Male gender[b]	12 (80.0%)	56 (76.7%)	84 (76.3%)	0.8
BMI (kg/m^2)[a]	25.1 ± 0.6	26.1 ± 0.4	26.2 ± 0.8	0.8
AST (UI)[a]	36.6 ± 1.1	38.7 ± 1.3	45.2 ± 1.5	0.1
ALT (UI)[a]	46.5 ± 1.9	53.8 ± 1.6	59.1 ± 1.4	0.2
GGT (UI)[a]	55.2 ± 2.4	51.1 ± 2.6	54.7 ± 2.8	0.9
Albumin (g/l)[a]	45.5 ± 0.5	44.3 ± 0.5	43.9 ± 0.7	0.4
Prothrombin level (% control)[a]	95.3 ± 0.3	93.0 ± 0.6	90.9 ± 1.0	0.05
Bilirubin (μM)[a]	10.8 ± 5.7	12.5 ± 4.9	12.6 ± 5.5	0.2
Blood iron (μM)[a]	37.0 ± 0.9	36.6 ± 0.8	36.6 ± 0.7	0.7
Ferritinemia (mg/l)[a]	1836 ± 98	1946 ± 123	1947 ± 134	0.6
Transferrin saturation (%)[a]	75 ± 0.8	81 ± 1.5	82 ± 1.8	0.9
Liver iron content (μmol/g)[a]	301 ± 14	322 ± 18	307 ± 15	0.8
Cirrhosis[b]	1 (6.7%)	21 (28.7%)	42 (38.1%)	0.01
HCC[b]	0 (0.0%)	3 (4.1%)	20 (18.2%)	0.005

[a]Mean ± SEM.
[b]Number (percentage) of patients.

Table 4. Features Associated with the Presence of Cirrhosis and Features Associated with Hepatocellular Carcinoma According to Multiple Logistic Regression Model

	Odds ratio (confidence interval) and/or p for features associated with cirrhosis		*Odds ratio (confidence interval) and/or p for features associated with HCC*	
	Univariate analysis	*Multivariate analysis*	*Univariate analysis*	*Multivariate analysis*
Age	NA[a] $p = 0.0002$	NA[a] $p = 0.0008$	NA[a] $p < 0.0001$	NA[a] $p = 0.0007$
Male gender	2.7 (1.2–6.3) $p = 0.01$	4.1 (1.5–10.8) $p = 0.004$	—	—
BMI	NA[a] $p = 0.009$	—	NA[a] $p = 0.004$	—
Cirrhosis	—	—	7.7 (2.8–20.7) $p < 0.0001$	5.8 (1.6–21.1) $p = 0.006$
(1- or 2Ala)-SOD2 alleles	3.8 (1.5–9.6) $p = 0.004$	3.1 (1.2–8.4) $p = 0.02$	1.9 (1.1–4.7) $p = 0.04$	—
2G-MPO genotype	1.8 (1.0–3.4) $p = 0.05$	—	6.2 (1.8–21.9) $p = 0.004$	7.1 (1.2–42.2) $p = 0.03$

[a]NA, not applicable. For the variables age and BMI, no odds ratio were calculated as both variables were not classified into groups, but were entered as continuous variables in the regression model.

two studies regarding the repartition of the SOD2 genotypes. A way to avoid the possible recruitment bias of case/control studies is to study the influence of such genetic features in prospective cohorts of patients. However, in the case of HH patients, therapeutic iron depletion is ethically required and expected to prevent cirrhosis development and the occurrence of most, although not all cases of HCC. In contrast, the role of the Ala-SOD2 allele on the prognosis of alcoholic cirrhosis has been clearly demonstrated in prospective cohort studies (13, 14, 26). In these studies, the occurrence of death or HCC during follow-up was markedly increased in cirrhotic patients with (1- or 2Ala)-SOD2 allele(s) (13, 14, 26) possibly due to the association of the Ala-SOD2 allele with a higher liver iron content. In HuH7 cells exposed to iron, transfection of the Ala-SOD2 variant increased cellular iron concentration compared to the Val-SOD2 variant, by modulating expression of several proteins involved in iron homeostasis (12). In cirrhotic alcoholic patients, likewise, the frequency of a mild iron overload progressively increased with the number (0, 1, or 2) of Ala-SOD2 allele(s) (14, 26). In the present study, however, liver

Table 5. Characteristics of 198 Hereditary Hemochromatosis Patients Classified According to Their Superoxide Dismutase 2 and Myeloperoxidase Genotypic Associations

	Group 1: 2Val-SOD2 alleles and (1- or 2A)-MPO alleles (n = 24; 12%)	*Group 2: 2Val-SOD2 alleles and 2G-MPO alleles (n = 20; 11%)*	*Group 3: (1- or 2Ala)-SOD2 alleles and (1- or 2A)-MPO alleles (n = 64; 32%)*	*Group 4: (1- or 2Ala)-SOD2 alleles and 2G-MPO alleles (n = 90; 45%)*	p
Age (years)[a]	47.6 ± 0.6	51.7 ± 0.7	49.3 ± 0.8	51.6 ± 0.7	0.2
Male gender[b]	20 (88.3%)	13 (65.0%)	48 (75.0%)	71 (78.8%)	0.8
BMI (kg/m^2)[a]	26.8 ± 0.7	26.7 ± 0.5	25.6 ± 0.9	26.1 ± 0.8	0.6
AST (UI)[a]	34.1 ± 2.1	41.3 ± 1.4	39.4 ± 1.6	46.1 ± 1.8	0.07
ALT (UI)[a]	54.1 ± 1.4	55.4 ± 1.5	52.0 ± 1.7	60.0 ± 1.1	0.05
GGT (UI)[a]	33.9 ± 2.8	43.0 ± 2.5	58.4 ± 2.7	57.4 ± 2.2	0.1
Albumin (g/l)[a]	45.1 ± 0.6	42.8 ± 0.3	44.4 ± 0.2	44.1 ± 0.6	0.3
Prothrombin level (% control)[a]	92.7 ± 1.8	91.5 ± 1.2	93.6 ± 1.7	90.8 ± 1.4	0.06
Bilirubin (μM)[a]	11.1 ± 3.8	9.0 ± 4.8	12.5 ± 5.2	13.4 ± 4.7	0.09
Blood iron (μM)[a]	36.4 ± 0.7	36.3 ± 0.9	36.8 ± 0.6	36.6 ± 0.5	0.9
Ferritinemia (mg/l)[a]	1467 ± 79	1470 ± 88	2102 ± 121	2054 ± 113	0.2
Transferrin saturation (%)[a]	82 ± 0.9	82 ± 0.6	80 ± 1.1	82 ± 1.2	0.9
Liver iron content ($\mu mol/g$)[a]	321 ± 11	230 ± 11	316 ± 15	325 ± 14	0.5
Cirrhosis[b]	3 (12.5%)	3 (15.0%)	19 (29.6%)	39 (43.3%)	0.0006
HCC[b]	1 (4.1%)	0 (0.0%)	2 (3.1%)	20 (22.2%)	0.0008

[a]Mean ± SEM.
[b]Number (percentage) of patients.

iron content of HH patients did not significantly differ according to the SOD2 genotypes, possibly because any mild effect associated with the Ala-SOD2 allele (Table 4) was masked by the high iron overload associated with homozygosity for the C282Y-HFE mutation in these patients.

In acute mitochondrial import and transfection experiments, the Ala-SOD2 variant was better imported into the mitochondrial matrix and achieved higher mitochondrial activity than the Val-SOD2 variant (24, 25). However, recent data raise the possibility that, in the presence of iron overload, the activity of MnSOD could be modified. In a model of HH ($HFE^{-/-}$ mice), hepatic iron overload was associated with an inhibition of mitochondrial manganese uptake and a reduced activity of MnSOD (8). Further, several yeast mutants with high mitochondrial iron, and one mutant with low cellular manganese levels, all misincorporated iron instead of manganese into the MnSOD active site (30). The iron-substituted MnSOD is characterized by increased stability, absent SOD activity, and gain of a radical-generating activity in the presence of hydrogen peroxide, probably because the iron-substituted MnSOD catalyzes the monoelectronic reduction of hydrogen peroxide into the hydroxyl radical (29). Should part of MnSOD be indeed iron-substituted in HH patients, then the spontaneous dismutation of the superoxide anion or the remaining manganese-associated MnSOD could form hydrogen peroxide, which could then be transformed into the hydroxyl radical by the iron-substituted MnSOD. Because the Ala-SOD2 allele is associated with a better importation of MnSOD than the Val-SOD2 allele, the Ala-SOD2 variant could give rise to a larger formation rate of the highly reactive hydroxyl radical by the iron-substituted enzyme. This increased ROS formation could stimulate stellate cell activation, thus promoting fibrogenesis. It could also increase DNA damage, thus leading to somatic mutations and the possible development of cancer clone.

Although the high activity-associated 2G-MPO genotype was not selected by the multiple logistic regression model (possibly due to the competitive risk associated with the Ala-SOD2 allele), this MPO genotype was also associated with the presence of cirrhosis in the present population, thus confirming a previous study (15). More importantly, this genetic trait was strongly associated with the presence of HCC in these patients despite the small number of patients ($n = 23$) in this subgroup. It is noteworthy that six of the cases of HCC developed in a noncirrhotic liver, a finding that is not unusual in HH patients (1). Interestingly, all these six cases occurred in patients with the 2G-MPO genotype, thus suggesting that the 2G-MPO genotype favors the development of liver cancer regardless of cirrhosis. Indeed, carriage of two copies of high expression G-MPO variant has been reported to be associated with various cancers (21), including hepatoblastoma (16), as well as cases of HCC that occurred during the prospective follow-up of alcoholic cirrhotic patients (14). As MPO activity is thought to be enhanced by iron (9), genotypic variations in MPO activity may be particularly important in HH patients. MPO is released by activated macrophages (10) and can form hypochlorous acid. This MPO-generated ROS may damage DNA bases and impair nucleotide excision repair, thus favoring the appearance of somatic mutations and the development of a malignant clone (28).

Finally, the study of the combined influence of the MPO and SOD2 genotypes showed that the rate of cirrhosis was 1.9-fold higher in patients combining both the at risk Ala-SOD2 allele and the at risk 2G-MPO genotype. Consistent with the view that the Ala-SOD2 allele favors cirrhosis (Table 4), and that both cirrhosis and the 2G-MPO allele are independent risk factors for HCC (Table 4), the rate of HCC was increased 8.2-fold in patients cumulating both the at risk Ala-SOD2 allele and the at-risk 2G-MPO genotype (Table 5). Overall, patients cumulating both (1- or 2 Ala)-SOD2 allele(s) and a 2G-MPO genotype represented 86.9% (20/23) of all cases of HCC. If this finding is further confirmed, it could help better understand the mechanism behind HCC development in HH, and may refine HCC screening strategies to individual HH patients.

In summary, the combination of the 2G-MPO genotype with carriage of at least one Ala-SOD2 allele was associated with a 1.9-fold higher risk of cirrhosis and an 8.2-fold higher risk of HCC in French HH patients.

Notes

Patients

We considered all patients who were consecutively referred to two hepatology units for liver biopsy performance in the setting of HH between January 1998 and December 2008, and who fulfilled the following inclusion criteria: (a) homozygosity for the C282Y-HFE mutation; (b) no infection by hepatitis B or C virus; (c) daily alcohol intake <30 grams; (d) residence in France; (e) Caucasian origin; (f) availability of frozen DNA; and (g) written informed consent for the use of frozen DNA.

For each patient, the following data were recorded on the day of the liver biopsy: gender, age, body mass index, serum bilirubin, albumin and prothrombin levels, serum alanine aminotransferase, aspartate aminotransferase and gamma-glutamyl transferase activities, serum iron, transferrin saturation, and ferritinemia.

Control subjects

To assess the prevalence of the SOD2, MPO, and GPx1 genotypes in the general population, we used a control group of 187 randomly selected French Caucasian blood donors with normal alanine adino transferase levels, and negative for hepatitis B surface antigen and anti-hepatitis C virus antibodies.

Liver histology and hepatic iron determination

Liver biopsy specimens were fixed in formalin and routinely processed. Four-micrometer-thick paraffin-embedded sections were routinely stained with hematoxylin/eosin, Masson trichrome, and Perls' Prussian blue and examined on a multipipe microscope by a trained pathologist unaware of genotypic and clinical data. The existence of cirrhosis was précised; liver iron was measured by atomic absorption spectrophotometry and expressed as μmol/g of dry liver tissue (6).

HCC diagnosis

In the setting of HCC screening procedures, all patients underwent physical examination, liver ultrasonography, and serum α-fetoprotein measurements before liver biopsy performance. When these investigations suggested a possible

diagnosis of HCC, computed tomodensitometry and/or guided liver biopsy were performed. HCC was diagnosed on either one of the following criteria: histological evidence, or, in case of tumor developed in cirrhotic liver, convergent demonstration of a focal lesion more than 2 cm in size and with arterial hypervascularization by two different imaging techniques, or the combination of one imaging technique showing this morphological aspect with an α-fetoprotein level of 200 ng/ml or more according to the Barcelona criteria (11).

DNA extraction, amplification, and genotyping

Genomic DNA was extracted from each patient's peripheral blood mononuclear cells using a commercially available kit according to manufacturer's instructions (Amersham, GE Healthcare). All patients gave written consent for blood sampling and genotyping. The use of left over 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.

We genotyped the Ala16Val-SOD2 (dbSNP: rs 4880), Pro198Leu-GPx1 (dbSNP: rs 1050450), −463G/A-MPO (dbSNP: rs 2333227), polymorphisms by allelic discrimination using fluorogenic probes, and the 5′ nuclease (TaqMan) assay. The Ala16Val-SOD2 and Pro198Leu-GPx1 polymorphisms were genotyped using the TaqMan SNP genotyping products: C___8709053_10 and 002–1770-CT, respectively (Applied Biosystems, Foster City, CA). Genotyping of the–463G/A-MPO single nucleotide polymorphism was performed by a TaqMan assay consisting of forward (5′-TCTTGGGCTG GTAGTGC-3′) and reverse (5′-GTATTTTTAGTAGATACAG GGTTTCA-3′) primers and allele-specific probes (TGAGG CGGGTGGATCACT) and (AGGCTGAGGCAGGTGGAT) labeled with VIC® and 6-FAM, respectively.

Polymerase chain reactions (PCRs; 25 μl) consisted of 1× TaqMan Universal PCR master mix (Applied Biosystems), 1× assay mix and 20 ng DNA. Real-time PCR was performed on a Step One Plus PCR system (Applied Biosystems) using a protocol consisting of incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles, denaturation at 92°C for 15 s, and annealing/extension at 60°C for 1 min. The FISH carboxy fluoresceine and VIC fluorescence levels of the PCR products were measured at 60°C for 1 min, resulting in the clear identification of all genotypes of SOD2, GPx1, and MPO on a two-dimensional graph.

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 (analysis of variance) was conducted to compare more than two means. Binary logistic regression analysis with forward and backward stepwise inclusion of variables was performed to assess the impact of laboratory and clinical data and of the studied genotypes on the presence of cirrhosis or HCC. Only variables significant on univariate analysis were included in the multivariate model. Statistical analysis used the SAS System Package version 8.02 (SAS Institute, Cary, NC). All reported *p*-values are two-tailed. Associations were first considered statistically significant at a two-tailed α of 0.05.

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Abbreviations Used

ALT = alanine adino transferase
AST = aspartate adino transferase
BMI = body mass index
GGT = gamma glutamyl transpeptidase
GPx1 = glutathione peroxidase 1
HCC = hepatocellular carcinoma
HFE = hemochromatosis gene
HH = hereditary hemochromatosis
MnSOD = manganese superoxide dismutase
MPO = myeloperoxidase
ROS = reactive oxygen species
SEM = standard error of the mean

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