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Polymorphisms in antioxidant defence genes and susceptibility to hepatocellular carcinoma in a Moroccan population

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Abstract
Reactive oxygen species have been related to the aetiology of cancer as they are known to be mitogenic and therefore capable of tumour promotion. The aim of this study was to assess the role of common variation in three polymorphic genes (MnSOD Ala-9Val, GPX1 Pro198Leu and CAT -262 C > T) coding for antioxidant defence enzymes in modulating individual susceptibility to hepatocellular carcinoma (HCC) using a case-control study (cases = 96 and controls = 222). PCR-RFLP and sequencing methods were used to determine the genotype. Overall, there were no associations between genotypes GPX1 and HCC risk (OR, 1.16; 95% CI, 0.56−2.42; $p = 0.685$). The MnSOD Ala/Ala and CAT TT genotypes were more frequent in HCC than in control ($p = 0.001$ and $p = 0.072$, respectively). Further analyses stratified by gender or HCV infection revealed that men and HCV-infected patients carrying CAT TT genotype had a higher risk to develop HCC when compared with controls (OR = 15.94; 95% CI, 3.48−72.92; $p < 0.000001$ and 12.01; 95% CI, 0.64–223.63, $p = 0.056$, respectively). Combined MnSOD Ala/Ala and GPx1 Leu/Leu had a synergistic effect on HCC risk, with an OR of 3.84 ($p = 0.029$). Furthermore an even more pronounced risk was observed when we combined MnSOD Ala/Ala and CAT TT (OR = 13.60, $p = 0.023$). It appears that variants in MnSOD, CAT or GPX1 have an influence on HCC risk in this cohort. Furthermore, it is possible that cumulative defects in protection from oxidative stress may result in increased risk of liver cancer in the Moroccan population.

Keywords: *Hepatocellular carcinoma, polymorphism, oxidative response, Morocco.*

Introduction

Oxidative damage caused by highly reactive compounds such as superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) plays a crucial role in the pathogenesis of many human diseases [1]. There is increasing evidence that the alteration of the cellular redox state with production of Reactive Oxygen Species (ROS) plays a crucial role in the various steps that initiate and regulate the progression of liver diseases, independently from the type of aetiologic agents. ROS are involved in the liver damage induced by alcohol, virus, alteration of lipids, carbohydrates and xenobiotics metabolism [2]. ROS and ROS-induced cytokines are known to trigger the apoptosis of some hepatocytes and therefore contribute to inflammation, regeneration, fibrogenesis and carcinogenesis [3–5]. The severity of ROS effects depends on individual characteristics such as age, obesity, alcohol intake, iron concentration as well as from endogenous intracellular and plasmatic antioxidant defences [6]. The imbalance between ROS production and antioxidant defences lead to a state of oxidative stress and adjust the levels of several biochemical mediators (principally cytokines) able to modulate tissular and cellular events such as apoptosis, fibrosis,

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cholestasis and regeneration, which characterize the different types of liver injury.

Inactivation and removal of these highly active ROS depend on reactions involving the antioxidant defence system [7,8]. The enzymes generally considered to be the frontline defence against ROS are the mitochondrial manganese superoxide dismutase (MnSOD), catalase (CAT) and glutathione peroxidase (GPX1). MnSOD catalyses the conversion of superoxide radicals to hydrogen peroxide, whereas CAT and GPX1 facilitate the further reduction of hydrogen peroxide to water and oxygen. By this chain of enzymatic events, most of the ROS in the cell are eliminated and potential damage is limited [9]. Activity levels of these enzymes are likely affected by functional polymorphisms in the genes encoding them. A polymorphism in MnSOD exists in codon 16 (rs4880), which is located at position -9 of the mature protein and results in the incorporation of either alanine (C allele) or valine (T allele) in the mitochondrial targeting sequence [10]. Recent experimental data indicate that the Ala-containing MnSOD is targeted into the mitochondria, whereas the Val form of the protein is partially arrested in the inner mitochondrial membrane [11].

The antioxidant enzyme glutathione peroxidase 1 (GPX1, EC 1.11.1.9) is part of the enzymatic antioxidant defence preventing oxidative damage to DNA, proteins and lipids, by detoxifying hydrogen and lipid peroxides. A polymorphism in the GPX1 gene (GPX1 Pro198Leu, rs1050450) encoding the isoenzyme GPX1, expressed in erythrocytes [12] as well as in several epithelial tissues including breast, has been associated with risk of lung [13] and breast cancer [14]. Hu and Diamond[15] found that the GPX1 198Leu enzyme had lower selenium-activation of GPX activity when transfected into MCF-7 cells. This indicates that the amino acid substitution may have a biological phenotype. A recent study showed that the variant Leu-allele was associated with a significant, although moderate, 5% lower erythrocyte GPX1 enzyme activity per allele, measured as a response to t-butyl hydroperoxide exposure *in vitro* [14].

The catalase gene contains 13 exons and is located on chromosome 11p13 [16]. Several polymorphisms in the catalase gene have been reported [17–19]. A common T/C polymorphism located at −262 (rs1001179) has a substantial impact on both the basal expression of catalase and the level of this enzyme in erythrocytes [20]. Individuals with variant CT or TT genotypes have significantly lower activity than those with CC genotypes in Caucasians [21].

In our previous study, we have found that MnSOD Ala-9Val is associated with higher risk of developing HCC [22]. In addition, ultimate levels of ROS are likely dependent not only on the generation of hydrogen peroxide by MnSOD, but also on the neutralization of H_2O_2 by catalase and glutathione peroxidase and we hypothesized that functional GPX1 Pro198Leu and CAT -262 C > T polymorphisms might be a genetic susceptibility factor for the development of HCC and, to our knowledge, there have been no investigations of associations between liver cancer risk and MnSOD, CAT and GPX1 polymorphisms. To test this hypothesis, we conducted a case-control study of HCC in a Moroccan population to evaluate potential associations between risk and variants in these enzymes, as well as potential interactions with ROS-related exposures.

Patients and methods

Clinical data

We studied 96 HCC patients and 222 control subjects. The controls did not have a previous diagnosis of any type of cancer. The detailed recruitment of patients and controls was described previously [22–24]. The procedures and purposes of the study were explained to all the subjects. After informed consent was obtained, each participant was interviewed and a structured questionnaire on demographic data and selected risk factors was given and facilitated by interviewers. The following risk factors were investigated in the questionnaire: heavy alcohol intake, smoking behaviour, non-insulin-dependent diabetes mellitus. Cases and controls accepted to be enrolled in a proportion of 98% and 80%. The major cause of refusal was merely a lack of interest for research work. All subjects were recruited in Western-Central Morocco (Rabat and Casablanca) in a population of mixed Berberic and Arabic ethnicity. Two groups of controls were recruited: (i) the first group among individuals coming for blood testing unrelated to liver pathology at the Pasteur Institute of Morocco; and (ii) the second group among HCV-infected patients with low grade liver disease. The severity of liver disease was routinely assessed by non-invasive methods. Controls were graded as 'low' grade when abdominal ultrasonographic examination confirmed the presence of a mild liver disease concomitant to moderately elevated plasma liver enzymes. These investigations were approved by the Ethics Committee of the Faculté de Medicine of Casablanca.

Serological markers for hepatitis viruses were tested with commercially available kits (Axsym, Abbott Diagnostics, Germany) for HBsAg, Hepatitis B early antigen (HBeAg), anti-HBe, anti-HBc IgG, anti-HBsAg and anti-hepatitis C virus (anti-HCV) for HCC patients and HBsAg and anti-HCV for control subjects.

SNP genotyping

Genomic DNA was isolated from peripheral leukocytes. The blood samples were submitted to digestion

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in SDS/proteinase K buffer at 37°C for 6 to 12 h, followed by two phenol and one chloroform extraction. DNA was then ethanol-precipitated and resuspended in TE buffer.

Genotyping of MnSOD Ala-9Val was performed by PCR-RFLP and sequencing methods as previously described [22].

Polymorphisms at codon 198 in the GPX1 gene were determined by using a polymerase chain reaction (PCR) restriction fragment-length polymorphism (RFLP)-based method as previously described [13,25]. DNA was amplified with primers 5'-TGTGCCCCTACGCAGGTACA-3' and 5'-CCCCCGAGACAGCAGCA-3'. 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 (Promega, France). The DNA fragments were separated by electrophoresis on 3% agarose gels.

CAT -262 C > T polymorphism was evaluated using a primer pair (forward 5'-TAAGAGCTGAGAAAGCATAGCT-3', reverse 5'-AGAGCCTCGCCCCGCCGGACCG-3' [20]. PCR was carried out in a final volume of 25 μl, containing 50 ng of genomic DNA, 20 pmol/μl of each primer, 0.5 unit of Taq DNA polymerase (Invitrogen, France), 200 μM of each dNTP and 1.5 mM MgCl2. The resulting 185-bp PCR product was digested with the restriction endonuclease Sma I (Invitrogen, France) at 30°C overnight according to the manufacturer's recommendation and digestion products were separated by gel electrophoresis in 3% NuSieve agarose stained with ethidium bromide. The digestion products reveal the presence of three different patterns: the TT homozygote showing an undigested product of 185 base pairs, the CC homozygote presenting a 155-bp and 30 fragments and the CT heterozygote characterized by fragments of 185 bp, 155 bp and 30 bp, respectively.

Approximately 30% of the RFLPs assays were repeated and no discrepancy was observed.

Statistical analysis

The characteristics of HCC and control subjects were compared using the two-sample *t*-test for continuous variables and the χ^2 test for categorical variables. The χ^2 test was used to compare genotype frequencies among subjects. The Hardy-Weinberg equilibrium (HWE) of MnSOD Ala-9Val, GPX1 Pro198Leu and CAT -262 C > T loci was tested separately in the patients and controls using the chi-square test. No deviation from Hardy-Weinberg equilibrium was detected in control group (all $p > 0.05$). The association between HCC and the genotypes was estimated based on an odds ratio (OR) and a 95% confidence interval (CI) using a multivariate logistic regression model. All ORs were adjusted for age, sex and HCV infection where it was appropriate.

We tested the null hypotheses of multiplicative gene–gene and gene–HCV infection interactions and evaluated departures from multiplicative joint effect models by including main effect variables and their product terms in the logistic regression model. For all tests, $p < 0.05$ was considered significant.

Results

Association between individual polymorphism and HCC risk

As shown in Table I, there were no statistically significant differences in the distributions of sex and age between patients and controls.

Table II presents the genotype distributions for the three polymorphisms analysed. In the control group, the *p*-values of HWE for the genotype frequencies of MnSOD Ala-9Val, GPX1 Pro198Leu and CAT -262 C > T were 0.405, 0.217 and 0.526, respectively. MnSOD Ala-9Val in HCC group respects HWE as well ($p = 0.680$). On the contrary, the genotype frequencies of GPX1 Pro198Leu and CAT -262 C > T in HCC group deviated significantly from those expected under the HWE ($p = 0.032$ and 0.001, respectively).

The proportions of MnSOD genotypes in HCC and controls were significantly different ($p = 0.001$). Taking Val/Val genotype for reference, OR (95% CI) for the Val/Ala and Ala/Ala genotypes presence in HCC were 1.72 (0.95−3.11; $p = 0.073$) and 2.89 (1.47−5.68; $p = 0.001$), respectively. The genotype frequencies GPX1 Pro198Leu in HCC patients and control group are presented in Table II and no significant difference in genotype distribution was observed ($p = 0.685$). On the contrary, CAT TT genotype was shown to be potentially a risk allele. Subjects carrying the TT had a 3.41 OR (95% CI, 0.94−12.45) of HCC development when compared with subjects carrying the more active CC genotype ($p = 0.072$).

Table I. Characteristics of HCC patients and control subjects.

Characteristics	HCC patients ($n = 96$)	Controls ($n = 222$)	*p*-value
Age (mean ± SD years)	59.3 ± 14.1	56.4 ± 10.1	0.093
Sex ratio (Male/Female)	1.53	1.33	0.987
HBsAg Positive	13 (13.2%)	7 (3.1%)	0.002
Anti-HCV Positive	56 (56.2%)	*a*	—
Hepatitis B and C	5 (5.1%)	1 (0.4%)	0.011
Alcoholic	3 (3.1%)	3 (1.3%)	0.375
NIDDM[b]	8 (8.2%)	3 (1.3%)	0.004

[a] Prevalence was not calculated because the control group includes two groups (163 healthy subjects and 59 patients with HCV).
[b] Non-insulin-dependent diabetes mellitus.

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Table II. Association between MnSOD, GPX1 and CAT genetic polymorphisms and risk of hepatocellular carcinoma.

Genotype	Controls (%)	HCC cases (%)	OR (95% CI)*	*p*-value
MnSOD Ala-9Val				
Val/Val	81 (36.5%)	21 (21.8%)	1.00	
Val/Ala	101 (45.5%)	45 (46.8%)	1.72 (0.95–3.11)	0.073
Ala/Ala	40 (18%)	30 (31.2%)	2.89 (1.47–5.68)	0.001
GPX1 Pro198Leu				
Pro/Pro	108 (48.6%)	50 (52.1%)	1.00	
Pro/Leu	88 (39.6%)	32 (33.3%)	0.78 (0.46–1.33)	0.367
Leu/Leu	26 (11.7%)	14 (14.5%)	1.16 (0.56–2.42)	0.685
CAT -262 C > T				
CC	173 (77.9%)	76 (79.2%)	1.00	
CT	45 (20.3%)	14 (14.6%)	0.71 (0.37–1.37)	0.302
TT	4 (1.8%)	6 (6.2%)	3.41 (0.94–12.45)	0.072

*Multivariate analysis adjusted for age, sex, HBV and HCV infection.

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The GPX1 Pro198Leu and CAT -262 C > T genotypes distributions in HCC cases and controls stratified by gender and chronic HCV infection are shown in Tables III and IV. Gender and hepatitis C infection did not distort the distribution of the genotypes of GPX1 Pro198Leu for patients when compared with controls (Table III). Overall, this situation is highly reminiscent of our previous results, showing an important interaction between HCV infection and MnSOD Ala/Ala genotype (OR, 5.09; 95% CI, 1.76−14.66) and a lack of effect of patient gender [22]. In contrast, there was significant correlation between HCC and CAT -262 C > T in male (OR = 15.94, 95% CI, 3.48–72.92; $p \leq 0.000001$, see Tables III and IV). Similarly, there was a strong trend associating TT genotype of CAT -262 C > T and HCV-infected patients (Table IV). Albeit non-significant, there was a 12-fold increase in risk for HCC development among HCV-infected patients homozygotes for the T allele (OR = 12.01, 95% CI = 0.64–223.63, Table IV).

Effects of gene-gene and gene-HCV infection interaction on HCC risk

Given that MnSOD, GPX1 and CAT genes are all active on the pathway of ROS detoxification, we hypothesized that an enhanced risk could be detected when unfavourable alleles are combined in a given patient. Consequently, statistical gene–gene interactions between the MnSOD and GPX1, MnSOD and CAT or GPX1 and CAT polymorphisms were examined (Table V). Adjusted ORs (95% CI) for the presence of HCC were obtained by logistic regression.

We observed that HCC patients carrying the MnSOD Ala/Ala genotype were also more often carriers of the the GPX1 Leu/Leu genotype than the controls (9.18 vs 2.25%, $p = 0.059$) suggesting a possible joint effect of MnSOD Ala/Ala and GPX1 Leu/Leu genotypes resulting in an increased risk of liver cancer. We also assessed the presence of an interaction between the MnSOD/GPX1 polymorphisms and HCV infection. We observed a non-significant

Table III. Distribution of GPX1 Pro198Leu and CAT -262C > T genotypes and associated odds ratio in relation to sex.

Genotype	Controls, *n* (%)	HCC cases, *n* (%)	OR (95% CI)*	*p*-value
GPX1 Pro198Leu				
Male				
Pro/Pro	60 (47.24)	30 (50)	1.00	
Pro/Leu	52 (40.94)	22 (36.67)	0.85 (0.44–1.64)	0.622
Leu/Leu	15 (11.81)	8 (26.67)	1.07 (0.41–2.80)	0.895
Female				
Pro/Pro	48 (50.53)	20 (38.46)	1.00	
Pro/Leu	36 (37.89)	10 (19.23)	0.67 (0.28–1.60)	0.361
Leu/Leu	11 (11.58)	6 (11.53)	1.31 (0.43–4.02)	0.638
CAT –262 C > T				
Male				
CC	107 (84.25)	47 (78.33)	1.00	
CT	18 (14.17)	9 (15)	1.14 (0.48–2.72)	0.770
TT	2 (1.57)	4 (6.67)	15.94 (3.48–72.92)	< 0.000001
Female				
CC	66 (69.47)	29 (80.55)	1.00	
CT	27 (28.42)	5 (13.89)	0.42 (0.15–1.20)	0.099
TT	2 (2.11)	2 (5.55)	2.28 (0.31–16.95)	0.411

*Multivariate analysis adjusted for age, HBV and HCV infection.

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Table IV. Distribution of GPX1 Pro198Leu and CAT -262C > T genotypes and associated odds ratio in relation to HCV infection.

Genotype	Controls, *n*. (%)	HCC cases, *n* (%)	OR (95% CI)*	*p*-value
GPX1 Pro198Leu				
Negative				
Pro/Pro	85 (52.79)	24 (60)	1.00	
Pro/Leu	61 (37.89)	13 (32.5)	0.75 (0.36–1.60)	0.462
Leu/Leu	15 (9.32)	3 (7.5)	0.71 (0.19–2.65)	0.607
Positive				
Pro/Pro	23 (37.70)	25 (45.45)	1.00	
Pro/Leu	27 (44.26)	19 (34.55)	0.64 (0.29–1.46)	0.295
Leu/Leu	11 (18.03)	11 (20)	0.92 (0.33–2.52)	0.871
CAT -262 C > T				
Negative				
CC	126 (78.26)	33 (82.5)	1.00	
CT	31 (19.25)	6 (15)	0.74 (0.28–1.92)	0.533
TT	4 (2.48)	1 (2.5)	0.95 (0.10–8.83)	0.967
Positive				
CC	47 (77.05)	43 (76.78)	1.00	
CT	14 (22.95)	8 (14.28)	0.62 (0.24–1.63)	0.335
TT	0 (0)	5 (8.93)	12.01 (0.64–223.63)	0.056

*Adjusted for age, HBV and sex.

trend for an increased risk among MnSOD Ala/Ala and GPX1 Leu/Leu or Pro/Pro genotypes carriers in HCV infected patients with OR of 4.50 (95% CI, 0.73–27.74) and 3.94 (95% CI, 0.63–24.73), respectively. Thus, the risk of HCC might be modified by MnSOD and GPX1 genotypes in interaction with HCV infection.

We proceed to the same analysis for MnSOD and CAT polymorphisms. Individuals homozygous Ala/Ala of MnSOD and TT of CAT have a 13.60-fold increase in HCC risk compared to Val/Val and CC carriers (Table VI). Moreover, when the risk associated with the MnSOD and CAT polymorphisms was further measured in the presence of HCV infection, the joint effects of susceptible genotypes were even more apparent for the Ala/Ala and TT genotype (OR = 11.48, 95% CI , 0.55−241.32; p = 0.036) as well as for the Ala/Ala and CC genotype (OR = 5, 95% CI , 1.45−17.27; p = 0.008). Symetrically, the association of Val/Val MnSOD to CT CAT alleles was, although non-significantly, apparently endowed of a protective activity against liver cancer development (OR = 0.15, 95% CI, 0.02−1.22, p = 0.064, Table VI).

Interestingly, a significant interaction was found between GPX1 and CAT allelic variants. Individuals heterozygous Pro/Leu of MnSOD and TT of CAT have a 18.79-fold increase in HCC risk compared to Pro/Pro and CC carriers (p = 0.005, Table VII).

Discussion

Hepatocellular carcinoma has the fifth incidence among malignant tumours worldwide and is the third cause of cancer-related deaths. Chronic hepatitis B, C and associated liver cirrhosis represent major risk factors for HCC development, being implicated in more than 70% of HCC cases worldwide. Malignant transformation of hepatocytes is believed to occur, regardless of the aetiological agent, through a process of increased liver cell turnover, induced by chronic liver injury and regeneration, in a context of inflammation and oxidative DNA damage [26]. It is believed that oxidative stress plays critical roles in the initiation and progression of hepatocarcinogenesis. In a previous study, Ngoka [27] shows that CAT, SOD and GSH-Px are strongly down-regulated at the protein level in a liver cancer cell line. In the current report, we

Table V. Risk of HCC for combinations of *MnSOD* and *GPX1* genotypes.

MnSOD Ala-9Val	*GPX1 Pro198Leu*	HCC, *n* (%)	Controls, *n* (%)	OR (95% CI)*	*p*-value
Val/Val	Pro/Pro	15 (15.30)	32 (14.41)	1.00	
Val/Val	Pro/Leu	8 (8.16)	38 (17.12)	0.50 (0.17–1.19)	0.104
Val/Val	Leu/Leu	1 (1.02)	7 (7.14)	0.30 (0.03–2.70)	0.264
Val/Ala	Pro/Pro	25 (25.51)	56 (25.22)	0.95 (0.44–2.06)	0.901
Val/Ala	Pro/Leu	15 (15.31)	33 (14.86)	0.97 (0.41–2.30)	0.944
Val/Ala	Leu/Leu	4 (4.08)	14 (6.31)	0.61 (0.17–2.17)	0.442
Ala/Ala	Pro/Pro	12 (12.24)	20 (9)	1.28 (0.50–3.28)	0.607
Ala/Ala	Pro/Leu	9 (9.18)	17 (7.66)	1.13 (0.41–3.11)	0.814
Ala/Ala	Leu/Leu	9 (9.18)	5 (2.25)	3.84 (0.10–13.45)	0.059

*Multivariate analysis adjusted for age, sex, HBV and HCV infection.

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Table VI. Risk of HCC for combinations of *MnSOD* and *CAT* genotypes.

MnSOD Ala-9Val	*CAT -262 C > T*	HCC, *n* (%)	Controls, *n* (%)	OR (95% CI)*	*p*-value
Val/Val	CC	21 (21.43)	58 (26.13)	1.00	
Val/Val	CT	1 (1.02)	18 (8.11)	0.15 (0.02–1.22)	0.064
Val/Val	TT	2 (2.04)	1 (0.45)	5.52 (0.48–64.13)	0.129
Val/Ala	CC	34 (34.69)	84 (37.84)	1.12 (0.59–2.12)	0.732
Val/Ala	CT	8 (8.16)	16 (7.21)	1.38 (0.52–3.70)	0.519
Val/Ala	TT	2 (2.04)	3 (1.35)	1.84 (0.29–11.80)	0.514
Ala/Ala	CC	21 (21.43)	31 (31.63)	1.87 (0.89–3.94)	0.098
Ala/Ala	CT	5 (5.10)	11 (11.22)	1.25 (0.39–4.04)	0.702
Ala/Ala	TT	2 (2.04)	0 (0)	13.60 (0.63–294.93)	0.078

*Multivariate analysis adjusted for age, sex, HBV and HCV infection.

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simultaneously determined three relatively common genetic variants related to oxidative stress (MnSOD Ala-9Val, GPX1 Pro198Leu and CAT -262 C > T) and evaluated their combined effect on HCC. First, we performed a conventional association analysis looking thereby for an association of each polymorphism and HCC. In our previous study, we have shown that patients with at least one Ala-MnSOD allele have a higher risk of hepatocellular carcinoma development than patients with Val/Val genotype [22].

Amongst numerous environmental factors that influence the risk for developing liver cancer, selenium intake is one possible candidate. So far, several studies have reported a significant reduction in liver cancer risk and increased selenium intake [28,29]. As selenium is an important element in glutathione peroxidases (GPXs), a major antioxidant enzyme family, it is reasonable to suggest that it could have chemopreventive effect through the function of GPXs that provide protection against oxidative damage of DNA. Significantly, selenium levels are frequently decreased in the Moroccan population, especially among its less affluent fractions. This situation is known to reduce global GPX activity and may explain partly its low impact compared with that of iron-dependent catalase on liver cancer risk in Morocco [30,31]. In a previous study, Sutton et al. [25] found a positive association between Leu allele and risk of developing HCC in French alcoholic patients with cirrhosis. However, in accordance with our findings, several other groups failed to demonstrate an association between codon 198 variants of GPX1 and cancer risk [32–34].

Catalase is a heme enzyme that has a major role in controlling H_2O_2 concentrations in human cells, by converting H_2O_2 into H_2O and O_2. Working together with other antioxidant enzymes, catalase plays an integral role in the primary defence against oxidative stress. The enzyme CAT is found mainly in the peroxisomes but may also appear in plasma. The current report is the first to evaluate the CAT polymorphism in relation to hepatocellular carcinoma risk. In the present case-control study, we found that patients with CAT TT genotype, resulting in a reduced activity, had a 3.41-fold increased risk of HCC (OR = 3.41, 95% CI, 0.94−12.45; $p = 0.039$). However, because 95% confidence interval marginally overlap with 1.0, our data warrant thus confirmation on a larger series of individuals and in others populations.

Our survey suggests a gender effect of the CAT -262 C > T polymorphism on HCC development. Women with TT genotype had a non-significant 2.28-fold increased risk for HCC (OR = 2.28, 95% CI, 0.31−16.95, $p = 0.411$), whereas the risk of HCC was much higher and highly significant in men with the same genotype (OR = 15.94, 95% CI, 3.48−72.92; $p < 0.000001$). It is possible that the mechanisms responsible for a protective effect

Table VII. Risk of HCC for combinations of GPX1and CAT genotypes.

GPX1 Pro198Leu	*CAT -262 C > T*	HCC, *n* (%)	Controls, *n* (%)	OR (95% CI)*	*p*-value
Pro/Pro	CC	39 (40.63)	82 (36.94)	1.00	
Pro/Pro	CT	10 (10.42)	22 (9.91)	0.95 (0.41–2.21)	0.915
Pro/Pro	TT	1 (1.04)	4 (1.80)	0.52 (0.05–4.86)	0.564
Pro/Leu	CC	13 (13.54)	69 (31.08)	0.39 (0.19–0.80)	0.009
Pro/Leu	CT	1 (1.04)	19 (8.56)	0.11 (0.01–0.85)	0.012
Pro/Leu	TT	4 (4.17)	0 (0)	18.79 (0.98–357.79)	0.005
Leu/Leu	CC	10 (10.42)	22 (9.91)	0.95 (0.41–2.12)	0.915
Leu/Leu	CT	3 (3.13)	4 (1.80)	1.57 (0.33–7.39)	0.560
Leu/Leu	TT	1 (1.04)	0 (0)	6.26 (0.25–157.29)	0.150

*Multivariate analysis adjusted for age, sex, HBV and HCV infection.

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vis-a-vis HCC development in women are due to a higher activity of the CAT compared with men [35]. Alternatively, gender differences can be related to different dietary habits between men and women [36], like a more regular consumption of fruit and vegetables or a lesser addiction to alcoholic beverages [37]. This latter hypothesis is, however, irrelevant to the Moroccan situation for obvious cultural reasons.

Our data show as well that the hepatocellular carcinoma risk is further modulated by the CAT -262 TT genotype in patients with HCV infection. Patients TT without HCV infection had a 0.95-fold risk of HCC (95% CI, 0.10−8.83), whereas those with HCV infection and TT genotype display a remarkable trend toward increased risk of liver cancer (OR = 12.01, 95% CI, 0.64−223.63; $p = 0.023$). It is possible that chronic HCV infection and its excess of ROS production synergize with the lower activity of TT genotype during liver cancer development.

Next, we assessed the combined effect of these polymorphisms on hepatocarcinogenesis based on the working hypothesis that the accumulation of several genetic variations could substantially influence the risk of HCC . In this study, we standardized the contribution of each genetic polymorphism and used the number of pro-oxidant alleles as an index of combined effect of these genes. Given that MnSOD and GPX1 genes are active on the pathway of detoxification of ROS from O^{2-} to H_2O_2 (MnSOD) and further to H_2O (GPX1) and the MnSOD Ala-9Val and GPX1 Pro198Leu polymorphisms influence the efficiency of this detoxification and it is reasonable to expect that their combined effects may be much more important than a single pro-oxydant allele. The combination of the two polymorphisms is, indeed, associated with a significantly increased liver cancer risk. Individuals homozygous for the Ala-9 allele of MnSOD and Leu198 allele of GPX-1 have a 3.84-fold increase in HCC risk compared to Val/Val and Pro/Pro carriers and the *p*-value for the interaction between these genotypes is 0.029. However, as mentioned above, the 95% CI includes the 1.0 value and hence precludes any firmer conclusion. This finding seems to be in line with the well-documented functional relevance of these SNPs in patients with HCC [25] as well as breast cancer [38]. Of note, this risk was more pronounced in patients with the same genotype and HCV infection (OR = 4.50, 95% CI, 0.73−27.74). Further *in vitro* or *in vivo* work should be carried out to examine the differences in ROS levels in cells containing variant GPX1 and MnSOD alleles and infected with HCV.

We examined associations between polymorphisms in MnSOD and CAT genes involved in the oxidative stress response and risk of HCC. Our findings support a major role for MnSOD Ala-9Val and CAT -262 C > T polymorphic association in the susceptibility to HCC. Indeed, this situation is explained by the higher prodution of H_2O_2 by the MnSOD Ala/Ala, accompanied by the lower detoxification capacity of CAT TT genotype. In accordance with this hypothesis, it has been suggested that over-expression of MnSOD could increase production of H_2O_2, which, if not subsequently neutralized and converted to H_2O and O_2, contributes to further generations of ROS and subsequent cell damage [39]. Interestingly, the combination of a low H_2O_2 production capacity (MnSOD Val/Val) to a moderate ability to destroy hydrogen peroxide (CAT CT) is associated in the Moroccan population with a trend to be protected from HCC (OR = 0.15, CI 0.02−1.22, $p = 0.045$).

In Moroccan patients, more than 56% of HCC are caused by the HCV infection alone. Increased production of ROS and other oxidants has been linked to liver damage during HCV infection [40,41]. In our study, stratification based on HCV infection status with combined effect between MnSOD Ala-9Val and CAT -262 C > T polymorphisms showed that patients with HCV are more likely to be carriers of the Ala/Ala and TT genotype (OR = 11.48, 95% CI, 0.55−241.32) and Ala/Ala and CC genotype (OR = 5, 95% CI, 1.45−17.27), although the latter is the only one to be significant. This situation may be explained in that HCV might not only increase ROS generation but also down-regulate certain antioxidant genes, as previously shown with human immunodeficiency virus [42]. In this regard, several surveys on HCV-infected patients have consistently detected a depletion in reduced glutathione, the main intracellular protector against oxidative stress [43,44]. Finally, the combination of a moderate ability H_2O_2 detoxification (GPX1 Pro/Leu) to a low capacity to destroy hydrogen peroxide (CAT TT) is associated in the Moroccan population with a trend to be predisposed to HCC (OR = 18.79, 95% CI 0.98−357.79, $p = 0.005$).

Identification of clinically relevant markers represents a crucial aspect of liver disease research since it will enable early detection of liver diseases and allow us to monitor the degree of liver damage, the response to pharmacological therapies and the development of new therapeutic approaches [45]. Our data provide some support to the hypothesis that polymorphisms that impair anti-oxidative capacity may influence HCC risk. We are, however, fully aware that some aspects clearly warrant further investigations. In summary, future analyses of above mentioned and additional polymorphisms and haplotypes in other oxidative stress genes within large pooled studies will help to clarify their role in hepatocarcinogenesis and may provide a mechanism for the relationship between oxidative stress and HCC. In addition, evaluation of polymorphisms in oxidative stress response genes may provide useful screening and/or prognostic markers for HCC.

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