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RESEARCH ARTICLE

Genetic polymorphisms in antioxidant enzyme genes and susceptibility to hepatocellular carcinoma in Chinese population: a case-control study

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Abstract An increased oxidant burden has been implicated in hepatocarcinogenesis, and several antioxidant enzymes counteract potential oxidative damage. So, polymorphisms in the genes encoding antioxidant enzymes may play an important role in the development of hepatocellular carcinoma (HCC). To test this hypothesis, we investigated the association of polymorphisms in antioxidant enzyme genes, including three superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GPx), with HCC in a Chinese population consisting of 434 HCC patients and 480 control subjects. Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Odds ratios (ORs) and 95 % confidence intervals (95 % CIs) were estimated by unconditional logistic regression. For the *ECSOD* Ala40Thr polymorphism, a significant association was observed between this polymorphism and HCC risk in non-hepatitis B virus (HBV) carriers but not in HBV carriers, and individuals with one 40Thr allele (Ala/Thr genotype) (OR=2.13, 95 % CI=1.25–3.64, *P*=0.006) or at least one 40Thr allele (Ala/Thr and Thr/Thr genotype) (OR=1.90, 95 % CI=1.15-3.15, *P*=0.012) showed significantly higher risk to HCC, compared with Ala/Ala genotype. No significant associations were observed between three other polymorphisms (*MnSOD* Ala16Val, *CAT*-262C/T, *GPx* Pro198Leu) and HCC susceptibility in both HBV carriers and non-HBV carriers. Furthermore, no other signs of combined effects, except for a combined effect of *ECSOD* Ala40Thr and *MnSOD* Val16Ala in non-HBV carriers, were observed for each combination of these four polymorphisms. In conclusion, our results indicate that the antioxidant enzyme gene polymorphisms at least partially contribute to the susceptibility to HCC.

Keywords Hepatocellular carcinoma · Antioxidant enzymes · Polymorphism · Susceptibility

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, especially in Eastern Asia and Southern Africa [1]. Hepatocarcinogenesis is a long-term multistage process with the involvement of both environmental factors and susceptibility genes [2]. Chronic hepatitis and liver cirrhosis associated with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, aflatoxin B1 exposure, cigarette smoking, and alcohol abuse are well-documented major environmental risk factors for hepatocarcinogenesis [3–7]. Furthermore, genetic polymorphisms of genes involving in different steps of carcinogenesis may also determine individual susceptibility to HCC [8]. The identification of susceptibility genes contributing to HCC may help to clarify the pathogenesis of hepatocarcinogenesis and improve the prevention and treatment of this malignancy [9–11].

Accumulating evidences have suggested that reactive oxygen species (ROS) from either endogenous or exogenous insults plays a crucial role in the initiation and promotion of hepatocarcinogenesis [12–16]. The antioxidant enzymes, which form the first line of defense against ROS, have been

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hypothesized to protect against carcinogenesis [14]. The main antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidases (GPx). SOD is a key antioxidant enzyme, responsible for the scavenging of superoxide anion—a precursor of all ROS [16, 17]. On the basis of functional relevance of antioxidant enzymes in the oxidative damage and hepatocarcinogenesis, we hypothesize that the antioxidant enzymes may be the excellent biological candidate susceptibility genes for the HCC. It is expected that the genetic variation within antioxidant enzymes could influence the effects of defense against oxidative stress, which in turn results in genotype-dependent differences in risk of HCC. Several polymorphisms in the *SODs*, *CAT*, and *GPx1* have been well characterized. Including A1934C in SOD1 (rs2234694), Ala16Val in *MnSOD* (rs4880), Ala40Thr (rs2536512), Arg213Gly (rs1799895) in *ECSOD*, C-262 T in *CAT* (rs1001179), and Pro198Leu in *GPx1* (rs1050450) [10, 18–21]. In the present study, we examined whether the polymorphisms within antioxidant enzymes have any bearing on the risk of HCC in Chinese population.

Materials and methods

Patients and controls

This case-control study consists of 434 incident patients with HCC and 480 control subjects. All the subjects were enrolled at the Affiliated Hospital of Luzhou Medical College. At recruitment, informed consent was obtained from each subject, and personal information on demographic factors, medical history, history of cigarette smoking and alcohol drinking, as well as family history of HCC were collected via structured questionnaire. This study was performed with the approval of the Medical Ethical Committee of The Affiliated Hospital of Luzhou Medical College.

Validation of polymorphisms

The six polymorphisms were validated by polymerase chain reaction (PCR) direct sequencing. The screening panel used for polymorphisms validation included DNAs from 96 individuals randomly chosen from the total control population of 480 subjects. The primers and conditions used for amplifying and sequencing the target region containing these six polymorphisms are available on request. Polymorphism candidates were identified by the PolyPhred program (available at http://droog.mbt.washington.edu/PolyPhred.html) and inspected by two observers. Polymorphism positions and individual genotypes were confirmed by reamplifying and resequencing the polymorphism sites from the opposite strand.

Genotyping of polymorphisms

Four polymorphisms, *MnSOD* Ala16Val, *ECSOD* Ala40Thr, *CAT* C-262 T, and *GPx1* Pro198Leu, were genotyped by PCR-based restriction fragment length polymorphism (RFLP) analysis. Genotyping was performed by staff blinded to the subjects' case-control status. The accuracy of genotyping data for polymorphisms obtained from PCR-RFLP analyses was tested by direct DNA sequencing of a 10 % masked, random sample of cases and controls and all results were in 100 % concordance.

Statistical analyses

Genotype and allele frequencies for the polymorphisms were determined by gene counting. The fitness to Hardy-Weinberg equilibrium was tested using the χ^2 test. The association between the genotypes and HCC risk was evaluated by multiple logistic regression analyses while controlling for confounding factors (including age, sex, status of smoking and drinking, and pack-years of smoking), and, the *P* values, odds ratios (ORs), and 95 % confidence intervals (CIs) were calculated. In view of the multiple comparisons in our study, the correction factor $n\times(m-1)$ (n loci with m alleles each) was applied to correct the significance level. An association was considered significant at a *P* value of less than 0.013 (i.e., 0.05 ÷ 4), and all statistical tests were two-sided. We also tested the null hypotheses of multiplicative gene-gene, gene-confounding factor (including age, sex, status of smoking and drinking, and pack-years of smoking) interactions and evaluated deviation from multiplicative interaction models by including main-effect variables and their product terms in the logistic regression model. These analyses were performed using SPSS software (version 9.0; SPSS Inc., Chicago, IL, USA).

Results

With the validation for the six polymorphisms in 96 individuals randomly chosen from the total control population of 480 subjects, we found that the *CuZnSOD* A1934C does not exist and the *ECSOD* Arg213Gly is very rare (with minor allele frequency 0.0052), whereas the minor allele frequencies of the polymorphisms *MnSOD* Ala16Val, *ECSOD* Ala40Thr, *CAT* C-262 T, and *GPx* Pro198Leu is 0.15, 0.36, 0.061, and 0.032, respectively. We therefore selected these four polymorphisms for the subsequent genotyping analysis.

The genotype distributions of the four polymorphisms are presented in Table 1. The observed genotype frequencies of

Table 1 Genotype and allele frequencies of 4 antioxidant enzymes polymorphisms in patients with HCC and controls

	HBV carriers		Non-HBV carriers	
Polymorphisms	Cases/controls	OR (95 % CI)*	Cases/controls	OR (95 % CI)*
MnSOD Ala16Val				
Val/Val	246/143	1.00 (reference)	88/216	1.00 (reference)
Ala/Val	63/36	1.07 (0.67–171)	15/71	0.55 (0.29–1.02)
Ala/Ala	9/6	0.94 (0.32–2.77)	1/7	0.33 (0.04–2.82)
Ala/Val+eAla/Ala	72/42	0.93 (0.59–1.44)	16/78	0.52 (0.28–0.95)
Ala allele	0.13/0.13		0.08/0.14	
ECSOD Ala40Thr				
Ala/Ala	123/74	1.00 (reference)	32/131	1.00 (reference)
Ala/Thr	158/79	1.19 (0.79–1.78)	56/126	2.13 (1.25–3.64)
Thr/Thr	31/30	0.61 (0.34–1.10)	13/35	1.39 (0.64–3.01)
Ala/Thr+Thr/Thr	189/109	1.03 (0.71–1.50)	69/161	1.90 (1.15–3.15)
Thr allele	0.35/0.38		0.41/0.34	
CAT C-262 T				
C/C	273/168	1.00 (reference)	92/264	1.00 (reference)
C/T	27/18	0.98 (0.52–1.87)	7/29	0.67 (0.27–1.65)
T/T	1/0	NA	0/1	NA
C/T+T/T	28/18	0.94 (0.50–1.78)	7/30	0.65 (0.27–1.60)
T allele	0.05/0.05		0.04/0.05	
GPx1 Pro198Leu				
Pro/Pro	282/179	1.00 (reference)	89/275	1.00 (reference)
Pro/Leu	12/9	0.76 (0.28–2.09)	7/18	1.48 (0.57–3.80)
Leu/Leu	0/0	NA	0/0	NA
Pro/Leu+Leu/Leu	12/9	0.76 (0.28–2.09)	7/18	1.48 (0.57–3.80)
Leu allele	0.02/0.02		0.04/0.03	

The frequencies of genotypes are indicated in absolute values. The number of samples genotyped varies because of genotyping failure for some individuals. The HBV carriers are subjects positive for both HBsAg and HBcAb for at least 12 months

CI confidence interval, *HCC* hepatocellular carcinoma, *NA* not applicable, *OR* odds ratio

*ORs and 95 % CIs were calculated by logistic regression and adjusted for age, sex, status of smoking and drinking, and pack-years of smoking. No correction was made for testing multiple polymorphisms

these polymorphisms conformed to the Hardy-Weinberg equilibrium among both cases and controls (all $P>0.05$). In the HBV carriers, on the basis of logistic regression analysis with adjustment for age, sex, status of smoking and drinking, and pack-years of smoking, there were no significant associations between these four polymorphisms and HCC risk. However, in non-HBV carriers, significant associations with the susceptibility to HCC were observed for the polymorphisms *ECSOD* Ala40Thr and *MnSOD* Ala16Val. For the *ECSOD* Ala40Thr, the Ala/Thr genotype (OR=2.13, 95 % CI=1.25–3.64, $P=0.006$) and the Thr allele carriers (Ala/Thr+Thr/Thr genotype) (OR=1.90, 95 % CI=1.15–3.15, $P=0.012$) showed significantly increased HCC risk compared with the Ala/Ala genotype. The associations remained significant even after correction for multiple comparisons. For the *MnSOD* Ala16Val, a decreased risk of HCC was found to be associated with the Ala allele carriers (Ala/Val+Ala/Ala genotype), with the OR being 0.52 (95 % CI=0.28–0.95, $P=0.034$), compared with the Val/Val genotype. However, after correction for multiple comparisons, the association was never again significant.

The associations between these four polymorphisms and the risk of HCC were further examined with stratification by age, sex, status of smoking and drinking, and pack-years of smoking in both HBV and non-HBV carriers. No significant interactions between these confounders and the polymorphisms were observed, even for the significantly associated polymorphism *ECSOD* Ala40Thr (all $P>0.05$, test for homogeneity, Table 2). These results therefore suggest that the confounding factors have no modification effect on risk of HCC related to the genetic polymorphisms.

The gene-gene interaction analyses were also performed for pairwise combination of four polymorphisms in our case-control data set. However, no signs of gene-gene interaction effects on HCC risk in both HBV and non-HBV carriers were observed (data not shown).

Table 2 Conbined effects and gene-gene interactions between four polymorphisms and HCC risk

ECSOD	MnSOD	Cases/controls	*P* value*	OR (95 % CI)*
All			0.052†	
Ala/Ala	Ala/Val+Ala/Ala	23/55		1.00 (reference)
Ala/Ala	Val/Val	129/150	0.012	2.035(1.172–3.532)
Ala/Thr+Thr/Thr	Ala/Val+Ala/Ala	61/65	0.003	2.642(1.403–4.975)
Ala/Thr+Thr/Thr	Val/Val	193/204	0.002	2.323(1.355–3.984)
HBV carriers			0.395†	
Ala/Ala	Ala/Val+Ala/Ala	21/16		1.00 (reference)
Ala/Ala	Val/Val	99/58	0.348	1.434(0.675–3.044)
Ala/Thr+Thr/Thr	Ala/Val+Ala/Ala	48/26	0.231	1.721(0.709–4.178)
Ala/Thr+Thr/Thr	Val/Val	138/82	0.405	1.366(0.655–2.847)
Non-HBV carriers			0.101†	
Ala/Ala	Ala/Val+Ala/Ala	2/39		1.00 (reference)
Ala/Ala	Val/Val	30/92	0.036	4.991(1.111–22.429)
Ala/Thr+Thr/Thr	Ala/Val+Ala/Ala	13/39	0.004	16.380(2.501–107.272)
Ala/Thr+Thr/Thr	Val/Val	55/122	0.004	9.020(2.038–39.923)

The number of samples genotyped varies because of genotyping failure for some individuals. The HBV carriers are subjects positive for both HBsAg and HBcAb for at least 6 months

OR odds ratio, *CI* confidence interval

**P* value, ORs, and 95 % CIs were calculated by logistic regression and adjusted for age, sex, status of smoking and drinking, and pack-years of smoking

† $P_{interaction}$ value

Discussion

In the present study, we examined the associations between the polymorphisms in four antioxidant enzyme genes and HCC risk in patients with or without persistent HBV infection, who were recruited at Fusui County and its surrounding regions at Guangxi province, a well-known high-risk region for HCC located in southern China. A significant association between the *ECSOD* Ala40Thr polymorphism and HCC risk was observed in non-HBV carriers even after correcting for multiple testing. Our results therefore confirmed the initial hypothesis that the polymorphism within certain antioxidant enzyme gene might play a role in mediating susceptibility to the development of HCC.

In a previous study by Ezzikouri et al. [22], it shows that the TT genotype of CAT C-262 T had increased risk to develop HCC in Moroccans (cases=96 and controls=222). However, in the current study, the polymorphism of CAT C-262 T was not associated the risk of HCC. The inconsistent results could be explained by several possible reasons, including the relatively small sample size, the different allele frequency, and the different LD relationships.

The genetic association between the ECSOD Ala40Thr polymorphism and susceptibility to HCC is biologically plausible. ECSOD is found in various tissues including blood vessels, skeletal muscle, and liver, and is considered to be the major extracellular scavenger of superoxide radical [23]. It has been reported that *ECSOD* gene delivery can protect $HepG_2$ cells against reactive oxygen species toxicity and reduce cell necrosis/apoptosis in vitro [24]. Furthermore, *ECSOD* gene therapy may protect liver against tissue damage, formation of large necrotic areas, and apoptosis after paracetamol overdose in mice [25]. Additionally, *ECSOD* gene delivery also protects the mice against lipopolysaccharide-induced acute liver injury [24].

However, no study has so far addressed the functional consequences of *ECSOD* Ala40Thr polymorphism in humans. This polymorphism is located in the amino-terminal domain, where it is thought to work for the tetramerization of the enzyme [26]. The corresponding amino acid in mouse to the Ala at codon 40 in humans is Ala, but Arg in rat, and it has also been reported that the exchange of the Ala by Ser did not affect the physical properties of the enzyme in mouse [27]. Therefore, the Ala40Thr polymorphism seems to be not a functional variant, but a genetic marker for the susceptibility to HCC. In a previous study, the Thr allele of Ala40Thr polymorphism has been shown to be associated with increased susceptibility to type 2 diabetes in the Japanese, supporting the possibility that the Thr allele is associated with the impaired function of ECSOD [28]. In the present study, we also found that the Thr allele is an at-risk allele for HCC (Table 1). Therefore, given the role of ECSOD in defending against oxidative stress, one might expect that individuals who carry the Thr allele and thus have impaired ECSOD activity over a

lifetime, may have a higher susceptibility of developing the HCC.

It is interesting that the association is observed only in non-HBV carriers, but not in HBV carriers. Previous studies showed that MnSOD can be induced by free radical challenge [29] and cigarette smoke [30]. In addition, HBV-transgenic mice with chronic active hepatitis display greatly increased hepatic oxidative DNA damage and increased incidence of HCC [31]. According to these observations, one may predict that there would be associations between polymorphisms of *SODs* and HCC risk in HBV carriers. But our result is reasonable under the assumption that aflatoxin B1 exposure, cigarette smoking, and alcohol abuse resulting in free radical challenge are the major etiology of HCC for non-HBV infected individuals. The findings of our study and the above give support to the hypothesis that there are gene-virus-environmental interactions in the pathogenesis of HCC, and associations between genetic polymorphisms in genes involved in ROS scavenging and HCC susceptibility would be affected by HBV or HCV infection.

The strength of this study is that its design and results include many of the features that are considered to be an ideal association study. These characteristics include a relatively large sample size, the genetic and environmental homogeneity of the study population, adequate adjustment for confounding factors, stringent Bonferroni correction to adjust for multiple comparisons, and exploring the gene-gene interactions, and so on. However, one of its limitations is the absence of information on dietary habits and of the subjects, restricting us from assessing the risk modified by diet. For instance, we should examine the effect of antioxidant intake as was done in the previous study.

In summary, our findings reveal an association between the *ECSOD* Ala40Thr polymorphism and susceptibility to non-HBV-related HCC in non-in a Chinese population. This association became much predominant when considering the combined effect of *ECSOD* Ala40Thr and *MnSOD* Val16Ala. These results may provide support for the importance of antioxidant enzymes in the pathogenesis of non-HBV-related HCC. Nevertheless, the biological function of the *ECSOD* Ala40Thr variants is still obscure. Further studies are needed to elucidate the exact molecular mechanism underlying this association and the interactions between the polymorphisms of antioxidant enzymes and plasma antioxidant status on HCC susceptibility.

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