ORIGINAL ARTICLE

Genetic variations of three important antioxidative enzymes SOD2, CAT, and GPX1 in nonalcoholic steatohepatitis

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Abstract

Background: Nonalcoholic steatohepatitis (NASH) is closely related to reactive oxygen species (ROS). Superoxide anion radicals, the main product of ROS, can be reduced by manganese superoxide dismutase (SOD2) to hydrogen peroxide, which is further reduced by catalase (CAT) and glutathione peroxidase (GPX) to water. We aimed to investigate the association between the most important genetic variants of *SOD2, CAT*, and *GPX1* and susceptibility to NASH.

Methods: A total of 126 adults with liver tissue-verified NASH, 56 patients with liver tissue-verified nonalcoholic fatty liver (NAFL), and 153 healthy controls were enrolled. Their DNA profiles were retrieved for genotype assessment of *SOD2* 47T>C (rs4880), *CAT* -262C>T (rs1001179), and *GPX1* 593C>T (rs1050450) variation.

Results: There were statistical differences between the *SOD2* and *CAT* genotypes across the NASH, NAFL, and control groups, but not *GPX1*. The NASH group had a significantly higher frequency of subjects with *SOD2* C allele (38.8%) compared with the NASL group (25.0%) and the controls (22.9%, $p = 0.010$). Similarly, the NASH group had a significantly higher percentage of subjects with *CAT* T allele (23.0%) compared with the NAFL group (10.7%) and the controls (7.2%, $p = 0.001$). For subjects with both the *SOD2* C allele and *CAT* T allele, 88.2% were in the NASH group. After adjusting for confounders, the *CAT* mutant T allele and *SOD2* mutant C allele were still the highest independent risk factors for NASH (odds ratio [OR] 3.10 and 2.36, respectively). In addition, there was a synergistic effect for those two alleles and the occurrence of NASH with an adjusted OR of 8.57 ($p = 0.030$).

Conclusion: The genetic variations of *CAT* and *SOD2* may increase the risk of NASH, which may aid in the screening of patients who are at high risk of NASH, and offer a potential anti-oxidant targeting route for the treatment of NASH.

Keywords: Catalase; Glutathione peroxidase; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Superoxide dismutase

1. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease around the world today.[1] Some NAFLD patients may progress to nonalcoholic steatohepatitis (NASH), fibrosis or cirrhosis, and even hepatocellular carcinoma (HCC).[1,2] NAFLD is associated with obesity, insulin resistance, diabetes mellitus (DM), hyperlipidemia, and metabolic syndrome.[1,2] The prevalence of NAFLD is found to increase worldwide as the number of people with the aforementioned comorbidities increases. The mechanisms leading to the development of NAFLD are complex. Hepatic steatosis has been proposed as an essential initiator of advanced NAFLD. However, as seen in many animal models of obesity, the accumulation of fat does not necessarily lead to necroinflammation or fibrosis.[3] In humans, only some people with the above-mentioned risk factors will develop advanced NAFLD and NASH.[3] Therefore, it seems other triggering factors are required to initiate the cascade of events that lead to NASH. Oxidative stress has been proposed as one pathogenic factor that could trigger the development of NAFLD.[3–5]

Reactive oxygen species (ROS) can lead to the increase of superoxide anion radicals that can form adducts with cellular nucleophiles, resulting in cell damage and a subsequent inflammatory response.[3–5] However, humans can ameliorate this damage through the effects of antioxidant enzymes, of which manganese superoxide dismutase (SOD2), catalase (CAT), and glutathione peroxidase (GPX) are the most important.[6] Superoxide anion radicals generated within mitochondria can be reduced by SOD2 to hydrogen peroxide, which is further reduced by CAT or GPX to water (Fig. 1). Functional genetic variations in the genes encoding these enzymes may affect their activities and in turn confer different susceptibilities to NAFLD and many other diseases.[7–9] It has been reported that the major single nucleotide polymorphism (SNP) of *SOD2* 47T>C (rs4880) may increase the enzymatic activity.[7–16] In contrast, the most important SNP of and *CAT* -262C>T (rs1001179)[17–25] and *GPX1* 593C>T (rs1050450) [26–31] may reduce the enzymatic activity. Our previous studies have shown that *SOD2* 47T>C genetic variants may increase the risk of drug-induced liver injury, NASH, and alcoholic cirrhosis in Chinese individuals.[10–12] However, little

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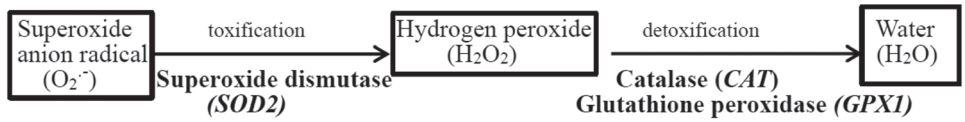

Fig. 1 The major process of antioxidation and detoxification by antioxidant enzyme, superoxide dismutase (SOD2), catalase (CAT), and glutathione peroxidase (GPX).

is known about the influence of the antioxidant enzymes CAT and GPX, on the development of NASH. The present study was undertaken to investigate the association between genetic variants in these three important antioxidant enzymes (SOD2, CAT, and CPX1) and an individual's susceptibility to NASH.

2. METHODS

2.1. Patients and controls

A total of 126 anonymous patients with NASH, 56 patients with NAFL, and 153 healthy adults without NAFLD were recruited from the Biobank of Taipei Veterans General Hospital, Taipei. The inclusion and exclusion criteria for NAFLD were based on guidelines from the American Association for the Study of Liver Diseases[1] and the Asia-Pacific Working Party on NAFLD.[2] The inclusion criteria for patients with NASH were (1) patients who had a liver biopsy with typical NASH findings and a combination of three lesions (steatosis, hepatocellular injury, and inflammation)[1,2] and (2) the steatosis should have a minimum of 5% hepatocytes containing fat droplets.[1,2] The exclusion criteria for NASH were (1) excess alcohol intake (more than an average of 10 g daily or 70 g weekly for women; 20 g daily or 140 g weekly for men)[1,2]; (2) systemic illness known to cause secondary fatty liver diseases, such as Wilson's disease, parenteral nutrition, Reye's syndrome, acute fatty liver of pregnancy, and some inborn error of metabolism; (3) taking medications that may cause steatosis, such as amiodarone, methotrexate, tamoxifen, corticosteroids, or valproates; and (4) hepatitis B or C virus, autoimmune liver diseases, hepatic malignancies, hepato-biliary infections, or biliary tract disease.

The inclusion criteria for patients with NAFL were (1) patients who had a liver biopsy with a typical finding of steatosis but without hepatocellular injury and inflammation[1,2] and (2) steatosis with a minimum of 5% hepatocytes containing fat droplets.[1,2] The exclusion criteria for this group were the same as for the NASH group. The inclusion criteria for the controls were adults with normal liver biochemistry and a normal abdominal ultrasound examination and no habitual alcohol consumption. The exclusion criteria were those with chronic hepatitis B or C virus infection, autoimmune liver diseases, metabolic liver diseases, and hepato-biliary malignancies.

The diagnosis, age, sex, and clinical data of the subjects were input and deposited to the Biobank of our hospital. Then the anonymous procedure was performed by the Biobank, but all the above-mentioned data except the identification and names were reserved and could be retrieved. The current study was approved by the Institutional Review Board of Taipei Veterans General Hospital (approval no. 2012-02-015B, 2015-12-013B, and 2016-07-011B).

2.2. Genotyping of catalase SOD2, CAT, and GPX

The DNAs from patients and controls were retrieved from the Biobank of Taipei Veterans General Hospital. They were genotyped for *SOD2* 47T>C (rs4880), *CAT* -262C>T (rs1001179), and *GPX1* 593C>T (rs1050450) variation using a validated polymerase chain reaction (PCR)-based assay with matrix-assisted laser desorption ionization-time of flight by Agena Mass ARRAY platform with iPLEX gold chemistry (Agena, San Diego, CA). The manufacturer's guidelines were followed and the specific PCR primer and extension primer sequences were designed using the Assay Designer software package (v.4.0, Agena) (Table 1). A total of 1 μL genomic DNA (10 ng/μL) was applied to the Multiplex PCR reaction in 5-μL volumes containing 1 U of Taq polymerase, 500 nmoL of each PCR primer mix and 2.5 mM of each dNTP (Agena, PCR accessory and enzyme kit). Thermocycling was performed at 94°C for 4 minutes, followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, followed by 72°C for 3 minutes. Unincorporated dNTPs were deactivated using 0.3 U shrimp alkaline phosphatase. The single base extension reaction used the iPLEX enzyme, terminator mix, and extension primer mix followed by 94°C for 30 seconds, and 40 cycles of 94°C for 5 seconds, 5 inner cycles of 56°C for 5 seconds, 80°C for 5 seconds, and then 72°C for 3 minutes (Agena, iPLEX gold kit). After the addition of a cation exchange resin to remove any residual salt from the reactions, 7 nL of the purified primer extension were loaded onto the matrix pad of a SpectroCHIP (Agena). The SpectroCHIPs were analyzed using a MassARRAY Analyzer 4, by clustering analysis with TYPER 4.0 software.

2.3. Statistical analyses

The observed and expected gene frequencies were compared using a chi-squared goodness-of-fit test to the Hardy-Weinberg proportion. Odds ratios (ORs) and confidence intervals (CIs) were calculated using logistic regression analyses. The chi-squared test with or without Yates' correction was used for the categorical data. One-way analysis of variance with Scheffe's post hoc multiple comparison was used for continuous data. The possible risk factors for NASH, such as age, body mass index (BMI), DM and hyperlipidemia were adjusted for the risk of NAFLD using multivariate logistic regression analysis. Interaction between *CAT* and *SOD2* genetic variations and the risk of NASH was also assessed using multivariate logistic regression analysis. The goodness-of-fit for the model containing the interaction term (*CAT* × *SOD2*) was compared with that of a reduced model containing indicator variables of the main effects of the *CAT* and *SOD2* genotypes. The statistical tests

Table 1

Primers for the genotyping

ID	Sequence
SOD2	
rs4880_forward	ACGTTGGATGTTGATGTGAGGTTCCAGGGC
rs4880_reverse	ACGTTGGATGTTTCTCGTCTTCAGCACCAG
rs4880_uep	GCCCAGATACCCCAAA
CAT	
rs1001179_forward	ACGTTGGATGCTGAAGGATGCTGATAACCG
rs1001179_reverse	ACGTTGGATGCAGCAATTGGAGAGCCTCG
rs1001179_uep	GCCCTGGGTTCGGCTAT
GPX1	
rs1050450_forward	ACGTTGGATGATCGAGCCTGACATCGAAGC
rs1050450_reverse	ACGTTGGATGTCATAGATGAAAACCCCCCC
rs1050450_uep	CCTGCTGTCTCAAGGGC

Uep = unextension primer.

Table 2

Clinical characteristics of patients with NASH, NAFL, and healthy controls

	NASH (n = 126)	NAFL (n = 56)	Controls (n = 153)	*p*
Gender (F/M)	55/71	17/39	74/79	0.067
Age, y	51.1 ± 9.0	50.5 ± 6.3	45.9 ± 11.0	< 0.001
Body mass index, kg/m^2	27.8 ± 3.3	25.8 ± 2.2	23.8 ± 2.7	< 0.001
ALT, U/mL	143.7 ± 45.9	94.4 ± 27.0	26.9 ± 5.9	< 0.001
AST, U/mL	116.9 ± 35.9	80.6 ± 27.5	31.9 ± 6.6	< 0.001
Diabetes mellitus	33 (26.2%)	20 (35.7%)	0 (0.0%)	< 0.001
Hyperlipidemia	37 (29.4%)	14 (25.0%)	0 (0.0%)	< 0.001

Variables are expressed as mean ± SD.
ALT = alanine aminotransferase; AST = aspartate aminotransferase; NAFL = nonalcoholic fatty liver; NASH = nonalcoholic steatohepatitis.

Table 3

Genetic variations of *SOD2*, *CAT*, and *GPX1* in patients with NASH, NAFL, and controls

Genetic variations	NASH (n = 126)	NAFL (n = 56)	Controls (n = 153)	*p*
SOD2				
TT	77 (61.2%)	42 (75.0%)	118 (77.1%)	0.019
TC	41 (32.5%)	10 (17.9%)	32 (20.9%)	
CC	8 (6.3%)	4 (7.1%)	3 (2.0%)	
With C allele	49 (38.8%)	14 (25.0%)	35 (22.9%)	0.010
CAT				
CC	97 (77.0%)	50 (89.3%)	142 (92.8%)	0.003
CT	26 (20.6%)	5 (8.9%)	11 (7.2%)	
TT	3 (2.4%)	1 (1.8%)	0 (0%)	
With T allele	29 (23.0%)	6 (10.7%)	11 (7.2%)	0.001
GPX1				
CC	96 (76.2%)	43 (76.8%)	124 (81.0%)	0.671
CT	26 (20.6%)	10 (17.9%)	26 (17.0%)	
TT	4 (3.2%)	3 (5.3%)	3 (2.0%)	
With T allele	30 (23.8%)	13 (23.2%)	29 (19.0%)	0.582
SOD2 C allele + CAT T allele	15 (11.9%)	2 (3.6%)	0 (0%)	<0.001

NAFL = nonalcoholic fatty liver; NASH = nonalcoholic steatohepatitis.

were based on two-tailed probability and a p value <0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL).

3. RESULTS

There were no statistically significant differences between the gender ratios of the NASH, NAFL, and control groups. However, the NASH and NAFL groups had an increased age, BMI, serum ALT and AST levels, and incidence of DM and hyperlipidemia compared with the control group (Table 2). The NASH group also had a higher BMI, ALT, and AST compared with the NAFL group. The *SOD2*, *CAT*, and *GPX1* genotypes under investigation were in Hardy-Weinberg equilibrium in the NASH, NAFL, and control groups (p > 0.05 in each group).

There were statistically significant differences in the incidence of *SOD2* TT, TC, and CC genotypes among the NASH, NAFL, and control groups (p = 0.019, Table 3). The NASH group had a higher frequency of subjects with *SOD2* C allele (38.8%) compared with the NAFL group (25.0%) and the controls (22.9%, Table 3). Similarly, there were statistically significant differences in *CAT* CC, CT and TT genotypes between the NASH, NAFL and control groups (p = 0.003, Table 3). The NASH group had a notably higher percentage of subjects with *CAT* T alleles (23.0%) compared with the NAFL group (10.7%) and the controls (7.2%, Table 3). However, there was no statistically significant difference between the frequency of *GPX1* genotypes or mutant alleles among the three groups (Table 3). For subjects with both the *SOD2* C allele and *CAT* T allele, 88.2% were in the NASH group.

Table 4

Multivariate logistic regression analysis of *SOD2* and *CAT* genetic variation in the susceptibility to NASH

Parameters	Odds ratio	95% confidence interval	*p*
CAT T allele	3.10	1.39-6.89	0.006
SOD2 C allele	2.36	1.31-4.25	0.004
Age	1.05	1.01-1.08	0.005
Body mass index	1.40	1.27-1.53	<0.001
Diabetes mellitus	1.67	0.71-3.95	0.241
Hyperlipidemia	2.16	0.89-5.26	0.09

NASH = nonalcoholic steatohepatitis.

Table 5

Interaction of *SOD2* and *CAT* genetic variation in the susceptibility to NASH

Parameters	Odds ratio	95% CI	*p*
CAT T allele X *SOD2* C allele	8.57	1.24-59.4	0.030
CAT T allele	0.18	0.01-2.42	0.194
SOD2 C allele	0.21	0.02-1.93	0.168
Age	1.05	1.01-1.08	0.006
Body mass index	1.40	1.28-1.54	<0.001
Diabetes mellitus	1.75	0.74-4.13	0.200
Hyperlipidemia	2.11	0.85-5.22	0.106

NASH = nonalcoholic steatohepatitis.

After adjustment for confounders (age, BMI, DM, and hyperlipidemia), the *CAT* mutant T allele and *SOD2* mutant C allele were significant risk factors for NASH (OR: 3.10 and 2.36, respectively; Table 4). Among the confounding factors, age and BMI were also found to increase an individual's susceptibility to NASH (Table 4).

Table 5 shows the synergistic effect of *CAT* T allele and *SOD2* C allele in susceptibility to NASH. The patients who possessed these two mutant alleles may have a significantly higher risk of NASH, with an adjusted OR of 8.57 (95% CI, 1.24-59.4, p = 0.030).

4. DISCUSSION

In this genetic association study, the most cited functional SNP of the three most crucial antioxidant enzymes in humans were assayed to elucidate their potential role in NASH. It was found that *SOD2* 47T>C (rs4880) and *CAT* -262C>T genetic variations were significantly associated with an increased risk of NASH.

The T to C substitution at position 47 in *SOD2* causes an alanine for valine substitution, which may modulate the helical structure of the mitochondrial targeting sequence, and thus increase the *SOD2* import into the mitochondrial matrix.[6–8] This SNP can cause a rise in enzyme activity and has been found to increase a patient's susceptibility to many diseases, including various cancers and liver diseases.[7–16] Some studies have shown

that *SOD2* rs4880 wild-type T allele is associated with a higher risk of NASH.[16,17] Interestingly, studies from France[7,8] and the present study have found that the *SOD2* mutant C allele may increase an individual's susceptibility to NASH. Different findings for the role of *SOD2* in fatty liver diseases exist between these studies. Namikawa et al[16] and Ahn et al[17] hypothesized that *SOD2* rs4880 C variant has a higher efficiency for detoxifying superoxide anion radicals and may decrease the risk of NASH. However, the French group speculated that although *SOD2* C variant may detoxify more superoxide anion radicals, it also produces more toxic intermediates and hydrogen peroxide.[7,8] The fast gathering of hydrogen peroxide may then overwhelm the subsequent detoxification process by GPX and CAT and induce liver injury.[7,8] Whether the liver injury induced by hydrogen peroxide is more severe than the injury caused by superoxide anion radicals is debatable. Further studies are warranted to determine the influence of superoxide anion radicals and hydrogen peroxide on NAFLD. The small sample size, different lifestyles, and ethnic differences affecting the *SOD2* genetic frequency are other possible explanations for the discrepancies between the studies.

CAT is the key enzyme for the removal of hydrogen peroxide after SOD2.[18–20] Numerous polymorphisms have been found in the promoter, 5′and 3′- untranslated regions, exons and introns of *CAT*. In the promoter region of the *CAT* gene, there is a common C to T substitution at position -262 (rs1001179).[18–20] This SNP may influence CAT transcription by modulating the transcription factor binding position and thus changing basal CAT expression in various cell types as well as the overall CAT level.[19] The association between this SNP and CAT concentrations in the blood was first demonstrated by Forsberg et al,[18] who showed that carriers of mutant T alleles display higher CAT levels compared with wild C alleles. In contrast, other studies have found that CC homozygotes had higher CAT activity compared with those with CT or TT genotypes,[19,20] and these findings are now generally accepted.

Significant correlations between *CAT* genetic variants and various diseases have been reported.[21–25] It has been shown that the *CAT* rs1001179 T variant plays a role in glucose disorders and may be a risk factor for metabolic diseases, such as impaired glucose tolerance, insulin resistance, DM, hypertension, and dyslipidemia.[15,21,22] This genetic variant was also found to be associated with an increased susceptibility to hepatitis C, liver cirrhosis, HCC, autoimmune liver disease, and many other cancers.[23–25] To the best of our knowledge, the preset study is the first to demonstrate an association between *CAT* -262C>T variation and NASH. Further studies in other ethnic populations with a larger sample size are warranted to validate this finding.

GPX1 is the main GPX in the mammalian liver, and it plays a significant role in preventing mitochondrial oxidative stress.[26] The major functional genetic variation of *GPX1* is a 593C>T polymorphism (rs1050450), which changes the proline to a leucine.[27] The presence of a leucine at this position has been shown to reduce enzyme activity by 40%.[26] This SNP has been implicated in the development of many common and complex diseases, including cancers and cardiovascular diseases.[27–31] The potential association between GPX1 and the risk of NAFLD was studied in a Chinese cohort,[30] which revealed that the *GPX1* T/T genotype was significantly higher in NAFLD cases, compared with healthy controls. The present study could not verify this association. This may be because at low hydrogen peroxide concentrations, GPX is responsible for its degradation. Whereas CAT plays an important role in removing higher intracellular hydrogen peroxide concentrations.[16] The present study focused on patients with NASH, who may have more severe steatosis/inflammation and higher hydrogen peroxide levels compared with those with NAFL. Therefore, the role of CAT may mask that of GPX.

Advanced NAFLD or NASH may progress to liver cirrhosis and HCC, which has now become the top liver disease worldwide. In addition to weight reduction, exercise, diet control, adequate treatment of DM and hyperlipidemia, many pharmacological therapies have recently been tried. However, at present, there is not a satisfactory treatment agent for NAFLD.[1,2] The present study may help support a potential therapeutic approach to this crucial liver disease.

A limitation of the current study was the limited number of enrolled cases. However, it is not easy to collect patients with NASH and NAFL that has been verified by liver pathology as in the present study. Abdominal sonograms were used as the major diagnostic tool in most of the previous relevant studies of NAFLD, but these cannot differentiate between NASH and NAFL. The gold standard for the diagnosis of NASH is liver pathology, as used in the present study. Nevertheless, further large-scale studies in other ethnic populations are warranted to confirm the association between *SOD2*/*CAT* genetic variations and NASH. The second limitation of this study was that NAFLD activity score (NAS) was not available in the pathological reports in most of the patients; therefore, we could not evaluate the association of genetic SNPs and the NAS severity in this study. The third limitation was that the enzymes activities were not measured in this study, which lessen the robustness of the study.

In conclusion, genetic variations of antioxidative enzymes *SOD2* rs4880 47T>C and *CAT* rs1001179 -262C>T may affect the disposition of ROS and increase the risk of advanced NAFLD and NASH in the Chinese population. These antioxidant enzymes and their SNPs may be a potential target for therapeutic approaches to NASH.

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