



MnSOD GENE POLYMORPHISM INCREASE THE RISK OF NONALCOHOLIC FATTY LIVER DISEASE IN WEST INDIAN POPULATION

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ABSTRACT

Genetic predisposition plays a role in the pathogenesis and progression of non-alcoholic fatty liver disease (NAFLD). Oxidative stress is major contributors in the pathogenesis of NAFLD. The aim of this study was to determine effects of MnSOD (Val16Ala) single nucleotide polymorphism (SNP) with the development NAFLD in West Indian population. 35 patients with NAFLD and 35 control subjects were enrolled matched according to age, gender and ethnicity. NAFLD was confirmed by ultrasonographic examination of the liver. Liver functions, blood glucose, serum insulin, lipid profile were assessed and HOMA-IR calculated. Genomic DNA was isolated from blood & Polymerase chain reaction- restriction fragment length polymorphism was used to determine SNP genotyping of MnSOD. In the present study BMI, ALT, AST, Fasting insulin, HOMA-IR, LDL-cholesterol, were significantly higher in patients with NAFLD than controls. The frequency of Val16Ala MnSOD polymorphisms in NAFLD patients was significantly higher in comparison with control subjects. The homozygous Ala/Ala genotype of the Val16Ala MnSOD was most frequent among patients with NAFLD (45.71%) as compared to control subject (8.57%). The highest Ala allele frequency was observed in the NAFLD patient group (61.43%) and this was statistically significant (p value < 0.001, OR = 0.23, 95% CI = 0.29- 0.68) compared to control healthy subjects (27.84%). These findings supported the idea that the Val16Ala MnSOD polymorphism contributes to the susceptibility to NAFLD in group of West Indian patients.

KEY WORD: NAFLD, MnSOD, Genetic polymorphism, Oxidative stress

INTRODUCTION

NAFLD (Non- Alcoholic Liver Disease) is an epidemic complex disease trait and one of the rapid emerging as the important cause of chronic liver disease globally. NAFLD is recognized as the most common cause of liver dysfunction worldwide. Clinically it is a chronic liver condition characterized by fat deposition in the liver that may cause hepatic inflammation and progressive scarring leading to nonalcoholic steatohepatitis (NASH) and irreversible liver damage (cirrhosis). Various risk factors such as insulin resistance (IR), obesity, type 2 diabetes mellitus, nutritional and life style have been associated with NAFLD. Pathogenesis of NAFLD is associated with insulin resistance, increased oxidative stress and abnormal fat metabolism but not fully understood.^[1]

Genetic factor play a significant role in the development and progression of NAFLD. The prevalence of the NAFLD varies markedly in diverse populations. It ranges between 20%-30% in the Western countries, 20%-30% in Europeans, 8% in Japanese and 25%-30% in Indians.^[2] With the socio-economic transition, Indian population vulnerable to non-alcohol related liver disease is rising rapidly. The occurrence of this disease increased to 67%– 75% in the obese population compared to 20%– 30% in the general population.^[3]

Role of oxidative damage in development of NAFLD has been reported. Apoptosis of hepatocytes and liver injury produced due to excessive free fatty acid oxidation leading to oxidative stress. Mitochondria are a major site for (reactive oxygen species) ROS species generation (90%).

Increased amounts of ROS generate lipid peroxidation likely damages intracellular membranes, leading to apoptosis and necrosis of hepatocytes. Free radicals and Reactive oxygen species are scavenged by Manganese superoxide dismutase (MnSOD) in mitochondria.^[4]

Genetic variability of MnSOD gene influence the activity and expression level may be associated with susceptibility for the development of NAFLD. Chromosome 6q25.2 contains Mn-SOD gene. Human Mn-SOD with dimorphism Ala-9Val (C1183T) and Val16Ala in mitochondrial targeting sequences (MTS) modify their structure configurations which influence the transport of enzyme to mitochondria. TT genotype in the *MnSOD* gene is associated with reduced efficiency in the transport of MnSOD in mitochondria which confers susceptibility for NAFLD.^[5] Reduced Mn-SOD activity due to SNP in Mn-SOD gene is good candidate modifier of NAFLD severity. Paradoxically, the role of *ala-SOD2* variant has been suggested in development of severe alcoholic liver disease in many studies but not all studies confirm this.^[6,7,8] Polymorphism in *val-SOD2* gene was associated with other oxidative stress related conditions including type II diabetic nephropathy in a West Indian population^[9] and in a Swedish.^[10]

Genome wide analysis has also investigated the role of various other candidate genes in development of NAFLD. Genetic variation in *ADIPOR2* SNP (rs767870) is also responsible for accumulation of large amount of fat in hepatocytes.^[11] Reduced plasma adiponectin levels in NAFLD patients have been associated with the C161T *PPARG* SNP in a Chinese population.^[12] Polymorphic variants including-G308A and -G238A of *TNF-α* gene have been shown to be strongly associated with the risk of developing NAFLD in Chinese population. *PNPLA3* gene with SNP rs738409 C/G (I148Met) is also correlated with the presence of NAFLD in Egyptian Patients.^[13] Product of *MTP* gene is involved in TG-VLDL assembly. G-493T genetic variation in promoter region of *MTP* gene was found to be associated with development of NASH in 63 Japanese biopsy-proven NASH patients.^[5] Therefore, recent research on NAFLD needs to focus on understanding genetic susceptibility which aid in better management of the disease.

Therefore, the present study was carried out to investigate the association of the SNP Val16Ala in Mn-SOD genes with the development of NAFLD in Western Indian population.

MATERIALS AND METHODS

Subjects: Total of 35 (23 females and 12 males) participants with Ultrasonographically as well histologically proven NAFLD and 35 healthy (nonsmoking, nonalcoholic subjects) subjects are enrolled in present study. All control subjects (Non-alcoholic subject) were recruited with normal liver function and no viral hepatitis infection by blood test.

Alcohol usage history and histology investigation suggestive of another liver disease are excluded from study. Written informed consent was obtained from all participants after explaining the objectives and details of the study. The study was approved by the Human Ethics committee of Shri A. N. Patel PG Institute of Charotar Education Society. Patients attending OPD and indoor in Department of Gastroenterology, Jeevandeep Hospital, Anand were screened for NAFLD. Consecutive patients with NAFLD was confirmed by histologically or ultrasound.

Routine laboratory investigation: All participated subjects were evaluated thoroughly at inclusion with anthropometric measurements (body mass index) and biochemical tests (liver function profile, HOMA-IR, serum insulin). The presence of fatty liver in all patients was confirmed by an ultrasound of abdomen and also to exclude any other hepatobiliary disease. Serum triglycerides, HDL-cholesterol, LDL-cholesterol, alanine transaminase and aspartate aminotransferase were estimated using commercial kits. Fasting serum insulin was determined using a commercial solid phase chemiluminescent enzyme immunometric assay. Insulin resistance was calculated for both patients and controls using the *HOMA-IR* value. The homeostasis model assessment method (*HOMA-IR*) = [fasting insulin (μ U/mL) $\times$ fasting glucose (mg/dL)/405].

DNA isolation: Blood was collected in the EDTA coated Vacutainer tube from all subjects and plasma was separated from blood cells by centrifuging at 3000rpm for 10 minutes. Genomic DNA was isolated using the G-Biosciences DNA extraction kit (OmniPrep™ Himedia Kit) from peripheral blood leukocytes from patient's and control's samples. Isolated genomic DNA was stored at -20 °C in TE buffer (pH-7.4) for the further analysis. The DNA yield and purity was determined by using U.V. Spectrophotometer at A260/A280 nm. Quality of DNA was also determined on 0.8% agarose gel electrophoresis.

Genotyping: Polymorphisms in MnSOD gene were detected by Polymerase chain reaction- Restriction Fragment length polymorphism (PCR- RFLP) in each DNA sample. Val16Ala polymorphic site (C-T substitution at 16th position) in MnSOD was amplified using following specified primer set describe previously.

Sense primer: 5'-GCTGTGCTTTCTCGTCTTCAG-3' and antisense primer: 5'-TGGTACTTCTCCTCGGTGACG-3'. The amplification reaction mixture (25 μ L) consisting of 200 ng genomic DNA, 500nM of each primer, 0.2 mM of each dNTPs, 1.0 units of Taq polymerase in PCR buffer containing 1.5 mM $MgCl_2$. The PCR reaction was carried out in Thermal Cycler (Thermo scientific) after an initial step of denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94° C for 30 s, 60° C for 30 s, (Annealing Tem), 72° C for 30 s (extension) in case of

Val16Ala. The PCR product of 267 bp has been verified through electrophoresis in 3% agarose gel. The amplified PCR product was digested for 2 hr at 60° C with restriction enzyme *Bsa*WI to detect Val16Ala polymorphism in Mn-SOD. Restriction fragments were then separated on 3% agarose gel or DNA PAGE and sized by comparison to a DNA step ladder.

Statistical analysis: Parametric quantitative data were presented as mean $\pm$ SD and compared by Student's *t* test. All data were analyzed using SPSS version 8.0 statistical package. Hardy-Weinberg equilibrium was determined for these polymorphisms within the control group using χ^2 test. Qualitative data were expressed as frequency and percentage. Odds ratio (OR) with 95% confidence Risk estimate was done by odds ratio. *P* value was considered significant at 0.05.

RESULTS

Demographic and Clinical Characteristics: The demographic and clinical data of the 35 patients with NAFLD and 25 healthy subjects are given in Table 1. A total 70 subject, including 35 NAFLD patients (26 M and 9 F) and 35 healthy control (20 M, 15 F) belonging to same ethnicity were analyzed for MnSOD gene polymorphism and its role in the development of Non-alcoholic fatty liver diseases (NAFLD). The mean age of NAFLD cases and control subject was 42 ± 9.7 and 48 ± 7.9 years respectively. NAFLD patients shown a significantly higher BMI (31.02 ± 1.14) compared to control subject (24.36 ± 3.97). Statistical analysis showed significant difference ($P < 0.05$) in AST and ALT activity between group of NAFLD patients and control group. Parameters of lipid profiling like serum TG, HDL-cholesterol, and LDL-cholesterol was found to be higher significantly compared to control subject. There was no significant difference in age found among control and NAFLD cases. Higher HOMA-IR value in patients with NAFLD (5.54 ± 4.55) compared to control subjects

(1.86 ± 1.19) indicates insulin resistance developed in patients with NAFLD.

Mn-SOD Polymorphisms: PCR product (207 bp) digested by *Bsa*WI results in two fragments (163 bp and 44 bp) when C $\rightarrow$ T substitution in at 16th nucleotide of (Val16Ala) of Mn-SOD gene. RFLP pattern of MnSOD gene with *Bsa*WI exhibited in Fig.1. Restriction patterns of PCR product by the RFLP method are presented in Table 2. The genotyping and allelic frequency of Val16Ala polymorphism of Mn-SOD gene in patients with NAFLD and controls is presented in Table 3. The genotype distribution of MnSOD gene (Ala9Val) variants were in Hardy Weinberg equilibrium in control group ($P < 0.05$). Allele distribution of Mn-SOD gene polymorphism was first analyzed using 2 x 2 tests. The significant value (*P* value), odd ratio with 95% Confidence Interval, Risk ratio, and Chi square are shown in Table 4 for comparative analysis of alleles and genotyping in study groups. Comparative analysis of resulting genotypes between patients and control showed significant association between Ala/Ala genotypes and NAFLD.

In case of MnSOD gene, a significantly higher frequency (45.71% vs. 8.57%) of homozygous Ala/Ala genotypes as well as Ala Alleles (61.43% vs. 27.84%) was observed in patients with NAFLD compared with control subject respectively. The Ala/Ala genotype frequency was significantly ($P < 0.001$, OR = 0.079, 95% CI = 0.07-0.61, RR = 0.21) higher than Val/Val genotypes in between NAFLD patients and control subject. The allele frequency distribution showed a significantly ($P < 0.001$, OR = 0.23, 95% CI = 0.29-0.68, RR = 0.44) higher frequency of the Ala allele in the NAFLD patient group as compared to control subject. In NAFLD cases, presence of Ala polymorphic variants was observed to be significantly associated with development of NAFLD.

Table 1: Demographic and Clinical Characteristics of control and NAFLD patients

Characteristics	Control	NAFLD patients
n (f/m)	25 (10/15)	25 (4/21)
Age (years)	42 ± 9.7	48 ± 7.9
Weight (kg)	62.81 ± 15.39	77.46 ± 16.92
BMI (kg/m^2)	24.36 ± 3.97	$31.02 \pm 1.14 \uparrow$
Systolic blood pressure (mmHg)	107 ± 11.81	112.56 ± 14.56
Diastolic blood pressure (mmHg)	64 ± 10.57	66.18 ± 5.10
Total cholesterol (mg/dL)	159.60 ± 20.99	$192.88 \pm 9.63 \uparrow$
Triglycerides (mg/dL)	108 ± 10.15	$135.53 \pm 6.83 \uparrow$
LDL-cholesterol (mg/dL)	82.32 ± 8.46	$102.01 \pm 12.44 \uparrow$
HDL-cholesterol (mg/dL)	62.65 ± 6.24	$42.23 \pm 9.29 \uparrow$
Fasting glucose (mg/dL)	83.68 ± 7.96	89.46 ± 8.96
Fasting insulin ($\mu\text{IU}/\text{Ml}$)	8.98 ± 5.16	$18.97 \pm 6.27 \uparrow$
AST (U/L)	24.25 ± 6.43	$36.17 \pm 5.43 \uparrow$
ALT (U/L)	19.93 ± 8.95	$45.26 \pm 11.51 \uparrow$
HOMA-IR	1.86 ± 1.19	$5.54 \pm 2.55 \uparrow$

Results are expressed as means $\pm$ SD in parenthesis. BMI; body mass index, AST: aspartate transaminase,

ALT: alanine aminotransferase, FBS: fasting blood sugar, LDL-c: low density lipoprotein, HDL-c: high

density lipoprotein, HOMA-IR (homeostatic model assessment-Insulin Resistance). All control subjects had

normal values. † $P < 0.05$ when compared to control group.

Table 2: PCR-RFLP protocol of polymorphism (Val16Ala) of MnSOD gene

Polymorphism	Set of Primers	Annealing Tem. (°C)	Size of PCR product	RFLP pattern
Val16Ala	Forward Primer: 5'-GCTGTGCTTTCTCGTCTTCAG-3' Reverse Primer: 5'-TGGTACTTCTCCTCGGTGACG-3'	60° C for 30 s	267 bp	VV : 267 bp VA : 267 bp, 163 bp, 44 bp AA : 163 bp, 44 bp

Table 3: Genotyping and allele frequency of Val16Ala polymorphism in MnSOD gene between control and NAFLD cases

Polymorphic genotype	Control	Preterm
Val/Val	19 (54.29)	08 (22.86)
Val/Ala	13 (37.14)	11 (31.43)
Ala/Ala	3 (8.57)	16 (45.71)
Alleles		
Val	51 (72.86)	27 (38.57)
Ala	19 (27.84)	43 (61.43)

Table 4: Association between the genotypes of Val16Ala polymorphism in MnSOD gene and risk of NAFLD

Genotypes /Allele	p-value	χ^2	OR (95% CI)	Risk ratio
VV vs. VA	0.23	1.43	0.49 (0.39 -1.24)	0.70
VV vs. AA	0.0003	13.31	0.079 (0.07-0.61)	0.21
V Allele vs. A Allele	< 0.001	16.67	0.23 (0.29-0.68)	0.44

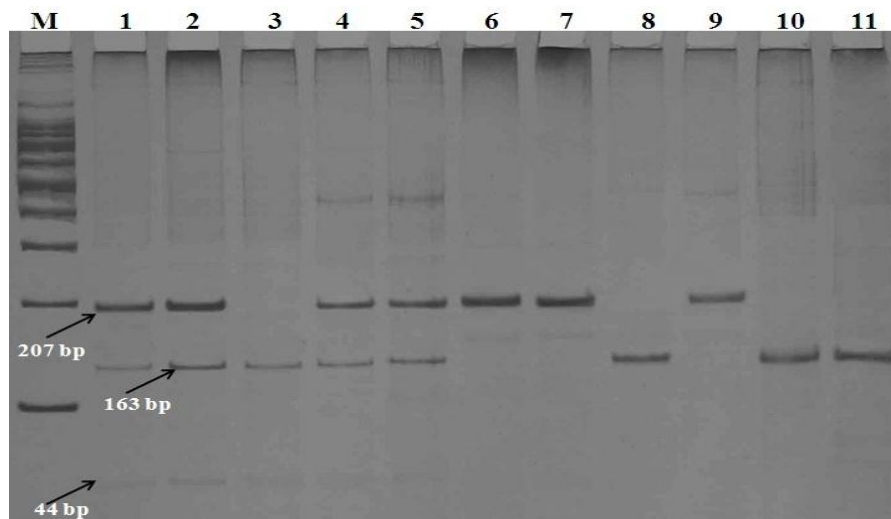


Figure 1: Genotyping of MnSOD gene (Val16Ala) polymorphism observed using PCR-RFLP technique after digestion with *Bsa*WI. Lane 1: 100 bp DNA Marker, Lane 1, 2, 4, and 5: heterozygous Val/Ala genotypes, Lane 6, 7, 9: homozygous Val/Val genotype, Lane 3, 8: homozygous Ala/Ala genotype.

DISCUSSION

There has been increasing attention that genetic factors are implicated in the pathogenesis and progression of NAFLD. Based on this, present study was carried out to determine role of Val16Ala MnSOD polymorphisms with NAFLD patients in a cross sectional study in West Indian population. This study shows a significant association between Val16Ala gene polymorphism and development of NAFLD to a high and significant level in the West Indian population for the first time.

Moreover, the BMI was found to be higher in NAFLD patients compared to control subjects in the present study. Body mass index is a known independent predictor of the degree of hepatic fatty infiltration. It is reported that higher BMI was only causative factor for NAFLD.^[14] The results of the present study indicated that carriers of the Ala allele had higher serum levels of transaminases (ALT and AST) and this was observed in the NAFLD patients and control subjects. A Subject with Ala allele with NAFLD had significantly higher serum levels of transaminases (ALT and AST) was reported in

the present study indicated this Ala allele affect the activity and level of this liver enzyme. Increased HOMA-IR value in patients with NAFLD in our data which is a key feature of the metabolic syndrome strongly associated with NAFLD.^[15] Trend toward higher levels higher level of BMI, AST and ALT, triglycerides, glucose, insulin and the homeostasis model assessment of insulin resistance was observed in NAFLD patients compared with healthy controls.^[16]

Perhaps, the most significant result showed here was related to the influence of the NAFLD and Val16Ala MnSOD polymorphism interaction on HOMA-IR value and insulin resistance. Potential effect of Val16Ala MnSOD polymorphism on HOMA-IR level and development of insulin resistance was analyzed previously could be correlated with result of the present study.^[17] Insulin resistance is a common contributory factor in the pathogenesis of NAFLD, but the primary mechanisms are far from being understood. Excess lipids accumulate determining high levels of TG, LDL-cholesterol found in subject with NAFLD that can trigger cell dysfunction and/or cell death, a response known as lipotoxicity.^[18] The increased LDL cholesterol levels observed in patients carrying the Ala allele are consistent with recent data indicating that Val16Ala genotype of SOD2 effects lipoprotein metabolism.^[19]

Biochemical mechanism of Val16Ala MnSOD polymorphisms which increase the susceptibility to NAFLD and its histological influence in hepatocytes during progression of NAFLD still remains to be fully elucidated. We found that the frequency of the Val16Ala MnSOD polymorphisms was significantly higher in patients with NAFLD than in controls. The homozygous Ala/Ala genotype was more frequent among the patients with NAFLD (61.43%) when compared to subjects. Higher incidence ($p=0.016$) of Val16Ala MnSOD polymorphism in NASH patients was reported in a study carried out by Namikawa *et al.*^[5] Our results are in agreement with results found by Trujillo-Murillo *et al.*^[20] who reported a higher frequency of Val16Ala MnSOD polymorphisms in Patients with Non-Alcoholic Steatohepatitis from Northeast Mexico. Accordingly, patients with NAFLD had a higher frequency of the Ala allele and the Ala/Ala genotype compared to controls in the present study. The contradictory results were also reported that homozygosity for a Valine to alanine substitution in the MTS doesn't influences the susceptibility to alcohol-induced liver fibrosis and also not associated with alcohol-induced oxidative stress.^[8] Along with MnSOD polymorphisms, other genetic factors like PNPLA3 and TNF- α promoter region G238A polymorphisms has confirmed it's the association with susceptibility to NAFLD and its progression.^[13, 21]

There is emerging evidence which indicates that Hepatic mitochondria play a vital role in the development and pathogenesis NAFLD.^[22] Valine-to alanine amino acid change due to the Val16Ala polymorphism change

affects the secondary structure of the MTS which leads to decreased formation of the active MnSOD. Thus, it induced accumulation of ROS within the cells.^[6, 8] Both homozygous genotypes (AA and VV) of MnSOD Val16Ala variation has been reported to correlate with increase the risk of oxidative stress-related pathological conditions might be due to an imbalance of MnSOD.^[23, 24] Increased production of ROS in cell can damage lipids, proteins and nucleic acids (particularly mtDNA) produced oxidative stress and ultimately apoptosis.^[25]

CONCLUSION

The results of the present study indicate that the genetic effect of MnSOD Val16Ala variation has a significant impact on the development of nonalcoholic fatty liver disease in a West Indian populations. These results provide genetic evidence for the importance of mitochondria-derived oxidative stress and pathogenesis of NAFLD. In conclusion, the MnSOD Val16Ala genotype is associated with the risk of developing NAFLD and its progression. The limitation of this study is the small number of NAFLD. Further studies are needed to explore the pathogenic mechanisms underlying this association with other possible genetic factors.

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