Journal of
Genetics and Genome Research

Zahreddine et al. J Genet Genome Res 2016, 3:024
Volume 3 | Issue 1
ISSN: 2378-3648

Original Article: Open Access

Susceptibility of Patients with Manganese Superoxide Dismutase Ala16val Genetic Polymorphism to Type 2 Diabetes Mellitus and its Complications in a Sample of Lebanese Population

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Abstract

Oxidative stress has been frequently associated with the development of type 2 diabetes mellitus. Manganese superoxide dismutase (MnSOD) is one of the most important enzymes responsible for the defense against oxidative damage in the mitochondria. A polymorphism in the second exon of the MnSOD at position 16 that changes Ala into Val, induces changes in the structural conformation and mitochondrial transport of MnSOD. This polymorphism affects the scavenger activity of the enzyme and generates high reactive oxygen species which could exacerbate the development of type 2 diabetes and/or its complications. Relationship between MnSOD polymorphism and type 2 diabetes mellitus and its complications remains uncertain. This study aims to investigate the association between Ala16Val MnSOD gene polymorphism and the susceptibility to type 2 diabetes mellitus (T2DM) and its complications in the Lebanese patients. For this purpose, PCR-based direct DNA sequencing reactions were performed for genotype analysis. A significant association between Ala16Val polymorphism with T2DM as 85.7% of the Val/Val homo zygotes were diabetic compared to 37.5% for the Ala/Ala genotype (p-value less than 0.01). In addition, Val/Val genotypes showed significant high mean HbA1c levels compared to their Ala/Ala genotype indicating that these patients might suffer from a poor diabetes control. Moreover, patients with Ala16Val were at a high risk from suffering diabetic complications that included retinopathy, nephropathy and cardiovascular complications.

Aim: Free radical-induced damage plays an important role in the onset of type 2 diabetes and the development of its complications. This study was undertaken to investigate the association between Ala16Val polymorphism in the MnSOD gene and the susceptibility to type 2 diabetes mellitus (T2DM) and its complications in a cohort group of the Lebanese population.

Settings and design: This case-control study was conducted on 59 patients with T2DM and 40 age-matched healthy unrelated controls, recruited from different regions of Lebanon.

Methods: Cases were confirmed as patients with diabetes clinically and obtained from Al Rai hospital and the Egyptian military hospital in Lebanon. Controls were healthy unrelated individuals with no history of diabetes or other genetic diseases. All subjects were genotyped for Ala16Val polymorphism (C to T) by PCR based reaction-DNA sequencing.

Results: A statistically significant difference was found when genotype and allele distribution of Ala16Val (C to T) polymorphism were compared between patients with diabetes and controls [P < 0.01 by chi-squared; allelic P < 0.01, OR (95% CI) = 4.068 (1.528-10.832)]. Patients with diabetes with Ala16Val polymorphism were shown to be at higher risk for developing complications including retinopathy, nephropathy and cardiovascular complications. Patients with diabetes with Ala16Val polymorphism showed high mean HbA1c blood levels indicating that these patients may suffer from a poor diabetes control.

Keywords

Type 2 Diabetes Mellitus, Oxidative Stress, Mnsod, Genetic Polymorphism

Introduction

Free radical-induced damage plays a significant role in the development of insulin resistance which is the main contributor to type 2 diabetes mellitus [1,2]. Antioxidant enzymes play an important role in limiting this oxidative stress burden and low activity of scavenger enzymes has been observed in patients with type 2 diabetes mellitus [3]. Manganese superoxide dismutase (MnSOD) also known as SOD2 is one of the key antioxidant defense systems against mitochondrial superoxide radicals, it catalyzes the conversion of superoxide radicals to hydrogen peroxide and molecular oxygen in the mitochondria [4]. Ala16Val is a single nucleotide polymorphism in the MnSOD mitochondrial targeting sequence; it affects the localization and efficiency of mitochondrial transport of MnSOD enzyme. It has been reported that the MnSOD Val variant generates a 30-40% less active MnSOD enzyme, suggesting that the homozygous AA genotype may have higher MnSOD activity than its VV counterpart [5]. Antioxidant enzymes protect against the rapid onset and progression of diabetic complications such as diabetic neuropathy, diabetic nephropathy and cardio-vascular complications, by reducing the excess of highly reactive free radicals. Defects and mutations in the genes encoding these enzymes may therefore lead to susceptibility to diabetic complications [6]. The excess generation of reactive free radicals due to chronic hyperglycemia causes oxidative stress which further exacerbates the development and progression of diabetes and its complications through modification of various cellular events in many tissues, including vessels, kidney, pancreatic beta cells and liver [7].

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Citation: Zahreddine AM, Moustafa ME, Chamieh HA (2016) Susceptibility of Patients with Manganese Superoxide Dismutase Ala16val Genetic Polymorphism to Type 2 Diabetes Mellitus and its Complications in a Sample of Lebanese Population. J Genet Genome Res 3:024

Received: January 12, 2016: **Accepted:** May 21, 2016: **Published:** May 24, 2016

Studies on diabetic mice treated with streptozotocin showed that MnSOD over expression in mitochondria plays a significant protective role in development of diabetic complication such as retinopathy [8]. Different studies investigated the association of the MnSODAla16Val polymorphism with several diseases including cancer, heart disease, hypercholesterolemia, Parkinson's disease and diabetes (In review (Bresciani *et al.*, 2013); Chistyakov *et al.*, 2001; Lee and Choi, 2006; Nakanishi *et al.*, 2008; Nomiyama *et al.*, 2003; Vanita, 2014) [6,9-13]. To our knowledge, the association of Ala16Val MnSOD single nucleotide polymorphism (SNP) with type 2 diabetes has not been studied in the Lebanese population before. We therefore conducted a case-control study to investigate the association MnSOD Ala16Val (C to T) with type 2 diabetes and its complications in Lebanon.

Materials and Methods

Study setting and design

A case control study involving 59 patients with type 2 diabetes mellitus recruited during one year prior to the study from Al Rai Hospital and Egyptian military hospital located at Beirut-Lebanon who meet ADA's criteria for a diagnosis of diabetes. All patients are of Lebanese nationality. An informed consent was signed by patients before collection of their blood samples after approval of the ethics board of research at the university. Demographic and clinical characteristics for patients are shown in table 1. Clinical data including the duration of the diabetes, their blood glucose level, their HbA1c levels, and the development of diabetic complications were obtained from the hospital records. Patients with diabetes included 26 males and 33 females, their ages ranged between 37 and 80 years. The mean duration of diabetes was 11.3 ± 7.8 years. Retinopathy was the most common complication among patients with diabetes (16 cases) while nephropathy was the least common complication among patients with diabetes enrolled in this study (5 cases), two patients had more than one diabetic complication. The mean blood glucose level was 182.4 ± 46.28 mg/dL and the main HbA1c was 8.2 ± 1.31% (Table 1). All patients were following a blood glucose control drug regimen. The control group consisted of 40 randomly selected unrelated healthy individuals with no history of genetic diseases. Control group had the following inclusion criteria: no diagnosis of diabetes or any comorbidity (hypertension, hyperlipoproteinemia), HbA1c < 5.6%, fasting blood glucose < 100 mg/dL. Control subjects included 17 males and 23 females, with similar age ranges as the patients group.

DNA extraction

DNA isolation was performed from 300 mL whole blood using a Flexi Gene DNA kit (QiagenGmBH D-40724).

Table 1: Demographic and laboratory characteristics of T2DM patients recruited for the study.

Gender (male/female)	26/33
Age (years)/ Mean age	37-80/ 60.115 ± 10.83
Nephropathy cases*	5
Retinopathy cases	16
Cardio-vascular complications	8
Duration of DM (years)	11.3 ± 7.8
Blood glucose level (mg/dL)	182.4 ± 46.28
HbA1c (%)	8.2 ± 1.31

Data are expressed as means ± S.E.

*two patients showed nephropathy and retinopathy complications.

Detection of the MnSODAla16Val polymorphism

The analysis of the MnSOD genotypes (NG-008729.1) was performed by polymerase chain reaction (PCR) followed by DNA sequencing. The following primers were used: Forward primer: 5'-AGC CCAGCCTGCGTAGAC-3', Reverse Primer 5'- CGT TCA GGT TGT TCA CG-3' DNA samples were amplified by PCR using 100-200 ng genomic DNA as an initial template. A 230 bp PCR product in the MnSOD gene was amplified that contains the Ala16Val SNP position. The DNA region amplified in the MnSOD gene represented the region between nucleotide 2160 to nucleotide 2392 in the sequence. The PCR assay was carried out using a REDTaq ReadyMix PCR reaction mix containing $MgCl_2$. The PCR reaction mix final volume was 40 µl and included 0.3 µM of each primer.

The PCR conditions were: 94°C for 4 min; 5 cycles of 94°C for 30 sec, 62°C for 50 sec, 72°C for 30 sec, followed by 38 cycles of 94°C for 10 sec, 59°C for 40 sec, and 72°C for 15 sec; final extension at 72°C for 5 min and then hold at 4°C. The PCR products (230 bp) for each sample were examined using 2% agarose gel electrophoresis.

PCR products amplified from genomic DNA were purified then examined by DNA sequencing. DNA sequencing was carried out by capillary electrophoresis in the Unit of Medical Genetics Facility, Saint Joseph University (USJ), Lebanon. The PCR products were sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Bio systems, Foster City, CA) under standard conditions according to the manufacturer instructions.

Statistical analysis

Data was analyzed using the statistical package SPSS (version 19). All values are represented as mean ± S.D. Genotype and allelic frequencies are presented as percentages and were computed and tested by Hardy-Weinberg equilibrium. The differences in genotype and allele frequencies between the cases and controls were tested using the chi square test. Differences in variables between normal and diabetic groups were performed by independent student's t-test. One-way ANOVA test was used to compare the effect of the gene polymorphism on continuous variables. The odds ratio (OR), 95% confidence interval (CI), and the corresponding p-value were calculated to describe the strength of the association. Associations were considered to be statistically significant if the Fisher's exact p-value was less than 0.05 and if the 95% CI excluded the value 1.0.

Results

Distribution of MnSOD SNP in T2DM patients and controls

To investigate the susceptibility of patients with MnSODAla16Val with type 2 diabetes, 59 patients and 40 control subjects were recruited to the study; their genomic DNA was extracted followed by a direct PCR-genotype analysis. Results show that the frequency of MnSOD SNPs showed that MnSOD Ala16Val genotype polymorphism was significantly associated with T2DM (Table 2). The Val/Val genotype was highly distributed among T2DM (85.4%) compared to normal subjects (14.3%); while the Ala/Ala genotype showed a higher distribution in normal subjects (62.5%) in comparison with T2DM (37.5%) with a p-value less than 0.001. The allele frequency distribution was in agreement with Hardy-Weinberg equilibrium expectations for normal subjects; however, they deviate from this

Table 2: Genotypic and Allele Frequency of MnSOD Single Nucleotide Polymorphisms (SNPs) in control group and Type 2 Diabetes Mellitus patients (T2DM). Chi-square test was used to determine significance at p < 0.05.

	Control (n = 40)	*T2DM (n = 59)*	*p-Value*	*OR (95% CI)*
Rs480 Genotype				
CC *(Ala)* (n = 16)	10 (62.5%)	6 (37.5%)		
CT *(Ala/Val)* (n = 55)	26 (47.3%)	29 (52.7%)		
TT *(Val)* (n = 28)	4 (14.3%)	24 (85.7%)	< 0.001	4.068 (1.528-10.832)*
Allele				
f(C)	57.50%	34%		
f(T)	42.50%	66%		

*Risk was calculated for a Val homozygote carrier

equilibrium for patients with diabetes. Allele frequencies showed a significant distribution for the C allele in normal subjects (57.5% for normal subjects compared to 34% in T2DM) in parallel to a higher frequency for the T allele for T2DM (66% for the T allele for T2DM compared to 42.5% in the normal subjects).

MnSOD Val Genotypes are correlated with poor diabetes control

In order to evaluate the possible effects of the Ala16Val polymorphism on the severity of type 2 diabetes, mean blood glucose levels and mean HbA1c levels were compared between the different MnSOD genotypes in patients with diabetes participated in this study. HbA1c levels were significantly higher in Val homozygotes ($p = 0.001$) when compared to Ala carriers (Figure 1 and Table 3) indicating that Val homozygotes are more susceptible to a poor diabetes control, whereas there were no significant differences found in mean fasting BGL among the Ala carriers and Val homozygotes (p value = 0.769) (Figure 1 and Table 3) although mean blood sugar in Val homozygotes was higher than the Ala homozygotes (Table 3). We also investigated whether the Ala16Val polymorphism would influence the age of onset of disease. The mean age of onset of type 2 diabetes was compared and no significance was found between the Ala carriers (CC and CT) and Val homozygotes (TT) of patients with diabetes ($p = 0.668$).

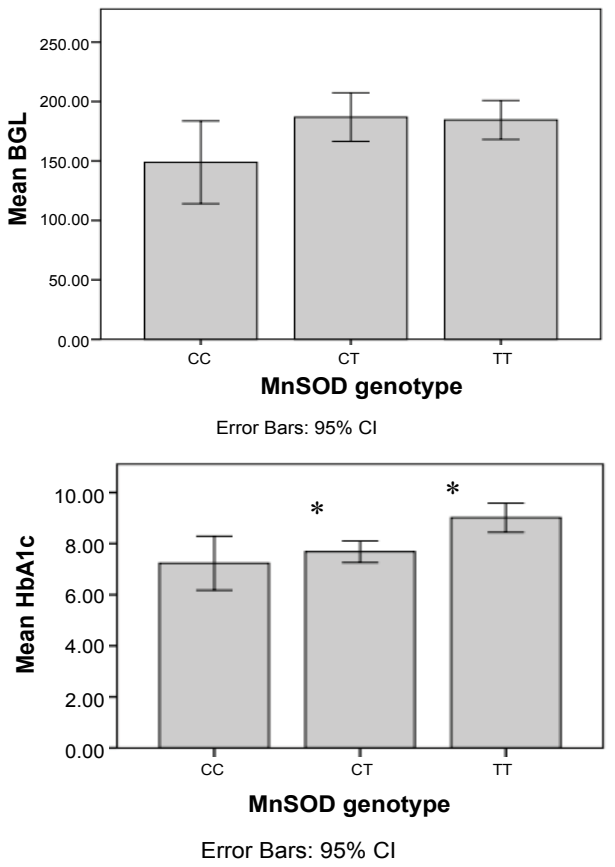

Figure 1: Comparison of the mean Blood glucose levels and HbA1C levels among T2DM patients. An asterisk indicates that there is a significant association between HbA1c and T2DM patients.

MnSOD Val Genotypes are significantly associated with the development of diabetic complications

All diabetic complications including retinopathy, nephropathy or cardio-vascular complications were compared between Ala carriers (CC and CT genotypes) and Val homozygotes (TT genotype) in patients with diabetes. Chi-square test showed that there is a significant difference between Ala carriers and Val homozygotes in the development of these complications ($p < 0.05$). 66.7% of the Val homozygotes T2DM patients showed diabetic complications compared to 16.7% for the Ala homozygotes and 34.5% for the Ala/Val heterozygotes. Among patients with diabetes suffering from complications, the frequency of the T allele was 78% compared to 22% for the C allele (Table 4).

Each of the diabetic complications was further compared separately between Ala carriers and Val homozygotes in T2DM patients. A high percentage of T2DM suffering from diabetic retinopathy, cardiovascular complications and nephropathy were found to be Val homozygotes (more than 60 percent in each case), however this result was not statistically significant (p-Value > 0.05) (Table 5).

Discussion

The current study investigated the association of the MnSOD Ala16Val polymorphism and the susceptibility to type 2 diabetes and its complications in the Lebanese population. The association of MnSOD Ala16Val polymorphism with type 2 diabetes mellitus and its complications is still controversial. This is mainly due to ethnic changes in allele frequencies from one population to another that depends on race and geographic location [14]. This study shows for the first time a significant association between Ala16Val gene polymorphism and T2DM to a high and significant level in Lebanese population. Indeed the Val/Val genotype was more common in patients with diabetes (85.7%) than in control group (14.7%) (p-value less than 0.01). A similar study on Japanese and Americans reported

Table 3: Correlation between the mean values of fasting BGL and HbA1c with MnSOD genotypes of T2DM patients. Data are expressed as mean ± SD. ANOVA was used to determine significance at $p < 0.05$.

Type 2 diabetic patients	*Genotype*			
	CC (Ala)(n = 6)	*CT (Ala/Val) (n = 29)*	*TT (Val) (n = 24)*	*P-Value*
Mean HbA1c(%)	7.23 ±1.00731	7.69 ± 1.01	9.01 ± 1.35	< 0.01
Mean BGL (mg/dL)	148.83 ± 33.14	186.9 ± 53.66	184.46 ± 38.73	0.189
Mean age of diagnosis	52.6 ± 10.72	49.17 ± 11.85	48.2083 ± 9.4	0.668

ANOVA test was used and data was expressed as mean ± SD and the significance was determined at $p < 0.05$

Table 4: Association of MnSOD Ala16Val polymorphism with diabetic complications chi-square test was used to determine significance at $p < 0.05$.

	No complications (n = 32)	***With complications (n = 27)***	***p-Value***	***OR (95% CI)***
Genotype				
CC *(Ala)* (n = 6)	5/6 (83.3%)	1/6 (16.7%)	0.021	4.364 (1.440-13.336)*
CT *(Ala/Val)* (n = 29)	19/29 (65.5%)	10/29 (34.5%)		
TT *(Val)* (n = 24)	8/24 (33.3%)	16/24 (66.7%)		
Allele				
f(C)	45.4%	22%		
f(T)	54.6%	78%		

*Risk was calculated for a Val homozygote carrier

Table 5: Association of MnSOD Ala16Val polymorphism with Diabetic retinopathy, nephropathy and cardiovascular complications. Chi-square test was used to determine significance at P < 0.05.

Type 2 diabetic patients	*Genotype*			*p-Value*	*OR (95% CI)*
	CC (Ala)(n = 6)	*CT (Ala/Val) (n = 29)*	*TT (Val) (n = 24)*		
DR+ (n = 16)	1/16 (6.3%)	5/16 (31.3%)	10/16 (62.5%)	0.115	1.92 (1.082-3.84)
DR- (n = 43)	5/43 (11.6%)	24/43 (55.8%)	14/43 (32.6%)		
DP+ (n = 5)	0/5 (0%)	2/5 (40%)	3/5 (60%)	0.563	1.543 (0.7-3.39)
DP- (n = 56)	6/56 (11.1%)	27/56 (50%)	21/56 (38.9%)		
CVD+ (n = 8)	0/8 (0%)	3/8 (37.5%)	5/8 (62.5 %)	0.320	1.678 (0.881-3.995)
CVD- (n = 51)	6/51 (11.8%)	26/51 (51%)	19/51 (37.3%)		

DR+: presence of diabetic retinopathy; DR-: no diabetic retinopathy; DP+: presence of nephropathy; DP-: no nephropathy; CVD+: presence of cardiovascular complications; CVD-: no cardiovascular complications.

that Ala16Val polymorphism might be associated with development of type 2 diabetes [11], however their study showed a weak association of the Ala16 Val polymorphism for the development of type 2 diabetes. The Ala genotype of MnSOD is more active for ROS scavenger activity in mitochondria than the Val homozygotes, thus Ala allele carriers might be protected from ROS induced diabetic pathology. We therefore examined their clinical characteristics and their diabetic complications. In our study, we observed that fasting blood glucose and HbA1c differed between the different genotypes; and moreover the Ala16 Val polymorphism was significantly associated with a high mean HbA1C levels. These results suggest that T2DM patients with Ala16Val polymorphism have a high genetic risk factor for diabetes control over a long period of time and should be closely monitored. A similar study demonstrated that the Val/Val genotype is associated with poor diabetes control in Czeck republic [3].

We also investigated the association between the MnSOD Ala16Val polymorphism and the development of all diabetic complications including retinopathy, nephropathy and cardiovascular complications. We showed that patients with diabetes with Ala16Val polymorphism are at a higher risk of developing complications in general to a significant level. A high frequency of Val genotypes was observed in the recruited patients with retinopathy, nephropathy and cardiovascular complications. Our study is the first study to examine all the complications together and their correlation with the MnSOD polymorphism. Previous studies have reported an association between type 2 diabetes and microangiopathy [3], and two other studies reported an association between type 2 diabetes and macular edema in Korean population and diabetic retinopathy in Indian, Northern Iranian and Japanese population [10-13,15].

The identification of genetic risk factors and susceptibility gene polymorphisms in the Lebanese population is essential for diabetes screening, prevention, and treatment. Our study shows that the MnSOD Ala16Val can be considered as a good biomarker for the susceptibility to T2DM and its complications in the Lebanese population.

Conclusions

Individuals with the Ala16Val MnSOD genotype are at increased risk in developing type 2 diabetes, and various complications. Ala16Val genotyping is a possible marker for type 2 diabetes prediction, and for monitoring disease progression. Further studies in larger population samples are needed to explain and confirm these findings.

Acknowledgments

The authors would like to express their gratitude to Professor Rajaa Fakhoury for the help she provided for the completion of this work.

Authors Contribution

A.Z collected blood samples, performed experiments and statistical analysis, wrote the manuscript. M.S performed statistical analysis and wrote the manuscript. H.C designed the study, interpreted the data, wrote the manuscript, and hold the main responsibilities for the work.

Author Disclosure Statement

No competing financial interests exist.

Ethics Statement

This study was approved by the Ethics in Research Committee at the University and was conducted according to the Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association Declaration of Helsinki.

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