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ORIGINAL RESEARCH

Association of Polymorphisms in Antioxidant Enzyme-Encoding Genes with Diabetic Nephropathy in a Group of Saudi Arabian Patients with Type II Diabetes Mellitus

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Introduction: An imbalance between reactive oxygen species (ROS) generation and the defence mechanisms underlying the activity of antioxidant enzymes has been demonstrated as the leading pathology in diabetes mellitus (DM)-related microvascular complications.

Purpose: This study aims to evaluate the association between polymorphisms in antioxidant enzyme-encoding genes: catalase (CAT); manganese superoxide dismutase (Mn-SOD); glutathione S transferase M1 (GSTM1); and GSTT1 glutathione S transferase T1 (GSTT1), and the risk of type II diabetic nephropathy (DN) in the Saudi population.

Patients and Methods: The present study involved 64 type II DM patients with nephropathy and 64 type II diabetes patients without nephropathy from the King Abdulaziz University (KAU) Hospital. They underwent real-time PCR genotyping for the Mn-SOD and CAT genes. Multiplex PCR was used to detect GSTM1- and GSTT1-null polymorphisms.

Results: A statistically significant difference was observed between the case and control groups with regard to polymorphisms in the CAT gene (P = 0.037), but not for polymorphisms in the Mn-SOD (P = 0.64) gene. In addition, a statistically significant association was observed between null polymorphisms of the GSTT1 and GSTM1 genes and DN in the case and control groups (P = 0.046 and P = 0.035, respectively).

Conclusion: Our results showed that the genetic ability to combat oxidative stress may play a major role in DN pathogenesis in Saudi type II DM patients. These polymorphisms in antioxidant enzyme-encoding genes could be used as independent genetic markers for the construction of risk prediction models for kidney-related complications in type II DM patients.

Keywords: diabetic nephropathy, gene polymorphism, oxidative stress

Introduction

Diabetes mellitus (DM) is a major multifactorial epidemic disease of this century, and it is affected by genetic and environmental factors.[1] The International Diabetes Federation reported approximately 536 million diabetic cases worldwide in 2021. This number may reach approximately 783 million by 2045.[2]

In Saudi Arabia, a study conducted revealed that the prevalence of diabetes is about 25%, making it one of the most frequently occurring diseases all over the world.[3]

Diabetic nephropathy (DN) is one of the most prevalent microvascular complications associated with DM.[4] The manifested symptoms include proteinuria, glomerular hypertrophy, reduction of glomerular filtration rate, and renal fibrosis accompanied by renal dysfunction.[5]

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The initiation and progression of vascular complications of diabetes, such as nephropathy, is caused by oxidative stress, where reactive oxygen species (ROS) production overrides the defensive antioxidant mechanisms.[6] ROS are produced by mitochondria, glucose oxidation, and cascade of enzymatic reactions, such as those involving (NAD[P]H) oxidase.[7]

ROS are removed by several enzymatic mechanisms. Superoxide dismutase (SOD) converts superoxidase $^{\bullet}O_2$ to H_2O_2, which is detoxified to H_2O by catalase present inside the lysosomes or by glutathione peroxidase (GPX) present inside the mitochondria.[8] Glutathione S-transferase (GST) is one of the most vital Phase II detoxification enzymes that protects cells against oxidative stress damage.[9]

Antioxidative enzymes are polymorphic. Genetic diversity in these genes causes individual variations in the genetic expression of the enzymes they encode; this affects the defensive activity of these enzymes against ROS.[10] Reduction of antioxidative capacity results in the insufficient elimination of ROS, increases the risk of microvascular dysfunction, and damage of cellular proteins, lipids, and nucleic acids.[11]

Catalase is a tetrameric haemoprotein that catalyses the breakdown of H_2O_2 into H_2O and O_2. The −262C/T single nucleotide polymorphism (SNP) is the most investigated SNP in relation to different types of diabetes.[12] The CT + TT genotype of catalase has been shown to increase blood catalase activity in type II DM patients. SOD2 is a critical defender against mitochondrial superoxide radicals. The C47T (Val16Ala) polymorphism is an important polymorphism in the SOD2 gene. The Val/Val genotype is associated with an increased risk of DN in both type I DM and type II DM.[13] GST represents a family of enzymes that are expressed as different isoforms encoded by a variety of genes.[14] The GST subtypes M1, T1, and P1 are involved in diabetes-related complications. Deletion polymorphisms of GSTM1 and GSTT1 have been widely investigated. The null variants of these genes have been associated with reduced GST activity, leading to reduced antioxidant defences.[15]

Studies investigating the relationship between antioxidant enzyme gene polymorphisms and DN, in different ethnic populations are inconsistent. This variation may be attributed to ethnic differences. Genetic associations are often inconsistent across ethnic groups owing to several polymorphic allelic frequencies and gene–gene interactions. Therefore, a genetic association may be consistent in a specific ethnic group and invalid for another ethnic group.[16]

Therefore, in this study, we aimed to explore the association of genetic polymorphisms in genes encoding the following antioxidant enzymes: CAT (catalase); Mn-SOD (manganese superoxide dismutase); glutathione S transferase M1 (GSTM1); and glutathione S transferase T1 (GSTT1), and the development of nephropathy in Saudi Arabian patients with type II DM. This group of antioxidant enzymes is representative of different types of antioxidative enzymes. SOD and CAT constitute the first line of defensive antioxidative enzymes.[13] In contrast, the GST family is responsible for the cytotoxic detoxification of ROS metabolites.[17]

Materials and Methods

Study Population

This study included 128 adult patients with type II DM recruited from King Abdulaziz University (KAU) Hospital in Jeddah between March 2019 and September 2020. The Research Ethics Committee of KAU approved this study (reference number 387–19).

The study population was divided into two groups: case group consisted of diabetes patients with nephropathy (n=64; 20 men and 44 women) and control group consisted of diabetes patients without nephropathy (n=64; 18 men and 46 women). Both groups were age- and sex matched.

Diagnosis of type II DM was based on the American Diabetes Association definition of diabetes and World Health Organization (WHO).[18]

DN was detected by screening for microalbuminuria from a random urine sample with a dipstick. By measuring the urine albumin-to-creatinine ratio in the second morning urine sample, if the result was positive with a value of more than 100 mg/g on two of three sample measurements, DN case was confirmed.[19]

Both, case and control group patients signed an informed consent form after understanding the purpose of the research. The samples were not used for any other purpose, and individual patient results were kept confidential.

Sample Collection

A 5-mL blood sample was collected into an EDTA-coated tube, and whole blood was used for DNA extraction. The blood sample was incubated at 4–6°C till extraction. DNA extraction from whole blood was performed using a mammalian genomic DNA extraction kit (Axygen Biosciences, California, USA), following the manufacturer's recommendations. The extracted DNA was stored at −80°C till the assay was performed.

All patients were subjected to real-time PCR genotyping for the Mn-SOD and CAT genes. Multiplex PCR was used to detect GSTM1 and GSTT1 null polymorphisms.

Mn-SOD and CAT Genotyping

Real-time PCR genotyping was performed to identify the Mn-SOD, Val16Ala, and CAT C-262T promoter polymorphisms.

Assay ID: C_8709053_10 was used to detect Val16Ala (Ref. SNP ID: rs4880), and assay ID: C_11468118_10 was used to detect CAT C-262T (ref. SNP ID: rs1001179).

The reaction mixture (5 µL) contained 0.125 µL TaqMan SNP genotyping assay, 2.5 µL TaqMan Universal PCR Master Mix, and 100 ng of extracted genomic DNA.

GSTM1 & GSTT1 Genotyping

GSTM1 and GSTT1 null polymorphisms were analysed using multiplex PCR, where multiple genes were co-amplified in the same reaction tube. For GSTT1 polymorphism, the sequences of the forward and reverse primers were 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′, respectively. For the GSTM1 null polymorphism, the sequences were as follows: forward 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and reverse 5′-GTT GGG CTC AAA TAT ACG GTG G-3′.

The PCR was carried out in a reaction volume of 50 µL; the reaction mixture contained 6 µL DNA, 5 µL 10X buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 2.5 U Taq polymerase, and 0.3 µM of each primer.

PCR amplification was performed using the following thermal profile: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min each, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

The human β-globin gene was used as the positive internal control. A third group of primers with the following sequences: forward 5′-GAA GAG CCA AGG ACA GGT AC-3′ and reverse 5′-TGG TCT CCT TAA ACC TGT CTT G-3′ were used for analysis of the β-globin gene. This was carried out in a separate reaction tube with the following thermal profile: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min each, annealing at 48 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

The PCR products were loaded onto a 2% agarose gel and analysed by horizontal electrophoresis.

Statistical Analysis

Descriptive statistics and cross-tabulations were used for the data analysis. Statistical Package for Social Studies software (SPSS version 25.0) was used for the statistical analyses. Genotype frequencies were compared between the case and control groups by chi-square (χ^2) test and odds ratio (OR). Multiple regression analysis was used to examine the relationship between the risk of DN development and polymorphisms of the 4 antioxidant enzyme-encoding genes.

All "P" values at 95% confidence intervals (CI) were calculated and $p<0.05$ was found to be statistically significant.

Results

The current study included 64 adult diabetes patients with nephropathy (63.9 ± 8.3 years), as the case group, and a control group (n= 64; 60.9 ± 9.9 years). The age (P = 0.309) or sex (P = 0.827) of the patients from the case and control groups did not show significant difference, as shown in Table 1.

For CAT gene polymorphisms, a statistically significant difference was detected between the case and control groups (χ^2 = 4.35, P = 0.037*) with respect to CAT genotypes (shown in Figure 1). Particularly, different allele and genotype

Table 1 Demographic Features (Age & Sex) of the Studied Groups

	Cases (n = 64)	Control (n = 64)	P-value
Age in Years (Mean/SD)	63.9± 8.3	60.9 ± 9.9	0.309
Sex (Male/Female)	20 (31.25%)/44 (68.75%)	18 (28.13%)/46 (71.87%)	0.827

frequencies of CAT gene polymorphisms were found between diabetes patients with, and those without, nephropathy, as shown in Table 2.

Regarding Mn-SOD gene polymorphisms, no statistically significant difference was observed between the case and control groups ($\chi^2 = 0.21$, P = 0.64), as shown in Figure 1. In addition, we did not find statistically significant differences between the frequencies of Mn-SOD alleles in diabetes patients with nephropathy and those without it, as shown in Table 3.

Furthermore, for GSTM1 null gene polymorphisms, a statistically significant difference was detected between the case and control groups ($\chi^2 = 3.99$, P = 0.046*), as shown in Table 4.

In addition, a statistically significant difference was observed between the case and control groups ($\chi^2 = 4.44$, P = 0.035*) with respect to the GSTT1 null polymorphism, as shown in Table 5.

The frequencies of the null GSTM1 and GSTT1 genotypes in the complicated DM group were 48/64 (75%) and 41/64 (64%), respectively (Figure 2).

Analysis of the PCR products of the GSTM1 and GSTT1 genes was performed using agarose gel electrophoresis. Multiplex-PCR analysis of GSTM1 and GSTT1 gene polymorphisms was performed; these results are shown in Figure 3.

To predict the risk of nephropathy development, multiple logistic regression was performed using the results of the polymorphisms in the four different antioxidant enzyme-encoding genes. These variables significantly predicted the risk of nephropathy development among diabetes patients, $F(4, 123) = 16.84$, $p < 0.0001$. All four variables showed significance for predicting the risk of DN ($p < 0.05$), as shown in Table 6.

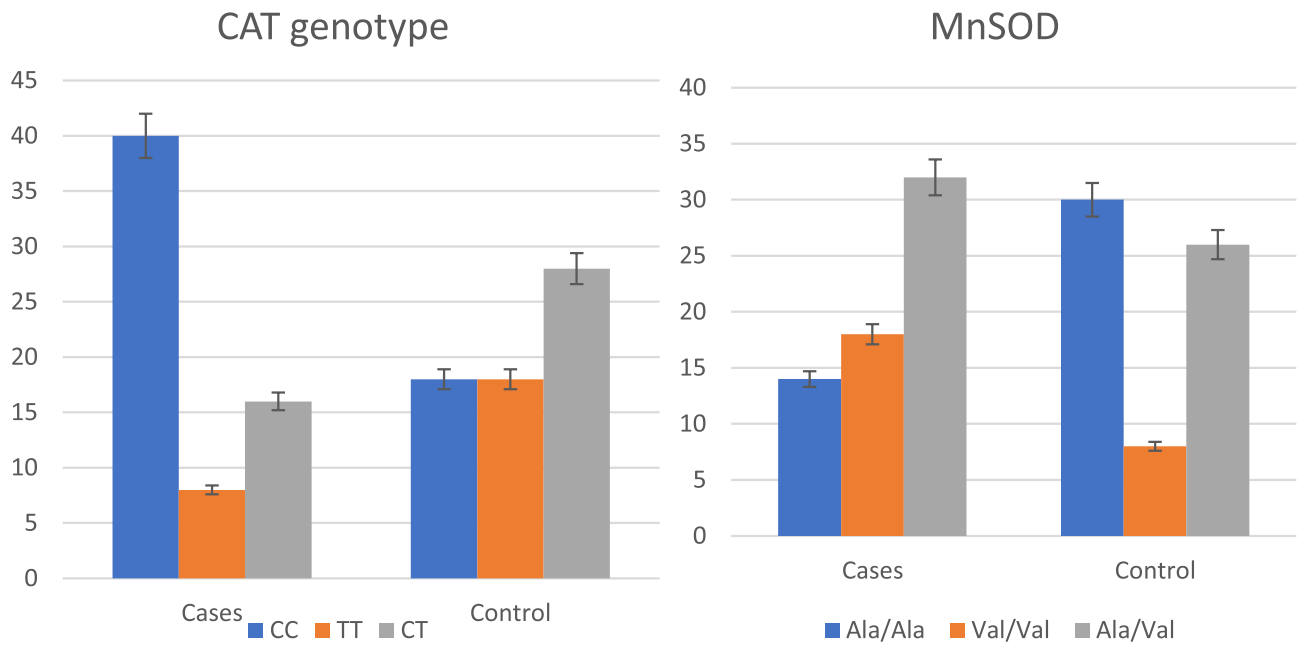

Figure 1 CAT & MnSOD Genotype among studied groups.

Table 2 Comparison of Genotype Frequency of CAT Polymorphism in Cases and Control Groups

CAT	Genotype			Allele Frequency	
	CC	TT	CT	C	T
Cases (n= 64)	40 (62.5%)	8 (12.5%)	16 (25%)	96 (75%)	32 (25%)
Control (n= 64)	18 (28.1%)	18 (28.1%)	28 (43.8%)	64 (50%)	64 (50%)
χ^2	4.35			OR= 3.0 95% CI: 1.77 to 5.09	
P- value	0.037*				

Note: *Statistically significant.
Abbreviations: χ^2, chi-square; OR, odds ratio; CI, confidence interval.

Table 3 Comparison of Genotype Frequency of MnSOD Polymorphism in Cases and Control Groups

MnSOD	Genotype			Allele Frequency	
	Ala/Ala	Val/Val	Ala/Val	Ala	Val
Cases (n= 64)	14 (21.9%)	18 (28.1%)	32 (50%)	60 (46.9%)	68 (53.1%)
Control (n= 64)	30 (46.9%)	8 (12.5%)	26 (40.6%)	86 (67.2%)	42 (32.8%)
χ^2	0.21			OR= 0.43 95% CI: 0.26 TO 0.72	
P- value	0.64				

Abbreviations: χ^2, chi-square; OR, odds ratio; CI, confidence interval.

Table 4 Frequency of GSTM1 Null Gene Polymorphisms in the Studied Groups

GSTM1 Genotype	Null (-)	Non Null (+)	χ^2	P-value	OR
Cases (n = 64)	48 (75%)	16 (25%)	3.99	0.046*	6.6
Control (n = 64)	20 (31.2%)	44 (68.8%)			95% CI: 3.04 to 14.32

Note: *Statistically significant.
Abbreviations: χ^2, chi-square; OR, odds ratio; CI, confidence interval.

Table 5 Frequency of GSTT1 Null Gene Polymorphisms in the Represented Groups

GSTT1 Genotype	Null (-)	Non Null (+)	χ^2	P-value	OR
Cases (n = 64)	41 (64%)	23 (36%)	4.44	0.035*	2.97
Control (n = 64)	24 (37.5%)	40 (62.5%)			95% CI: 1.45 to 6.09

Note: *Statistically significant.
Abbreviations: χ^2, chi-square; OR, odds ratio; CI, confidence interval.

Discussion

To the best of our knowledge, this is the first study to explore the genetic variations in antioxidative enzyme-encoding genes in Saudi patients with type II DM and DN. DM causes increased ROS production and the suppression of defensive antioxidant mechanisms. Oxidative stress has been reported to be an important risk factor for the initiation and progression of diabetic complications.[20]

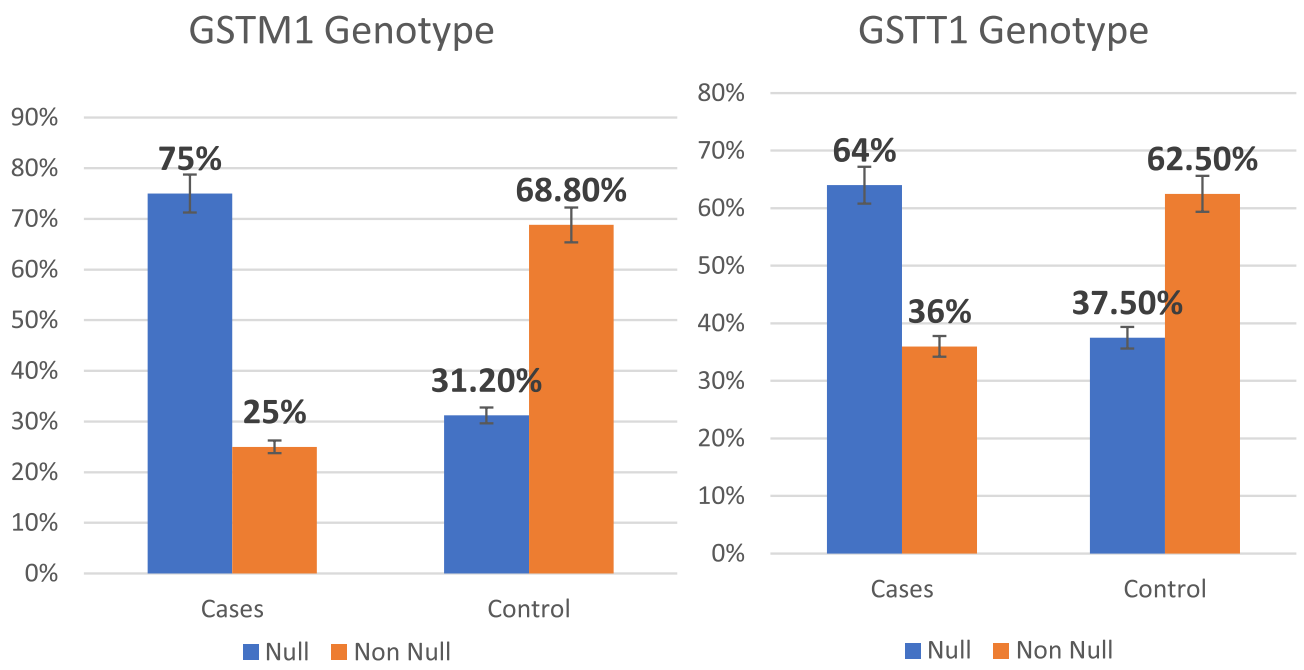

Figure 2 Frequency of GSTM1 & GSTT1 null polymorphism in cases and control groups.

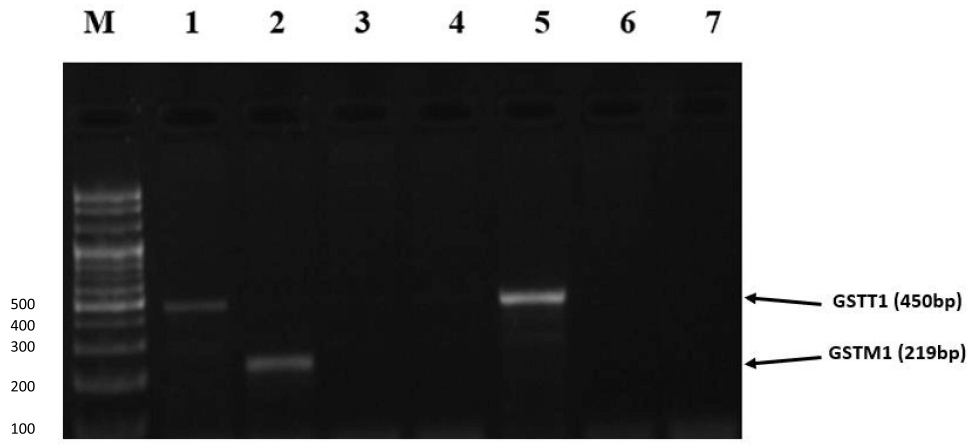

Figure 3 GSTM1 & GSTT1 gene polymorphism analysis in studied cases.(Electrophoresis of PCR products showed the presence of GSTM1 gene in lane 2, while GSTT1 gene was present in lane 1 and 5).

CAT and SOD constitute an important line of defence against ROS; antioxidative protective mechanisms are mainly affected by polymorphisms in these genes.[21] CAT, which is present in peroxisomes, catalyses the decomposition of hydrogen peroxide to the less reactive molecules oxygen and water. CAT is located on chromosome 11; the p13. polymorphism of exon 9–262C/T in the CAT gene was studied in diabetes patients with complications such as retinopathy, nephropathy, and cardiovascular disease.[12]

In the present study, we examined the relationship between polymorphisms in the CAT gene and the risk of DN in Saudi population. A statistically significant difference was observed between the case and control groups in this regard.

This finding was in agreement with a study performed in 2015 among type II DM patients from Slovenia, which reported a relationship between polymorphisms of the CAT gene and the progression of late-stage renal failure.[21]

In addition, the CAT −262C/T polymorphism promotes hypertriacylglycerolemia in Chinese patients.[22] However, another study reported the absence of a relationship between the CAT-262 C/T polymorphism and microvascular diabetic complications in Caucasian patients with type II DM.[23]

Table 6 Multiple Logistic Regression and Estimation of Significance of the Four Different Antioxidant Enzyme Genes Polymorphism

Variables (Genes Polymorphism)		P value		95% CI	
CAT		0.0009		−0.23 to −0.06	
MnSOD		0.0102		0.03 to 0.19	
GSTM1		<0.0001		−0.50 to −0.21	
GSTT1		<0.0001		−0.47 to −0.17	
Analysis of Variance	**SS**	***DF***	**MS**	**F (DFn, DFd)**	**P value**
Regression	11.32	4	2.831	F (4, 123) = 16.84	P<0.0001

Abbreviations: SS, sum of squares; *DF*, degree of freedom; MS, mean square; Dfn, degree of freedom for the numerator of the F ratio; DFd, degree of freedom for the denominator of the F ratio; CI, confidence interval.

Several studies have reported the absence of an association between the catalase −262C/T genotype and type I DM risk.[24] However, other studies have showed an association between catalase C allele and the risk of diabetic neuropathy development.[25]

Genes from the SOD family catalyse the dismutation of $^{\bullet}O_2^-$ to H_2O_2 and oxygen. This family comprises intracellular the enzymes SOD type 1 (Cu-Zn-SOD), mitochondrial SOD type 2 (Mn-SOD), and extracellular SOD type 3 (EC-SOD).[26]

Exon 2 of the SOD2 gene, A16V(C/T) (rs4880) functional polymorphism causes a structural change in the mitochondrial targeting domain, lowering the antioxidative capacity and limiting post-transcriptional transport of the protein. A valine to alanine substitution was shown to enhance the activity of Mn-SOD in the mitochondria, resulting in reduced risk of coronary artery disease.[27]

In the current study, no statistically significant relationship was detected between the genetic polymorphism of Mn-SOD and DN among the studied groups. This is in agreement with a study conducted by Klen et al, who did not report any association between Mn-SOD polymorphisms and renal function and microvascular complications.[21]

In addition, there was no association found between Mn-SOD polymorphisms and renal complications in the DIABHYCARGENE and DIABHYCAR cohort studies.[28] Another study showed no association between the Val/Val Mn-SOD polymorphism and non-smoking patients with DN; however, an association was present between this polymorphism and DN in diabetes patients who were smokers.[29]

However, a contradictory study showed a significant reduction in Mn-SOD activity in the presence of the Mn-SOD Ala16Val polymorphism in diabetes patients with albuminuria.[30] The Mn-SOD, Val16Ala polymorphism was also reported to be associated with nephropathy in type II DM.[31]

Mollsten et al reported an association between the rs4880 T-allele and the risk of DN in Finnish, Swedish, and Danish prospective studies on diabetes patients.[32,33]

Glutathione-S-transferases (GSTs) are located in the cytosol, where they play an important role in metabolising xenobiotics, catalysing the active conjugation of xenobiotics with GSH, in addition to the detoxification of reactive electrophiles.[12]

In humans, cytosolic and membrane-bound GSTs are divided into the alpha, mu, pi, kappa, sigma, theta, omega, and zeta isoenzymes.[34] Genes encoding GST enzymes have polymorphic traits that lead to modifications in enzymatic activity.[35]

In the present study, a statistically significant relationship was observed between null polymorphisms in the GSTT1 and GSTM1 genes and DN in the studied groups.

Many studies have been performed to identify the association between these polymorphisms and the risk of developing DN among different ethnic groups.

The results of 25 meta-analysis studies showed that the null genotype of GSTT1 enhanced the risk of development of complications in patients with type II DM, in contrast to the null genotype of the GSTM1 gene.[36] Another study showed that the GSTM1-null genotype increases the risk of DN.[20]

In addition, Fujita et al reported the absence of a significant representation of GSTM1 null genotypes in patients with DN, compared with the case in patients with diabetes only.[37] Moreover, a study conducted in the Brazilian population did not detect any association between the null polymorphism of the GSTM1 and GSTT1 genes and DN development.[38] However, a study performed in an Iranian population showed that the GSTT1 and GSTM1 null genotypes increased the risk of late-stage renal diseases.[39]

Many studies have investigated the effect of GST null polymorphisms on type II DM microvascular complications and have shown contradictory results; these contradictions may be attributed to the heterogeneity of the populations studied. Accordingly, further studies are required to evaluate the effects of these polymorphisms.

Conclusion

The present study detected a statistically significant relationship between CAT, GSTM1, and GSTT1 polymorphisms and DN development. The synergistic effects of polymorphisms in different antioxidant enzyme-encoding genes significantly predicted the risk of nephropathy in patients with type II DM. These results show that the genetic ability to combat oxidative stress may play a major role in DN pathogenesis in Saudi patients with type II DM.

The polymorphisms of antioxidant enzyme-encoding genes in our study could be utilised as independent genetic markers for the construction of a risk prediction model for kidney-related complications in type II DM patients. However, further studies with a larger sample size are required to evaluate and validate these genetic markers objectively.

Statement of Ethics

Ethical approval of this study was obtained from the Biomedical Ethics Research Committee, KAU, Saudi Arabia (Reference No 387-19).

The study complies with the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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