Plant Archives Vol. 20, Supplement 2, 2020 pp. 1537-1543 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

GENETIC POLYMORPHISM IN *NOS* GENE AND CORRELATED WITH PEROXYNITRITE LEVEL VARIANT IN T2DM OF IRAQI PATIENTS

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Abstract

The effect of the genetic polymorphism of three SNPs rs1549758 (T132C), rs1799983 (T517G), rs1007311 (A414G) of endothelial nitric oxide synthase gene (*NOS3*) as a risk factor for the complication of T2DM showed that rs1549758 (T132C) of *NOS3* gene aligned with reference sequence in (NCBI BLAST) where TC heterozygous polymorphic percentage appeared in 40% of patients compared to 50% in healthy controls. On the other hand, the mutant homozygous (CC) percentage was 60% in patients and 50% in healthy controls. There was a significant (p-value 4.392) between the TC, CC genotypes in both of T2DM patients and controls. This variation causes synonymous (silent) mutation without changing the amino acid (Aspartic acid GAT and GAC). Accordingly, this may not represent a risk factor to T2DM.Variant rs1799983 (T517G) appeared patients as TG heterozygous polymorphic 46.67% and mutant homozygous GG 53.33%. Furthermore, results showed both TG heterozygous polymorphic and mutant homozygous GG 50% in controls. These differences in TG and GG were not significant between patients and controls. This variation cause missense mutation by changing Aspartic acid (GAT) to Glutamic acid (GAG), however, both amino acid have the same properties (mono amino dicarboxylic acid, polar, hydrophilic, ionisable, acidic group). Again, whether this mutation can considered as a risk factor for T2DM, is still unknown. Variation rs1007311 (A414G) presented in patients, it appeared 33.33% in AA non-polymorphic while 46.67% as a heterozygous polymorphic AG and 20% appeared as homozygous mutant GG. While appeared in controls 40% as a non-polymorphic AA and 60% were heterozygous polymorphic AG. Both AG and GG variants were shown to be significant (p-value 0.0277, 7.250) respectively. It was noted, that variation located in the intron region, which may not be involved in influencing.

Keywords **:** Genetic polymorphism, oxide synthase gene (NOS3), T2DM, DNA extraction.

Introduction

Diabetes mellitus (DM) is possibly one of the oldest diseases known to man. Type 2 DM was qualified as a component of metabolic syndrome Type 2 DM (Olokoba *et al.,* 2012).

DM is a serious, chronic and complex illness described by hyperglycemia that resulted from the pancreatic β-cells generates deficient insulin, a hormone that regulated blood glucose (Okur *et al.,* 2017).

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune case that required the lifelong administration of insulin, resulting in absolute deficiency of pancreatic insulin production. Regular and lifelong insulin administration is therefore necessary to inhibit hyperglycemia metabolic decompensating and life-threatening diabetic ketoacidosis (DKA) (Iqbal *et al.,* 2018).

The oxidative stress outcome of raising free radical production or minimized activity of antioxidant defenses or both. Persistent hyperglycemia source increased production of free radicals, especially reactive oxygen species (ROS), for all tissues (Sheikhpour, 2013). Reactive nitrogen species (RNS) means all oxidation states and reactive adducts of the nitrogenous nitric oxide synthase (NOS) products, from nitric oxide (NO) to nitroxyl (NO^-), Snitrosothiol (RSNO), and peroxynitrite ($OONO^-$), as production of the reaction between NO and O_2^-. ROS and RNS have critical biological role necessary for normal physiology. Overproduction or insufficiency of ROS and RNS may result in impaired homeostasis and related pathology (Pitocco *et al.,* 2010). Nitric oxide, a metabolite of L-arginine to L-citrulline processing via endothelial (NO) synthase (eNOS), is liberated by the endothelium (Kearney, 2013). The eNOS gene is described on human chromosome 7q35–36, and includes 26 exons and 25 introns, the eNOS G894T polymorphism, a coding position variant, effects in a Glu298Asp substitution and reduction the NO ratios (Luo *et al.,* 2014).

Genetic factors play a significant function in the expansion of type 2 diabetes. Endothelial nitric oxide synthase (*NOS3*) gene is accountable for the bioavailability of nitric oxide, and endothelial function (Moguib *et al.,* 2017). NO production can be affected by polymorphisms in the *NOS3* gene. The gene is situated on chromosome 7q35–36 and include 26 exons exceed 21 kb. It has been described that the G894T polymorphism in exon 7 of the *NOS3* gene alterations the amino acid substitution of the NOS3 protein, which involves the structural modifications (Corapcioglu *et al.,* 2010).

Various polymorphisms have been described in the *NOS3* gene, however, the Glu298Asp (rs1799983) polymorphism in exon 7 was the only prevalent variance that leads to amino acid substitution in the mature protein. In this polymorphism, the guanine at location 894 is substituted by thymine leading to an alteration in the amino acid at location 298 from glutamate to aspartate (Gad *et al.,* 2012).

The G894T polymorphism of *NOS3* arising from G to T conversion at nucleotide 894 of exon 7 of the gene is one of *NOS3* the most clinically significant polymorphisms. It has been suggested that there is a substantial reduction in the quantity of eNOS or its enzymatic activity in the presence of *NOS3* TT genotype and the *NOS3* Asp298 protein has increased susceptibility to intracellular proteolytic cleavage relative to the *NOS3* Glu298 protein leading in decreased NO levels (Rahimi *et al.,* 2012).

NOS3 has been the most extensively studied in both CVD and other illnesses. Analysis described NOS3SNPs

rs1549758 for Coronary heart disease (CHD) and rs3918226 and rs3918227 for hypertension. SNP rs1549758 is in strong linkage disequilibrium (LD) with exon 7 SNP Glu 298 to Asp which is also recognized as rs1799983 (Levinsson *et al.,* 2014).

Materials and Methods

Subjects

The study was done on 60 patients with type 2 Diabetes Mellitus (T2DM) from National Diabetes Center, Al-Mustansirya University. Type 2 Diabetes Mellitus patients included 32 men and 28 women with the age range of 35-70 years. Another blood samples were collected from 30 non diabetic healthy controls, they include 15 men and 15 women with the age range of 30- 68 years. The range of disease duration was between two months to15years.

DNA extraction

The genomic DNA of the investigated samples were extracted using Genaid Kit according to the manufacturer's instructions (Geneaid Biotech, Taiwan). The concentration and purity of DNA were measured by a nanodrop (BioDrop μLITE, BioDrop Co., UK), while the DNA integrity was checked by a standard 0.8% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 μg/ml) in TAE (40 mM Tris-acetate; 2 mM EDTA, pH 8.3) buffer, using a 1 kb ladder as a molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea). The isolated DNA was used as a template for PCR.

PCR

One PCR fragments were selected for amplification, which covered three exons within the *NOS3* gene. The details of these primer's pairs were shown in (Table 1). The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using *AccuPower* PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). Each 20μl of PCR premix was contained 1 U of Top DNA polymerase, 250 μM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Daejeon, South Korea). The amplification was begun by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94˚C, annealing (58–61 ˚C for 1 min), and elongation at 72˚C, and was finalized with a final extension at 72°C for 10 min. Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 100-bp ladder (Cat # D-1010, Bioneer, Daejeon, South Korea) as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only one clean and sharp band in order to be submitted into sequencing successfully.

Table 1. One specific primers pairs selected to amplify *NOS3* genetic locus within the human genomic DNA sequences.

Primer	Sequence (5′-3′)	Amplicon size	GenBank Accession Number	Annealing temperature
NOS3-F	GCTCTGACCAGCTCTTTC	999 bp	NG_011992.1	55°C
NOS3-R	CTTGTCCTCAGTTCCCATTTA			

DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from one direction, forward direction, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Checking the novelty of SNPs

The observed SNP was submitted to the dbSNP database to check their originality. Each particular SNP was highlighted according to its place in the reference genome. Subsequently, the determination of the presence of previous SNP was performed by viewing its corresponding dbSNP position. Then, the dbSNPs position for the detected SNP was documented.

Results

Within this locus, 30 samples were included, which had shown about 999 bp amplicons length of the *NOS3* locus, which is positioned within chromosome 7 and encodes for nitric oxide synthase 3, which responsible on producing the smallest signaling molecules known as nitric acid (Forstermann and Sessa, 2012). Before sending the *NOS3* amplicons to sequencing reaction, it was made sure that all the amplified amplicons had shown sharp, specific, and clean bands. The sequencing reactions indicated the confirmed identity of the amplified products by performing NCBI blastn (Zhang *et al.,* 2000). Concerning the 999 bp PCR amplicons of the *NOS3* gene, NCBI BLASTn engine has shown extremely high sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of about 99% of homology with the expected target that completely covered a portion of the *NOS3* gene, including exons 7 – 9 respectively. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. NG_011992.1), the exact positions and other details of the retrieved PCR fragment were identified (Fig. 1).

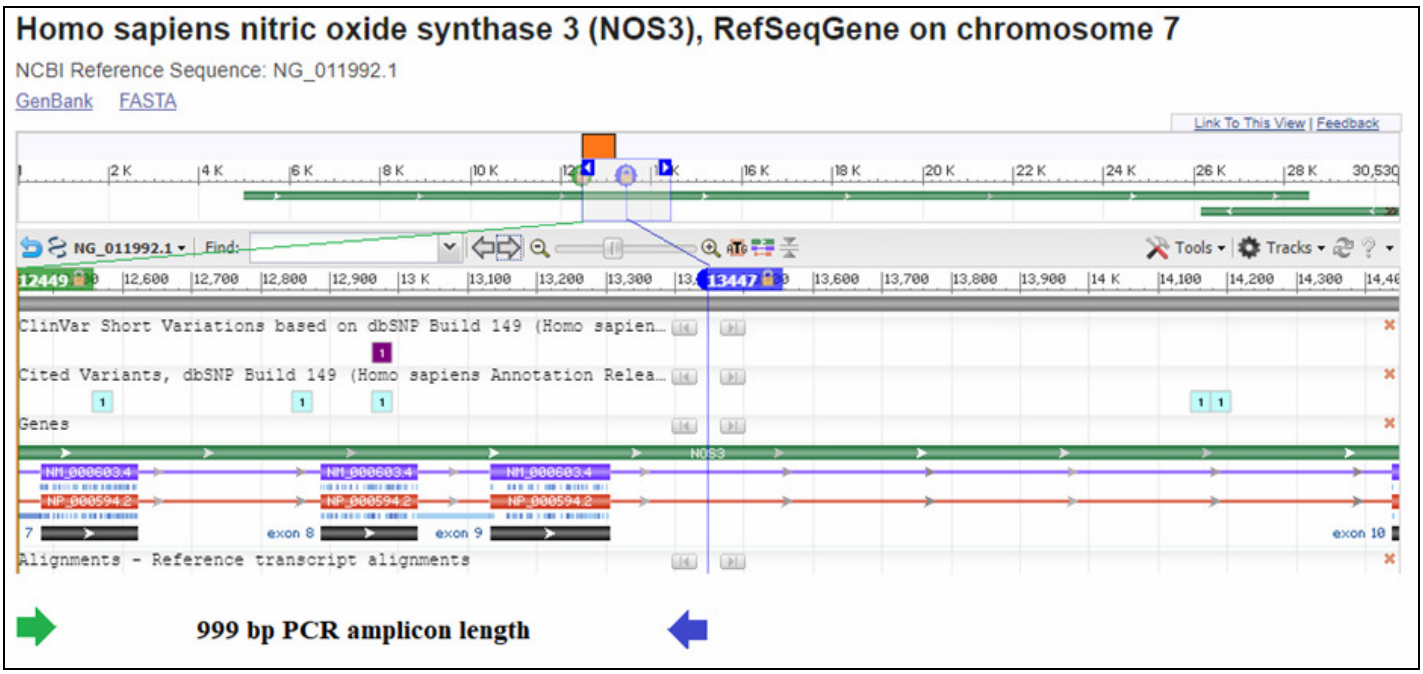

Fig. 1 : The exact position of the retrieved 999 bp amplicons that entirely covered a portion of the *NOS3* genetic sequences (acc. no. NG_011992.1). The green arrow refers to the starting point of this amplicons, while the cyan arrow refers to its end point.

Alignment of amplified exon (7-8) of *NOS3* gene

The alignment results of the 999 bp samples revealed the presence of 3 genetic variations variably distributed in some of the analyzed samples in comparison with the referring *NOS3* genetic sequences.

The sequencing chromatograms of the observed substitution variants, as well as their detailed annotations, were documented. The chromatogram details of the observed variants were shown according to their positions in the PCR amplicons, in which T132C shown two polymorphic patterns, heterozygous (C/T), and mutant homozygous (C/C). Whereas A414G exerted all three different polymorphic patterns, including normal homozygous (A/A), heterozygous (G/A), and mutant homozygous (G/G), while T517G exhibited only two polymorphic patterns, including heterozygous (G/T), and mutant homozygous (G/G). As seen in (Fig. 2).

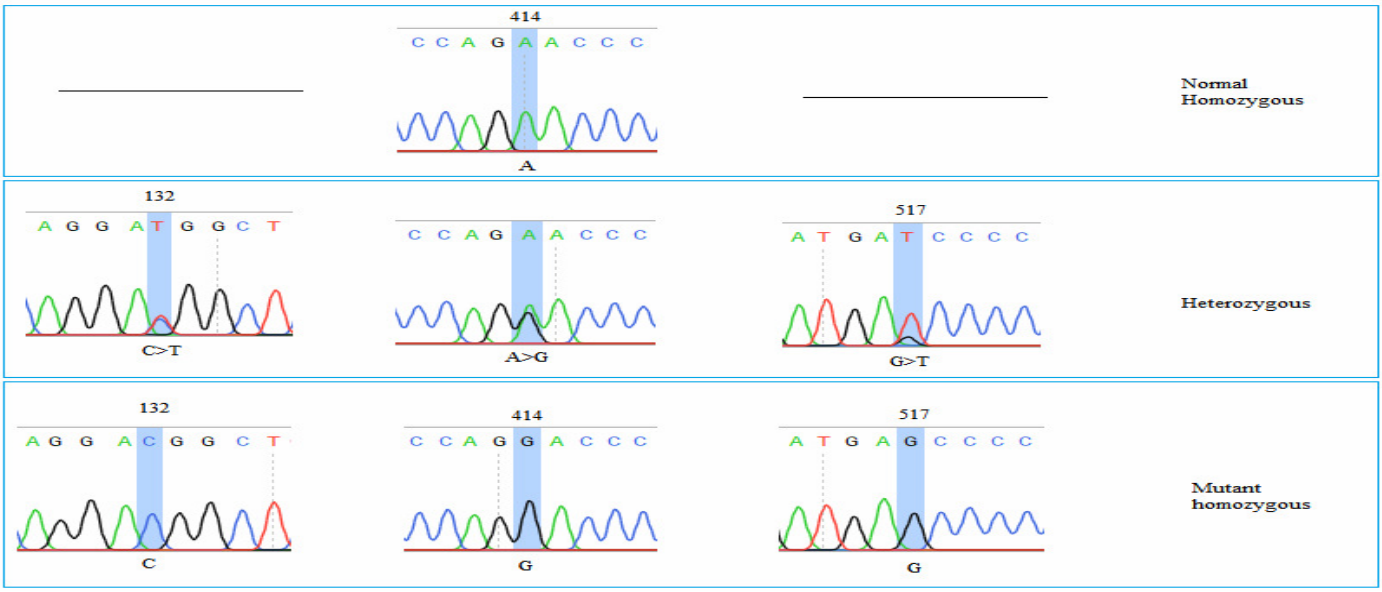

Fig. 2: The DNA chromatogram patterns of the observed substitution mutation of the 999 bp amplicons within the targeted *NOS3* genomic DNA sequences. The observed substitution mutations were highlighted according to their positions in the PCR products. The symbol ">" refers to a particular substitution mutation.

To elucidate the positions of the observed SNPs with regard to their deposited SNP database of the sequenced 999 bp fragment, the corresponding position of the *NOS3* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of the observed SNP, a graphical representation was performed concerning the *NOS3* dbSNP database within chromosome 7 (GenBank Acc. no. NG_011992.1). By reviewing the dbSNP engine, it was found that all these three SNPs were found to be previously known SNPs, namely rs1549758, rs1007311, and rs1799983 (Fig. 3). However, both rs1549758 and rs1799983 was found to be positioned in the coding portions of exon 7 and exon 8 respectively, while rs1007311 SNP was found to be located in an intronic position between both referred exons.

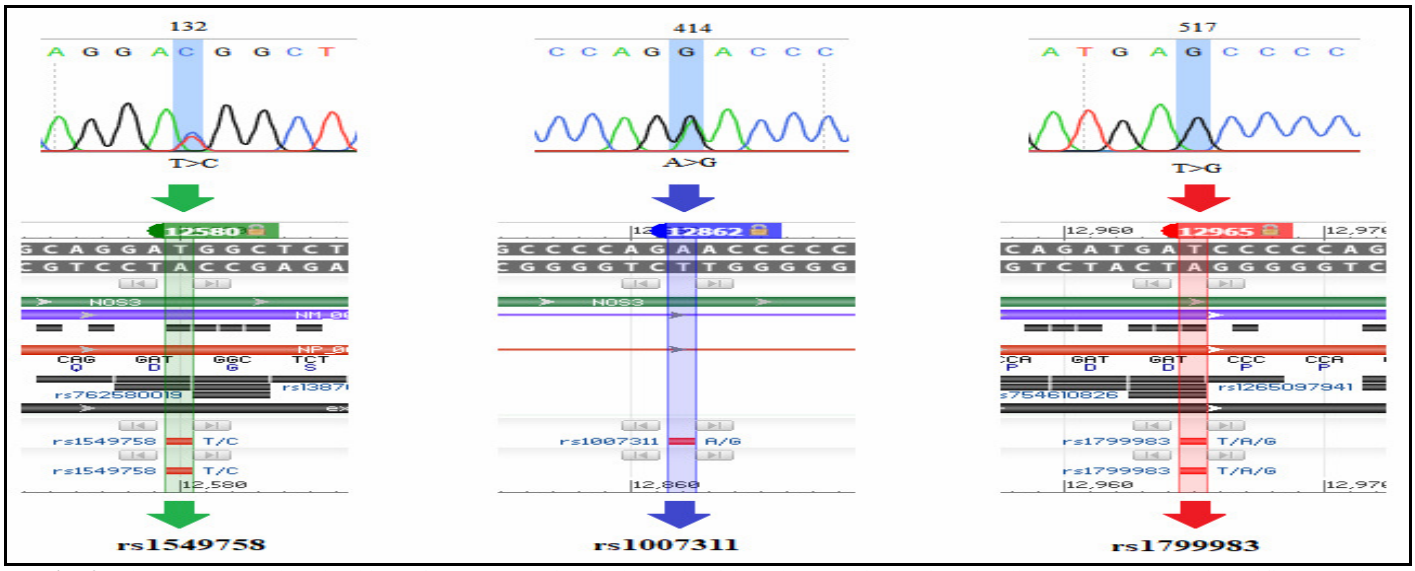

Fig. 3: The SNP's novelty checking of *NOS3* genetic single nucleotide polymorphism using the db SNP server. The identified SNP is marked with a green color.

Distribution of genotype and allele frequency of rs1549758 (T132C)

For rs1549758, the distribution in Table (2), frequencies of TT, TC and CC genotypes were (0) 0.00%, (6) 40%, and (9) 60% in the patients, and (0) 0.00%, (5) 50% and (5) 50% in the controls, respectively. There was no significant effect on the distribution of the TT genotype frequencies between T2DM patients and controls, and a significant effect in TC and CC genotype between patients and controls P-value (4.392).

Table 2: Distribution of genotype and allele frequency of rs1549758 (T132C)

Genotype: rs1549758 (T132C)	Patients No. (%)	Control No. (%)	P-value	O.R. (CI)
TT	0 (0.00%)	0 (0.00%)	1.00 NS	-
TC	6 (40.00%)	5 (50.00%)	4.392 *	0.702 (0.72-1.57)
CC	9 (60.00%)	5 (50.00%)	4.392 *	0.702 (0.73-1.62)
Total	15 (100%)	10 (100%)	1.00 NS	---
Chi-Square (χ^2)	12.33 **	10.25 **	0.21 NS	---
Allele Frequency				
T	0.20	0.25	---	---
C	0.80	0.75	---	---
* (P<0.05), ** (P<0.01), NS: non-Significant.				

Distribution of genotype and allele frequency of rs1007311 (A414G)

The distribution of rs1007311 genotype frequencies of the patient group is shown in Table (3), the highest genotype was the AG found in 7 patients (46.67%) followed by AA found in 5 patients (33.33%) and GG genotype found in 3 patients (20.00%). In addition, the highest genotype was the AG (6) 60.00%, followed by AA (4) 40.00% and GG genotype not found in the controls (0.00).

There was a significant effect on the distribution of the AG genotypic frequencies between T2DM patients and controls p-value (0.0227) and highly significant in GG genotype that found in T2DM patients only p-value (7.250).

Table 3: Distribution of genotype and allele frequency of rs1007311 (A414G)

Genotype: rs1007311 (A414G)	Patients No. (%)	Control No. (%)	P-value	O.R. (CI)
AA	5 (33.33%)	4 (40.00%)	0.069 NS	0.073 (0.58-1.49)
AG	7 (46.67%)	6 (60.00%)	0.0277 *	0.759 (0.75-1.62)
GG	3 (20.00%)	0 (0.00%)	7.250 **	1.197 (0.86-1.64)
Total	15 (100%)	10 (100%)	---	---
Chi-Square (χ^2)			---	---
Allele Frequency				
A	0.57	0.70	---	---
G	0.43	0.30	---	---
* (P<0.05), ** (P<0.01), NS: non-Significant.				

Distribution of genotype and allele frequency of rs1799983 (T517G)

The distribution of rs1799983 genotype frequencies of the patient group is shown in Table (4), the highest genotype was the GG found in 8 patients (53.33%) followed by TG found in 7 patients (46.67%) and TT genotype not found in patients and control (0.00%), the genotype was GG 5 (50.00%) and TG 5 (50.00%) found in control. There was no significant effect of this genotype TT, GG and TG frequencies between T2DM patients and controls P-value (1.00), (0.2095) respectively.

Table 4: Distribution of genotype and allele frequency of rs1799983 (T517G)

Genotype rs1799983 (T517G)	Patients No. (%)	Control No. (%)	P-value	O.R. (CI)
TT	0 (0.00%)	0 (0.00%)	1.00 NS	-
TG	7 (46.67%)	5 (50.00%)	0.2095 NS	0.217 (0.48-1.53)
GG	8 (53.33%)	5 (50.00%)	0.2095 NS	0.217 (0.48-1.53)
Total	15 (100%)	10 (100%)	---	---
Chi-Square (χ^2)	11.53 **	10.25 **	---	---
Allele Frequency				
T	0.23	0.25	---	---
G	0.77	0.75	---	---
** (P<0.01), NS: non-Significant.				

Relationship between rs1549758 (T132C), rs1007311 (A414G) and rs1799983 (T517G) with Peroxynitrite in T2DM

As demonstrated in Table (5), there was a significant decrease (P=0.736) in the mean of peroxynitrite in T2DM patients with TC genotype (32.67 ± 4.86) when compared with the CC genotypes (41.11 ± 15.01) in rs1549758 (T132C). Furthermore, there was a significant decrease (p=0.818) in the mean of peroxynitrite in T2DM patients who carried TG genotype (30.71 ± 4.55) when compared with the GG genotype (43.87 ± 16.73) in rs 1799983 l(T517G).

Table 5: Relationship between rs1549758 (T132C), rs1007311 (A414G) and rs1799983 (T517G) with Peroxynitrite in T2DM patients.

Mutation	Genotype	No.	Mean ± SE (nmol/ml)	LSD (P-value)
rs1549758 (T132C)	TC	6	32.67 ± 4.86	33.29 NS (0.736)
	CC	9	41.11 ± 15.01	
rs1007311 (A414G)	AA	5	30.40 ± 6.50 b	35.15 * (0.0392)
	AG	7	30.00 ± 4.12 b	
	GG	3	68.00 ± 25.09 a	
rs1799983 (T517G)	TG	7	30.71 ± 4.55	32.15 NS (0.818)
	GG	8	43.87 ± 16.73	
* (P<0.05), NS: non-Significant, a: high mean, b: low mean.				

While there was a significant increase in the mean of peroxynitrite in T2DM patient who carried GG genotype (68.00 ± 25.09 a) when compared with the AG genotypes (30.00 ± 4.12 b) and AA genotype (30.40 ± 6.50 b) in rs1007311 (A414G).

To summarize the results obtained from the sequenced 999 bp fragments, the exact positions of the observed variations were described in the NCBI reference sequences (Table 6).

Table 6: The pattern of the observed mutation in the 999 bp amplicons of the *NOS3* gene in comparison with the NCBI referring sequences (GenBank acc no. NG_011992.1). The symbol "P" refers to the patient sample number, while "C" refers to the control sample number.

Sample No.	Native	Allele	Zygosity status	Position in the PCR fragment	Position in the Reference genome	Amino acid substitution	Variant summary
C17, C22, C76, C77, C79, P9, P10, P19, P31, P35, P37, P39, P48, P51	T	C	Homozygous	132	12580	Synonymous (silent) variant Asp258Asp	rs1549758
C21, C23, P4, P7, P50	T	C	Heterozygous				
P35, P37, P51	A	G	Homozygous	414	12862	Non (intronic variant)	rs1007311
C21, C22, C23, C81, P4, P10, P31, P48	A	G	Heterozygous				
C17, C22, C76, C77, C79, P10, P19, P31, P35, P37, P39, P48, P51	T	G	Homozygous	517	12965	Missense variant Asp298Glu	rs1799983

Discussion

This study was done to address the potential of peroxynitrite-mediated mechanisms and the damaging effects of peroxynitrite in protein oxidation/nitration, in hopes of finding novel therapeutic approaches to mitigate peroxynitrite-related oxidative stress processes. A considerable body of evidence implicates formation of peroxynitrite as a critical pathogenic element in diabetic endothelial dysfunction. Previous studies have shown that the adverse effects of diabetes on impaired endothelial function have been positively associated with rises in oxidative stress and peroxynitrite formation as specified by nitrotyrosine. Increases in oxidative and nitrative stress were postulated to estimate for declines in NO and subsequent formation of peroxynitritis in coronary and aortic vessels (Tawfik *et al.,* 2006; Romero *et al.,* 2008). The previous results confirm with previous experimental research indicating that hyperglycemia could increase NO production or reduce its bioactivity leading to the enhanced superoxide formation (Adela *et al.,* 2015).

The possible contribution of peroxynitrite and its derived species has been intensely investigated by a number of groups studying various complications. These complications may lead to more serious diseases such as ulcers, renal failure, cataract, and blindness, as well as endothelial dysfunction, atherosclerosis, and myocardial injury (Obrosova *et al.,* 2005).

This study showed that the polymorphism of NOS3 was independently associated with the complication of T2DM which may be occurring due primarily to lifestyle factors and genetics. Type 2 diabetes mellitus is the most common form of diabetes and is characterized by disorders of insulin action and insulin secretion, hence a number of lifestyle factors are known to be important in the development of T2DM (Ripsin *et al.,* 2009). Several genes have been investigated for T2DM susceptibility, as well as the *NOS3* gene has been a candidate as a risk genetic factor for T2DM (Bressler *et al.,* 2013).

In additions, rs1549758 has been associated with several metabolic dysfunctions, such as glaucoma (Jeoung *et al.,* 2017), pulmonary hypertension, heart disease and hypertension (Levinsson *et al.,* 2014) and other syndromes (Duzkale *et al.,* 2013).

Whereas the intronic rs1007311 was not reported to be significant according to ClinVar database. However, several manuscripts were described this SNP with regard to several diseases, such as chronic mountain sickness (Buroker *et al.,* 2017), high altitude sickness (Buroker *et al.,* 2012), non-Hodgkin lymphoma (Han *et al.,* 2009).

As in the case of rs1549758, rs1799983 has also been found to be associated as a risk factor with several dysfunctions according to ClinVar database, but, rs1799983 has been studied extensively and many accumulated manuscripts have described the possible association of this SNP with several diseases, such as male infertility (Vučić *et al.,* 2018), enterocolitis in preterm infants (Szpecht *et al.,* 2018), gastric cancer (Zhu *et al.,* 2018). This is due to the larger effect of this SNP on the resulting protein structure as it includes an amino acid substitution from Asp to Glu at the 298th amino acid position.

With regard to rs1007311, it was found that there was no clinical significance for this intronic variant according to ClinVar database. However, several manuscripts have described this variant in terms of its possible association with several metabolic syndromes (Amankwah *et al.,* 2012).

In order to study the association of genetic polymorphism in *NOS3* gene with susceptibility complication in T2DM in Iraqi population, the result showed that was no relationship between rs1549758 (T132C) and rs1799983 (T517G) with peroxynitrite level. However, there was a relationship between rs1007311 (A414G) with peroxynitrite level high in GG genotype (68.00 ± 25.09 a) then in AA genotype (30.40 ± 6.50 b) and AG genotype (30.00 ± 4.12 b).

In conclusion, in this study there was no significant difference in the circulating peroxynitrite level between patients and controls. The variant rs1799983 had an influence on the fate of amino acid substitution but whether its contribution to T2DM risk is still unknown.

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