

GENETIC POLYMORPHISM IN ADIPONECTIN GENE IN IRAQI PATIENTS WITH TYPE-II DIABETES MELLITUS

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SUMMARY

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. This study was conducted to investigate the relationship between genetic polymorphism in adiponectin gene and the incidence of Type-II diabetes mellitus (T2DM) in a sample of Iraqi patients. Genetic polymorphism was studied in the variation site rs2241766 of adiponectin gene (*ADIPOQ*) located on the long arm of chromosome three (3q27), which regarded as a risk factor for the incidence of T2DM. First of all, genomic DNA was extracted from

blood samples for T2DM patients and healthy controls with a concentration ranged between 200-400 µg/ml and purity ranged between 1.5-2.0, then exon 2 of *ADIPOQ* was amplified using specific primers. Results of electrophoresis on 2% agarose gel for the amplified products showed that there is an amplified fragment with a molecular size 372 bp was obtained. Results of sequencing for the amplified fragment showed that 60% of healthy controls are (TT) non-polymorphic in the site of variation of exon 2 when aligned with the reference sequence in NCBI data base, while it was found that 40% of healthy controls are polymorphic heterozygous (TG) in the same site. Results also showed that 67.6% of T2DM patients are (TT) non-polymorphic in the same site of *ADIPOQ* exon 2, while 33.3% of those patients were polymorphic heterozygous (TG), and there is no significant difference between the homozygous variations in both groups of healthy controls and T2DM patients which refers that this polymorphism was not regarded as a risk factor for the incidence of T2DM in Iraqi patients under study.

KEYWORDS: The metabolic syndrome, *ADIPOQ*, polymorphism, Type-II diabetes mellitus, case-control study, Iraq.

INTRODUCTION

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces, Hyperglycemia, or increase in blood sugar, is a common effect of uncontrolled diabetes and over time it leads to serious damage to many of the body system, especially the nerves and blood vessel.^[1] During the last years the prevalence of diabetes has increased dramatically in many parts of the world and the disease is now a worldwide public health problem. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030.^[2] There are three main types of DM are recognized Type1 DM or insulin depended diabetes mellitus (IDDM), Type2 DM or non-insulin depended diabetes mellitus (NIDDM) and Gestational Diabetes Mellitus (GDM). Other specific types of diabetes are caused by specific genetic defects of β -cells function or insulin action, the pancreas diseases and drug or chemical also induced diabetes mellitus.^[3]

Type-II diabetes is the result of failure to produce sufficient insulin and insulin resistance. Elevated blood glucose levels are managed with reduced food intake, increased physical activity, and eventually oral medications or insulin. Type-II diabetes is caused by a combination of genetic factors related to impaired insulin secretion and insulin resistance and environmental factors such as obesity, overeating, lack of exercise, and stress, as well as aging. It is typically a multifactorial disease involving multiple genes and environmental factors to varying extents.^[4]

Adipose tissue was considered to play an important role in the pathogenesis of diabetes, as well as obesity by secreting a variety of secretory proteins.^[5] Adiponectin is one of the major adipocyte secretory proteins most abundantly found in human plasma, with potent roles in insulin sensitivity in muscle and liver, regulating energy homeostasis and glucose tolerance.^[6,7] Adiponectin is a product of the *ADIPOQ* gene, which is located on human chromosome 3q27 that modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation.^[8,9]

Many single nucleotide polymorphisms (SNPs) have been detected in *ADIPOQ* gene, which are associated with a variety of disorders. Most of the disorders are part of metabolic

syndromes e.g. impaired glucose tolerance, obesity, dyslipidemia and T2DM. The association of SNP +45 T >G in the adiponectin gene with Type-II diabetes and insulin resistance has been investigated in several populations.^[10] According to those findings, this study was aimed to determine the relationship between the genetic polymorphism and in *ADIPOQ* and the incidence of T2DM in a sample of Iraqi patients.

MATERIALS AND METHODS

Subjects

Venous blood was obtained after overnight fasting, and processed at the National Diabetes Center, Al-Mustansirya University in Baghdad governorate, in the period between October 2016 and November 2016.

Extraction of Genomic DNA

Total genomic DNA was extracted from 15 blood samples collected from T2DM patients, and from 15 blood samples collected from healthy volunteers. DNA extraction was carried out by using G-spin™ Blood gDNA silica membrane system supplied by iNtRON Biotechnology/KOREA.

Genotyping of the *ADIPOQ* +45 T > G polymorphism

The *ADIPOQ* +45 T > G polymorphism (rs2241766) was genotyped by polymerase chain reaction (PCR). The polymorphism was amplified using the following primers (11): forward, 5'-GCAGCTCCTAGAAGTAGACTCTGCTG-3' and reverse, 5'-GCAGGTCTGTGATGAAAGAGGCC-3'. Polymerase chain reaction was performed in a total volume of 25µl mixture containing 1.5µl DNA, 10µl forward primer, 10µl reverse primer, 5µl deoxynucleotide triphosphate (dNTP), 5 µl MgCl₂, 16.5µl molecular biology grade D.W., 5µl of *Taq* DNA polymerase and 16.5 µl of ddH₂O. The amplification conditions were as follows: 94 for 3 min, followed by 35 cycles of 40 sec. at 94, 1 min at 62 °C and 1 min at 72, and ending with a single 7 min extension step at 72. The PCR products were run on 2% agarose gels electrophoresis and viewed under an UV transilluminator to evaluate overall amplification efficiency.

Amplification products for exon 3 of *ADIPOQ* gene was sequenced, then sequences of these products was compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *ADIPOQ* gene, using Bioedit software.

Statistical analysis

Continuous variables are presented as means \pm standard deviation (SD) and difference between the cases and controls was compared using Student's *t*-test for normally distributed variables or non-parametric Mann-Whitney *U* test for non-normally-distributed ones. Test for Hardy Weinberg equilibrium and comparison of allele and genotype distribution between MS cases and controls were assessed by chi-square test. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated using the Mantel-Haenszel method. Multiple logistic regression analysis was performed to estimate an association of the +45 T > G polymorphism with the risk of MS. A *p* value less than 0.05 was considered statistically significant. All data analyses were performed using SAS version 9.2 software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Demographic characteristics of 15 T2DM patients and 15 controls are shown in Table 1. Mean age of the cases and controls were 58.65 ± 1.10 years and 41.4 ± 2.27 years, respectively.

Genomic DNA was first extracted from blood samples of T2DM patients and healthy controls. Results illustrated in figure (1) showed the clear bands for genomic DNA extracted from each blood samples with a concentration ranged between 200 and 400 ng/ μ l, while the purity was ranged between 1.5 and 2.0. This purity and concentration were suitable and recommended for further genetic analysis by using PCR technique. Polymerase chain reaction was used to amplify exon 2 of *ADIPOQ* in the extracted genomic DNA under the optimum amplification conditions by using specific primers shown previously.

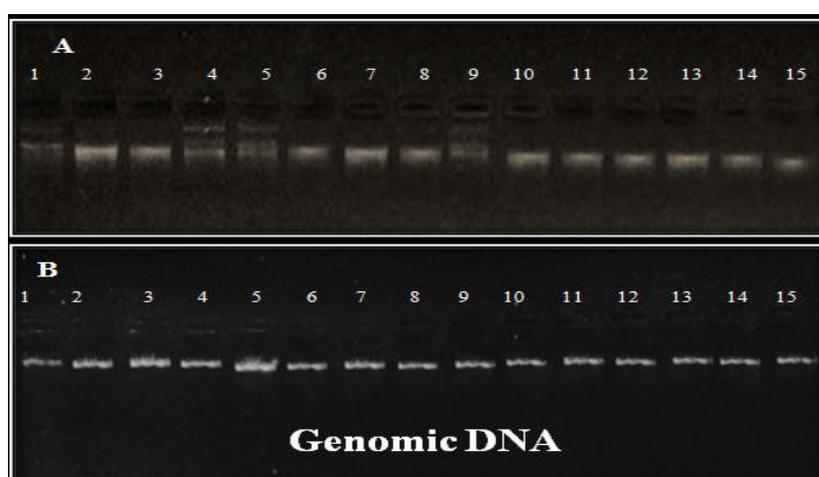


Figure (1): Electrophoresis of genomic DNA on agarose gel (1.0%) for 75 minutes hour at 5 v/cm. (A): DNA extracted from blood sample of healthy controls (B): DNA extracted from blood sample of patients with Type two Diabetes.

Sequencing of Amplified Exon 2 of *ADIPOQ* gene

In order to study the association of genetic polymorphism in *ADIPOQ* (rs2241766) with susceptibility to T2DM in Iraqi population, PCR products for exon 2 were sequenced. Results illustrated in figure (2) showed the complete nucleotide sequence of this exon in healthy controls and T2D patients and the position of the expected SNP rs2241766 that may be associated with the incidence of the disease were defined.

A	
1	GCAGCTCCTAGAAAGTAGACTCTGCTGAGATGGACGGAGTC 40
41	CTTTGTAGGTCCCAACTGGGTGTGTGTGTGGGGTCTGTC 80
81	TCTCCATGGCTGACAGTGCACATGTGGATTCCAGGGCTC 120
121	AGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTC 160
161	TGCCCCGGTCATGACCAGGAAACCACGACTCAAGGGCCCCGG 200
201	AGTCCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGTTGG 240
241	ATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCC 280
281	CAGGCCGTGATGGCAGAGATGGCACCCCTGGTGAGAAGGG 320
321	TGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTCTTT 360
361	CATCACAGACCT 372
B	
1	GCAGCTCCTAGAAAGTAGACTCTGCTGAGATGGACGGAGTC 40
41	CTTTGTAGGTCCCAACTGGGTGTGTGTGTGGGGTCTGTC 80
81	TCTCCATGGCTGACAGTGCACATGTGGATTCCAGGGCTC 120
121	AGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTC 160
161	TGCCCCGGGCATGACCAGGAAACCACGACTCAAGGGCCCCGG 200
201	AGTCCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGTTGG 240
241	ATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCC 280
281	CAGGCCGTGATGGCAGAGATGGCACCCCTGGTGAGAAGGG 320
321	TGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTCTTT 360
361	CATCACAGACCT 372

Figure (2): Nucleotide sequences and amino acid of *ADIPOQ* gene of exon 2. Yellow letter indicates the position of expected *ADIPOQ* SNP in T2DM patients in Iraq. A: Sequence from healthy controls group, B: Sequence from T2DM patients group.

Alignment of amplified Exon 2 of *ADIPOQ* Gene

Results illustrated figure (3) showed that the nucleotide sequence for *ADIPOQ* exon 2 in healthy controls and it was found that only 9 (60%) out of 15 controls were genotyped as non-polymorphic homozygous TT in comparison with reference sequence of NCBI. and was given ID: NG 021140.1 from 15320-15620 number of nucleotide from *Homo sapiens* adiponectin gene, with score 556 and expect 155. This indicates that there is a high degree of similarity between both sequences which give greater confidence, while the other six healthy controls (40%) genotyped as polymorphic heterozygous (TG), where thymine was substituted

with guanine in the site of variation rs2241766 of ADIPOQ gene exon 2 as illustrated in figure (4).

Score	Expect	Identities	Gaps	Strand
556 bits(301)	8e-155	301/301(100%)	0/301(0%)	Plus/Plus
Query 1	CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	60		
Sbjct	15320 CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	15379		
Query 61	CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGTCATGACCAG	120		
Sbjct	15380 CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGTCATGACCAG	15439		
Query 121	GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	180		
Sbjct	15440 GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	15499		
Query 181	TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	240		
Sbjct	15500 TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	15559		
Query 241	GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	300		
Sbjct	15560 GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	15619		
Query 301	T 301			
Sbjct	15620 T 15620			

Figure (3): A representative sequence alignment of *ADIPOQ* gene of exon 2 of non-polymorphic healthy controls (sbjct) aligned with reference sequence (Query) of *ADIPOQ* gene exon 2 obtained from Gene Bank. Yellow letters indicate the position of expected SNP.

Score	Expect	Identities	Gaps	Strand
551 bits(298)	4e-153	300/301(99%)	0/301(0%)	Plus/Plus
Query 1	CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	60		
Sbjct	15320 CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	15379		
Query 61	CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGGCATGACCAG	120		
Sbjct	15380 CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGGTCATGACCAG	15439		
Query 121	GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	180		
Sbjct	15440 GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	15499		
Query 181	TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	240		
Sbjct	15500 TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	15559		
Query 241	GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	300		
Sbjct	15560 GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	15619		
Query 301	T 301			
Sbjct	15620 T 15620			

Figure (4): A representative sequence alignment of *ADIPOQ* gene of exon 2 of polymorphic heterozygous T2DM patient (sbjct) compared with reference sequence (Query) of *ADIPOQ* gene exon 2, obtained from Gene Bank. Yellow letters indicate the position of expected SNP

These results agree with other study who found that the frequency of TT and TG genotypes were 70.4% and 25.9% in healthy controls respectively, while the other GG are 3.7% in Han Chinese population.^[11]

Results of nucleotide sequence for *ADIPOQ* exon 2 in Iraqi patients with T2DM illustrated in figure (5) showed that 10 (66.66%) out of 15 patients were genotyped as non-polymorphic homozygous TT and not significantly difference with healthy controls, Thymine nucleotide was located in the same position as in healthy controls without any base substitution to other nucleotides compared to reference sequence in this sample of patients.

Score	Expect	Identities	Gaps	Strand
556 bits(301)	8e-155	301/301(100%)	0/301(0%)	Plus/Plus
Query 1	CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	60		
Sbjct 15320	CTGGGTGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	15379		
Query 61	CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGTCATGACCAG	120		
Sbjct 15380	CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCGGTCATGACCAG	15439		
Query 121	GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCAAGGGGGCCTGCACAGGT	180		
Sbjct 15440	GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCAAGGGGGCCTGCACAGGT	15499		
Query 181	TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	240		
Sbjct 15500	TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	15559		
Query 241	GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	300		
Sbjct 15560	GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	15619		
Query 301	T	301		
Sbjct 15620	T	15620		

Figure (5): A representative sequence alignment of *ADIPOQ* gene of exon 2 of non-polymorphic heterozygous T2DM patients (sbjct) compared with reference sequence (Query) of *ADIPOQ* gene exon 2, obtained from Gene Bank. Yellow letters indicate the position of expected SNP.

On the other hand five out of 15 T2DM patients 33.3% genotyped as polymorphic heterozygous in the site of polymorphism with no significant difference with polymorphic heterozygous T2DM patients as shown in figure (5).

Score	Expect	Identities	Gaps	Strand
551 bits(298)	4e-153	300/301(99%)	0/301(0%)	Plus/Plus
Query 1	CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	60		
Sbjct	15320 CTGGGTGTGTGTGTGGGGTCTGTCTCTCCATGGCTGACAGTGCACATGTGGATTCCAGGG	15379		
Query 61	CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCCGGGCATGACCAG	120		
Sbjct	15380 CTCAGGATGCTGTTGCTGGGAGCTGTTCTACTGCTATTAGCTCTGCCCCGGGCATGACCAG	15439		
Query 121	GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	180		
Sbjct	15440 GAAACCACGACTCAAGGGCCCGGAGTCTGCTTCCCCTGCCCAAGGGGGCCTGCACAGGT	15499		
Query 181	TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	240		
Sbjct	15500 TGGATGGCGGGCATCCCAGGGCATCCGGGCCATAATGGGGCCCCAGGCCGTGATGGCAGA	15559		
Query 241	GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	300		
Sbjct	15560 GATGGCACCCCTGGTGAGAAGGGTGAGAAAGGAGATCCAGGTAAGAATGTTTCTGGCCTC	15619		
Query 301	T	301		
Sbjct	15620 T	15620		

Figure (6): A representative sequence alignment of ADIPOQ gene of exon 2 of polymorphic heterozygous T2DM patients (sbjct) compared with reference sequence (Query) of ADIPOQ gene exon 2, obtained from Gene Bank. Yellow letters indicate the position of expected SNP.

This study showed that the ADIPOQ +45 T > G polymorphism was independently associated with T2DM. Type-II DM is due primarily to lifestyle factors and genetics. Type-II is the most common form of diabetes and is characterized by disorders of insulin action and insulin secretion, A number of lifestyle factors are known to be important to the development of Type-II DM.^[12] These are physical inactivity, sedentary lifestyle, cigarette smoking and generous consumption of alcohol.^[13] Obesity has been found to contribute to approximately 55% of cases of T2DM. The increased rate of childhood obesity is believed to have led to the increase in T2DM in children and adolescents, Environmental toxins may contribute to the recent increases in the rate of T2DM. A weak positive correlation has been found between the concentration in the urine of bisphenol A, a constituent of some plastics, and the incidence of T2DM.^[14] As shown in table (2), frequencies of TT, TG and GG genotypes were 66.66%, 33.33% and 0.00% in the patients, and 60%, 40% and 0.00% in the controls, respectively. There was no significant difference in distribution of the TT and TG genotypic frequencies between patients and controls. According to these results the homozygous TT genotype considered as predominant genotype within the population of this study and the presence of

TG genotype in control samples reveal that it has no effect on the disease occurrence. The absence of GG genotype considered it as infrequent genotype within the studied group. This study showed that the *ADIPOQ* +45 T > G polymorphism was independently associated with T2DM. Both genetic and environmental factors contribute to the development of MS.^[15,16] Some studies have provided evidence that the *ADIPOQ* +45 T>G polymorphism is associated with MS components.^[17-19] Both genetic and environmental factors contribute to the development of MS.^{27,28} Some studies have provided evidence that the *ADIPOQ* +45 T>G polymorphism is associated with MS components.^[15-17] However, a number of studies have mainly focused on the association of the polymorphism with one or two components of MS, such as obesity or/and T2DM.

Table (2): Distribution of frequencies of genotype between patients and controls.

Genotype/ Allele	Patients N=15	Control N=15	Chi-square
TT	10(66.66%)	9(60%)	2.674 NS
TG	5(33.33%)	6(40%)	2.674 NS
GG	0(0.00%)	0(0.00%)	0.00 NS
T	0.83	0.80	
G	0.17	0.20	

In China a study reported that frequencies of TT, TG and GG genotypes in T2DM patients were 47.7%, 40.3% and 12%, and its frequencies in healthy controls were 48.6%, 44.8% and 6.6% respectively.^[20] While another study in China results of T2DM patients were 52.4%, 39.5% and 8.0% and 49.7%, 40.1% and 10.1% for healthy controls, respectively.^[21] in Indian population results of T2DM patients were 78.6%, 21.3% and 0.0%, while in healthy controls were 93.3%, 5.3% and 1.3%, respectively.^[10] While studying the relationship between *ADIPOQ* gene +45T>G polymorphism in Taiwan results showed 49.4% of TT genotype, 46.1% of TG genotype and 4.5% for GG genotype in T2DM patients.^[22] In Italy results were 73.5% for TT genotype, 23.2% for TG genotype and 3.1% for GG genotype in T2DM patients while in healthy controls results were 71.3% for TT genotype, 25.0% for TG genotype and 3.5% for GG genotype.^[23] In addition to study in Iranian population results showed 70.9% for TT genotype, 26.1% for TG genotype and 2.9% for GG genotype in T2DM patients and its frequencies in healthy controls were 67.6%, 27.1% and 5.2% for TT, TG and GG genotypes respectively.^[24] Another study in Saudi Arabia by showed 73.8%, 24.1% and 2.0% in T2DM patients and 70.1%, 26.8% and 3.0% in healthy controls for TT, TG and GG genotypes respectively.^[25]

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