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Association of PPARG gene polymorphism (Pro 12 Ala) with the risk of type 2 diabetes mellitus (T2DM) incidence in sample of Iraqi patients

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Abstract: Diabetes mellitus is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycaemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both . *PPARG* gene variant (pro 12 ala) has been linked to risk of development of diabetes mellitus . this study was carried out to examine whether the polymorphism of *PPARG* gene are correlated with the incidence of type 2 diabetes mellitus (T2DM) in Iraqi population. Diabetic patients (n=50) and apparently healthy control subject (n=50), were enrolled genotyping of *PPARG* gene SNP (rs1801282) were determined by using Taqman genotyping assay. The results showed that the distribution of genotypes and alleles frequencies at (rs1801282) SNP of *PPARG* gene, as related with CC , CG and combined CG+CC genotypes, G allele seem to be a protective allele, therefore, the presence of both heterozygous and homozygous mutants may reduce the risk of T2DM (the frequency of CG+GC mutants were 68% in apparently healthy control individuals and 50% in T2DM patients). In contrast, there is wild CC genotype (50 versus 32% in T2DM and control group, respectively, X2=6.93; P<0.01; OR=1.272).

Keywords: type2 diabetes mellitus , *PPARG* , Pro 12 Ala, in silico study.

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Introduction:

Diabetes mellitus is recognized as a public health problem of pandemic proportions. Data compiled by the World Health Organization (WHO) in 2014 indicated that the global prevalence of diabetes was close to 10% among adults aged 18 years and above.(1)

If present trends persist, projection estimates ominously predict that over half a billion people will develop diabetes within the next two decades, with Asian countries contributing more than 60% of the world's diabetic population . (2)

Diabetes is currently the fastest-growing epidemic and has been ascribed to a collision between genes and the environment. Worldwide prevalence figures estimate that there were 382 million people living with diabetes in 2013 and that by 2035 this number will have risen to 592 million (3).

The genetic background of T2DM has shown to be a cocktail as described by Freeman and Cox who suggested that understanding the basis of the genetic traits of T2DM can help identify new therapeutic targets, which represents one of the most promising strategies for the long-term treatment success (4).

Scientists have used their tools for exploring the genetic background of T2DM including "Genome Scans" and "Association studies". The association studies, including Genome Wide Association (GWA) Studies , are case-control studies that investigate the

relationship of particular disease status with certain alleles, genotype (or haplotype) or most commonly a set of single-nucleotide polymorphisms (SNPs). (5). The GWAS which were first successfully conducted in 2005 is a major tool for identifying different biological pathways, understanding the pathophysiology of several complex (multifactorial) diseases and developing drug therapies (5).

Several association studies have identified some key genes for human T2DM susceptibility of which the peroxisome proliferator-activated receptor gamma (*PPAR*-γ) and the KCNJ11 are the most promising genes (6).

Peroxisome proliferator-activated receptors (*PPARS*) are ligand-activated transcription factors that are part of the superfamily includes receptors for steroid hormones, thyroid hormones, retinoic acid and fat-soluble vitamin A and D. The primary role of *PPAR*S is to regulate glucose, fatty acid and lipoprotein metabolism, energy balance, cell proliferation and differentiation, inflammation and atherosclerosis (7).

There has been conflicting results about the association of Pro12Ala mutation of *PPAR*- γ gene with T2DM in different populations (8,9) .This study theorize that this variant is linked to the risk of T2DM in Iraqi people as well due to its possible impacts on insulin action and/or signaling pathways and that the inconsistent findings concerning its association with diabetes around the world is a reflection of the multifaceted interactions between this polymorphism and various environmental factors (including the different fatty acids contents in people diet world-wide) and/or between this sequence variation and other genetic factors.

In fact, there have been few studies on the association of Pro12Ala (rs1801282) of *ppar*-γ gene with T2DM in Iraqi people So, this study aims to investigate these associations in sample of adult Iraqi people of Arabic ethnicity.

Materials and Methods:

Whole Blood samples were collected from T2DM patients (n=50) and apparently healthy subjects as control (n=50). The T2DM patients were chosen from private clinics in Baghdad. Those patients who were identified by physicians as having T2DM, the criteria to diagnose diabetes were based on WHO guidelines.A subject was considerd have diabetes if his/her fasting glucose level was >126 mg/dl (7.0 mmol/l) in addition of symptoms of diabetes (1).Questionnaire that includes information about age, gender, family history, for all subjects‟ weight, BMI had measured.

Peripheral blood samples of T2DM and control groups were collected in EDTA anticoagulated tubes, Blood samples were collected by vein puncture from the T2DM patients and apparently healthy controls. Then, 2.5 ml of blood was kept in EDTA anticoagulant tubes in refrigerator to be a source for DNA extraction. 2.5 ml kept for biochemical investigations.

Total genomic DNA was extracted from the whole fresh blood using genomic DNA extraction kit (WizPrep™ DNA Extraction Kit), Nanodrop (2000 C apparatus, Thermo Scientific, USA) was used to estimate the purity and the concentration for DNA samples. The purity of DNA should be between 1.7-1.9 (10). Genotyping analyses were performed using Real Time PCR (The software program was used to analyze the

genotypes comes from Rotor gene Q. QIAGEN Company), TaqMan fluorescent oligonucleotide probes and primers (Alpha DNA Ltd., Canada) were prepared according to William *et al.*(11), and stored lyophilized at - 20°C. The sequence of each probe and primer used in the allelic discrimination experiments are shown in table (1).

Primer and probe sequence were matched by the bioinformatic programs (NCBI). The probe prepared for the wild type was labeled with FAM at the 5′ end and MGB at the 3′ end. While the mutant allele detecting probe (SNP) was labeled with VIC at the 5′ end and MGB at the 3′ end, the normal wild type, mutant genotype and the heterozygous genotype are shown in figure(1).

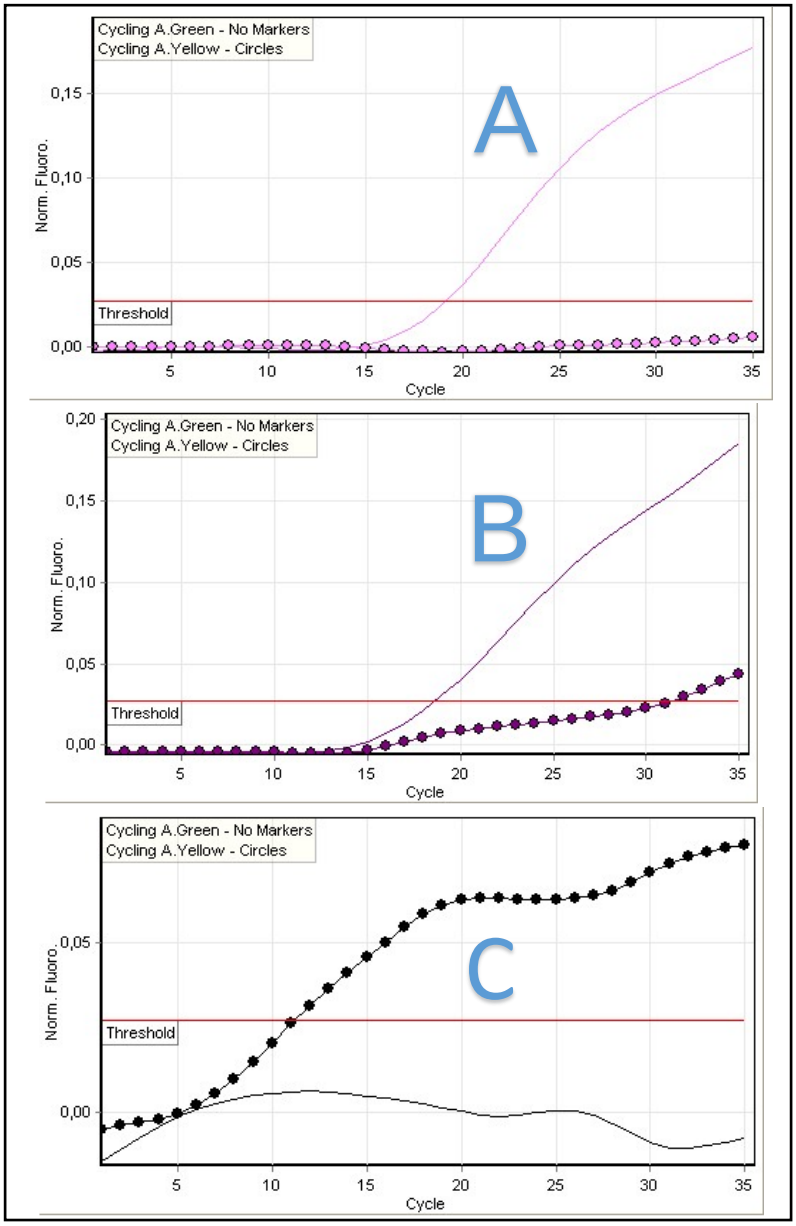

Figure (1): The (A) Homozygouse wild genotype (reacted with FAM only), (B) heterozygous genotype (reacted with FAM and VIC) and (C) Homozygouse mutant genotype (reacted with VIC only) .

Table (1): Primers and Probes used in the study designed by express ® software v 2.0

Primer/probe	Sequence (5′ →3′ direction)
rs1801282	
Forword	AAACCCCTATTCCATGCTGTTATG
Reverse	GCAGACAGTGTATCAGTGAAGGAATC
FAM- probe	CTCCTATTGACCCAGAAA
VIC-probe	TCCTATTGACGCAGAAA

The System of Statistical Analysis - SAS (2012) program was used to evaluate the difference factors in parameters in this study. The test of Chi-square and Odd ratio were used for significant difference between percentage and least significant difference (LSD) test was used for significance comparison between means.

Results and discussion:

1. Subjects data:

A total of one hundred blood samples were collected from type 2 diabetic patients (n=50) and apparently healthy subjects as a control (n=50).

Results presented in table 2 related to information of different criteria that obtained from the questionnaire forma answered by patients and control subjects.

The age results revealed that most patients with T2DM (43/50) were aged more than 45 years old. Results of table 2 showed that gender has no significant contribution on the incidence of T2DM among the 50 diabetic patients involved in the present study (male: female=24:26).

The present analysis of BMI showed that (20/50) of control subject and (10/50) of T2DM patients were healthy weight (BMI 25-29) while 48% of apparently healthy subjects (control) were overweight (BMI was equal to 30-34) .in addition, the number of obese subjects was in T2DM patients three-fold that of apparently healthy subjects (15 versus 6, respectively). The BMI was used in a wide variety of contexts as a simple method to assess how much an individual body weight departs from what is normal or desirable. Forty-two percent of T2DM patients were suffer from the disease since less than 5 years (21/50) while more than 5 years for 58%(29/50). Forty-five out of fifty subjects were with family history as related with T2DM.

The characteristics of the study subjects stratified according to diabetic and non-diabetic status (control), as defined by IDF criteria , are displayed in table (2).

Table (2): The distribution of the basic characteristics of subjects in both study groups.

characteristics		Control N (%)	T2 DM N (%)
Age	<46	6(12%)	7(14%)
	46-55	23(46%)	21(42%)
	>55	21(42%)	22(44%)
Gender	male	23(46%)	24(48%)
	female	27(54%)	26(52%)
BMI	healthy 25-29	20(44%)	10(20%)
	over weight 30-34	24(48%)	25(50%)
	Obese 35-39	6(12%)	15(30%)
Duration	<5 years	-	21(42%)
	>5 years	-	29(58%)
Family history	Positive	35(70%)	45(90%)
	Negative	15(30%)	5(10%)

2. Biochemical characteristics:

The biochemical characteristics of the T2DM patients and apparently healthy subjects (control) are summarized in table (3).

Fasting blood sugar concentrations were significant in T2DM patients ($p<0.01$) higher than those of control group (161.54 *versus* 96.24 mg/dl, respectively). Also, the HbA1c values were significant in T2DM patients ($p<0.01$) higher than those of control group (9.98 compared with 4.49, respectively).

Serum low density lipoprotein (LDL) concentrations were significantly ($p<0.01$) decreased in T2DM patients when compared with those of apparently healthy subjects (60.74 *versus* 98.56 mg/dl, respectively).

Serum HDL, cholesterol and triglyceride concentrations were increased ($p<0.01$) in T2DM patients when compared with apparently healthy control subjects (124.24, 215.48 and 240.7 versus 60.3, 174.14 and 101.76 mg/dl, respectively).

Serum levels of FBS, HbA1c, LDL, HDL, Cholesterol and TG in diabetic patients versus control subjects are presented in table (3).

Table (3): The distribution of the biochemical characteristics of subjects in both study groups.

Parameters(mg/dl)	Control Mean±S.E.	T2DM Mean±S.E.	P-value Mean±S.E.
FBS	96.24 ± 1.66	161.54 ± 3.95	0.0001 **
HbA1c	4.49 ± 0.09	9.98 ± 0.28	0.0001 **
LDL	98.56 ± 2.83	60.74 ± 3.54	0.0001 **
HDL	60.30 ± 1.98	124.24 ± 4.41	0.0001 **
Cholesterol	174.14 ± 4.19	215.48 ± 7.71	0.0001 **
Triglyceride	101.76 ± 4.55	240.70 ± 10.07	0.0001 **

**** significant at 0.01 level.**

3. DNA concentration and purity:

In this study, DNA extraction method depend on lysis of red blood cells and remove the undesirable contaminants such as protein and RNA in addition to degradation of the cell membrane of white blood cells.

The quantification of DNA by Nanodrop, measured DNA concentration reached between 5.5 and 9 ng/µl and the purity range was between 1.7 and 1.9 as recommended. These results revealed that the fresh blood samples yielded enough DNA concentration.

4. Primer and probe design:

Primers and probes that used in this study for genotyping of pro12ala (rs1801282) SNP in *PPARG* gene (NG-011749.1) was designed by using primer express ® software v 2.0 then checked using NCBI as shown in figures (2).

5. Polymorphisms of *PPARG* gene:

SNP genotypes were determined by TaqMan genotyping assay using the primers and probes shown in figure 2 and the results were obtained and classified according to the FAM and VIC dyes as shown in the figure (3).

Homo sapiens peroxisome proliferator activated receptor gamma (PPARG), RefSeqGene on chromosome 3
NCBI Reference Sequence: NG_011749.1
GenBank Graphics
>NG_011749.1:68723-68811 Homo sapiens peroxisome proliferator activated receptor gamma (PPARG), RefSeqGene on chromosome 3
Pro12Ala (rs1801282C>G)

AAACCCCTATTCCATGCTGTTATGGGTGAAACTCTGGGAGATTCTCCTATTGACCCAGAAAG
CGATTCCTTCACTGATACACTGTCTGC

AAACCCCTATTCCATGCTGTTATGGGTGAAACTCTGGGAGATTCTCCTATTGACGCAGAAAG
CGATTCCTTCACTGATACACTGTCTGC

sequences of forward and reverse primers **FAM and VIC probes rs1801282 SNP position.**

Figure (2): Checking the designed primers and probes of Pro12Ala (rs1801282) by using NCBI.

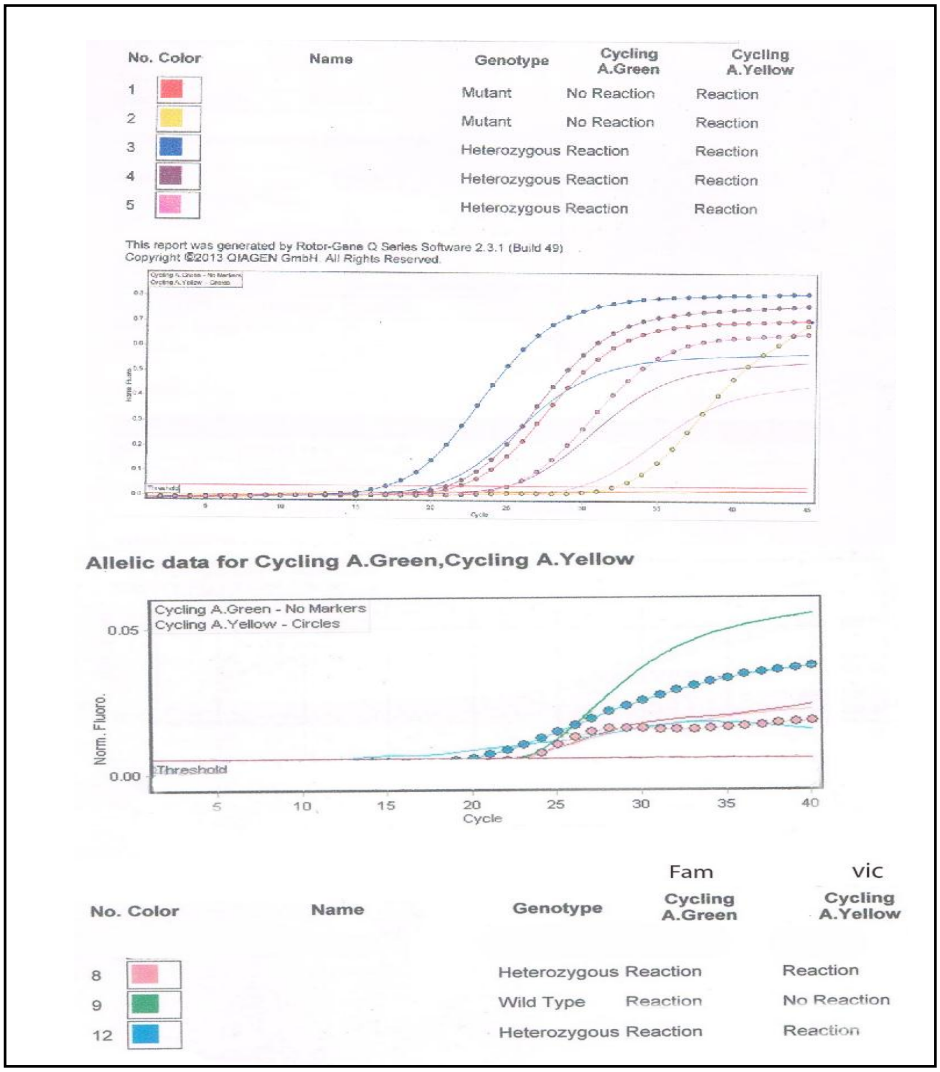

Figure (3): TaqMan SNP genotyping assay using real time thermocycler. Wild type genotype reacted with FAM only, heterozygous genotype reacted with both FAM and VIC, while mutant genotype reacted with VIC only.

Pro12Ala SNP of *PPARG* gene:

This SNP was subjected to both *in silico* and molecular studies.

1. In silico study:

Many web servers were used for in silico prediction for Pro12Ala SNP (18061282) of *PPARG* gene.

A. Provean:

PROVEAN (protein variation effect analyzer) is a tool to predict the functional effects of amino acid substitution. It uses an alignment-based score approach . In this study, the SNP pro12 ala (rs1801282) of *PPARG* gene was subjected to PROVEAN (provean.jcvi.org/seq-submit.php). The provean score of the SNP Pro12Ala (rs1801282) of *PPARG* gene was -1.279 then the prediction of this variant was neutral because the score is above – 2.5.

B. polyphen-2 :

PolyPhen2 (Polymorphism Phenoty ping v2) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. The PolyPhen-2 score can be interpreted as follows: 0.0 to 0.15: Variants with scores in this range are predicted to be benign.0.15 to 1.0: Variants with scores in this range are possibly damaging. 0.85 to 1.0: Variants with scores in this range are more confidently predicted to be damaging. The PolyPhen-2 score of the SNP Pro12 Ala (rs1801282) of *PPARG* gene was 0.000 therefore this mutation is predicted to be benign.

C. I-Mutant 2.0 :

I-Mutant 2.0 can predict the stability change of the mutated protein structure. The DDG values were computed at 25 C and p H =7. If the DDG value (RI) is more than zero, the protein stability decreases and when DDG value is less than zero, protein stability increases. In this study, the SNP pro12 ala (rs1801282) of *PPARG* gene was subjected to I-mutant. The DDG (Rrliability index) of rs1801282 SNP of *PPARG* gene was equal to -1.40 then the predicted protein stability of this variant was increase because DDG value is less than zero.

D. PhD-SNP:

PhD-SNP is a tool used to predict of human deleterious single nucleotide polymorphisms(PhD-SNP). In this study the pro12ala SNP of *PPARG* gene was subjected to PhD-SNP. The prediction value was equal to 7 then the predicted human deleterious of this variant was neutral polymorphism .

2. Molecular study:

The studied variant (rs1801282) is located within short taq sequence (STS) upstream exon 3 of *PPARG* gene. The distributions of genotypes and alleles frequencies of this SNP are shown in table 4.

As related with CC (wild) genotype, the frequency was in patients with T2DM significantly ($p<0.01$) higher than in apparently healthy controls (50 versus 32%, respectively; X2= 6.932; OR= 1.272). Also, the frequency of CG (heterozygous) genotype was significantly ($p<0.01$) higher in apparently healthy control

when compared with T2DM patients (54 versus 36%, respectively; X2= 6.932; OR= 1.272). There was no significant difference in the frequency of GG (homozygous) genotype between apparently healthy controls and T2DM patients. Generally, the frequency of combined (heterozygous + homozygous) genotypes was significantly ($p<0.01$) higher in controls when compared with T2DM patients (68 versus 50%, respectively; X2= 6.932; OR = 1.272). The frequencies of C and G alleles were 0.59 and 0.41 in apparently healthy subjects and 0.68 and 0.32 in diabetic patients, correspondingly.

In contrast, there is wild CC genotype (50 versus 32% in T2DM and control group, respectively, X2=6.93; $P<0.01$; OR=1.272).

Table (4): Comparison of the genotype and allele frequency of *PPARG* gene polymorphism Pro12Ala between control group and patient group.

Genotypes rs1801282	Frequencies (%)		χ^2	Odd ratio (95% CI)
	Control (No=50)	T2DM (No=50)		
CC	32%(n=16)	50%(n=25)	6.932 **	1.272 (0.89-1.62)
CG	54%(n=27)	36%(n=18)	6.932 **	1.272 (0.89-1.62)
GG	14%(n=7)	14%(n=7)	0.00 NS	0.00 (0.88- 1.90)
CG+GG	68%(n=34)	50%(n=25)	6.932 **	1.272 (0.89-1.62)
C	0.59	0.68	---	---
G	0.41	0.32	---	---

**** significant at 0.01 level; NS: No significant.**

Yen *et al*. (12) firstly reported the Pro12Ala polymorphism of *PPARG* gene. The CCA to GCA variation at codon 12 of the *PPAR*-γ2 specific exon is common sequence variation with a reduced functional capacity as a transcriptional factor for a large number of genes .(12,13)

Because of the conflicting results about the association of Pro12Ala mutation of *PPARG* gene with T2DM in different populations (14,15), the present study was conducted to investigate the association of Pro12Ala variant of *PPARG* gene with T2DM incidence in Iraqi population.

In silico results of Pro12Ala variant in the present study were in agreement with the molecular results as related with its association with T2DM. According to in silico study, the effect of Pro12Ala variant was neutral. As shown in the results, the risk of T2DM is associated with C allele of Pro12Ala (Pro allele). This result is agreeing with many studies that found an association between C allele of Pro12Ala variant and the risk of T2DM. Studies showed a 1.25-fold increase in the diabetic risk in subjects with C (wild-type) allele (16,17,18,19,20,21,22,23). Ho *et al*.(24) found that C allele of Pro12Ala variant is the risk allele for T2DM. Egyptian study supported an association between C allele of Pro12Ala and T2DM.(25)

Other studies indicated that Pro12Ala variant of *PPARG* gene was significantly associated with the increase of predisposition to T2DM like Finns (26), Taiwanese (27), Turkish (28), Egyptian (25), Russian (29), Indian (30), American (31), and Iranian (32) populations. Sanghera *et al*. (33) observed that the Pro12Ala polymorphism of *PPARG* gene contributed to the risk of developing

T2DM in 554 Indian Sikhs. Ghoussaini *et al*. (34) found a significant association between Pro12Ala variant and T2DM. Some studies have found an increased risk for T2DM in subjects with G allele .(35)

While, other studies showed an association between *PPARG* gene polymorphism and reduction of risk of T2DM, which has been found in different ethnic populations such as Danish (36), Scotts (37), Japanese (38), Korean (39), Caucasians (40), and Spanish (41). Deeb *et al*. (13) reported a 75% risk reduction for diabetes conferred by the G allele of Pro12Ala variant. Altshuler *et al*. (16) reported that G allele of the codon 12 polymorphism was associated with decreased of T2DM. The protective association of the G allele with T2DM was confirmed in the present study. Combined evidences suggest that the G allele exerts a protective effect with carriers of this allele causing a decreased diabetes risk . (42,43,44,45,46)

Al-Naemi and Ahmad (47) proposed that G allele of Pro12Ala variant improves the peripheral insulin sensitivity by reducing the release of insulin-desensitizing free fatty acids, TNF-α and resistin in addition to the increased release of adiponectin (an insulin-sensitizing hormone). These effects will ultimately enhance peripheral glucose uptake and inhibit the hepatic glucose production (48).

The minor allele (G allele) frequency of this common gene variant is widely variable worldwide. It was found to range from 5.9 to 21.6 % among Caucasian ethnicities, and from 1.7 to 9.3 % among people of East Asian descent such as Chinese and Japanese(49).

In contrast to our results, the heterozygous CG genotype revealed no significant association to T2DM as reported by previous studies on German (50), Tunisian (51), Qatarian (52), Chinese (53), and Italian populations (54).

Several reports failed to reproduce a significant association (55,56).while (51,52) showed no association between diabetes and Pro12Ala in Tunisian subjects and in the Qatari consanguineous population, respectively.

According to these results, G allele seems to be a protective allele, therefore, the presence of both heterozygous and homozygous mutants may reduce the risk of T2DM in Iraqi population.

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