Original Article

Relationship of Insulin Gene (SNP rs3842752G>A), TCF7L2 Gene (SNP rs12255372 G>T), and SLC30A8 Gene (SNP rs13266634T>C) with Serum Insulin Level in Type II Diabetic Patients in Iraq

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Abstract

Background: Type II diabetes is a health challenge all over the world, with both environmental and genetic factors involved in its occurrence. Moreover, its genetic factors have diversity among population groups and races in the world. **Objectives:** Our study aims to find the association of SNPs(rs3842752G>A- Insulin Gene , rs12255372 G>T- TCF7L2 Gene, and rs13266634T>C- SLC30A8 Gene), with type II diabetes mellitus and with serum insulin levels in patients with type II diabetes in Iraq. **Materials and Methods:** In total, 88 individuals (34 nondiabetics and 54 type 2 diabetics millets) participated from Baghdad. Insulin concentration was measured using an enzyme-linked immunoassay. The genotype of the SNP rs3842752 in the insulin gene was analyzed using the RFLP technique. While the genotypes of SNP rs12255372 in the TCF7L2 gene and the SNP rs13266634 in the SLC30A8 gene were analyzed using the SSCP-genotyping technique. Sequence analysis was performed by Macrogen Inc., South Korea. **Results:** The results of the current study showed that there was a significant difference ($P < 0.05$) for insulin concentrations between the T2DM group and the ND group. In addition, the results of the genetic analysis showed the presence of alleles (G and A) in rs3842752, (C and T) in rs13266634 and in rs12255372 were (G and A), which indicated the presence of the allele (A) in Iraqis as a substitute for the allele (T), which was related to type II diabetes in many studies in the world. On the other hand, the results of the Hardy-Weinberg equilibrium test showed that there was a high significant difference ($P < 0.0001$) between the frequency of the (C and T) alleles in the SLC30A8 gene, which may indicate the functional role of the (C) allele in susceptibility to type II diabetes in Iraqis. However, no significant differences were seen between the distributions of alleles and genotypes as they did not indicate the relationship with type II diabetes, and no significant difference was seen in insulin concentrations between carriers of genotypes at the three single nucleotide polymorphisms in the current study. **Conclusions:** We concluded from the results of the current study that the single nucleotide polymorphism rs3842752, which did not appear to be related to type II diabetes and had no effect on insulin levels in Iraqis, may have a different functional role that may need more research to determine it. Moreover, Previous studies around the world showed that single nucleotide polymorphisms (SNPs rs13266634 and rs12255372) are related to type II diabetes and have an effect on insulin levels, but the failure to show the same results in the current study may be ,due to the small size of the studied sample, in addition, the single nucleotide polymorphisms in the current study may have a different functional role in the Iraqis. However, the current results need other studies with a larger number of participants, perhaps from other Iraqi provinces, to interpret and support the current results.

Keywords: Insulin concentration, single nucleotide polymorphism-SNP, type 2 diabetes

Introduction

Type II diabetes is a metabolic illness. Insulin resistance plus a gradual disruption in the functions of beta-pancreatic cells that cause insulin deficiency are the most

Access this article online

Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_551_23

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Submission: 14-May-2023 **Accepted:** 06-Jul-2023 **Published:** 23-Jan-2026

How to cite this article: Rabie BH, Hasan AH, Kamil ZH. Relationship of insulin gene (SNP rs3842752G>A), TCF7L2 gene (SNP rs12255372 G>T), and SLC30A8 gene (SNP rs13266634T>C) with serum insulin level in type 2 diabetic patients in Iraq. Med J Babylon 2025;22:1110-6.

important manifestations,[1,2] which represent decreased tissue sensitivity to insulin, impaired production, or compensatory secretion of insulin.[3] Metabolism and genetic diversity are heterogeneous etiologies for the progress of type II diabetes.[4,5] Insulin is a hormone essential for the health of all vertebrate species.[6] It is located on chromosome 11 on the short arm, and it is a small gene consisting of 1425 base pairs divided into three exons and two introns. The first exon consists entirely of five untranslated (5′UTR) sequences, but the second and third exons contain the sequences encoding the proinsulin precursor protein, which are translated into 110 amino acids.[7,8] Insulin genes (single nucleotide polymorphisms [SNPs]) may be related to a susceptibility of diabetes.[9,10]SNP rs3842752G>A is located in the insulin gene at position chr11:2159843 (GRCh38. p14) and later located in the mRNA (3′UTR).[11]There is an association of rs3842752 with type 1 diabetes circulation glucose level.[9,12]The presence of this SNP in the 3′untranslated region (3′UTR) of the INS mRNA, probably causes mRNA instability.[13]TCF7L2 acts as a gene repressor and activator, many of its variants being associated with the presence of diabetes.[14] It is located on chromosome 10q25.3, and it contains 17 exons.[15,16]This Transcription factor is 7-like 2, a member of the transcription factors family for T-cell, and lymphoid as a factor for the enhanced binding (TCF/LEF) that attach to DNA by a high-mobility group domain-HMG[17,18] This gene regulates biosynthesis and insulin secretion at the level of B cells in the pancreas.[19] rs12255372 (G/T) is an intronic (SNP) of the TCF7L2 gene that is closely related to type 2 diabetes millets (T2DM).[20,21] SLC30A8, a member of the zinc transporter (ZnT) family, is encoded by the SLC30A8 gene located on chromosome 8q24.11 and has a length of 37 kb, with eight exons.[22] The expression of zinc transporter 8 (ZnT8) is unique to islets, as it regulates the concentration of zinc in cells by transporting zinc from the cytoplasm to secretory vesicles and also acts as a zinc sensor.[22,23] Zinc plays an important role as a second cellular messenger in insulin signaling pathways for the process of insulin synthesis, storage in secretory vesicles, secretion when needed, and thus blood glucose homeostasis.[24]In type 2 diabetes, ZnT8 plays a role as a mediator in transporting zinc to insulin-secreting granules. Moreover, genome-wide association studies (GWAS) reported that the presence of the mutation in the ZnT8 gene (SLC30A8) is associated with T2DM.[18,25] Furthermore, the meta-analysis also indicated that the rs13266634 polymorphisms are among the genetic markers that most confirm susceptibility to T2DM.[26,27] The aim of the current study is to find the association of (SNPs) in insulin, TcF7L2, and SLC30A8 genes with type 2 diabetes mellitus and with serum insulin levels in patients with type 2 diabetes in Iraq.

Materials and Methods

Study population

This study was conducted in the consulting clinic lab of Baghdad Teaching Hospital, Baghdad, Iraq, and the Laboratories of the College of Science, University of Babylon. Volunteers attended from December 2021 to February 2022 in the consulting clinic laboratory at Baghdad Teaching Hospital. The study included 88 subjects: 54 T2DM patients with a BMI greater than 27.8 for both genders and 34 nondiabetics (ND control group). Their ages ranged from 40 to 55 for both genders in T2DM and ND groups. Subjects with systemic diseases and oral diseases were excluded. In addition, patients with type 2 diabetes taking insulin were excluded, and BMI over 27 was excluded for nondiabetics (control group).

Collecting of blood samples

Blood samples for both groups were taken in the early morning from 8 am until 11 am after fasting for at least 8 h. Venous blood (3 mL) was collected using (Gel and clot activator tube— 5 mL) to extract serum for both diabetic (T2DM group) and the nondiabetics (ND control group), 2 mL of blood was collected from a vein using (K3 EDTA) tubes for DNA extraction. Serum was obtained from whole blood samples by allowing the samples to coagulate for one hour at room temperature before centrifuging for 20 min at 1000 × *g*. The isolated serum was kept in Eppendorf tubes frozen at -20°C until the tests were carried out.

Measurements

An estimation of the serum insulin concentration was measured in serum samples for both T2DM and ND (control group) according to sandwich enzyme-linked immunosorbent assay (sandwich ELISA) by ELISA kit Elabscience-USA.

Genotyping of SNPs

The insulin gene snprs3842752 G>A was genotyped by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) technique. In contrast, the single-strand conformation polymorphisms-SSCP genotyping technique was used to detect SNPs for both a TCF7L2 gene polymorphism rs12255372A>G and a SLC30A8 gene polymorphism rs13266634 T>C. The selection of genotyping technique according to Hashim and Al-Shuhaib.[28] The primers that were used to detect (SNPs) of the insulin gene, TCF7L2 gene, and SLC30A8 gene are shown in Table 1. The design of PCR primers according to the protocol of Hashim *et al.*[29]

DNA was extracted from the frozen blood samples for both T2DM and ND (control group) using the extraction kit (ReliaPrep™ Blood g DNA Mini-prep System-PromegaUSA). Reaction conditions such as

Table 1: Primers used to detect single nucleotide polymorphisms (SNPs)

Insulin gene SNP	Forward5′-CCCCCAAGACACACAGACG-3′
rs3842752 (G/A)	Reverse complement 5′-GTGTCTCCCTGACTGTGTCC-3′
TCF7L2gene SNP	Forward 5′-TCATAGGGGTCTGGCTTGGA-3′
rs12255372 (G/T)	Reverse complement 5′-TTTGGAGCTGGGGAGAGAGT-3′
SLC30A8 geneSNP	Forward 5′-GGAGTCAGAGCAGTCGCC-3′
rs13266634 (T/C)	Reverse complement 5′-ACTGACGGTGTGACTGAGCTA-3′

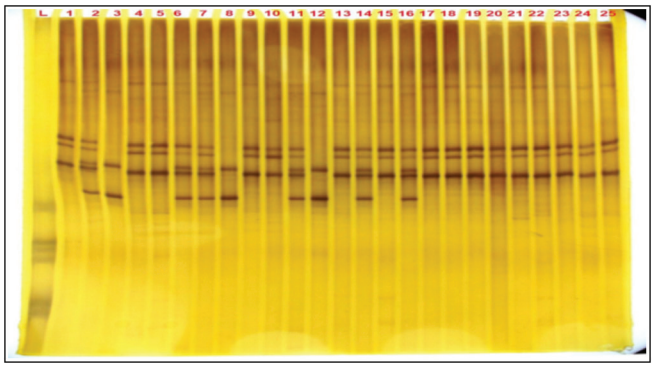

Figure 1: SSCP genotyping of rs13266634. Lane L DNA ladder; lanes 3, 8, and 12 A pattern; lanes 1, 4, 5, 9, 10, 13, 15, 17, 18, 19, 20, 21, 22, 23, 24, and 25 B pattern; lanes 2, 6, 7, 11, 14, and 16 C pattern. DNA sequencing reveals that A, B, and C patterns represent TT, CC, and CT genotypes of rs13266634, respectively

thermal cycles, contents of the PCR, RFLB, and SSCP reaction mixture were optimized to obtain high-quality PCR products. Twenty microliters (20 µl) PCR reaction was conducted. The RFLB-PCR products were digested overnight with restriction enzyme. We used 0.25 µl of PstIrestriction endonuclease for rs3842752 (G/A). The digestion products were separated by 2% agarose gels and stained with ethidium bromide. Agarose gel (2%) was prepared according to the method of Samboork and Russell.[30] and a 100 bp DNA ladder was used to identify the size of the resulting RFLP products. A horizontal electrophoresis device was used at 100 V and 50 mA for 1 h. The agarose gel template was then photographed and analyzed In order to obtain results. The PCR products of SNP rs13266634 (T>C) and rs12255372 (G/T) were genotyped usingSSCP technique. Sample preparation and loading on an acrylamide gel, according to Sambrook and Russell[30] and Badi *et al.*[31] The gel was stained and fixed according to the method described by Byun *et al.*[32]Then, the gel was photographed to select the samples for genotype analysis [Figures 1 and 2]. Two amplicons were selected from each DNA pattern were produced by polyacrylamide gel electrophoresis of rs13266634 a SLC30A8 gene polymorphism, rs12255372 aTCF7L2

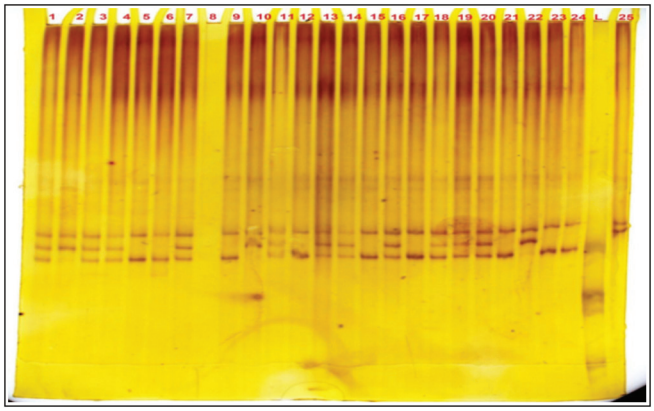

Figure 2: Genotyping of rs12255372 by PCR-SSCP technique. Lane L DNA ladder, lanes 2, 10, 22, and 25 A pattern; lanes 5, 6, 9, 12, 15, 17, 21, 23, and 24 B pattern; lanes 1, 3, 4, 7, 11, 13, 14, 16, 18, 19, and 20 C DNA pattern. DNA sequencing reveals that A, B, and C patterns represent TT, GG, and GT genotypes of rs12255372, respectively

gene polymorphism. And sent for DNA Sequencing by Macrogen Inc. Geumchen, Seoul, South Korea.

Sequence analysis

The sequences of the selected amplicons were analyzed by the Macrogen Inc. Geumchen, Seoul, South Korea. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

Statistical analysis

All statistical calculations were performed by using SPSS Version 23 and Microsoft Excel. All insulin concentrations were displayed as mean ± SE. For data that were not normally distributed, the Mann–Whitney test, the Kruskal–Wallis test, and the Spearman correlation test were used. Assess the categorially association variables and genetic association performed using Hardy–Weinberg equilibrium, according to Solé *et al.*[33]

Ethical considerations

After reviewing the research plan, research subject information, and approval form, the Ethics Committee in the Department of Life Sciences, College of Science, University of Babylon, Iraq, approved it according to document number z220101 on January 17, 2022. Oral consent was taken from the contributors (two study groups) before sampling was taken from them.

Results

The results of genetic analysis and sequencing analysis showed that the genotypes of Iraqis in the rs3842752 were (G/G, G/A, A/A), rs13266634 were (C/C, C/T, T/T), and in the rs12255372 were was (G/G, G/A, A/A), and, recording

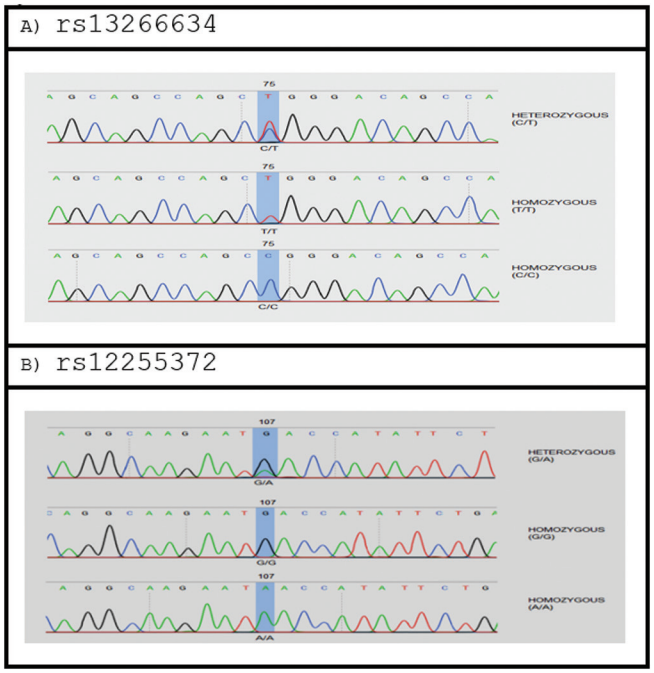

Figure 3: The pattern of the detected SNPs within the DNA chromatogram of the *SLC30A8* and *TCF7L2*geneas shown in branches A and B, respectively. The identified SNPs were highlighted according to their positions in the PCR amplicons

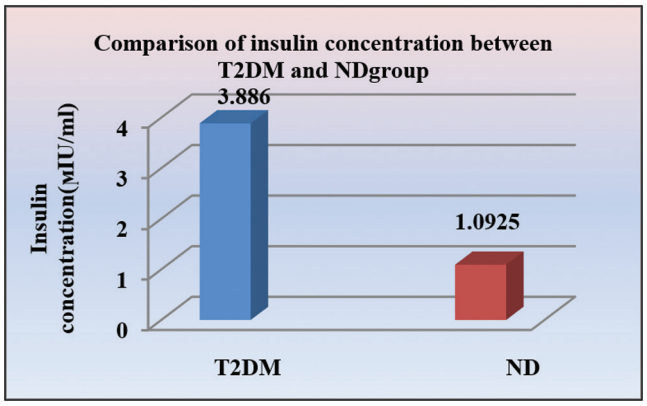

Figure 4: Comparison of insulin concentration between the T2DM and the ND group. T2DM: type 2 diabetics millets, ND: nondiabetics (control group)

the appearance of the allele (A) in Iraqis as a substitute for the minor allele (T) according to sequence analysis [Figure 3]. The comparison between insulin concentrations showed a highly significant difference (P-value = 0.000) [Figure 4] between the T2DM group (3.886 ± 0.681) and the ND control group (1.0925 ± 0.0494), statistically significance at $P \leq 0.05$ (Mann–Whitney U test). Odds ratio analysis for single alleles in SNPs [Table 2] did not show any significant difference ($P = 6.2$), ($P = 0.46$), and ($P = 0.1624$) for SNPs (rs3842752), (rs12255372), and (rs13266634), respectively.

In the exact test of Hardy–Weinberg equilibrium [Table 3], there were no significant differences in the distribution of genotypes within all subjects, ND group, and T2DM group ($P = 0.31$), ($P = 0.69$), ($P = 0.34$), respectively, for the snp rs3842752, nor snp rs12255372 for the same groups above ($P = 0.19$), ($P = 0.72$), ($P = 0.052$), respectively, which means that it is within Hardy–Weinberg equilibrium. On the other hand, the results of the current study showed that there was a highly significant difference between the groups, all subjects, ND group, T2DM group, (P <0.0001), ($P = 0.014$), (P = <0.0001), respectively, for snp s13266634 Where it indicates it is not within Hardy–Weinberg equilibrium.

Odds ratio analysis for the genotypes [Table 4] showed that there were no significant differences between the genotypes for any of the SNPs (snp rs3842752), (snp rs12255372), (snp rs12255372).

Also, the results of the current study [Tables 5] did not show any significant difference ($P > 0.05$) for insulin concentrations between the carriers of genotypes in the three single nucleotide polymorphisms.

Discussion

We conducted this study to identify the genotypes of (SNPs) insulin, TCF7L2, and SLC30A8 genes and their relationship to insulin levels of Iraqis T2DM and ND groups. Figure 4 shows an increase in the level of insulin in the T2DM group compared to the ND group, indicates

Table 2: Odds ratio analysis of the single alleles in each single nucleotide polymorphism

Allele	ND (control)		T2DM		OR (95% CI)	*P*-value
	Count	Proportion	Count	Proportion		
Insulin gene snp: rs3842752						
G	49	0.72	74	0.69	0.84 (0.43–1.65)	0.62
A	19	0.28	34	0.31	1.19 (0.61–2.31)	
TCF7L2gene snp: rs12255372						
G	41	0.6	59	0.55	0.79 (0.43–1.47)	0.46
A	27	0.4	49	0.45	1.26 (0.68–2.34	
SLC30A8 snp: rs13266634						
C	48	0.71	65	0.6	0.63 (0.33–1.2)	0.1624
T	20	0.29	43	0.4	1.59 (0.83–3.04)	

OR: odds ratio, T2DM: type 2 diabetics millets, ND: nondiabetics (control group), SNP: single nucleotide polymorphism

Table 3: Hardy–Weinberg equilibrium test

Insulin gene snp: rs3842752-exact test for Hardy–Weinberg equilibrium ($n = 88$)

	GG	GA	AA	G	A	*P*-value
All subjects	45	33	10	123	53	0.31
ND	18	13	3	49	19	0.69
T2DM	27	20	7	74	34	0.34

TCF7L2gene snp: rs12255372—exact test for Hardy–Weinberg equilibrium ($n = 88$)

	GG	GA	AA	G	A	*P*-value
All subjects	25	50	13	100	76	0.19
ND	13	15	6	41	27	0.72
T2DM	12	35	7	59	49	0.052

SLC30A8snp: s13266634—exact test for Hardy–Weinberg equilibrium ($n = 88$)

	CC	CT	TT	C	T	*P*-value
All subjects	47	19	22	113	63	<0.0001
ND	20	8	6	48	20	0.014
T2DM	27	11	16	65	43	<0.0001

T2DM: type 2 diabetics millets, ND: nondiabetics (control group), SNP: single nucleotide polymorphism

Table 4: Odds ratio according to genotypes

Insulin gene snp: rs3842752

Model	Genotype	ND	T2DM	OR (95% CI)	*P*-value
Codominant	G/G	18 (52.9%)	27 (50%)	1.00	0.83
	G/A	13 (38.2%)	20 (37%)	1.03 (0.41–2.57)	
	A/A	3 (8.8%)	7 (13%)	1.56 (0.35–6.82)	
Dominant	G/G	18 (52.9%)	27 (50%)	1.00	0.79
	G/A–A/A	16 (47.1%)	27 (50%)	1.12 (0.48–2.66)	
Recessive	G/G-G/A	31 (91.2%)	47 (87%)	1.00	0.55
	A/A	3 (8.8%)	7 (13%)	1.54 (0.37–6.41)	
Overdominant	G/G–A/A	21 (61.8%)	34 (63%)	1.00	0.91
	G/A	13 (38.2%)	20 (37%)	0.95 (0.39–2.30)	

TCF7L2gene snp rs12255372

Model	Genotype	ND	T2DM	OR (95% CI)	*P*-value
Codominant	G/G	13 (38.2%)	12 (22.2%)	1.00	0.15
	G/A	15 (44.1%)	35 (64.8%)	2.53 (0.94–6.81)	
	A/A	6 (17.6%)	7 (13%)	1.26 (0.33–4.84)	
Dominant	G/G	13 (38.2%)	12 (22.2%)	1.00	0.11
	G/A–A/A	21 (61.8%)	42 (77.8%)	2.17 (0.84–5.57)	
Recessive	G/G–G/A	28 (82.3%)	47 (87%)	1.00	0.55
	A/A	6 (17.6%)	7 (13%)	0.70 (0.21–2.28)	
Overdominant	G/G–A/A	19 (55.9%)	19 (35.2%)	1.00	0.056
	G/A	15 (44.1%)	35 (64.8%)	2.33 (0.97–5.61)	

SLC30A8snp s13266634

Model	Genotype	ND	T2DM	OR (95% CI)	*P*-value
Codominant	C/C	20 (58.8%)	27 (50%)	1.00	0.44
	C/T	8 (23.5%)	11 (20.4%)	1.02 (0.35–3.00)	
	T/T	6 (17.6%)	16 (29.6%)	1.98 (0.66–5.95)	
Dominant	C/C	20 (58.8%)	27 (50%)	1.00	0.42
	C/T–T/T	14 (41.2%)	27 (50%)	1.43 (0.60–3.40)	
Recessive	C/C–C/T	28 (82.3%)	38 (70.4%)	1.00	0.2
	T/T	6 (17.6%)	16 (29.6%)	1.96 (0.68–5.66)	
Overdominant	C/C–T/T	26 (76.5%)	43 (79.6%)	1.00	0.73
	C/T	8 (23.5%)	11 (20.4%)	0.83 (0.30–2.34)	

T2DM: type 2 diabetics millets, ND: nondiabetics (control group), OR: odds ratio

Table 5: Comparison ofT2DM insulin concentrations between genotypes

Insulin gene SNP rs3842752G>A			
Genotype	***N***	**Insulin concentration (MIU/mL)**	***P*-value**
GG	27	3.481 ± 0.988	0.390
GA	20	4.411 ± 0.969	
AA	7	3.765 ± 2.568	
TCF7L2 gene SNP rs12255372 G>T			
Genotype	***N***	**Insulin concentration (MIU/mL)**	***P*-value**
GG	12	7.257 ± 2.302	0.809
GA	35	2.899 ± 0.560	
TA	7	2.857 ± 1.366	
SLC30A8 gene SNP rs13266634T>C			
Genotype	***N***	**Insulin concentration (MIU/mL)**	***P*-value**
CC	27	4.107 ± 1.008	0.580
CT	11	3.99 ± 1.581	
TT	16	3.361 ± 1.181	
N	54		

Data have been displayed as the mean and standard error, *n*: number, T2DM: type 2 diabetics millets, Kruskal–Wallis test at *$P \leq 0.05$, *r*: correlation coefficient, Spearman correlation **$P \leq 0.05$

that in early type 2 diabetes, insulin secretion increases as a response to the high level of glucose in the blood, although there is resistance from the cells to the action of insulin. Later, as the disease progresses, the pancreatic beta cells will fail to compensate, and blood glucose will continue to rise.[34]With regard to the snp rs3842752 in the insulin gene, we did not find an association with type 2 diabetes, and this agrees with Massarenti *et al.*'s study.[10] And some other previous studies.[35,36]We also did not find an association of the same SNP with the level of insulin in the blood of both T2DM and ND groups, It is possible that the presence of this rare snp at the site of the *INS* mRNA (3′UTR), which are not included in the mRNA sequences in the translation step, and this requires more studies to identify the role of this SNP in the expression and translation of the insulin gene.[37]The results related to the sequence analysis of rs12255372 and the chromatogram report [Figure 3] showed that the alternative allele of rs12255372 is (A) in Iraqis. Instead of the alternative allele (T), which appears in many studies in many countries and races.[38]On the other hand, there were no significant differences in the frequency of alleles (G) and (A) between T2DM and ND groups, and there is no statistically significant difference in the insulin concentrations with the genotypes and even with the single allele (A) between the two study groups, which indicates the need for more studies on this SNP(rs12255372) in the Iraqi society, perhaps by taking a larger sample size[39] than the sample size in our current study to reach results that represent a comprehensive picture that may support our results or may explain the nonassociation of the allele (A) as a risk factor for susceptibility to diabetes. Where the sample size was significantly larger in the studies that showed an association of snp rs12255372 with type II diabetes[40] than in our sample size. Finally, for SNP rs13266634, our results in Table 3 showed no significant differences in odds ratio analysis between alleles nor between genotypes, nor in comparison of carriers of the three genotypes with insulin concentration [Table 4 and 5], respectively. Despite the strong association of allele (G) with susceptibility to type 2 diabetes in many previous studies around the world and its lack of association in other studies.[26] This confirms the need to understand the function of this allele and its role in the occurrence of type II diabetes in Iraqis Genetic variations have a major role in increasing the chance of disease by affecting gene expression and protein production, and their functional roles may vary according to countries and races. Therefore, it is useful to diagnose the functional role of single nucleotide polymorphisms related to type II diabetes and other diseases in Iraqis, and to establish a database for this purpose.

Conclusion

SNPs had an important role in susceptibility to type 2 diabetes. Our results in this study showed the identity of the genotypes of the SNPs studied in Iraqis and showed the presence of an Iraqi-specific allele (A) in snp rs12255372. But proving the role of these SNPs in type II diabetes and their effect on insulin production needs additional studies, perhaps with a larger number of participants, to support and interpret our results.

Financial support and sponsorship

Nil.

Conflict of interest

There is no conflict of interest.

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