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Investigating the Relationship between rs7903146 Polymorphism Genotypes of TCF7L2 Gene and Visfatin Serum Level in Patients with Type 2 Diabetes

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ABSTRACT

This study showed that T allele is a risk allele for both homozygous and heterozygous in rs7903146 polymorphism genotyping in TCF7L2 gene. Here, 132 individuals were investigated (87 of them were patients with type 2 diabetes and 45 other were healthy, respectively). DNA was extracted from whole blood samples via a purification kit; then in order to the determination of quality, it was electrophoresised on agarose gels. Genotyping method was performed through high-resolution melt (HRM) real time polymerase chain reaction (RT-PCR). The visfatin level was determined based on enzyme-linked immunosorbent assay (ELISA) method. According to the achieved results, increasing the plasma levels of visfatin in diabetics patients (10.73 ± 5.38) compared to the control group (6.73 ± 1.88) and their changes based on the separation of genotypes also confirms that T allele is the probable risk factor (p-value < 0.05). In addition, the presence of the T allele is related to increasing triglyceride (TG) levels of blood. Therefore, adipose tissue burning and its reduction (along with the releasing of TG in the blood) decreased the source of visfatin, therefore increasing the TG in the presence of T allele (the development of diabetes) is associated with decreased plasma levels of visfatin. According to this study, evaluating plasma levels of visfatin and TG levels in blood is useful in prognosis of type 2 diabetes.

Key words: TCF7L2 gene, rs7903146 polymorphism, Type 2 diabetes, Visfatin.

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1. INTRODUCTION

Type 2 diabetes is characterized by three pathophysiological abnormality including insulin secretion disorders, peripheral insulin resistance and production of excess glucose by the liver (1-3). The number of patients with type 2 diabetes is rapidly increasing so that in 1985 the number of these patients was about 30 million, but in 2010 this number increased to 285 million (4, 5). The prevalence of this disease in the world is high (6 percent) and is rising continuously (6). It is estimated that in 2030 the number of diabetics will be about 438 million (7). Diabetes in the long term, will lead to heart disease, kidney disease, stroke and damage to the eye (2, 3, 8). Due to the high prevalence of this type of diabetes and a lot of side effects created by it, studying and research about this disease is essential to find early detection methods and learn more about it (9, 10). Both genetic and environmental factors contribute to create clinical presentation of type 2 diabetes. TCF7L2 is a member of T cell family transcription factor that is located on chromosome 10q 25.2 (11, 12). This gene plays a critical role in WNT signaling pathway through regulating cell proliferation and differentiation (13). Changes in expression of TCF7L2 gene lead to reduced insulin secretion and increase the risk of diabetes ultimately (14). Various polymorphism regions have been identified on TCF7L2 gene that their relation with impaired insulin secretion, glucose production and insulin resistance through direct effects on pancreatic beta cells has been proven (15, 16). Diabetogenic effect of TCF7L2 rs7903146 gene variation is related to reduced insulin secretion, impaired processing of insulin, reducing the effects of

glucagon-like peptide 1 (GLP-1) and increasing production of hepatic glucose (17, 18). Accumulation of excess fat due to secreted adiponectin from adipose tissue or changes in insulin signaling lead to interfering in glucose uptake affected by insulin and insulin resistance will be occurred according to compensatory increasing in insulin levels (19). Visfatin is an adipokine that has been discovered recently and the amount of it is high in visceral fat (20-22). The plasma levels of visfatin are increased along with obesity. Although major of visfatin Visfatin is produced in visceral fat but it is also produced in skeletal muscles, liver, bone marrow and lymphocytes. Biological characteristics of Visfatin are similar to some cytokines, so that it has anti-apoptosis properties and increases cell proliferation (23). Visfatin (molecular weight: 54 kDa) is synthesis colony-stimulating factor in isolated beta precursor cells from lymphocytes which was presented in 1994 and introduced as this name by Fukuhara et al in 2005 (20). Recently, according to enzymatic function of visfatin in the biosynthesis of nicotinamide dinucleotide it is also called nicotinamide phosphoribosyl transferase (24, 25). Levels of visfatin are changed in different diseases such as diabetes, kidney disease, bone disease and cancer. Therefore it is considered as a diagnosis and prognosis factor (20-23, 25). Evaluating the relationship between various genotypes of single nucleotide polymorphisms (SNPs) in TCF7L2 gene with the serum level of visfatin will help to complement the information in this field (26). In this study, the relationship between this hormone (visfatin) and rs7903146 polymorphism genotyping in TCF7L2 gene was investigated in in patients with type 2 Diabetes.

2. MATERIALS AND METHODS

2.1. Materials and reagents

All chemical materials such as ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA $2H_2O$), NaOH, Tris base, Ethanol, Boric acid and other reagents were purchased from Sigma (USA) and Merck (USA). Taq DNA Polymerase (50 $U.\mu L^{-1}$), deoxynucleotide triphosphates (dNTPs) and Tris-borate-EDTA (TBE) Buffer (10X) were purchased from Bioron (Germany). Loading Dye (Fermentas, Germany), Agarose (Cinagene, Iran), Type-it HRM PCR Kit (Qiagen, Germany) and Visfatin kit were purchased from ZellBio GmbH, Germany and used in stable conditions. Used primers sequences were produced by Bioneer biotechnology company (South Korea).

2.2. Apparatus

Several devices were used to do experiments, including Gel Electrophoresis System (Payagene, Iran), Gel Document (Vilber–Lourmat, France), Rotor Gene 6000 Real-Time PCR Machine (Corbett Research, South Africa) and ELISA reader (Hiperion, Germany).

2.3. Procedure for preparation of solution and buffer

100 ml of EDTA $2H_2O$ (0.5 M, pH. 8) was prepared and sterilized by autoclaving for using in all related experiments. TBE Buffer (10X) was prepared using 108 g Tris base, 55 g Boric acid and 40 ml of EDTA $2H_2O$ (0.5 M, pH. 8) that were dissolved in 960 ml double-distilled water.

2.4. Sampling

In this study, 2-hour post-prandial (2-HPP) blood sugar samples were collected in order to ensure the absence of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) in individuals; namely individuals were selected as control group that their 2-HPP blood sugar were normal. This research is a case-control study. Patient and control groups were selected so that all factors such as age, sex, race, etc. were almost close together. According to the statistical rules about the sample size, to compare two ratios at 95% confidence interval, power of 80% and the considered outcome ratio in the case (10%) and control (30%); groups were selected as 87 diabetic patients and 45 controls. In patient group, 87 samples (32 males and 55 females) were collected from diabetic patients. In the other group, 45 healthy individuals (21 males and 24 females) were selected as controls that did not have diabetic patients in their first degree relatives (FDR). 4 cc venous blood were taken from each individual in case and control groups, 2 cc of it were poured in tubes containing EDTA (anticoagulant substance) and other 2 cc were poured in tubes without EDTA.

2.5. Extraction of DNA from whole blood samples

Extraction of DNA from whole blood samples was performed via an extraction kit according to offered protocols (27). After extracting the DNA, extracted samples as a solution were stored frozen at -20 ° C. The resulting solution was used for qualitative and quantitative evaluation of DNA and also for evaluation of PCR reactions.

2.6. Electrophoresis

In order to ensure the quality of DNA, 1 µl of genomic DNA was electrophoresised on 1% agarose gel.

2.7. Designing primers for HRM-RT-PCR

For each mutation, a pairs of forward and reverse primers were designed using the Beacon Designer software (version: 7.91). Designed primers were purchased from Bioneer company. The designed primers sequences are listed in Table 1.

Table 1. Designed primers sequences

Mutation	Primers sequences	Amplicon length
TCF7L2 (rs7903146)	Forward: GGAGCCGTCAGATGGTAATGC	171 (bp)

	Reverse: CCTTCCCTGTAACTGTGGTTTCTC	

Here, the aim of performing PCR was isolating the fragment containing the considered polymorphism of whole genome and amplification of it to numerous. The used materials in PCR and amounts of them are listed in Table 2.

Table 2. Used materials in PCR and their amounts

Component	Volume
HRM PCR Master Mix × 2	12.5 µl
10X Primer Mix	2 µl
RNase-free water	8.5 µl
Template DNA (added at step 4)	2 µl

After completion of PCR, the temperature was decreased to 65 ℃ and every 2 seconds, was increased 1 ℃. With increasing temperature, the two strands of produced DNA via PCR were separated and DNA binding dyes were released. Therefore the amount of detected fluorescence by the PCR device, was decreased gradually. This process continued until 95℃ and melting curve was plotted through related software using the recorded fluorescence changes in this interval time (Time for increasing temperature from 65 ℃- 95 ℃). After normalizing, the related melting curves with each mutation were analyzed by software, and related pattern for each one was determined. After the HRM, by comparing the curves of samples together or with normal samples (sequenced) the differences were identified. In curve samples, each peak represents the melting point of a PCR product that this work was performed through measuring the fluorescence changes at different temperatures.

2.8. Determination visfatin concentration by ELISA

In order to determine the concentration of visfatin, the 96 well ELISA kits were used. Reagents, samples and standards were prepared in accordance with ELISA protocols (28). After using the kit, optical density (OD) results of samples were read by using an ELISA reader at 450 nm.

2.9. Analysis of data

SPSS version 22 and MS Excel version 2010 were used to analyze obtained data. All analyzes were performed according to biostatistical instructions.

3. RESULTS AND DISCUSSION

The presented results in Table 3 show Student's t-test for two independent groups at a significance level of 5%. As it can be seen, the average of visfatin was significantly higher in diabetics patients (10.73) compared to the control group (healthy) (6.73). In Figure 1, the average of visfatin was shown for both healthy and diabetic groups.

Table 3. Determination and comparison of demographic and biochemical factors between diabetic and healthy individuals

Biochemical Factors	Patients	Healthy	*p-value*
FBS	183.84± 65.66	89.27±14.04	0.001
Blood Sugar	275.33±97.72	119.09±13.09	0.001
Creatinine	0.971±0.172	0.869±0.112	0.001
Cholesterol	180.85±45.91	161.29±32.44	0.012
TG	170.52±81.31	99.29±61.38	0.001
LDL	97.88±37.82	101.98±21.36	0.503
HDL	45.23±11.45	56.78±37.75	0.009
HA1C	7.68±1.35	4.72±0.351	0.001
Visfatin	10.73±5.38	6.73±1.88	0.001

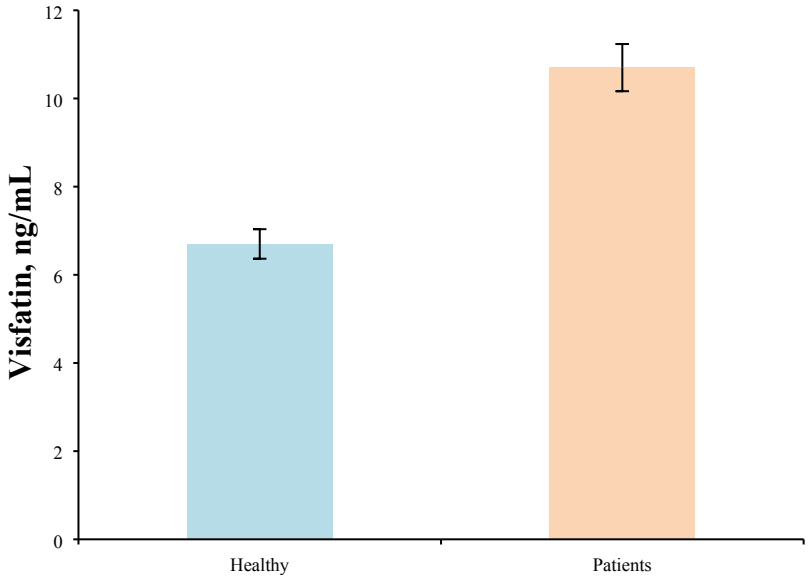

Figure 1. The average of visfatin for both healthy and diabetic groups

The results of determination and comparison the average of biochemical factors for healthy allele and risk allele in both studied groups are offered in Table 4 according to analysis of variance (ANOVA) test at a significance level of 5%. Results showed that any significant difference in the average of assessed biochemical factors between the healthy allele and risk allele in both healthy and diabetic groups was not found (p-value > 0.05).

Table 4. Determination and comparison the average of biochemical factors for healthy allele and risk allele

Biochemical Factors	Healthy		p-value	Patients		p-value
	CC	CT+TT		CC	CT+TT	
FBS	88.26±4.06	90.12±1.76	0.999	186.2±23.37	183.3±6.94	0.997
Blood Sugar	119.5±3.04	118.7±2.58	1	253.2±33.41	280.3±9.97	0.589
Creatinine	0.890±0.027	0.850±0.020	0.817	1.02±0.062	0.961±0.018	0.526
TG	98.81±12.52	99.71±13.45	1	155±23.57	174±9.31	0.800
cholesterol	158.5±6.73	163.7±6.80	0.977	163.5±12.40	184.8±5.28	0.256
LDL	105.7±5.11	98.7±3.93	0.891	81.8±11.15	101.5±4.21	0.137
HDL	64.2±11.55	50.3±2.87	0.204	50.8±4.48	43.1±1.08	0.721
HA1C	4.7±0.080	4.76±0.070	0.994	7.46±0.478	7.73±0.143	0.808
Visfatin	7.14±0.390	6.84±0.407	0.998	11.55±1.57	10.47±0.614	0.661

The average of visfatin for risk allele and healthy allele in both groups is shown in Figure 2.

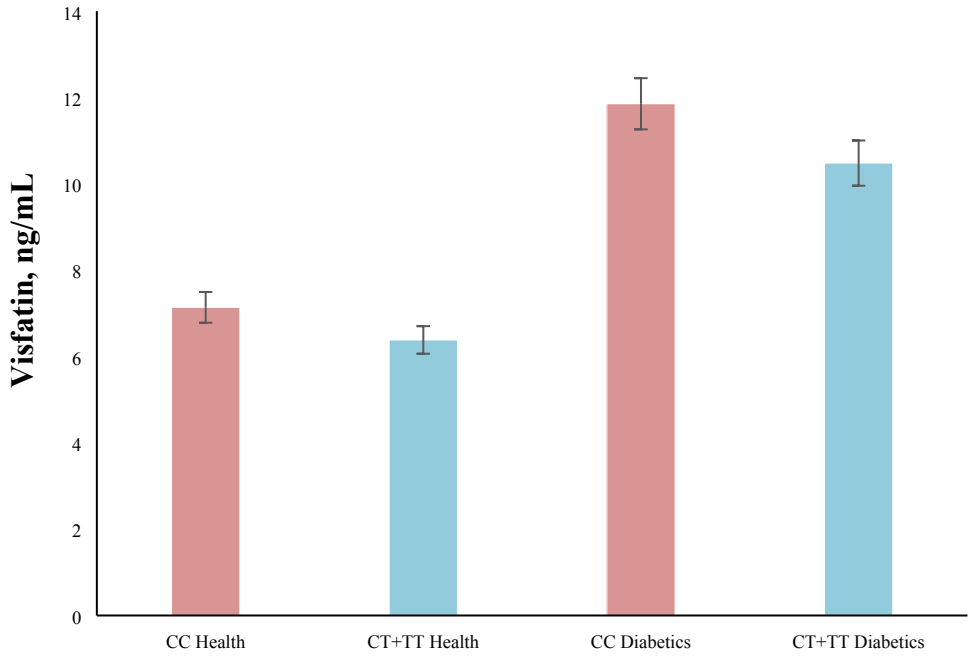

Figure 2. The average of visfatin for risk allele and healthy allele

The results of determination and comparison the average of biochemical factors for genotypes of TCF7L2 gene in both studied groups are offered in Table 5 according to ANOVA test at a significance level of 5%. The average of visfatin had a significant difference among all six studied groups (p-value < 0.05). Plasma levels of this hormone were higher in diabetic patients for all three genotypes compared to control group. The average of visfatin for genotypes of TCF7L2 gene in both groups is shown in Figure 3.

Table 5. Determination and comparison the average of biochemical factors for genotypes of TCF7L2 gene

Biochemical Factors	*Healthy*			*Patient*			*p-value*
	CC	CT	TT	CC	CT	TT	
FBS	88.28±4.06	90.41±1.85	89.43±4.30	186.2±23.37	192±11.61	177.9±8.65	0.001
Blood Sugar	119.5±3.04	117.5±2.57	121.8±6.50	253.2±33.41	290.4±14.57	274.1±13.41	0.001
Creatinine	0.890±0.027	0.835±0.022	0.888±0.040	1.02±0.062	0.933±0.028	0.977±0.023	0.005
TG	98.81±12.52	89.23±13.32	125.1±32.82	155.01±23.58	172.2±12.83	175.1±12.90	0.001
cholesterol	158.6±6.73	158.4±6.83	176.4±17.38	163.5±12.40	183±8.52	185.8±6.80	0.057
LDL	105.7±35.11	97.23±3.27	102.3±11.45	81.81±11.15	101.2±6.31	101.7±5.64	0.340
HDL	64.19±11.55	50.53±2.68	49.71±7.82	50.81±4.48	45.63±1.67	42.95±1.41	0.039
HA1C	4.67±0.080	4.80±0.074	4.66±0.160	7.46±0.478	7.93±0.300	7.61±0.138	0.001
Visfatin	7.14±0.389	6.58±0.510	5.91±0.690	11.55±1.57	11.66±0.829	9.76±0.850	0.001

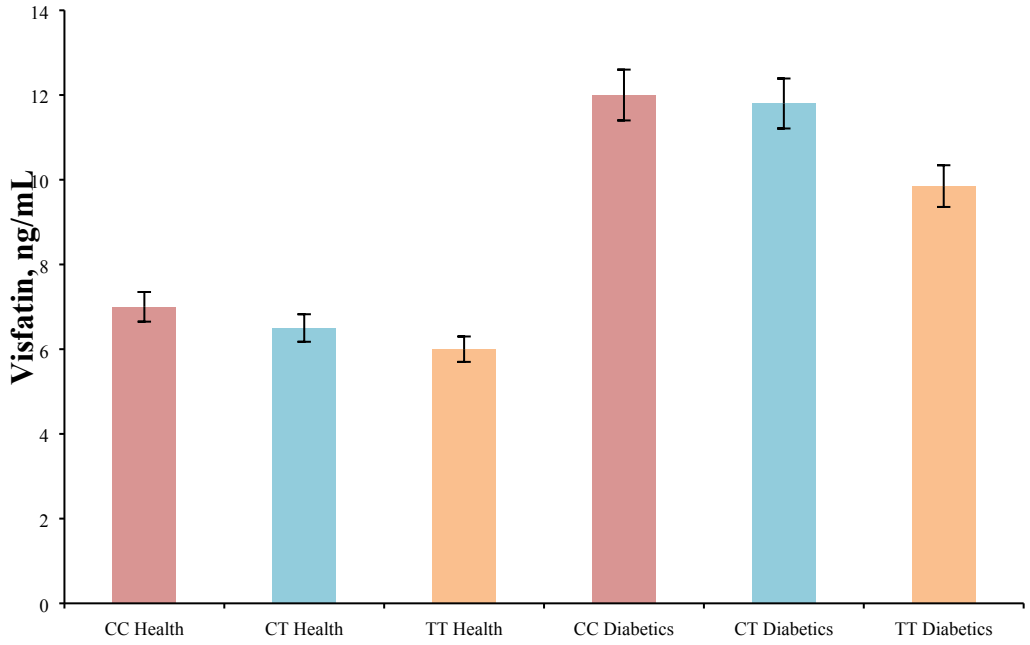

Figure 3. The average of visfatin for genotypes of TCF7L2 gene

The results of investigating the frequency for genotypes of TCF7L2 gene in both studied groups according to chi-squared test (χ^2 test) at a significance level of 5% is offered in Table 6. A significant difference in incidence frequency for genotypes of TCF7L2 gene in both studied groups was observed. So that in the control group, CC genotype with frequency of 21 (46.67) was the most common genotype, but in diabetics patients, TT genotype with frequency of 44 (50.57) was the most common (p-value < 0.05).

Table 6. Determination and comparison the frequency distribution for genotypes of TCF7L2 gene

Group	*Genotype*	*Frequency*	*Percent*	*p-value*
	CC	21	46.67	
	CT	17	37.78	
Healthy	TT	7	15.55	
	CC	16	18.40	
	CT	27	13.03	
Patient	TT	44	50.57	0.001

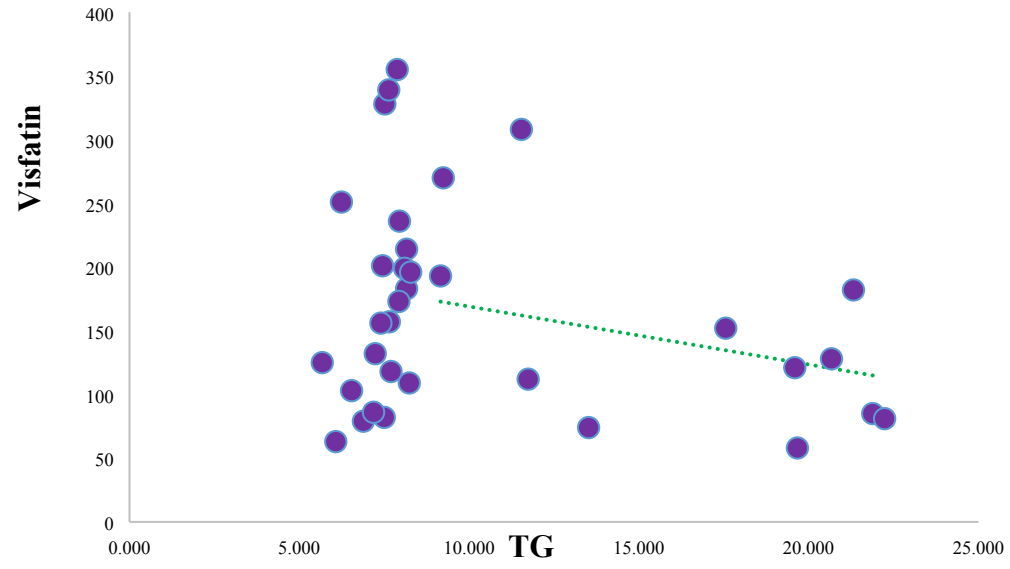

Figure 4. The scatter diagram of visfatin against TG in diabetic patients

According to considered regression model, it was found that there was an inverse relationship between visfatin levels against TG levels in patients, So that with one unit increasing of TG level, visfatin is reduced 4.55 units averagely. In addition, the data analysis of TG indicates that the presence of risk allele (T allele) is associated with increasing TG levels of blood and therefor adipose tissue burning and its reduction (along with the release of TG in the blood) decreased the source of visfatin. Thus, increasing the TG level in the presence of T allele (development of diabetes) is associated with decreasing of plasma levels of visfatin. WNT is a family of secreted glycoproteins that are efficient on cell growth through autocrine and paracrine mechanisms (29, 30). There are several different mechanisms about involving of WNT signaling pathway in secretion of insulin and it's performance. WNT signaling pathway via nuclear receptors and TCF7L2 is essential for secretion of GLP-1 (by endocrine intestinal L cells) (31). Thus, changing in this pathway can lead to reduction of GLP-1, thereby the reduced secretion for this hormone can have a negative impact on secretion of insulin after a meal and also on generation new beta cells from their ductal progenitor cells (32). Plasma levels of visfatin are directly related with insulin resistance. Visfatin is efficient in improvement of glucose tolerance and adjustment of insulin resistance through stimulating insulin receptors (33). It also improves lipid disorders associated with diabetes via affecting metabolism of lipids (34). Visfatin secretion in visceral adipose tissue is more toward subcutaneous adipose tissue (20). Along with obesity, the plasma levels of visfatin are increased. By binding visfatin to insulin receptors, they become activated therefor, the absorption of glucose and the development of obesity facilitated (35). In this study, according to the results of investigating rs7903146 polymorphism genotyping in TCF7L2 gene in both healthy and patients groups, it is found that the incidence frequency of genotypes for this gene between both groups had significant difference. This result is also confirmed by previous researches that show so far more than 36 gene locus have been identified which are involved in diabetes (36, 37). In a study, Michele et al tried to investigate six associated genes with type 2 diabetes and their known polymorphisms for each of them in an African-American population. Their results showed significant relationship between two polymorphisms located in introns of three genes of TCF7L2 and the risk of type 2 diabetes. Accordingly, rs7903146 polymorphism increased the risk of type 2 diabetes as 1.51 times (OR= 1.51; P= 3.77×10^{-6}) (38). In another study, increased rs7903146 variants risk and the risk of diabetes through the defection of insulin secretion were investigated in people who had IGT or IFG. This study showed that insulinogenic index was 40% reduced in the TT homozygotes compared to CC homozygotes (39). Genotypes CT / TT of rs7903146 polymorphism increased the risk of type 2 diabetes sharply compared to CC genotype in two independent Swedish and Finnish groups. The expression of TCF7L2 in human pancreas in type 2 diabetes specifically increases to 5 times for TT genotype (15). According to Table 5, it is noteworthy that changes in plasma levels of visfatin are according to distinguish genotypes in the healthy and diabetics groups, so that in both groups, in the presence of T allele, the visfatin levels was reduced and also drastic reduction of its levels in TT diabetes is justifiable according to changes in adipose tissue (producer of visfatin). In Figure 4, it is clear that according to the insulin-like role of visfatin and its performance in reducing blood sugar and improving insulin resistance, it had more secretion with further progress of diabetes to have optimal performance. But the presence of the risk allele (T allele) led to reduced level of visfatin and this issue is justifiable based on an inverse relationship between visfatin and TG blood levels.

4. CONCLUSION

This study clearly showed the increasing of plasma levels of visfatin in diabetic patients and its changes based on genotype identification is also confirmed that T allele is a risk factor. In the presence of this allele in type 2 diabetes, the visceral adipose tissue is more breakdowns. Then the TG level increases and due to that the visceral fat is the main source for producing of visfatin, the plasma levels of this hormone in the presence of T allele are reduced. This reduction indicates the exacerbations of diabetes in individuals carrying this allele. Therefore, plasma measurement of visfatin is helpful in prognosis and determination of the diabetes progression.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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