Investigating *Visfatin gene* Polymorphism *rs4730153* with Insulin Resistance and Non-Alcoholic Fatty Liver Diseases in Iranian Population

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Abstract

Background: Visfatin is known as one of the adipokines associated with the development of inflammation, but its role in the pathogenesis of nonalcoholic fatty liver is less known so far. We aimed to investigate the association between *visfatin gene* polymorphism *rs4730153* and insulin resistance and non-alcoholic fatty liver disease (NAFLD).

Methods: This case-control study was performed on 80 patients with NAFLD as well as 80 healthy participants as controls referred to Amir Al-Momenin and Bouali hospitals in Tehran. Genotyping was performed using PCR-RFLP method. Plasma concentrations of visfatin and insulin were measured using ELISA kit. The fasting blood glucose, TC, TG, LDL-C, HDL-C, ALT, AST, SBP, DBP, and BMI levels were measured using the standard methods. Statistical analysis was also performed using SPSS software.

Results: A significant difference was found in Visfatin level in the patients with NAFLD compared to this level in healthy individuals. The levels of HDL-C and LDL-C in healthy individuals and triglyceride in patients for GG, AG, and AA genotypes carriers also were significantly different. There was a significant relationship between *rs4730153* polymorphism and insulin resistance; however, no association was found between this polymorphism and NAFLD. Notably, Visfatin showed a significant association with age (all individuals), body mass index (healthy individuals), insulin, and HOMA (in patients).

Conclusion: Visfatin levels reduced in patients with NAFLD. Moreover, rs4730153 polymorphism was indicated to be associated with both lipid metabolism and insulin resistance, but no association was found between this polymorphism and nonalcoholic fatty liver disease.

Keywords: Visfatin; Insulin resistance; NAFLD; *rs4730153*

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease is a big challenge in recent years (1) and became a global public health problem (2). Correspondingly, its prevalence is increasing due to obesity, lifestyle modification, and the decreased physical activity level

among populations. Non-alcoholic fatty liver disease is characterized by impaired fat metabolism, diabetes, hypertension, and elevated liver enzymes. Insulin resistance, oxidative stress, and inflammation, which probably play important and key roles in the development and progression of NAFLD (3,4). Additionally, some interactions between many environmental and genetic factors could consequently induce NAFLD (5). The compounds synthesized and secreted from Adipose tissue are called adipokines (6). Although visfatin as adipokines whose role in the pathogenesis of NAFLD is less known and controversial, there is a significant relationship between visfatin and NAFLD (7). Furthermore, visfatin increases the activation of leukocytes, synthesis of adhesion molecules, and the production of proinflammatory cytokines (8). It is also involved in insulin function and glucose metabolism (9). Moreover, visfatin binds to the insulin receptor at a location different from the insulin site, and then activates the insulin receptor. The insulin-mimetic actions of visfatin are involved, which stimulate glucose uptake by insulin-sensitive cells (adipocytes and myocytes) and inhibit the release of glucose from liver cells (10- 12). Furthermore, visfatin is known as a pre-B-**cell colony-**enhancing factor, which plays an enzymatic role in nicotinamide adenine dinucleotide (NAD) biosynthesis (13,14), so it consequently regulates insulin secretion from beta cells through NAD production (9). Visfatin is associated with secreted insulin and insulin resistance (15, 16).

The *visfatin* gene is located between q22.1 and q31.33 on chromosome 7 and contains 10 introns and 11 exons (17, 18). This polypeptide, which has a molecular weight of 52 kDa, is made up of 491 amino (14, 19). Inflammatory cytokines like visfatin interfere with insulin signaling and pathways, thus the genes which regulate these types of cytokines are associated with insulin resistance (13), inflammation, type 2diabetes, and obesity (20). Several studies have demonstrated the relationship of visfatin with fasting blood sugar (14), triglyceride (14, 10), lipid profiles, and IR (8). Moreover, the studies conducted on mononucleotide polymorphisms in the *visfatin* gene have

shown that polymorphisms may alter the expression of the *visfatin* gene, so they are associated with metabolic factors and the disease (20). *rs4730153* polymorphism, located in the intron of the *visfatin* gene, is associated with glucose, lipid metabolism, and visfatin levels (21).

We aimed to investigate the relationship of *visfatin* gene *rs4730153* Polymorphism with Visfatin levels, insulin resistance, and non-alcoholic fatty liver disease.

Materials and Methods

Study population

The present case-control study was performed on 160 participants referred to Amir Al-Momenin and Bouali hospitals in Tehran, Iran. The minimum age of these participants was 18 yr old; the maximum age was 60, and the mean age was 40.4. This study included 80 patients with non-alcoholic fatty liver, with a mean age of 44.3 yr old, including 37 women (46.2%) and 43 men (53.8%). In this regard, the final diagnosis of non-alcoholic fatty liver was identified and then confirmed by ultrasound. Eighty healthy individuals with a mean age of 36.6 yr old, including 53 women (66.2%) and 27 men (33.8%), were selected as the controls.

The number of the included individuals was calculated based on the sample size estimation formula. This study period was from Sep 2018 to Jun 2019, which was done in terms of the Helsinki Memorandum of Understanding.

The study was approved by the ethics committee of Islamic Azad University, Tehran Medical, under the code of IR.IAU.TMU.REC.1396.293.

In this study, all the included participants were Iranians, and non-Iranians were not enrolled. Moreover, the inclusion criteria were having no history of medication used to treat metabolic disorders, having no weight loss diet, and having no regular exercise program (three months before their referral). Moreover, the exclusion criteria were having a history of metabolic drug use, alcohol use, acute illness, pregnancy, kidney disease, other liver diseases, heart disease, cancer, immune diseases, infection, hypertension, and other known diseases.

To comply with ethical standards, the objectives and reasons for performing this study were explained to the included participants, and in case of any complete satisfaction, the general and medical questionnaire, including personal information, history of drug usage, diet, and other information, was filled by each person. Accordingly, all the questionnaires are available. Thereafter, the variables of height, weight, and blood pressure were measured using the standard method and body mass index was then calculated using the following formula: division of weight in kilograms by height squared in square meters) (22). The blood samples (10 ml) were taken from the included participants after overnight fasting for about 12-14 hours. Five ml of the obtained samples were poured into a tube containing anticoagulant EDTA (Ethylene diamine tetraacetic acid) for DNA extraction and the remaining 5 ml was poured into a tube without anticoagulant for serum preparation, to measure biochemical variables.

Clinical and biochemical measurements

Glucose was measured using Pars Azmoun glucose diagnostic kit by photometric method. Cholesterol (CHOL) and triglyceride (TG) levels were then measured by enzymatic colorimetric and the amount of cholesterol (HDL-C) was measured by sodium phosphotungstenate precipitate. Subsequently, Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedwald formula CHOL - HDL - TG / 5 = LDL (mg/dl) (1). Additionally, Pars test kit and BT3500 Biotechnica model were used to measure alanine aminotransferase and aspartate aminotransferase enzymes. Accordingly, Vaspin level was measured at 450 nm using ZellBio ELISA kit, Germany (ZB-10921-H9648). In addition, Insulin level was also measured using ELISA kit, Mercodia (01-110-1103). Insulin resistance was calculated by applying the formula HOMA-IR = fasting serum glucose (mmol /L) and fasting serum insulin (microunit/L)/22.5(7).

Genotyping

To determine the genotype, genomic DNA was extracted from whole blood tubes obtained from the subjects, containing anticoagulants using the salting-out method (3). Thereafter, carrier fragment 4730153rs polymorphism was amplified by PCR (Polymerase chain reaction) method. For this purpose, two primers made by Pishgam Biotech Company were used. Forward primer: 5'_GGTATGGTTGACCCAGCTAC_3', Reverse primer: 5'_CAGATTTACTTAGGCAGA-CACTTGA_3'. PCR method was performed with total volume of 25 µl put in a 0.2 ml microtube in terms of the following concentrations: 1 µl of DNA (50-100ng/ µl), 12.5 µl of Mastermix, 1µl of each primer (10 pm/µl), and 9.5 µl of distilled water, all of which were poured into the microtubes, and then transferred to a thermocycler. The thermocycle program was set at 40 cycles. Accordingly, the program included initial denaturation for 5 min at 96 °C, then 40 cycles for each cycle consisting of 3 steps, denaturation for 35 sec at 96 °C, the connection of primers for 35 sec at 61 °C, the expansion step was performed for 35 sec at 72 °C, and the final expansion was performed for 4 min at 72 °C. Genotypes were determined by RFLP (Restriction fragmentation length polymorphism) and fermentas RsaI restriction enzyme. One band for the AA genotype had a fragment length of 157bp, GG genotype with two bands with lengths of 90 bp and 67 bp, and AG genotype consisted of three bands with lengths of 157 bp, 90 bp, and 67 bp.

Statistical analysis

Statistical analysis was performed using SPSS 20 software (IBM Corp., Armonk, NY, USA). Quantitative variables were examined for normality using the Kolmogorov-Smirnov test. If data were not normally distributed, Mann-Whitney test was used for comparing the case and control groups; otherwise, independent t-test was used. Thereafter, qualitative factors were assessed using Chi-square or Fisher's exact test. Spearman correlation coefficient was also used to evaluate the relationship between Visfatin and clinical and anthropometric variables. The Statistical significance level was considered as less than 0.05.

Results

There were significant differences in age, body mass index, HDL, triglyceride, insulin, HOMA, visfatin, liver enzymes AST and ALT, and systolic blood pressure between the patients with NAFLD and the controls. *P*-value less than 0.05 was considered statistically significant (Table 1).

Table 1: Demographic and clinical characteristics of the study participants

Variable	NAFLD (n=80)	Non-NAFLD (n=80)	P-value
Age (yr)	44.4±9.6	36.6±10	**<0.001**
BMI (kg/m²)	28.7±4.6	25.3±4.2	**<0.001**
HDL (mg/dL)	35.1±12.1	46.5±17.5	**<0.001**
LDL (mg/dL)	94.1±30.8	92.6±26.5	0.765
Cholesterol (mg/dL)	170/3±43.5	166.7±34.2	0.575
Triglyceride (mg/dL)	128(108-185)	94(75.5-161)	**<0.001**
FBS (mg/dL)	92.1±13.8	89.1±8.9	0.111
Insulin (mu/L)	12.7(9-21.5)	9.3(5.7-15.1)	0.012
HOMA	2.8(2.1-4.8)	2.2(1.3-3.5)	0.006
Visfatin (ng/mL)	14.9±12.1	24.2±10.3	**<0.001**
AST (IU/L)	25(21-31.5)	16(14-19)	**<0.001**
ALT (IU/L)	32.5(20-42.5)	16(13-22.5)	**<0.001**
SBP (mm Hg)	12.3±1.9	11.4±1.8	0.024
DBP (mm Hg)	8.1±0.7	7.9±0.4	0.093

The relationship of visfatin with variables was examined using Spearman's correlation, in the healthy and the NAFLD groups, the results of which are shown in Table 2. Age showed a significant inverse relationship with visfatin (in all the subjects).

Table 2: Spearman rank correlation coefficient of the visfatin with different parameters

Variable	Cases and control		Control		Case	
	The correlation coefficient	P-value	The correlation coefficient	P-value	The correlation coefficient	P-value
Age (yr)	-0.212	0.012	-0.063	0.641	-0.123	0.278
BMI (kg/m²)	0.054	0.515	0.334	0.006	-0.115	0.309
HDL (mg/dL)	-0.149	0.098	0.126	0.417	-0.127	0.262
LDL (mg/dL)	-0.022	0.809	0.118	0.451	-0.028	0.802
Cholesterol (mg/dL)	0.057	0.523	0.015	0.920	0.058	0.612
Triglyceride (mg/dL)	-0.035	0.700	0.128	0.397	0.136	0.230
FBS (mg/dL)	-0.104	0.213	-0.185	0.140	0.039	0.729
Insulin (mu/L)	0.099	0.236	0.035	0.784	0.270	0.015
SGOT	-0.076	0.444	0.260	0.209	0.099	0.380
SGPT	-0.037	0.707	0.256	0.217	0.114	0.314
SBP (mm Hg)	-0.049	0.622	0.229	0.271	0.012	0.919
DBP (mm Hg)	-0.122	0.214	-0.154	0.463	-0.070	0.540
HOMA	0.066	0.432	-0.017	0.892	0.267	0.017

Moreover, in healthy individuals, a significant relationship was observed between visfatin and body mass index (*P*=0.006, r=0.334), and in patients, there was a significant relationship between visfatin, insulin (*P*=0.015, r=0.270), and HOMA

(*P*=0.017, r=0.267). Genotype counts and allele frequencies in female and male cases and controls are shown in Table 3. In this regard, Genotype frequencies of the *rs4730153* differed significantly among the subjects of the case group (*P*<0.05). Biochemical and anthropometric variables in *rs4730153* genotypes were separately studied in healthy individuals and patients (Table 4). The variables of HDL and LDL in healthy individuals and triglyceride in patients had significant associations with *rs4730153*. In this study, no significant differences were observed in serum levels of visfatin in the subjects with AA, AG, and GG genotypes of the *rs4730153* single nucleotide polymorphism (*P*≥0.05).

Table 3: The distribution of studied variables in each genotype of rs4730153 in case and control groups

Variable		*AA*	*AG*	*GG*	*P-value*
Gender	Control	4/8	13/29	10/16	0.816
man / Woman	Cases	7/3	14/29	20/4	0.001
Age (year)	Control	37.10±10.0	37.7±9.7	34.2±10.5	0.753
	Cases	43.5±8.6	45.2±10.4	44.2±8.6	0.85
BMI (kg/m2)	Control	24.8±3.9	28.6±4.5	25.2±3.6	0.887
	Cases	28.2±4.4	30.2±10.3	29.5±5.2	0.326
FBS (mg/dL)	Control	84.8±9.9	93.1±8.9	90.9±8.7	0.150
	Cases	88.5±8.9	93.1±16.2	91.7±10.9	0.639
HDL (mg/dL)	Control	60.5±37.2	43.1±9.2	44.5±10.4	0.029
	Cases	33.0±4.1	37.2±14.1	32.7±12.6	0.639
LDL (mg/dL)	Control	86.9±20.1	100.5±24.4	81.1±28.6	0.038
	Cases	99.2±24.9	87.9±34.9	101.3±24.6	0.200
Cholesterol (mg/dL)	Control	160.8±40.8	172.0±26.8	158.4±31.9	0.369
	Cases	160.8±40.8	165.9±47.4	180.8±37.7	0.327
Triglyceride (mg/dL)	Control	168(79-185)	94(77.5-144)	89(58-143)	0.117
	Cases	111(78-149)	120(95-181)	163.5(111.2-210)	0.047
AST (IU/L)	Control	13.2±3.0	16.5±4.8	17.1±4.4	0.197
	Cases	24.1±5.7	27.1±9.7	26.3±6.9	0.601
ALT (IU/L)	Control	15.2±6.4	17.6±6.5	21.3±10.9	0.254
	Cases	28.7±8.6	38.1±19.3	33.1±16	0.235
SBP (mm Hg)	Control	9.8±4.4	11.7±0.7	11.7±0.8	0.064
	Cases	12.3±1.1	12.5±1.9	11.9±2.2	0.587
DBP (mm Hg)	Control	7.8±0.4	7.9±0.4	167.0±44.8	0.751
	Cases	8.1±0.5	8.1±0.8	8.1±0.6	0.966
Visfatin (ng/mL)	Control	23.9±10.8	25.8±8.6	21.7±12.3	0.354
	Cases	14.9±12.5	15.5±12.3	13.6±12	0.831
Insulin (mu/L)	Control	12.7(8.9-16.4)	9.2(5.4-15.5)	8.6(5.9-14.6)	0.293
	Cases	12.6(7.9-13)	13.1(6.9-26.3)	11.1(7.4-21.1)	0.711
HOMA	Control	2.7(1.8-3.3)	2.0(1.2-3.8)	1.9(1.1-2.5)	0.151
	Cases	2.8(1.8-3.2)	3.4(1.6-6.6)	2.6(1.5-4.7)	0.548

Regression analysis for alleles and genotypes in the healthy individuals and the patients with fatty liver showed that none of the *rs4730153* genotypes plays a role in the development of fatty liver disease (*P*≥0.05). Moreover, *rs4730153* showed no significant difference in terms of the genotypes distribution frequencies between the insulin resistance and non-insulin resistance groups (*P*=0.09); however, alleles were found to have a significant relationship with insulin resistance. The

frequency of allele G was more in the HOMA**<**2.6 group compared to the HOMA≥2.6 group (64.1% vs. 52.8 *P*<0.05) and it also had an association with insulin resistance (*P*=0.042).

Table 4: Distribution of genotypes of visfatin's SNP in *rs4730153* in the research group

Genotypes and Alleles	NAFLD	Non-NAFLD	OR (95%CI)	P-value
AA	10(45.5)	12(54.5)	0.85(0.34-2.09)	0.717
AG	43(50.6)	42(49.4)	1.14(0.61-2.14)	0.674
GG	24(48.0)	26(52.0)	0.94(0.48-1.84)	0.858
A	63(49.9)	66(41.2)	1.13(0.56-2.26)	0.733
G	91(59.1)	94(58.8)	1.18(0.48-2.92)	0.717
Genotype and Allel	HOMA**<**2.6	HOMA≥2.6	*P*-value	
AA	8(9.4)	14(19.4)		
AG	45(52.9)	40(55.6)	0.090	
GG	32(37.6)	18(52.0)		
A	61(35.9)	68(47.2)	0.042	
G	109(64.1)	76(52.8)		

Discussion

This study was performed to explore the possible association among *rs4730153* visfatin SNP and anthropometric, biochemical parameters, and insulin resistance in the control and NAFLD groups. In the current study, the genotype of *rs4730153* was found to have no association with visfatin levels, NAFLD and HOMA-IR, but allele was associated with HOMA-IR. Moreover, we observed **no** association between NAMPT rs4730153 and anthropometric and biochemical variables, except HDL and LDL in the healthy individuals, and triglyceride in the patients. According to the current study, Visfatin was found to be positively correlated with BMI in healthy individuals, and with insulin and HOMA in the subjects with NAFLD.

Visfatin is a protein secreted by adipose tissue that could mediate proinflammatory properties. Accordingly, its increase has been reported in some diseases, including type 2 diabetes, obesity, cardiovascular disease, and non-alcoholic fatty liver disease (23). Many studies have been previously performed on the relationship of visfatin with diabetes. According to these reports, visfatin levels are increased in patients with diabetes, but in some studies, an increase in visfatin was only observed in diabetic patients infected for a long time (22).

Visfatin concentrations are significantly higher in obese adults (23), obese adolescents with insulin resistance (24), and obese individuals with type 2 diabetes compared to healthy individuals (10). However, plasma visfatin levels were significantly lower in obese individuals compared to the controls with normal weight (6).

Few studies have been conducted to clarify the association of visfatin with fatty liver. Plasma levels *of* visfatin were higher in patients with fatty liver disease compared to healthy individuals (7). In chronic inflammatory processes like NAFLD, visfatin plays a protective role in NAFLD (8). In some studies, similar to the present study, in healthy individuals, higher levels of visfatin were observed compared to nonalcoholic fatty liver disease. By interpreting these results, the level of visfatin has a negative and significant correlation with α-TNF-; therefore, in patients with NAFLD, along with increasing the expression of TNF-α, the level of visfatin decreases. (8). However, Mousavi et al. (7) and researchers who performed studies on the Turkish population observed no difference in visfatin levels in the subjects with NAFLD compared to the controls (8).

In our study, similar to other studies, visfatin levels decreased with aging in the patients with NAFLD (7, 10). Moreover, in healthy individuals,

visfatin showed a positive and significant relationship with body mass index as well as in patients with insulin and HOMA, but Mousavi's study found no association among visfatin level and BMI and systolic blood pressure (7). The results of research conducted on the association between visfatin and BMI were also inconsistent with ours. Correspondingly, most studies have reported a significant positive correlation among them (25). Visfatin is positively associated with increased triglyceride storage in preadipocytes and stimulation of fat synthesis (14). Therefore, in some studies, a significant relationship was shown between the level of visfatin and triglycerides and cholesterol, while similar to our study, no correlation was found among these factors (7, 26). Similar to our results, in some studies, visfatin was indicated to be significantly associated with insulin resistance (HOMA/IR) and insulin (6, 9). Contrary to our findings, some researchers have found a significant association between visfatin and FPG (9) and HDL (10, 19). The reason for the difference in studies' results may be related to genetic changes (6).

In the present study, the levels of HDL and LDL in healthy individuals and triglycerides in the patients were significantly different at the level of *rs4730153* genotype; however, no correlation was found between *rs4730153* polymorphism and other variables. Similar to our results, no association was found among this polymorphism and HOMA and visfatin levels (20), but contrary to our findings, in other populations, a significant relationship was found among *rs4730153* polymorphism and glucose, insulin, HOMA index (20, 19), and visfatin level (21). Similar to our results, *rs4730153* polymorphism was effective onregulating lipid metabolism in obese Chinese children and adolescents (21). However, Korner and Johansson in their study, contrary to our results, showed no association between *rs4730153* polymorphism and lipid metabolism (19, 21). The reason for this discrepancy in these studies is not clear yet, but discrepancies in a race, number of individuals examined in different studies, and techniques used (ELISA visfatin kit) may be considered as the possible reasons, as well (20). Our results similar

to some studies showed that *rs4730153* polymorphism were not associated with NAFLD (27), but visfatin showed a significant relationship with age and in the subgroups with body mass index, insulin, and HOMA.

Conclusion

The *rs4730153* polymorphism in the *visfatin* gene was also shown to be associated with both lipid profile and insulin resistance, but no association was found between this *rs4730153* polymorphism and NAFLD. Visfatin is one of the adipokines whose role in the pathogenesis of NAFLD is less known so far, so investigating the association of this protein with fatty liver is needed in different populations and larger volumes, in order to identify its mechanism and to develop appropriate treatment strategies.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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