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Screening rs2241766 Polymorphism as a Susceptibility Index Parameter in Obese Patients with a High Level of Cholesterol

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ABSTRACT

Obesity is a multifactorial disorder that has increased dramatically in recent years in developing countries. The disproportion of energy uptake and expenditure, which is the result of several factors such as diet, behavior, environment, as well as metabolic and genetic factors, could eventually lead to this disease. Mutations in genes that regulate appetite and metabolism could affect various aspects of obesity. Adiponectin protein, the product of the *ADIPOQ* gene, is an essential adipokine for controlling energy homeostasis and fat storage and is secreted in white adipose tissues. It is an insulin-sensitive hormone whose blood concentration declines in obesity. In this study, we examined the association of rs2241766 +45 T>G polymorphism of the *ADIPOQ* gene with obesity and blood levels of High-Density Lipoprotein (HDL), Low-Density Lipoprotein (LDL), Triglyceride (TG), and total cholesterol in the population of Borujen City. In this case-control study, blood samples were collected from people referred to Tamin Ejtemaee Laboratory of Borujen who suffered from obesity problems. Triglyceride, total cholesterol, LDL, and HDL levels were analyzed in the patient's blood using the alpha6 auto-analyzer. In the next step, total DNA was extracted from the blood samples and subjected to Restriction Fragment Length Polymorphism (RFLP) methods to investigate the rs2241766 polymorphism of the *ADIPOQ* gene. According to the results of this study, there is no significant correlation between any alleles and genotypes of this polymorphism with obesity (Chi-square *p-value* was 0.82 and 0.85 respectively for alleles and genotypes, odds-ratio 1.155, 95% CI= 0.32-4.14). There is no significant correlation between the blood lipid characteristics of the patients and the genotypes of the studied polymorphism. According to our results, it seems that the alleles and genotypes of the *ADIPOQ* gene rs2241766 do not affect the etiology of obesity in our samples.

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Introduction

Obesity is one of the most prevalent unfavorable situations in populations that increases the risk of various harmful disorders such as cardiovascular diseases, type 2 diabetes, hypertension and stroke, hypertriglyceridemia, osteoarthritis, hypercholesterolemia, asthma, and certain types of cancer (Greenberg and Obin, 2006). Many factors participate in the etiology of obesity, e.g., lifestyle, diet, physical activity, and genetics (Kadouh and Acosta, 2017; Weinsier *et al*., 1998). Interpersonal variability in the obesity phenotype is due to multiple genes and environmental factors (Bouchard, 2008). Nowadays, the genetic basis of obesity has been illustrated by scientists in more detail while it has not yet been possible to present a full picture of human gene loci involved in conditions leading to obesity.

Adipose tissue is a vital endocrine organ that secretes several factors, adipokines or

adipocytokines. A number of these factors are involved in the development of obesity through their effect on food absorption, lipid and carbohydrate metabolism, and many other processes (Yu *et al.*, 2012). Adipocytes exclusively secrete an adipokine protein with 244 amino acids called adiponectin, adipocyte complement-related protein, or adipoQ. This protein plays a critical role in cardiovascular homeostasis and metabolic (AlSaleh *et al.*, 2012; Greenberg and Obin, 2006; Lim *et al.*, 2014). Adiponectin protein increases the oxidation of fatty acids and insulin sensitivity and decreases the circulation of free fatty acids. In addition, this adipokine impedes monocyte adhesion to endothelial cells and represses lipid accumulation in human monocyte-derived macrophages (Ambroziak *et al.*, 2018).

The adiponectin level in the plasma of healthy subjects is 5-30 µg/ml, around 0.01% of the whole plasma proteins (Elghazy *et al.*, 2019). Plasma adiponectin level negatively correlates with Body Mass Index (BMI), insulin resistance, and human obesity (Lim *et al.*, 2014; Wanders *et al.*, 2012). This adipokine concentration is also associated with decreased plasma Triacylglycerol (TAG) (Greenberg and Obin, 2006) and increased HDL-C concentrations (AlSaleh *et al.*, 2012; Persson *et al.*, 2010; Petrone *et al.*, 2006). Furthermore, Serum adiponectin level is highly heritable (~50%) and associated with the adiponectin gene (*ADIPOQ*), emphasizing the importance of investigating the *ADIPOQ* gene as one of the candidates of obesity etiology and hence metabolic syndromes (Warodomwichit *et al.*, 2009). The *ADIPOQ* gene (also known as GBP28, APM1, or ACRP30) is about 15.8 kb long with three exons (Breitfeld *et al.*, 2012). There are 683 records for Single Nucleotide Polymorphisms (SNPs) in the NCBI database for the human adiponectin locus (www.ncbi.nlm.nih.gov), 33 of which have been cited in PubMed (Kaftan and Hussain, 2015). This gene is on chromosome 3q27, a primary genomic region associated with metabolic syndrome, obesity, T2D, and CVD (Karmelić *et al.*, 2012; Persson *et al.*, 2010; Ramya *et al.*, 2013). The rs2241766 is one of the most commonly studied SNPs and a synonymous T to G substitution in exon 2 of the *ADIPOQ* gene (Melistas *et al.*, 2009). Rs2241766 polymorphism was found to affect adiponectin secretion (Ji *et al.*, 2015; Potapov *et al.*, 2008; Tu *et al.*, 2014). Association studies have implied a substantial correlation between the rs2241766 polymorphism and obesity (Ergören *et al.*, 2019). Adiponectin is one of the adipokines playing a significant role in lipid homeostasis (Vasseur *et al.*, 2006). Disturbances in lipid levels of blood and lipoprotein metabolism are recurrently recognized cases of Cardio-Vascular Disease (CVD) risk factors (Wanders *et al.*, 2012). It was found by a recent meta-analysis that the decrease of total cholesterol by one mMol/L reduces the risk of coronary and other cardiovascular conditions by 20 to 25% (Johansson *et al.*, 2009). Being overweight could increase the risk of Congenital Heart Disease (CHD) by approximately 45% through its adverse influences on blood pressure and cholesterol levels (Bogers *et al.*, 2007). Also, many studies indicated that high TGs levels play a part in a group of metabolic disorders called atherogenic dyslipidemia, characterized by higher mean LDL-C levels (130–159 mg/dL) and TGs (>150 mg/dL), small LDL particles, and lower levels of high-density lipoprotein cholesterol (HDL-C <35 mg/dL) (Wanders *et al.*, 2012). Johansson reported a significant correlation between plasma adiponectin levels and clinical (systolic blood pressure or SBP, waist circumference or WC, diastolic blood pressure or DBP, and BMI) and metabolic variables (TG, HDL-C, GLUC) (Johansson *et al.*, 2009). According to previous studies, there were no differences in plasma lipids and adiponectin concentrations between obese and non-obese children (Breitfeld *et al.*, 2012). Ethnic differences and environmental interactions of potential genes might explain the inconsistencies in the cited studies. In this study, we investigated the association of the rs2241766 polymorphism of *the ADIPOQ* gene with people affected by high weight and disturbance in plasma biochemical factors in Borujen City.

Materials and Methods

Sampling and Biochemical Analyses

The sampling process was performed on 100 patients who were referred to Borujen Tamin Ejtemaee Clinic due to obesity problems. Also, 50 people who did not have obesity problems

were subjected to blood collection as a control group. The study protocol was approved by the University of Mazandaran Sciences ethics committee #IR.UMZ.REC.1399.031 and all participants in this study have informed the research and completed the relevant consent form. In the first step, we determined a full-fasted lipid profile for each subject, including total cholesterol, triglyceride, HDL-C, and LDL-C levels that were measured by the auto-analyzer (Alpha 6, ISFAHAN SANJESH equipment |Co. IR).

DNA Extraction and Genotyping

Total DNA was extracted from the whole blood using a phenol-chloroform (reagents Merck, Germany) method (Butler, 2011; Di Pietro *et al.*, 2011). Forward and reverse primers were designed based on the sequence extracted from the NCBI gene database (Accession No. NM_001177800) by Gene runner software (version 6.5.51 Beta). Forward primers: 5′-CACACAGGGAATAATGCTAAG-3′ and reverse primer: 5′-CCTTTCTCACCCTTCTCACC-3′ were designed in such a way that the area involved in the rs2241766 polymorphism. PCR was performed on a total volume of 25 µl. Each reaction contained genomic DNA (0.1-1 µg/ml), dNTPs (50 µmol/L), forward and reverse primers (0.4 µmol/L of each primer), MgCl2 (1.5 mmol/L), and one U Taq DNA polymerase (Cinagen Co. Iran). The PCR program was performed for 35 cycles (94 ºC for 30 sec, 57.5 ºC for 30 sec, and 72 ºC for 30 sec) with a 5-minute initial denaturation at 94 ºC and 5-minute final extension at 72 ºC (Biorad thermal cycler, USA). The amplicon length was 432 bp. The PCR product was digested with *Sma* I (Fermentas, Canada) restriction enzyme.

Following the digestion, the presence of each allele was determined according to the fragment sizes: allele T, 432 bp; allele G, 115, and 217 bp (Fig. 1b). The PCR and their digestion products were analyzed by 1.5% agarose gel (Sinaclon, Iran) electrophoresis, stained by a green viewer (Pishgam, Iran), and visualized directly under ultraviolet illumination. A single undigested 432 bp band indicated the presence of homozygous T/T, the emergence of two 217 and 115 bp bands represented homozygous G/G, and the presence of three 432, 217, and 115 bp bands indicated T/G heterozygote genotype in rs2241766 location.

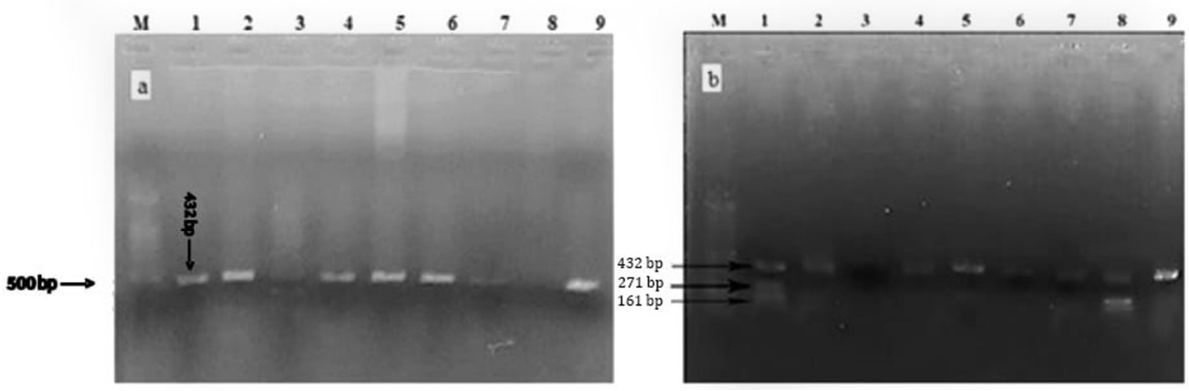

Fig. 1. The result of PCR-RFLP: a) Result of enzymatic digestion of PCR products in RFLP tests of some control subjects after optimization, and enzymatic cleavage; b) Result of enzymatic digestion of PCR products in RFLP tests of some obese patients after optimization, and enzymatic cleavage. In these figures, the 432 bp fragment is the T allele index, and the two 271 bp and 161 bp bands are seen in the G allele index at the rs2241766 position. M: 100 bp DNA marker, 1.5% agarose gel. The numbers show the sample subjects.

Statistical Analysis

All statistical analyses were performed using R Software, version 4.0.3. The normality distribution of the collected data was examined through Shapiro-Wilk Test. The non-parametric statistical analysis method included Man-Whitney U. The categorical data were tested using the chi-squared test, odds-ratio test, and proportion test.

Results

Demographic Statistics and Parameters

This case-control study includes 100 obese patients in the treatment group and 50 non-obese individuals in the control group. The means and standard deviations of obesity parameters and lipid levels are presented in Table 1 for both the obese and non-obese groups. The obese subjects were significantly older than the controls (*p-value*=0.003). All biochemical characteristics we studied, including total cholesterol, HDL, LDL, and triglyceride levels, were significantly higher in obese patients compared to the control group (*p-value*< 0.001, *p-value*= 0.001, *p-value*= 0.002, *p-value* < 0.001, respectively).

Table 1. Comparison of biochemical and physical parameters in both obese and control groups

Parameter	Obese (n = 100)	Nonobese (n = 50)	*P-value*
Age	50.19 ± 13.42	43.16 ± 13.43	0.003*
BMI	23.05 ± 1.6	39.77 ± 5.03	0.02*
Total cholesterol	236.05 ± 31.55	188.92 ± 62.95	<0.001*
Triglyceride	238.96 ± 99.68	184.92 ± 72.25	0.001*
HDL	41.57 ± 11.85	46.88 ± 10.35	0.002
LDL	146.55 ± 31.18	105.05 ± 55.09	<0.001*

BMI= *Body Mass Index*; LDL= Low-Density Lipoprotein; HDL= High-Density Lipoprotein; *: *p-values* < 0.05, reaches statistical significance.

Genotype Distribution and Allele Frequencies

The genotype and allelic frequency distributions of the *ADIPOQ* rs2241766 T>G gene polymorphism are presented in Table 2. According to the results, there is a significant correlation between the heterozygous T/G genotype of individuals and the obesity status of the subjects (*p-value* <0.001). Our results didn't imply any significant correlation between the G allele and obesity of the participants (*p-value*= 0.585). The odds ratio was 1.55 with 95% CI: 0.32-4.14.

The results showed that no genotypes of the given SNP are associated with obesity risk. This conclusion was also confirmed by the phi-coefficient, contingency coefficient, and Cramer's V test (all were 0.018), and the likelihood ratio (*p-value* 0.85).

Comparison of Gene Polymorphisms with Clinical Parameters

To study the correlation between the different genotypes of the rs2241766 and the given blood biochemical parameters, the ANOVA and Kruskal-Wallis tests were used for normal and abnormally distributed data, respectively (Table 3). The analysis of the association of different genotypes of the subjects and biochemical characteristics of their blood could not support any significant relationship between the genotypes and the LDL, HDL, triglyceride, and total cholesterol levels.

Table 2. A comparison of genotype and allele distribution of *ADIPOQ* rs2241766 T>G polymorphism in obese and non-obese subjects

Genotype/ Allele	Obese (n=100)	Nonobese (n=50)	*P-value*	Estimate	lower	Upper
Genotypes						
TT	69 (69%)	37 (74%)	0.941	1.0000000	NA	NA
TG	24 (24%)	9 (18%)	0.666	0.7275	0.1708	3.436418
GG	7 (7%)	4 (8%)	Ref	0.9005914	0.2479154	3.772920
TG+GG	93 (93%)	46 (92%)	0.073			
G	7 (7%)	4 (8%)	0.927			
Alleles				**Total allele frequency**		
T (ref. allele)	162 (81%)	83 (83%)		0.81		
G	38 (19%)	17 (17%)		0.18		

*: *p-values* < 0.05, reaches statistical significance.

Table 3. Evaluation of the relationship between biochemical parameters and each of the *ADIPOQ* rs2241766 G and T alleles in the obese group.

Parameters	T/T	T/G	G/G	*P-value*
Total Cholesterol	237.68 ± 30.52	232.13 ± 37.94	233.43 ± 15.92	0.364
Triglyceride	245.39 ± 108.28	229.21 ± 84.27	209 ± 43.99	0.708
HDL	40.88 ± 10.51	41.83 ± 11.41	47.43 ± 22.82	0.380
LDL	147.72 ± 29.12	143.87 ± 39.32	144.20 ± 21.33	0.857

*: *p-values* < 0.05, reaches statistical significance.

Discussion

In the current study, we investigated the association between one prevalent and widely studied SNP rs2241766 +45 T>G of *ADIPOQ* gene and obesity risk and levels of blood biochemical parameters, including total cholesterol, HDL, LDL, and triglyceride levels. A detailed analysis of the 3q27 chromosomal region showed that various alleles of the *ADIPOQ* gene are the primary determinants for adiponectin levels (Breitfeld *et al.*, 2012; Lindsay *et al.*, 2003; Siitonen *et al.*, 2011). It is estimated that the genetic variants can explain 50-60% of the changes in adiponectin levels in blood circulation (Breitfeld *et al.*, 2012). This fact points to the *ADIPOQ* gene as a prominent candidate gene in studies about the etiology of obesity, body weight, and consequent metabolic syndromes (Divella *et al.*, 2017). In different population studies, some researchers concluded that the rs2241766 +45 T>G polymorphism was not associated with obesity (Al-Daghri *et al.*, 2012; Payab *et al.*, 2017) while some researchers in other studies reported an association between them (Park *et al.*, 2011). Therefore, it is plausible that specific SNPs in *ADIPOQ* genes (*e.g.,* SNP rs2241766) are associated with some features of metabolic syndromes, such as obesity and higher levels of blood lipids, especially insulin sensitivity (triglycerides, LDL cholesterol, and total cholesterol) (Enns *et al.*, 2011). The effects of this SNP appear to be fully integrated with the ethnic background. It can probably be explained by different genetic backgrounds and the influence of different environments on various human populations (Enns *et al.*, 2011; Gu, 2009). Therefore, we studied the relationship between this polymorphism and obesity and a few biochemical characteristics in the population of Borujen in Iran. This study again showed that the origin of the subjects should be carefully considered, especially when performing genetic analysis because the genetic varieties do not necessarily follow the same pattern in different populations.

We found that, in the population we studied, neither the rs2241766 alleles nor its different genotypes are associated with obesity, and any other serum risk factors for this medical condition. This SNP is a synonymous variant. However, many studies showed the association of its recessive GG genotype with diseases *e.g.* (Han *et al.*, 2020; Zhao *et al.*, 2017). Data are controversial about the probable effect of the rs2241766 genotypes on obesity. Some indicate the predisposing effect for GG genotype (de Luis *et al.*, 2018; Wu *et al.*, 2014), others cannot find any correlation between its alleles and central obesity (Barliana *et al.*, 2019; Muiya *et al.*, 2013), while another study suggests that this SNP is not associated with BMI yet playing a role in the pattern of fat distribution in the body (Guzman-Ornelas *et al.*, 2012). Although we did not find a significant correlation between the GG genotype and obesity, the *p-value* = 0.07 was near the significant level. Therefore, it is plausible to suppose that, in a larger sample size, especially in the control group, we might find a significant relationship between the GG genotype and obesity. Another drawback of our work is that our case and control groups were not age-matched (*p-value* = 0.002). It can be ignored because the difference in alleles and SNP genotypes between the case and control groups was insignificant (*p-value* = 0.97 and 0.85 for genotypes and alleles, respectively) and also, this could interfere with our conclusions if we found a significant association between the SNP and obesity.

In the study of total cholesterol, LDL, HDL, and triglyceride parameters, significant differences were observed such as higher total cholesterol, triglyceride, and LDL levels and lower HDL

levels among patients. These results are consistent with the level of the above-metioned parameters obtained in many other studies (Bermúdez-Cardona and Velásquez-Rodríguez, 2016; Satoh *et al.*, 2008; Wang and Peng, 2011). The importance of biochemical parameters, including triglycerides, HDL, LDL, and total cholesterol levels, concerning obesity, has been considered in many studies. For example, Feingold and Grunfeld (Feingold and Grunfeld, 2018) reported in 2018 that 60 to 70 percent of obese people show a high level of blood lipids, indicating abnormal LDL, HDL, or triglyceride levels in the blood. Wang and Peng studied the relationship between the HDLc levels and obesity and found a negative relationship between the blood HDLc levels and obesity, *i.e.*, obese people have lower HDLc levels than normal subjects (Wang and Peng, 2011). This study (like our study) suggested that a reduction in such a parameter could be considered a prognostic factor for obesity. In a study, Subramanian also mentioned hypertriglyceridemia as a common abnormality in obesity and metabolic syndrome, which is in line with the importance given to the triglyceride parameter in our study (Subramanian and Chait, 2012). Klop *et al.* stated that obesity augments the risk of CVD due to the risk factors, such as elevated triglyceride and LDL levels and lowered HDLc levels (Klop *et al.*, 2013).

In our study, none of the rs2241766 +45 T>G genotypes was associated with the biochemical factors analyzed; in contrast, Ergören *et al.,* found that the rs2241766 +45 T>G was slightly associated with total cholesterol and LDL-C levels in obese individuals and, also the G allele of this polymorphism was correlated with the total cholesterol and LDL-C levels in the obese group ($p = 0.003$) (Ergören *et al.*, 2019). These results could be obtained under the influence of secondary conditions that arose after the obesity process. However, this justification based on metabolic pathways needs further investigation. Also, in this study, the variances of biochemical characteristics have been investigated between all samples in the form of two fixed genotypes. The lack of significant differences in the *p-values* of the indicators among the participants indicates accurate sampling, i.e., this indicates that factors such as hormonal diseases and environmental and occupational factors were not involved in the obesity of the subjects. The studied polymorphisms do not show a significant relationship between these characteristics, which might be because they do not affect the adiponectin levels or other pathways compensate for defected adiponectin levels. In other words, according to other studies, factors such as glucagon, leptin, and irisin will compensate for or modify the changes of adiponectin (Landecho *et al.*, 2019; Rabiee *et al.*, 2020). Finally, it should be considered that unstudied polymorphisms involved in the imbalance of association with the genetic variants of adiponectin may also play a role in changes in plasma levels of adiponectin (Breitfeld *et al.*, 2012).

Conclusion

Our study showed that there is a significant correlation between the biochemical characteristics, including total cholesterol, triglycerides, HDL, and LDL, and the occurrence of obesity phenotype although the cause-and-effect relationship of these factors with obesity is not proven. Our study also showed that the T/G genotype in the rs2241766 position and the incidence of obesity are not correlated. These results are confirmed and supported by many studies. Our results did not show a significant correlation between the different genotypes of patients with serum levels of total cholesterol and triglyceride and HDL and LDL. The lack of relevance of these characteristics can be related to their multifactorial nature such as diet and nutrition. Also, our results did not show a significant allelic correlation between obese and control groups which might be affected by limited sampling.

Conflicts of interest

The authors declared no conflicts of interest.

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