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Original Article

Lack of association between rs1800795 (-174 G/C) polymorphism in the promoter region of interleukin-6 gene and susceptibility to type 2 diabetes in Isfahan population

Reza Ghavimi, Mohammadreza Sharifi[1], Mohammad Ali Mohaghegh[2], Hossein Mohammadian, Saedeh Khadempar[3], Hamzeh Rezaei[4]

Departments of Pharmaceutical Biotechnology, [1]Genetics and Molecular Biology and [2]Parasitology and Mycology, Isfahan University of Medical Sciences, Isfahan, [3]Department of Biology, Kurdistan Science and Research Branch, Islamic Azad University, Sanandaj, [4]Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

Abstract

Background: Type 2 diabetes mellitus (T2DM) is an inflammatory autoimmune disease that mostly affects older adults. The etiology of T2DM includes both genetic and environmental factors. rs1800795 (−174 G/C) single nucleotide polymorphism (SNP) linked with autoimmune disorders predispositions, identified by Genome-Wide Association Study among genes, which immunologically related is considerably over signified. The goal of this study was to evaluate the association between rs1800795 (−174 G/C) polymorphisms in the promoter of interleukin-6 (IL-6) gene with susceptibility to T2DM in a subset of the Iranian population.

Materials and Methods: In this case–control study, 120 healthy subjects and 120 patients with T2DM were included. Genomic DNA obtained from whole blood samples and the polymerase chain reaction was used to amplify the fragment of interest contain rs1800795 SNP, restriction fragment length polymorphism method was applied for genotyping of the DNA samples with *NlaIII* as a restriction enzyme. SPSS for Windows software (version 18.0, SPSS, Chicago, IL, USA) was performed for statistical analysis.

Results: No significant differences were found between healthy controls and T2DM patients with respect to the frequency distribution of the cytokine gene polymorphism investigated. Odds ratio, adjusted for sex, age, and smoking status has displayed similar outcomes.

Conclusion: These results indicated that the rs1800795 SNP is not a susceptibility gene variant for the development of T2DM in the Isfahan population. Further studies using new data on complex transcriptional interactions between IL-6 polymorphic sites are necessary to determine IL-6 haplotype influence on susceptibility to T2DM.

Key Words: Genome-wide association study, interleukin-6 gene, polymorphism, type 2 diabetes mellitus

Address for correspondence:
Dr. Mohammadreza Sharifi, Department of Genetics and Molecular Biology, Isfahan University of Medical Sciences, Hezar Jarib Street, Isfahan, Iran.
E-mail: mo_sharifi@med.mui.ac.ir
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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders in which there are high blood sugar levels over a prolonged period. Two main types of diabetes

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are type 1 DM (T1DM) and type 2 DM (T2DM). T2DM is the most common form of diabetes that affects 85–90% of all diabetic peoples, characterized by hyperglycemia (high blood sugar) in the context of insulin resistance and relative lack of insulin.[1,2] The fast growing prevalence of T2DM has been a leading public health crisis that causes significant morbidity and mortality worldwide.[3] T2DM is a multifactorial disease that both genetic risk factors with many predisposing genes as well as environmental factors have been identified as important causes.[4] Many risk factors associated with T2DM have been reported such as age, sex, obesity, insulin resistance, hyperlipidemia, hypertension, and smoking.[5,6] Genetic susceptibility to T2DM is likely to be under monogenic and polygenic manner, but the majority of the genes involved is yet to be identified.[7,8] Over the past decade, many efforts have been invested in the exploration for genes predisposing to an autoimmune disease such as T2DM.[9] Recently, the notable advance was made in the detection of susceptible genes in which single nucleotide polymorphisms (SNPs) are involved in autoimmune diseases. Thus, many association studies of immune associated genes described for autoimmune disorders. Based on the function of the gene, most of these genes are supposed to be related to the pathogenesis of diseases. One of the susceptibility genetic variants that related to autoimmune diseases is rs1800795 SNP in the interleukin-6 (IL-6) gene. Several studies have investigated whether a promoter region polymorphism in the gene encoding IL-6, might enhance susceptibility to some autoimmune disorders.[10-14] It was demonstrated that polymorphisms within the promoter or other critical regulatory regions of cytokine genes can modulate the transcriptional activity that results in inter-individual differences in the levels of cytokine production. These variations may increase susceptibility to autoimmune diseases. As T2DM is considered as an immune-mediated disease, polymorphism in inflammatory cytokines seems to be reasonable candidates for investigation.[15] The functionally relevant promoter polymorphism IL6 -174 G > C was reported to be associated with circulating IL-6 levels,[16] T2DM,[17] insulin resistance,[18] obesity,[19] and cholesterol levels.[20] Patients with T2DM have significantly higher levels of IL-6 compared to those without diabetes.[21] IL-6 is a key anti-inflammatory and pro-inflammatory cytokine produced mainly by T-cells and macrophages but also from renal cells, muscle cells, osteoblasts, and adipocytes, and plays significant roles in the regulation of the immune response, inflammation, and hematopoiesis.[22,23] IL-6 is also a key regulator of the acute-phase response associated with insulin-resistant states including T2DM.[24] rs1800795 (–174 G/C) polymorphism in the promoter of IL-6 gene has been positively correlated with some autoimmune disorders and other authors have investigated this polymorphism inT2DM,[10] systemic lupus erythematosus (SLE),[11] systemic sclerosis,[25] and multiple sclerosis (MS).[13] Given complex genetic effects and multifaceted gene–environment interaction nature of T2DM, frequencies of genetic SNP polymorphisms are different among ethnic populations.[26] Since this study has not been carried out on the Iranian population, thus, the purpose of current study is to specify whether the –174G/C polymorphism (rs1800795) SNP in the IL-6 gene is associated with the susceptibility to T2DM in Isfahan population.

MATERIALS AND METHODS

The case–control study was conducted to assess the association between rs1800795 SNP and T2DM on Isfahan population, a city located in the central region of Iran. The studied population, including 120 T2DM patients that referred to endocrine and metabolism research center and 120 healthy control subjects who referred to Isfahan Transfusion Organization (control group). All patients had clinically diagnosed T2DM, and the healthy subjects had no history of T2DM or other autoimmune disorders. The population under study were composed of 67 (55.8%) women and 53 (44.2%) men for controls and 69 (57.5%) women and 51 (42.5%) mean for patients. Written informed consent was obtained from all patients and controls, and the study was approved by the University Ethics Committee.

DNA extraction and single nucleotide polymorphism genotyping

Peripheral blood samples related to patient and control groups were collected in tubes containing ethylenediaminetetraacetic acid as an anticoagulant, and then, using the DNG plus DNA extraction Kit (CinnaGen, Iran) DNA was extracted from whole blood samples. DNA quality was analyzed by ultraviolet (UV) absorption at 260 and 280 nm and also by agarose gel electrophoresis. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was applied for genotyping. The fragment comprising the site of restriction enzyme *NlaIII* at –174G/C was amplified using the following primers:

Forward: 5'-TGACTTCAGCTTTACTCTTTG-3', Reverse: 5'-CTGATTGGAAACCTTATTAAG-3'. The primer was synthesized by Pishgam, Tehran. These specific PCR primers amplified a 198-bp fragment in which there is a specific restricted site to determine the different alleles of the rs1800795 SNP. Eppendorf thermal cycler (Eppendorf, Hamburg, Germany)

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was applied for PCR under the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 5 min. Then, PCR products were digested with 1 unit of *NlaIII* restriction enzyme (Reaction Volume15 ML) (Fermentas, Lithuania). After 4-h incubation at 37°C, 198 bp PCR product was cut with *NlaIII* into four fragments 168, 119, 49, and 30 bp in length [Figures 1 and 2]. The resulting products were visualized by 3.5% agarose gel electrophoresis. Fragment size of 119 and 49 bp indicated the presence of a wild-type homozygous CC genotype, two 30 bp and 168 bp fragments represented the presence of homozygous GG genotype (due to limitation of agarose gel in the detection of fragments that are smaller than 50 bp, 30 bp fragment was invisible) and three fragments of 168, 119, and 49 bp displayed the presence of heterozygous CG genotype. In the final step, visualization of the products was performed with UV light. To estimate the genotyping error rate, we performed both random duplications in 20% of the samples.

Statistical analysis

SPSS for Windows software (version 18.0, SPSS, Chicago, IL, USA) was used for statistical analysis. Frequencies of alleles and genotypes for Hardy–Weinberg equilibrium were tested using the Chi-square analysis. To estimate distributions of risk allele/genotype-specific odds ratios (ORs), 95% confidential intervals (CIs), and analogous *P* values after adjustment for gender, age, as covariates between cases, and logistic regression analysis were applied. All continuous variables were expressed as the mean ± standard deviation. Student's *t*-test was used to compare the continuous variables between the T2DM and control groups. Pearson's v2 test was used to evaluate the difference in the prevalence of T2DM among genotypes. $P < 0.05$ was considered as a significant association.

RESULTS

Demographic and clinical features of the participants include case and control subjects in the studied population and the association with T2DM is demonstrated in Table 1. No major differences were observed between the two groups concerning gender ($P = 0.774$), age (50.2 ± 9.5 years for controls and 51.3 ± 9. 9 years for cases), and ($P = 0.312$). Compared with the controls, the cases had lower physical activity ($P = 0.001$) but had a higher body mass index ($P = 0.01$). There was no significant relation to smoking ($P = 0.314$) [Table 2]. Genotypes were effectively typed in all subjects and did not deviate from the distribution expected by the Hardy–Weinberg equilibrium. The RFLP detection system is schematized in Figure 2. The results of the RFLP analysis were confirmed by randomly selected samples for sequencing [Figures 3-5]. The frequencies of the G/G, G/C, C/C, and genotypes of rs1800795 SNP were 24.1, 53.4, and 22.5% in controls, and 33.3, 51.6, and 15% of cases, respectively, the frequency of the G allele in cases (78.9%) was more than that in the healthy control group (71.4%) [Table 2]. No statistically significant differences were found between T2DM patients and healthy controls with respect to the – 174 G > C genotype or allele distribution. For all of alleles and genotypes of the rs1800795 polymorphism, results were not associated with the risk for T2DM in the population under study [Table 2]. Our data indicate that the – 174 IL-6 genotypes have no role in susceptibility to study T2DM. In addition, when we compared the CC and GC genotypes against GG genotype as reference, the association between rs1800795 SNP genotypes and T2DM risk was examined in subgroups of both subjects stratified by gender, age (under and over 40 years), and smoking status [Table 3]. The adjusted OR for the GG and GC + CC genotypes was 0.783 (95% CI: 0.281–1.951, $P = 0.346$) in males, and 0.473 (95% CI: 0.179–1.353, $P = 0.172$) in females, which is indicative of no significant association. Also, we did not find a significant relation for under and over 40 years groups (OR = 0.612, 95%

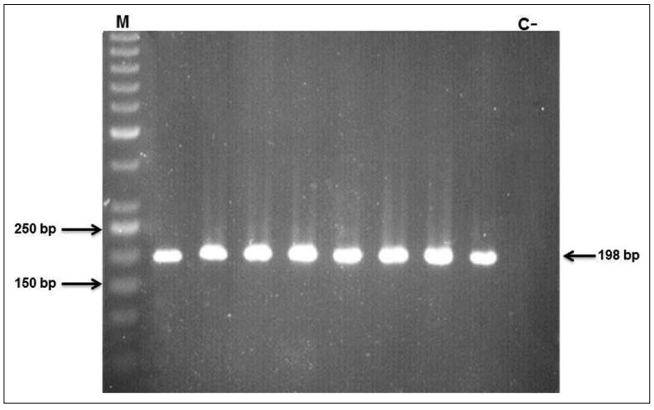

Figure 1: 198 bp polymerase chain reaction product of interleukin-6 gene polymorphism (rs1800795). Last lane is a negative control

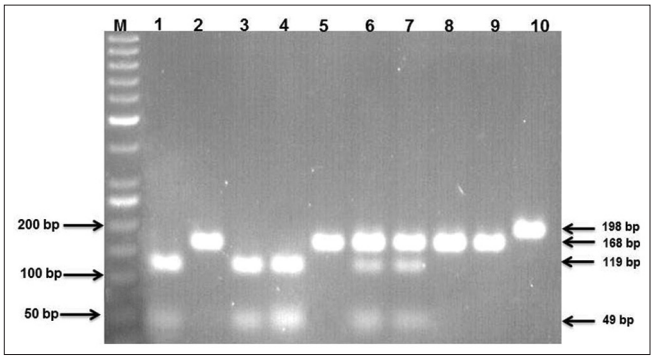

Figure 2: Enzyme digestion. M (50 bp marker); 1, 3, 4 are (CC genotypes); 2, 5, 8, 9 are (GG genotypes); 6, 7 are (GC genotypes) and lane 10 is undigested producing

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Table 1: Demographic features in cases and normal controls

Variables	Controls (n=120)	Cases (n=120)	P
Age (mean±SD)	50.2±9.5	51.3±9.9	0.312
BMI (kg/m^2)	27.3±4.3	28.5±4.8	0.01
Gender (%)			
Male	53 (44.2)	51 (42.5)	0.774
Female	67 (55.8)	69 (57.5)	
Smoking (%)			
Ever	18 (15)	21 (17.5)	0.305
Never	102 (85)	99 (82.5)	
Physical activity (%)			
Few	50 (41.6)	75 (62.5)	0.001
Moderate	46 (38.4)	34 (28.3)	
Much	24 (20)	11 (9.2)	

BMI: Body mass index, SD: Standard deviation

Table 2: Allele and genotype distribution of rs1800795 SNP in cases and controls and their association with T2DM in this study

Groups	Cases n (%)	Controls n (%)	P*
Allele frequency (rs1800795)			
C	98 (40.8)	118 (49.1)	0.086
G	142 (59.2)	122 (50.9)	
Genotype frequency			
GG	40 (33.4)	29 (24.1)	0.153
GC	62 (51.6)	64 (53.4)	
CC	18 (15)	27 (22.5)	

*P<0.05, SNP: Single nucleotide polymorphism, T2DM: Type 2 diabetes mellitus

Table 3: Stratification analysis of rs1800795 genotype frequency in cases and controls

Group	Genotype (%)		OR (95% CI)	P*
	GG	GC/CC		
Overall				
Case	40 (33.3)	80 (66.7)	0.591 (0.298-1.172)	0.165
Control	29 (24.1)	91 (75.9)		
Male				
Control	18 (32.1)	38 (67.9)	0.783 (0.281-1.951)	0.346
Case	13 (22.4)	45 (77.6)		
Female				
Control	22 (34.3)	42 (65.7)	0.473 (0.179-1.353)	0.127
Case	16 (25.8)	46 (74.2)		
Age <40				
Control	16 (35.5)	29 (64.5)	0.612 (0.215-1.520)	0.201
Case	13 (26)	37 (74)		
Age >40				
Control	24 (32)	51 (68)	0.591 (0.281-1.491)	0.144
Case	16 (22.8)	54 (77.2)		
Ever smokers				
Control	8 (40)	12 (60)	0.346 (0.281-1.491)	0.408
Case	6 (24)	19 (76)		
Never smokers				
Control	32 (32)	68 (68)	0.635 (0.323-1.379)	0.354
Case	23 (24.2)	72 (75.8)		

*P<0.05. OR: Odds ratio, CI: Confidence interval

CI: 0.215–1.520, P = 0.201 and OR = 0.591, 95% CI: 0.281–1.491, P = 0.144, respectively), and the genotype distribution. In a stratification analysis of smoking, adjusted OR for the GG and GC + CC genotypes was 0.346 (95% CI: 0.281–1.491, P = 0.408) in ever smokers and 0.635 (95% CI: 0.323–1.379, P = 0.354) in never smokers. Thus, there was no statistically significant evidence for an association between IL-6 – 174 G>C and these variables.

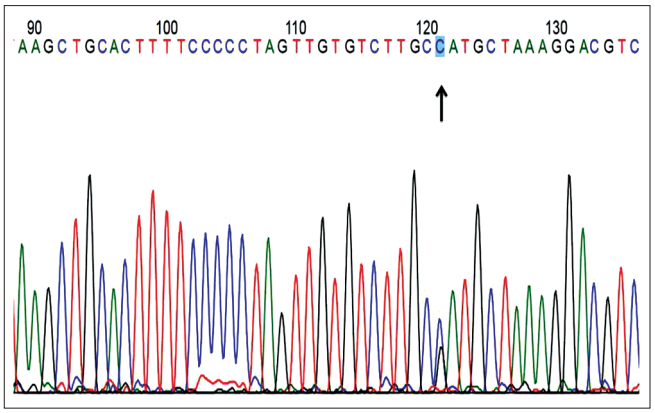

Figure 3: A sequencing chromatogram showing GC genotype of rs1800795 G>C

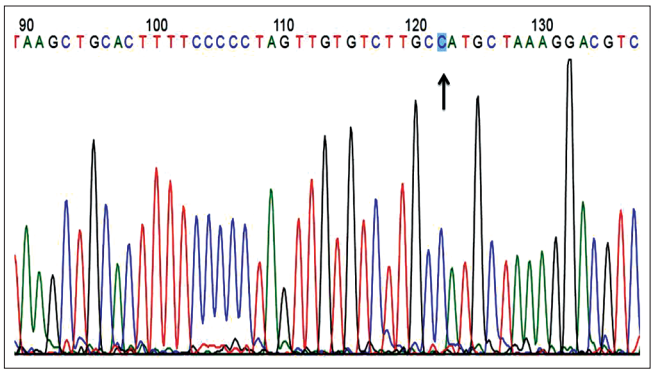

Figure 4: A sequencing chromatogram showing CC genotype of rs1800795 G>C

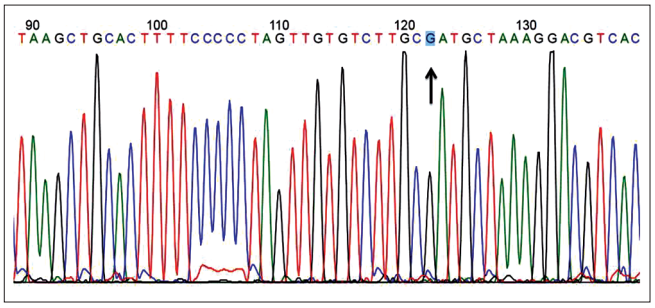

Figure 5: A sequencing chromatogram showing GG genotype of rs1800795 G>C

DISCUSSION

T2DM is a multifactorial disorder. While we know about environmental risk factors for T2DM, identification of the genetic factors of T2DM has

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proved to be challenging. Thus, the identification of gene-environmental interaction factors responsible for susceptibility to T2DM is a promising approach to establish novel and efficient ways of the prevention.[27] It has been demonstrated that levels of IL-6 are increased in various autoimmune disorders, including T2DM and play an important role in the pathophysiology of T2DM.[28] Transcription of IL-6 is regulated by different factors binding to the promoter region so this may cause variations in expression levels of IL-6 and may possibly play a role in susceptibility to different inflammatory and autoimmune diseases.[29] In the present study, polymorphism in the IL-6 gene (rs1800795 –174 G>C) was examined in order to investigate a possible association between specific polymorphic profile and T2DM susceptibility. The rs1800795 polymorphism in the IL-6 gene has been positively correlated with some autoimmune diseases and other authors have investigated this polymorphism with respect to type 2 diabetes,[10] SLE,[11] systemic sclerosis,[25] and MS.[13]

In a study of MS patients carried out on IL-6 promoter polymorphism (rs1800795) by Mirowska-Guzel *et al.*,[13] IL-6 –174 G>C polymorphism was found to be relevant for population risk of MS. Percentage of C allele carriers were higher in MS group (53%) than in controls (38%) ($P < 0.0001$, OR = 1.88, 95% CI 1.4–2.5). IL-6 –174 CC genotype occurred more than twice as often in patients (29.4%) as for healthy controls (12.4%) ($P < 0.00001$, OR = 2.86, 95% CI 1.7–4.9). Another study by Sfrent-Cornateanu *et al.*,[12] carried out on this polymorphism in systemic sclerosis patients showed that the GG homozygosis was found to be associated with a higher degree of disease activity and disability in systemic sclerosis patients. In a study of SLE patients carried out by Chua *et al.*,[11] significant association was observed at homozygous G genotype in patients ($P < 0.00000000625$, OR = 7.33, 95% CI 3.5–15.05), whereas the heterozygous G/C genotype was significant in the controls. In another study by Illig *et al.*,[10] carried out in type 2 diabetes patients on rs1800795 polymorphism showed that GG genotype was found to be associated with type 2 diabetes ($P < 0.0096$, OR = 1.51, 95% CI 1.11–2.07). In our investigation, the rs1800795 SNP genotype distribution and allele frequency among the control and case groups was not statistically different. Furthermore, when patients and controls were stratified on the basis of gender, age, and smoking status, no association was found between these variables and genotype distribution. Therefore, this polymorphism has not any interaction with these variables in our population, so no evidence was obtained to suggest that –174 G>C polymorphism in IL-6 gene is a susceptibility factor for the development of T2DM in a subset of Iranian population. Further investigation using new data on transcriptional interactions between IL-6 polymorphic sites are necessary to determine IL-6 haplotype influence on susceptibility to T2DM.

CONCLUSIONS

In the present study, polymorphism of IL-6 in the promoter region did not have significant association with T2DM susceptibility. We were unable to reproduce previous studies which indicated that polymorphism at the IL-6 promoter region correlates with susceptibility to autoimmune diseases. The reasons for this could be due to differences in the ethnicity of patients in the current study and those studies, environmental risk factors, the sample size that was used in the studies, or to other risk factors as yet undetermined. Further evaluation in this area is still needed.

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Conflicts of interest

There are no conflicts of interest.

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