Original Article

Association Study of Transcription Factor 7-like 2 (TCF7L2) Gene Polymorphisms and Haplotypes with Type 2 Diabetes in the South-West of Iran

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Abstract

Background & Aims: Type 2 diabetes mellitus (T2DM), one of the costliest chronic diseases of our time, is a multifactorial and heterogenic disease with a complex etiology. Among all the T2DM related genes, the SNPs of the transcription factor 7-like 2 (*TCF7L2*) gene have been recognized as the strongest genetic risk factors for T2DM in different ethnic groups by several studies. The aim of this study was to investigate the possible association between *TCF7L2* gene polymorphisms (rs290487, rs11196205, rs7903146, and rs12255372) and the risk of T2DM in a population from Khuzestan province, South-West of Iran.

***Materials & Methods*:** In this case-control association study, we studied 146 patients with T2DM and 146 healthy subjects. Genotyping for rs290487 and rs11196205 were done by Tetra-Primer ARMS-PCR and genotyping for rs7903146 and rs12255372 were carried out using PCR-RFLP. Statistical analyses were carried out using SPSS v.25.

***Results*:** For rs290487 polymorphism, TT and TC genotypes were not observed in patients and controls, and all of the subjects showed only CC genotype. The C allele of rs11196205 polymorphism was associated with T2DM (OR = 1.393, 95% CI = 1.005-1.932, p-value = 0.046). Two other studied polymorphisms, rs7903146 and rs12255372, had no significant differences in the genotype and allele frequencies between the two groups. Three-variant haplotypes of *TCF7L2* gene were analyzed using the PHASE software. There was no significant difference between control group and case group for haplotype distribution.

***Conclusion*:** Our results suggest that there is a significant association between rs11196205 polymorphism and T2DM, but no association is observed between the three other polymorphisms (rs290487, rs7903146, and rs12255372) with T2DM in the studied population. Moreover, no risk haplotype is reported in our population.

Keywords: Association study, *TCF7L2* gene, Type 2 diabetes mellitus, rs11196205, rs7903146, rs12255372, rs290487, Haplotype.

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Introduction

The prevalence of diabetes is rising across the globe, and the number of people with diabetes aged 20–79 years is predicted to rise to 642 million by 2040 globally (1). According to the fourth Iranian plan (2011) for the "periodic National Survey of Risk Factors for Non-

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Communicable Diseases (SuRFNCD-2011)", the national prevalence of diabetes is estimated to be 11.4% (approximately 4.5 million) of the adult population in the country (2); while over a quarter of this population in 2011 was not diagnosed. Therefore, with this rate, it is appraised that in 2030, 9.2 million Iranian individuals will have diabetes. (3). Several studies demonstrated that the most common appearance of diabetes, which involves 95% of the diabetic population, is Type 2 diabetes mellitus (T2DM). Type 2 diabetes as an epidemic disease, is one of the costliest chronic diseases of our time and a major challenge in modern society. Patients with T2DM suffer from hyperglycemia which happens via several mechanisms including insulin resistance disorder in peripheral tissues, devastated secretion of insulin and soared glucose production by the liver. So, T2DM is a heterogeneous group of metabolic disorders (4,5). T2DM is multifactorial in nature, and genetic and environmental risk factors including obesity, sedentary lifestyle, small or large birth weight and stressful lifestyle can play major roles in the development of T2DM as a complex genetic disorder (6–8). Although it is thought that environmental factors such as lifestyle changes may lead to type 2 diabetes, only in the presence of genetic risk factors for this condition, the person gets the disease; for this reason, much effort has been made to identify the genes and the loci associated with this disease (9,10). By associating regions of the genome with disease susceptibility, more than 100 loci influencing T2DM risk have been identified so far (11). Among all the T2DM related genes, genetic variants in the transcription factor 7 like 2 (*TCF7L2*) gene have been identified as the strongest genetic risk factors for T2DM in multiple ethnic groups (12). *TCF7L2*, also known as *TCF4* locus, is located on chromosome 10q25.2–25.3; a kind of transcription factor (high mobility group box-containing), which participates in the Wnt signaling pathway, is expressed by this gene with 215.9 kb longs (13,14). It is demonstrated that this signaling pathway has a great impact on the development process and comprises a complex network of protein interactions that regulate intercellular communications at multiple levels (15). *TCF7L2* gene comprises 17 exons (16). Interestingly, it has been reported that none of the exonic variants of the *TCF7L2* gene are associated with T2D disorder; however, all of the *TCF7L2* SNPs located in intronic regions are associated with T2D disorder (6). rs7903146 at the *TCF7L2* gene is located in the third intron. rs11196205 and rs12255372 are located in *TCF7L2* intron 4. rs290487 is located in the LD block (LD block 6) near the 3' end of *TCF7L2* gene, spanning from intron 7 to intron 13 (14 kb in length). Although the T2DM-associated SNPs are located in non-coding regions, it is not clear if these SNPs or a vsariant in strong linkage disequilibrium (LD) with these, play a role in alternative splicing, gene expression or protein structure (16–18). The aim of the current study was to investigate the association of *TCF7L2* gene polymorphisms (rs290487, rs11196205, rs7903146, and rs12255372) with T2DM, in a population consisting of diabetic and non-diabetic individuals, from South-West of Iran (Khuzestan province).

Materials and Methods

Study subjects:

In this case-control study, a total of 292 individuals, including 146 unrelated adult T2DM patients (75 men and 71 women; age 52.62 ± 9.241years) who were selected from those referred to the Diabetes Clinic and Golestan Hospital in Ahvaz, and 146 unrelated controls (71 men and 75 women; age 56.59 ± 7.230years) who were selected from patients referred to Valiasr Hospital in Khoramshahr and Golestan Hospital of Ahvaz (Table 1). The patient group with T2DM were recognized based on ADA criteria, according to which FPG _ 126

mg/dl or 2-hPG (2h Plasma Glucose) _ 200 mg/dl or RBG (Random Blood Sugar) _ 200 mg/dl described as diabetic. Subjects with diabetes were chosen as samples that were taking medicines to treat diabetes. In the control group, subjects with no family history of diabetes among their first-degree relatives were carefully monitored. Subjects in both studied groups were living in Khuzestan province. The consent form to participate in this study was signed by all subjects, before blood sampling. The project was approved by Special Committee of the Genetic Department of Shahid Chamran University on January 25, 2016 with 20/9/686 code for considering the ethical issues of patients and controls who participated in sampling.

DNA extraction:

Approximately 3-5 ml peripheral blood was collected from all subjects in EDTA tubes and stored in -40 °C for DNA extraction. Leukocyte Genomic DNA was extracted from whole blood samples by non-enzymatic salting out method. The extracted DNA was quantified by Nano Drop and agarose electrophoresis (stored at -20 °C until genotyping).

Genotyping of *TCF7L2* SNPs:

All participants were genotyped for four SNPs of *TCF7L2* gene including rs290487(C/T), rs11196205(C/G), rs7903146(C/T), and rs12255372(G/T). Genotyping for rs290487 and rs11196205 were carried out using TETRA ARMS PCR technique and for rs7903146 and rs12255372 were done by PCR-RFLP method. The results were confirmed by direct sequencing. Master Mix PCR was provided from Ampliqon Company and oligonucleotide primers were prepared from the Pishgam Company. Table 2 shows the primers specific for each polymorphism. The reaction conditions for PCR cycling of different genes are shown in Table 3. The size of DNA fragments amplified for rs290487 (285 bp control fragment, 134 bp C allele, 210 bp T allele) were suitable for separation on 1.5% agarose gel. The size of DNA fragments amplified for rs11196205 (434 bp control fragment, 253 bp C allele, 235 bp G allele) were suitable for separation on 3% agarose gel. Genotyping rs7903146 was amplified by M-PCR (Miss match primer PCR; we used a Miss match base in the third position from 3' end of forward primer in order to make restriction site for the restriction enzyme *RsaI*). *TCF7L2* primers were designed based ona previous study(19). PCR products (201 bp) were verified on 1.5% agarose gel. The 201 bp products were digested with *RsaI* restriction enzyme for 4 hours at 37°C followed by 3% agarose gel electrophoresis. T-allele was not cleaved by *RsaI* and gave a 201 bp band, and C-allele was cleaved into two bonds 175 bp and 26 bp. A 226 bp fragment was amplified. PCR products were verified on 1.5% agarose gel. The 226-bp products were digested with *Tsp509I*(*Tas*I) restriction enzyme at 65°C for 10 hours. For T allele, the *TSP509*I enzyme has two restriction sites (17 bp, 75 bp, and 134 bp), however, G allele has one restriction site (75 bp and 151bp).

Statistical analysis:

Data for continuous variables were expressed as mean ± standard deviation (SD). To evaluate the normality of data for age, diabetes duration, B.M.I, FPG, HBA1c, TG and TC, Kolmogorov-Smirnov and Shapiro-Wilk have been used, and base on the results of these tests, T test or Mann Whitney test were used to analyze the significance of differences for these features between case and control groups. The genotype and allele frequencies of *TCF7L2* gene polymorphisms among patients and controls are shown as number (percentage) and were compared with the χ^2 test. Logistic regression was used to find the association between *TCF7L2* gene polymorphisms with T2DM and to calculate Odds Ratios (ORs), 95% confidence intervals (95% CIs) and corresponding p-values. $p < 0.05$ was considered statistically significant. All these analyses were performed using SPSS v.25. The frequencies of haplotypes were calculated using SNPAnalyzer 2.0 and PHASE v 2.1 software.

Haplotypes with an estimated frequency <0.05 were excluded from the analysis. Moreover, $\chi 2$ test was used to determine the significant differences of haplotypes frequencies between two groups.

Results

The clinical and demographic features of the participants are presented in Table 1. Our results illustrated statistically noticeable differences in criteria for diabetes between T2DM patients and control individuals (p=0.000). Regarding the Table 1, the average age of patients in control group was higher than the average age of patients ($p < 0.003$); in addition, the mean of triglycerides and BMI were significantly higher in patient group than the control group ($p = 0.000$). However, there were no statistically significant differences between T2DM patients and control for ethnicity (Arab and non-Arab), sex, and total cholesterol ($p > 0.05$). In this study, 146 T2DM patients and 146 controls were tested for four selected SNPs of *TCF7L2* gene (rs290487, rs11196205, rs7903146, and rs12255372). The results of the PCR product of TETRA ARMS-PCR for rs290487 and rs11196205 and digestion results of PCR products for rs7903146 and rs12255372 are shown in Figs 1–4. For the first selected SNP (rs290487), TT and TC genotypes were not observed in patients and controls, and all of the subjects showed only CC genotype. The prevalence of genotypes and allele frequencies of rs11196205, rs7903146, and rs12255372 polymorphisms of *TCF7L2* gene in diabetic patients and controls are shown in Table 4. In case of the second selected SNP (rs11196205), a significant difference between genotypic frequencies of cases and controls is demonstrated by a general comparison (Table 4). Also, according the logistic regression analysis, by considering GG genotype as a basis, the subsequent results were gained for genotype CG: OR = 0.959, 95%CI = (0.513– 1.794), p-value = 0.048, and for CC genotype the following results were obtained: OR = 2, 95%CI = (0.976–4.098), p-value = 0.047. The frequencies of C and G alleles in patients with diabetes were 58.2% and 41.8%, respectively, and in the healthy subjects the frequency for each allele was 50% (OR = 1.393, 95%CI = 1.005-1.932, p-value = 0.046). So, there was a significant difference between both groups; nevertheless, for two other studied polymorphisms, rs7903146 and rs12255372, no significant differences were observed in the genotype and allele frequencies between case and control groups. In case of the third selected SNP (rs7903146), a general comparison between genotypic frequencies of cases and controls did not show a significant difference. The odds ratios for CC and CT genotypes were 1.897 (95% CI, 0.850 - 4.238, p-value = 0.118) and 0.8 (95% CI, 0.467 - 1.371, p = 0.418), respectively, compared with TT genotype. The frequencies of alleles C and T were 45.2% and 54.8%, respectively. In the control group, the frequencies of alleles C and T were 41.8% and 58.2%, respectively (OR = 1.595, 95%CI = 0.829-1.15, p-value = 0.404). In the case of the fourth selected SNP (rs12255372), a general comparison between genotypic frequencies of cases and controls did not show a significant difference. Moreover, by using logistic regression analysis in which GG genotype was considered as a basis, these data were gained for genotypeTT: OR = 2.143, 95% CI = (1.001-4.586), p-value = 0.083 and for genotype GT: OR = 1.003, 95%CI= (0.602-1.671), p-value = 0.992. The prevalence of G and T allele frequencies in diabetic patients were 56.2% and 43.8% and in the individuals of control group were 62.3% and 37.7%, respectively (OR = 1.291, 95%CI = 0.928-1.798, p-value = 0.130). The haplotype analysis was performed. The frequency rates of five common haplotypes of three SNPs in TCF7L2 gene (rrs11196205, rs7903146, and rs12255372) are shown in Table 5. Haplotypes with estimated frequency <0.05 were excluded from the analysis. Regarding the

Table 5, there was no significant difference between control and case groups for haplotype distribution.

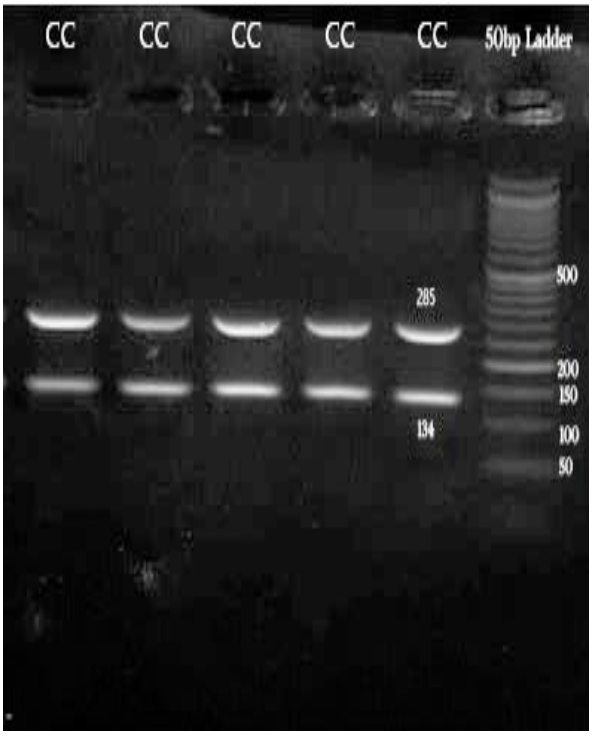

Figure (1). Agarose gel electrophoresis (1.5%) of PCR product of Tetra-Primer ARMS-PCR for rs11196205 in some individuals; CC homozygote is observed as two fragments (134 bp and 285 bp).

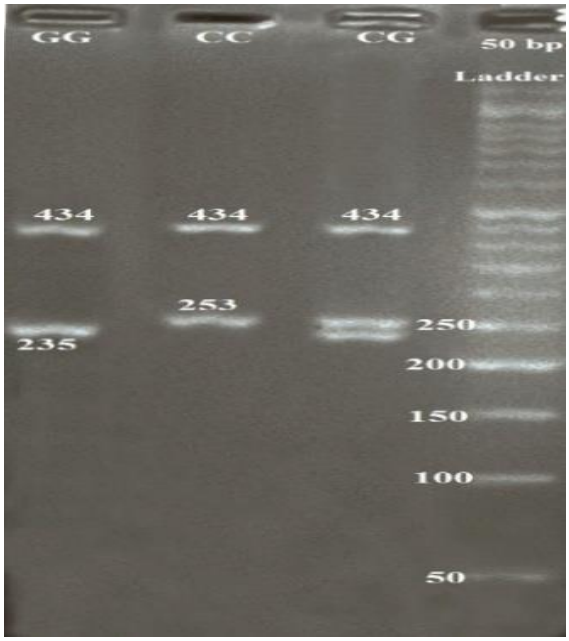

Figure (2). Agarose gel electrophoresis (3%) of PCR product of Tetra-Primer ARMS-PCR for rs11196205 in some individuals; GG homozygote is observed as two fragments (235 bp and 434 bp) and CC homozygote is observed as two fragments (253 bp and 434 bp) and heterozygote (CG) is observed as three fragments (235 bp, 253 bp, 434 bp).

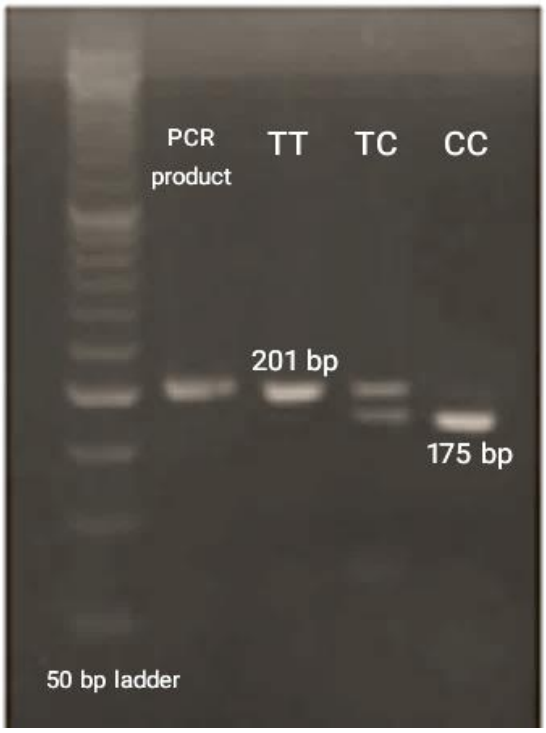

Figure (3). 3% agarose gel showing results for rs7903146 in some individuals; TT homozygote is observed as a fragment (201 bp) and CC homozygote is observed as two fragments (26 bp and 175 bp) and heterozygote (TC) is observed as three fragments (26 bp, 175 bp, 201 bp).

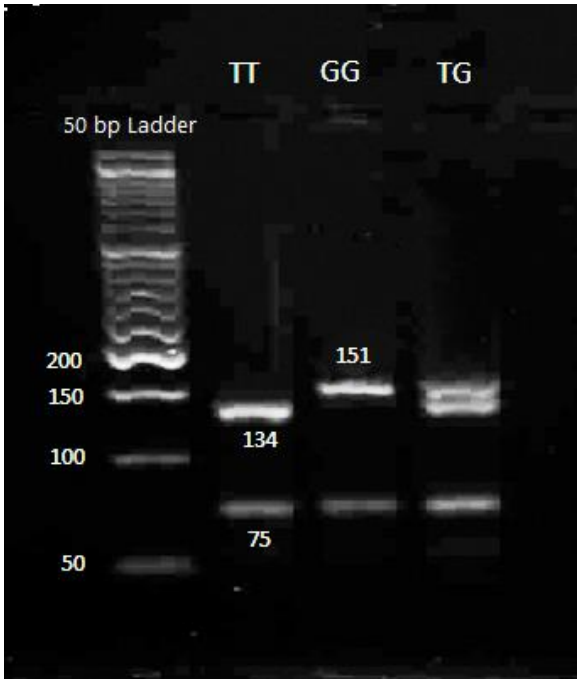

Figure (4). 1.5% agarose gel showing results for rs12255372 in some individuals; TT homozygote is observed as three fragments (17 bp, 75 bp and 134 bp) and GG homozygote is observed as two fragments (75 bp and 151 bp) and heterozygote (TG) is observed as four fragments (17 bp, 75 bp, 134 bp and 151 bp).

Table (1). Demographic and clinical characteristics of the cases and controls

Characteristic		Groups Control N=146	Diabetes N=146	P-value
Gender	Female (%)	75 (51.4)	71 (48.6)	0.640
	Male (%)	71 (48.6)	75 (51.4)	
Ethnicity	Non-Arab (%)	51 (39.4)	49 (33.6)	0.805
	Arab (%)	95 (65.1)	97 (66.4)	
Mean Ages (years)		56.59 ± 7.230	52.62 ± 9.241	0.003
Diabetes duration (years)		-	6.38 ± 3.96	-
Mean B.M.I (kg/m2)		26.46 ± 4.27	28.85 ± 5.05	0.000
FPG (mg/dl)		91.40 ± 6.365	170.048 ± 80.157	0.000
HBA1c (%)		-	8.319 ± 2.345	-
TG (mg/dl)		123.07 ± 57.34	144.63 ± 56.060	0.000
TC (mg/dl)		183.6 ± 53.42	177.061 ± 56.059	0.057

BMI: body mass index, FPG: fasting plasma glucose, HbA1c: hemoglobinA1c, TG: triglyceride, TC: total cholesterol

Table (2). Primer sequences of each SNP

Primer 5'-3'	
rs290487	
forward outer	ATCTGAACAGCTTCCCAATCTGCTCA
reverse outer	CCAACCAAGTACATTAGCCAGGACAG
forward inner	AACCCAGTACAAATCATGGTGACACAAC
reverse inner	GATCAAACACCTTTCTCATTTTCAATTTTCCA
rs11196205	
forward outer	TAGATTGTCTCCTTTTGTTTCTGCTAC
reverse outer	TAAACATCTGACCTTGAAGCCTACC
forward inner	CTGAAAGTTCTCAACATTTATAACTGCC
reverse inner	CAACCATAACTCTCTTACATACTGGTC
rs7903146	
forward primer	TTAGAGAGCTAAGCACTTTTTAGGTA
reverse primer	GATGAAATGTAGCAGTGAAGTG
rs12255372	
forward primer	GAGGTGTACTGGAAACTAAGGC
reverse primer	GAGGCTGAATCTGGCACTCA

Table(3). PCR Conditions for Amplifying Each polymorphism.

polymorphism	step 1						step 2		
	Initial Denaturation						Secondary Denaturation		
	Cycle		Temperature		Time		Cycle	Temperature	Time
rs290487	1		95˚C		5 min		33	95˚C	30 sec
rs11196205	1		95˚C		5 min		30	95˚C	30 sec
rs7903146	1		95˚C		4 min		35	95˚C	30 sec
rs12255372	1		94˚C		4 min		35	94˚C	35 sec
	step 2						step 3		
	Annealing			Extension			Secondary Elongation		
	Cycle	Temperature	Time	Cycle	Temperature	Time	Cycle	Temperature	Time
rs290487	33	64	30 sec	33	72	30 sec	1	72	10 min
rs11196205	30	58	30 sec	30	72	15 sec	1	72	5 min
rs7903146	35	58	30 sec	35	72	45 sec	1	72	5 min
rs12255372	35	66	35 sec	35	72	35 sec	1	72	7 min

Table (4). Genotype and allele frequencies of the rs11196205, rs7903146 and rs12255372

Polymorphism	Genotype	Groups		χ^2	P	Allele	Groups		χ^2	P
		Control (%) N=146	Diabetes (%) N=146				Control (%)	Diabetes (%)		
rs11196205	CC	28 (19.2)	48 (32.9)	7.132	0.028	C	146 (50.0)	170 (58.2)	3.972	0.046
	CG	90 (61.6)	74 (50.7)							
	GG	28 (19.2)	24 (16.4)			G	146 (50.0)	122 (41.8)		
rs7903146	CC	13 (8.9)	26 (17.8)	5.657	0.059	C	122 (41.8)	132 (45.2)	0.697	0.404
	CT	96 (65.8)	81 (55.5)							
	TT	37 (25.3)	39 (26.7)			T	170 (58.2)	160 (54.8)		
rs12255372	GG	50 (34.2)	45 (30.8)	4.795	0.091	G	182 (62.3)	164 (56.2)	2.298	0.130
	GT	82 (56.2)	74 (50.7)							
	TT	14 (9.6)	27 (18.5)			T	110 (37.7)	128 (43.8)		

Table (5). Results of association analyses of haplotypes for the *TCF7L2* locus

Haplotype	Groups		χ^2	P
	Control	Diabetes		
GTG	0.36601	0.30318	0.054955	0.815
CCT	0.26073	0.28204	2.436554	0.118
CTG	0.1473	0.11584	0.186299	0.666
CCG	0.06347	0.09718	0.786325	0.375
CTT	0.05052	0.08712	0.918367	0.338

Discussion

Our study was carried out on a subset of the Iranian population from Khuzestan province (including Arab and non-Arab ethnics), in which the potential association between T2DM and four variants of *TCF7L2* gene was investigated. The results of this study propose that there is a notable association between rs11196205 polymorphism and type 2 diabetes, but three other polymorphisms (rs290487, rs7903146, and rs12255372) are not associated with the risk of this disorder. In addition, there is not any consequence for specific risk haplotype in our population. In all GWAS studies, *TCF7L2* gene clearly showed the major impact size with an odds ratio of 1.37, and according to meta-analyses that were conducted by Florez and also Cauchi et al. the *TCF7L2* gene is a strong susceptible gene for T2DM in different ethnic groups (7,8,20). According to a meta-analysis conducted by Peng et al., although there was no notable association for rs290487 polymorphism and type 2 diabetes, considerable associations were found between this disorder and rs11196205, rs12255372, rs7903146 polymorphisms (21). The rs7903146 polymorphism, among all *TCF7L2* gene SNPs, shows the most remarkable association with type 2 diabetes risk in almost all groups, except in some ethnic populations such as some groups of Arabs (22–26). In

the studies conducted in several areas of Iran (Golestan, Ilam, Isfahan, and Rafsanjan), significant associations were reported between rs7903146 polymorphism and T2DM; in another study (in Jahrom), nevertheless, this polymorphism showed lack of association with T2DM (19,22,27–29). The association of rs12255372 polymorphism with T2DM was initially reported in European and Caucasian populations (30–33). Consequently, replicated studies showed significant positive associations (21). Whereas in some ethnic populations like China (18) and the United Arab Emirates (34), no significant association was found. SNP rs12255372 was investigated within the Iranian population and showed different results. In contrast, a study in Jahrom (22) based on a negative association between rs12255372 and T2DM, a significant association was found between T2DM and this variant in Gorgan (28), Ilam (27) and Mashhad (35). The association of rs11196205 polymorphism with T2DM has been reported by many studies in different populations(6,36,37). According to the meta-analysis by Luo et al., the association between rs11196205 polymorphism with T2DM risk was shown in the population of East Asia (38); while some studies have reported weak or no association with this disease(17,39). In 2012, Saravani et al. conducted a study on a population from southeast of Iran. In which, a significant association was observed between rs11196205 polymorphism and type 2 diabetes (40). Our findings for rs11196205 polymorphism illustrated that CC and CG genotypes were associated with T2DM as a risk factor; the C allele of this polymorphism, moreover, was associated with the risk of type 2 diabetes. There is little data about the association between T2DM and rs290487 SNP. Peng et al. showed that rs290487 polymorphism was reported only in East Asian studies (21). In a study by Chang et al, in a Chinese population, rs290487 SNP was associated with T2D. In this study, in comparison with the TT genotype, the odds ratio for the CC genotype was 1.51 (1.10 -2.07; p = 0.0085) and for the CT genotype was 1.36 (95% CI 1.08-1.71; p = 0.0063) (18). Nonetheless, a meta-analysis conducted by Ren et al., in the Chinese population, demonstrated no association between type 2 diabetes and rs290487 polymorphism (41). A study on rs290487 polymorphism was also conducted in Japanese subjects and no association was detected between SNPs and T2DM (42). In Iran, the association of rs290487 polymorphism with T2DM was investigated in the Kurdish ethnic group and a significant association was found between SNP and T2DM (27). Therefore, regarding different studies that have been done until now, the association of *TCF7L2* SNPs with type 2 diabetes is confirmed in some populations.

In conclusion, due to the difficulty of identifying and estimating the contribution of different variants in different genes that are effective in T2DM, investigating the role of genetic factors in the progress of T2DM is very complicated, like other heterogeneous multifactorial disorders. The genetic analysis of T2DM, moreover, needs to understand the interactions between these genes and variants as well as their accumulative impacts (4). The difference and diversity in results of mentioned studies and the present study may be influenced by many factors such as ethnic differences (the role and effect of genetic varieties in different ethnic groups), different geographical and climatic regions, differences in the lifestyle of individuals in different societies, differences in sample size in different studies, differences in diagnostic criteria, differences in sampling methods (in terms of demographic characteristics such as age and sex) and differences in genotyping methods. In this study, due to time limitation, we could not collect more samples; so it is recommended to investigate these polymorphisms with more samples; so that, we can conclude more accurately about the association or non-association of these polymorphisms with type 2 diabetes in the population of

Khuzestan province. In addition, similar studies with consideration environmental factors can be carried out.

Acknowledgments

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval

Ethical approval: All procedures performed in study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent: Informed consent was obtained from all individual participants included in the study.

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