ORGINAL ARTICLE

Investigating the rs2237892 and rs231362 Polymorphisms of *KCNQ1* Gene Associations with Type 2 Diabetes in an Iranian Population (Yazd Province)

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Abstract

Objective: Type 2 diabetes (T2DM) is a worldwide prevalent metabolic disorder and the cause of many morbidities and mortalities. KCNQ1 gene encodes α-subunit of voltage-gated potassium (K+) channel which plays a role in insulin secretion in the pancreas, thus its variants may confer susceptibility to diabetes. Recognition of genetic variants involved in T2DM could help the early diagnosis and prevention of the disease. The main purpose of this paper was to investigate the frequencies of rs231362 and rs2237892 polymorphisms of KCNQ1 gene in T2DM patients and comparing these frequencies with normal subjects in an Iranian population from Yazd province, Iran.

Materials and Methods: This case-control study was conducted on 166 patients with T2DM and 168 normal subjects. After obtaining the informed consent, 5 ml peripheral blood was taken from the cases and controls and then DNA was extracted. The molecular investigation was done using 4-primer ARMS PCR and PCR-RFLP methods.

Results: Statistical analysis showed that GG genotype [OR= 3.9 (2.1-7.1), *P*-value< 0.001] and G allele [OR=2.85 (2.07-3.93), *P*-value< 0.001] frequency of rs231362 polymorphism was significantly different between case and control groups. While rs2237892 polymorphism did not show any differences between the two groups.

Conclusion: The result of this study showed that GG genotype and G allele of rs231362 polymorphism can be related to T2DM susceptibility in the population under study.

Keywords: Type 2 diabetes, *KCNQ1*, Polymorphism, ARMS PCR,

Introduction

Diabetes mellitus includes a group of metabolic disorders which is characterized by high blood sugar and dysfunction of carbohydrate, fat, and protein metabolisms that could be the results of deficiency in insulin secretion or insulin

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function or both (1,2). Both beta-cell insufficiency of insulin secretion and insulin resistance incorporate in blood sugar increase which are consequences of environmental and genetic factors (3,4).

The pandemic of T2DM is one of the most important problems in the 21st century worldwide. The global prevalence of diabetes (20-79 years) is estimated to rise from 8.8% in 2015 to 10.4% in 2040, and the number of people with diabetes is predicted to increase from 415 million in 2015 to 642 million in 2040. The Middle East and North Africa are among the regions that are projected to experience the highest growth rate in the number of diabetes (5). In the Middle East and North Africa region, Iran with approximately five million adult diabetic patients (20-79 years of age) has the highest level after Egypt and Pakistan (6). Yazd province is among the areas with the highest prevalence in the country, which can be explained by genetics, lifestyle, and ecological condition, although more studies are needed to be done for confirmation of these causes (7,8). This high prevalence of diabetes and its complications lead to high treatment expenses. So early diagnosis and development of suitable strategies can help to decrease these burdens (9).

Many genes have been discovered to be related to T2DM so far (10). There are different approaches for detecting genes involved in diabetes. Since 2007, a new technology of whole-genome sequencing has made a lot of progress in genetic research of T2DM. Up until now, the detected genes by this method mainly have been related to dysfunction of pancreatic beta cells, but they are only explaining less than 5% of T2DM heritability (11,12).

KCNQ1 gene effect on T2DM was first discovered in 2008 in a Japanese population through a genome-wide association (GWA) study, and after that was repeated in other populations (11,13-16). *KCNQ1* is known as the most effective gene in T2DM after *TCF7L2* (10,17). This gene encods a voltage-dependent K+ channel, which is expressed in many organs like heart, ear, kidney, intestine, and pancreas. The product of *KCNQ1* regulates important physiological functions such as repolarization of heart tissue after action potential and transportation of salt and water through epithelial tissue (18-20). *KCNQ1* has 404120 bp, includes 17 exons and its location is 11p15.5-p15.4 (21,22). The two most common regions of the *KCNQ1* gene identified until now which are related to T2DM, are introns 11 and 15. Both regions are shown to have an effect through deficiency of pancreatic cells (13,15,16).

In this study, we examined two polymorphisms in the introns 11 and 15 of the *KCNQ1* gene in an Iranian population; rs231362 which is located in intron 11 and its significant effect first was found in an European population, and rs2237892 which is placed in intron 15 and shown a significant effect in the Asian population (11).

Materials and Methods

Subjects

In this case-control study, the case group included 166 patients with T2DM who referred to the Yazd Diabetes Center, and the control group comprised of 168 healthy individuals who went for a routine checkup to the Central Yazd Laboratory (so case and control were ethnically matched). The minimum sample size was determined assuming CC genotype frequencies 58% for diabetic patients, and 43% for the control group considering a 95% confidence level, power of 80%, and margin of error no more than 15%. The case group was selected based on the ADA (American Diabetes Association) diagnosis criteria. The inclusion criteria for the control group was the age of more than 40 years (the most likely onset age of type 2 diabetes), fasting plasma sugar less than 100 mg/dl (considered normal in ADA diagnosis criteria), and no diabetic history in the individual and in her/his first-degree families (to reduce the possibility of being diabetic in the future).

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After the Ethics Committee of Shahid Sadoughi University of Medical Sciences approval that is in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards and taking informed consent from all individuals, 5 ml peripheral blood were taken in EDTA tube to prevent clotting. The genomic DNA was extracted and kept at -20 °C. Then, the samples were genotyped using ARMS-PCR and PCR-RFLP methods. Biochemical factors data were collected from the individuals' blood test results recordings, and height, weight, and blood pressure were determined in all individuals.

DNA extraction

The DNA was extracted by Biospin Whole blood Genomic DNA Extraction Kit (BioFlux, China) according to the instruction of the kit. DNA quality and quantity were assessed by 1% agarose gel electrophoresis and spectrophotometry respectively.

Primer designing

Two types of software were used for designing primers. PRIMER1 online software (http://primer1.soton.ac.uk/primer1.html) was used to design primers for genotyping rs231362 polymorphism by tetra-ARMS PCR technique. The primers for this method include 2 outer primers and 2 inner primers. Clone Suit Mgr was applied to design rs2237892 polymorphism primers for the PCR-RFLP method. Then primers were BLASTed to ensure that they are unique. The primers and their melting temperatures (Tm) are shown in Table 1.

Rs231362 genotyping

The PCR reaction was performed in a total volume of 20µL, containing 2µL DNA template, 2µL 10x Buffer (Feldan Company), 1µL 10mM dNTP mix (Feldan Company), 2µL of each primer, 0.1µL Taq DNA polymerase (5U) (Feldan Company), and 6.9µL distilled water. Temperature condition was as follows: first denaturation temperature at 94ºC for 5 min, then 30 cycles of denaturation 94ºC, annealing 68ºC, and extension 72ºC each for 30 seconds, and the final extension at 72ºC for 5 min. The PCR products were run on 2% agarose gel containing 4µL safe DNA stain in TBE1x buffer at 100v for 30 min. Afterward, they were visualized under UV light. Fig. 1 shows agarose gel result of rs231362 polymorphism of *KCNQ1* gene, which was genotyped by tetra-primer ARMS-PCR. As this figure demonstrates, the amplicon (product of outer primers) size is 396 bp (i), and allele A shows the band with size 250 bp (ii) and G allele has a band with size 202 bp (iii). In homozygote AA we will have 396 bp and 250 bp bands, homozygote GG will show 396 bp and 202 bp bands, and heterozygote GA has 396, 250, and 202 bp bands.

To confirm the results of tetra-ARMS PCR method, we randomly (simple random sampling) selected six samples including two of each genotype (AA, GG, and AG) to be sequenced. Sample preparation had been done including doing PCR with two outer primers (that were used for ARMS PCR before, Table 1), then PCR product and 20 µL of forward outer primer along with temperature and product length data had been sent for sanger sequencing. The sequencing results were analyzed with Nucleotide BLAST and

Table 1. The primers and their Tms (melting temperatures)

Parameters	Primer sequence (5'- 3')	Tm (ºC)	Primer length (Nu)
rs231362			
Forward inner primer (A allele)	GTAGCTCACCTGCCTTTGACCCTGCCCA	76	28
Reverse inner primer (G allele)	ACCATGGTCCTCTTCCCTCGCCCGTAAC	75	28
Forward outer primer	GGGGGTTGGAGGCAGAATCAGTGGACTG	75	29
Reverse outer primer	ATGTGGGCTGTGTGTCTCAGCCAACAGC	75	28
Rs2237892			
Forward	GTCAGGAATGGCGTCCTTGTG	65	21
Reverse	GGGCTGGTAGGGAACAACTG	64	20

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Chromas version 2.4. All of the sequencing outcomes were in concordance with our results.

Rs2237892 genotyping

For rs2237892 polymorphism, PCR was carried out in a total volume of 20µL comprising of 1µL DNA template, 1µL of each primer, 10µL 2x Taq polymerase Master mix (2mM MgCl2), and 7µL distilled water. PCR was performed at the thermal condition including an initial melting temperature of 94ºC for 5 minutes, followed by 30 cycles of denaturation 94ºC, annealing 60ºC, and extension 72ºC each for 30 seconds, and 5 minutes of final extension in 72ºC. PCR products were visualized on 1% agarose gel. All PCR products showed the 414 bp band, which is our amplicon size.

The enzyme used for the amplicon digestion for determining rs2237892 polymorphism was Msp1. The cutting site of this enzyme is 5' C˅CGG 3'. As a result of digestion, C allele made 284, 87 and 43 base pair fragments, and T allele produced 371 and 43 base pair pieces. Restriction Fragment Length Polymorphism (RFLP) reaction was performed in a total volume of 20 µL containing 7.5µL distilled water, 2µL 10x Tango buffer, 10µL PCR reaction mixture containing the product, and 0.5µL of the Msp1 enzyme. The mixture was incubated in 37ºC for 16 hours. The digestion results were visualized on 2% agarose gel containing safe DNA stain under UV light (Figure 2).

Statistical analysis

Our data were analyzed by Chi-square, Fisher exact, and odd ratio (with 95% confidence intervals) tests using SPSS 19. For haplotype study, Haploview 4.2 software was used. The *P*-value< 0.05 was considered statistically significant.

Ethical considerations

This study has been approved by the Research Committee of Shahid Sadoughi University of Medical Sciences in Yazd. (Ethics code: IR.SSU.REC.1399.167523.)

Results

In this study, there were significant differences between case and control groups in systolic blood pressure (*P*-value= 0.007), diastolic blood pressure (*P*-value< 0.001), LDL (*P*-value< 0.001), HDL (*P*-value< 0.001), cholesterol (*P*-value= 0.004), and triglyceride (*P*-value= 0.004) (Table 2).

Genotype and allele frequencies of rs231362 and rs2237892 polymorphisms of the *KCNQ1* gene were calculated and compared in case and control groups. As it can be seen in Table 3, genotype frequency of rs231362 showed a significant difference between case and control

Table 2 Demographic and biochemistry features

Parameters	Case n=166	Control n=168	*P*-value
Male	45.2%	50%	
Female	54.8%	50%	
Age (years)	58.2 (± 9.4)	55.31(± 10.4)	0.008
Systolic blood pressure (mmHg)	133 (± 15.3)	128 (± 16.8)	0.007
Diastolic blood pressure (mmHg)	80 (± 6.7)	83 (± 10.4)	0.001>
Height (meter)	1.63 (± 0.09)	1.62 (± 0.16)	0.3
Weight (kilograms)	73 (± 11.5)	73.3 (± 13.5)	0.8
BMI (Kg/m^2)	27.3 (± 4.5)	27.8 (± 4.9)	0.3
FBS ($^{mg}/_{dl}$)	173 (± 48.8)	92.1 (± 6)	0.001>
HDL ($^{mg}/_{dl}$)	43.4 (± 11.6)	56.3 (± 19.1)	0.001>
LDL ($^{mg}/_{dl}$)	104.6 (± 29)	119.4 (± 36.5)	0.001>
Chol ($^{mg}/_{dl}$)	185.7 (± 35.8)	199.1 (± 44.9)	0.004
TG ($^{mg}/_{dl}$)	201.1 (± 123)	164.7 (± 94.1)	0.004
HbA1c (%)	9.69 (± 1.54)	NA	NA
2hpp($^{mg}/_{dl}$)	258.68 (± 75.87)	NA	NA

BMI: Body mass index, FBS: Fasting blood sugar, HDL: High-density lipoproteins, LDL: Low-density lipoproteins, Chol: Cholesterol, TG: Triglyceride, HbA1c: Glycated hemoglobin, 2hpp: Two- Hour Postprandial Glucose, NA: Not Available (Fisher exact tests was used and mean ± standard deviation calculated and *P*-value< 0.05 considered significant)

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groups, even after adjusting for age, gender and body mass index (BMI) (*P*-value< 0.001). Assessing the odd ratio has been shown that the GG genotype with OR= 3.9 increases the risk of T2DM in relation to the GA genotype, and the AA genotype has a protective role (OR= 0.2). In addition, as shown in Table 3, the allelic frequency was significantly different between case and control groups (*P*-value< 0.001) and with an odds ratio of 2.85, the G allele increases the type 2 diabetes risk.

Neither genotype nor allele frequencies of rs2237892 polymorphism showed a significant difference between case and control groups (Table 3).

Two loci haplotype frequency was measured and compared between case and control groups using Haploview 4.2 software. AC and GC haplotype frequencies had a significant difference in case and control groups (*P*-value< 0.001), although determining the odds ratio by SPSS 19 software showed that none of the haplotypes make further susceptibility for T2DM (Table 4).

Assessing of Linkage Disequilibrium (LD) block by Haploview 4.2 software showed that the two polymorphisms are at a distance of 148 kb from each other, and there is no linkage disequilibrium between them (D'= 0.162, LOD= 0.11, R^2= 0.002).

Discussion

It is established that there are many genetic variants in different populations in geographic regions and with different ethnicities. Various genetic components can lead to different

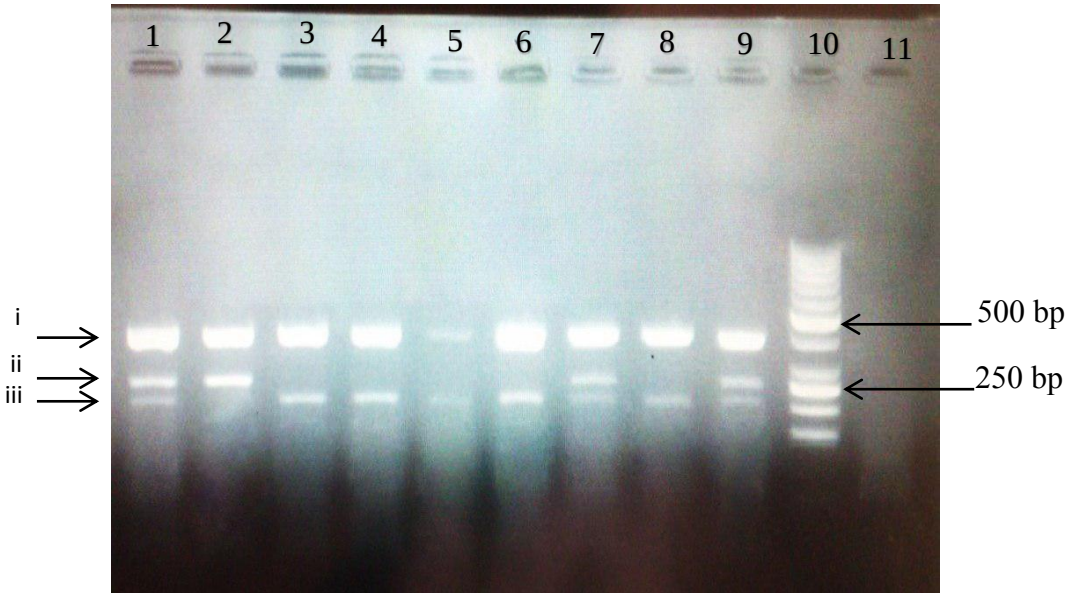

Figure 1. Gel electrophoresis image of tetra-primer ARMS -PCR results of rs231362 polymorphism. 50 bp ladder was used. amplicon (product of outer primers) size is 396 bp (i), allele A shows the band with size 250 bp (ii), G allele has the band with size 202 bp (iii). Lane1,7, 9= GA, lane2= AA, lane3, 4, 5, 6, 8= GG, lane10= DNA ladder, lane11= negative control

Table 3. Frequencies of genotypes and risk alleles of rs231362 and rs2237892 polymorphisms

SNP	Group	Allele frequency n (%)		*P*-value	Genotype n (%) (logistic regression model, analysis adjusted for age, gender, and body mass index)			*P*-value
		G	A		AA	GA	GG	
rs231362	Normal	111 (34.3%)	213 (65.7%)	<0.001	70 (43.2%)	73 (45.1%)	19 (11.7%)	<0.001
	Diabetic	189 (59.8%)	127 (40.2%)		23 (14.6%)	80 (51%)	54 (34.4%)	
OR (95% CI)		2.85 (2.07- 3.93)			0.2 (0.1- 0.4)	1	3.9 (2.1-7.1)	
		C	T		TT	CT	CC	
rs2237892	Normal	312 (94.5%)	18 (5.5%)	0.66	1(0.6%)	16(9.7%)	148(89.7%)	0.71
	Diabetic	284 (95.3%)	14 (4.7%)		0(0%)	14(9.5%)	134(90.5%)	
OR (95% CI)		1.17 (0.57-2.3)			0.000	1	0.68(0.3-1.2)	

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prognoses and different responses to drugs. Therefore, many studies are investigating polymorphisms of causative genes in many diseases including T2DM in various populations.

In this study, we evaluated two *KCNQ1* gene polymorphisms frequencies in type 2 diabetic patients in Yazd population of Iran and compared them with healthy non-diabetic controls. Two polymorphisms, rs231362 which is located in intron 11, and rs2237892 which is placed in intron 15 were chosen and examined.

Assessing genotype and allele frequency of rs231362 showed that with the *p*-value less than 0.001, this polymorphism may have a significant role in conferring T2DM susceptibility to Iranian population of Yazd province. It seems diabetes risk conferred by the G allele is 2.85 times more than the A allele (OR= 2.85). The GG genotype with OR= 3.9 (2.1-7.1) increases the risk of T2DM, while the AA genotype with OR= 0.2 (0.1-0.4) has a protective effect. No other Iranian study has been done on this polymorphism so far.

Calculating of rs2237892 polymorphism frequency demonstrated that there is no association between this polymorphism and T2DM in our population. Three different studies have been performed on different populations in Iran. All of them had a lower sample size from our study. The results of the two of them were in concordance with ours. In contrast to our outcome, one of the studies showed a significant relationship between this polymorphism and T2DM (later will be discussed) (23-25).

These polymorphisms have been assessed in other populations as well. The effect of rs231362 polymorphism was first observed in the European population, in a GWA study which shows that this polymorphism had a

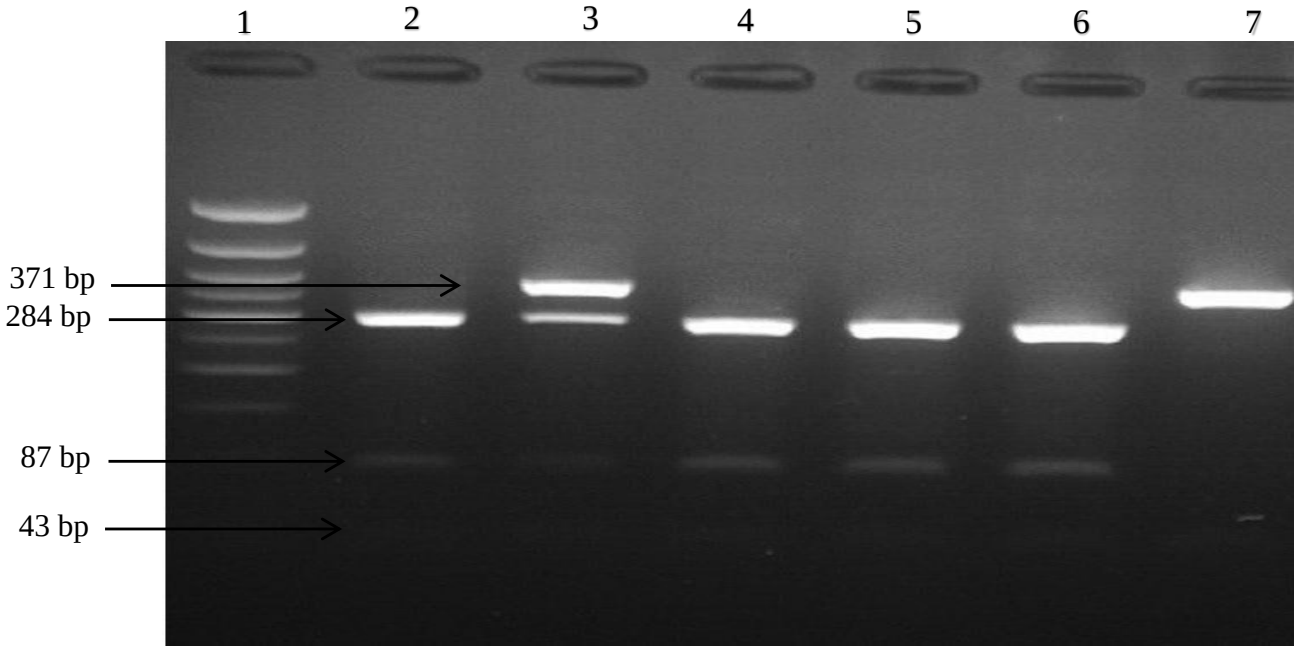

Figure 2. Effect of Msp1 enzyme on PCR products of rs2237892 polymorphism. 50 bp ladder was used. C allele shows 284, 87 and 43 base pair bands, T allele has 371 and 43 base pair bands: lane1= DNA ladder, lane2,4,5,6= CC, lane3= CT, lane7= TT

Table 4. Comparison of frequencies of two-loci haplotypes (rs231362/rs2237892) in case and control groups

Haplotype	Case % (n= 166)	Control % (n= 168)	OR	*P*-value
AC	0.388	0.626	1.85 (0.18-18.4)	1.57×10^{-9}
GC	0.565	0.319	5.3 (0.53-53.5)	3.68×10^{-10}
GT	0.033	0.024	4.5 (0.25-80.5)	0.5468
AT	0.014	0.031	1	0.1939

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significant effect on diabetes susceptibility, which has the same effectiveness compared to our study (13). Another study on Indian population living in India and America showed that the G allele with *p*-value=0.002 and OR= 1.24 is effective but their effectiveness was not as high as our study (26). A study in Pakistan showed no connection between this polymorphism and diabetes (27). In Saudi Arabia, a significant relation was found between the risk allele G and diabetes but compare to our study it showed less association (28). In China, same as our study, the risk allele G showed a significant effect. However in Chinese study, the GA and GG genotypes didn't show any significant difference in giving the risk of diabetes, but our work confirms the protective role of the AA genotype, OR=0.2 (0.1-0.4) in T2DM (29). Finally, this polymorphism in Japan showed nominal association (30). Two different studies of the African American population showed different results, one had a significant relation, and the other showed no significant association (31,32).

In contrast to our study, the studies that have been performed so far all showed some level of association between rs2237892 and diabetes susceptibility. The significant effect of this polymorphism first was found in a GWA study in Japan (15). A meta-analyze study in India showed that rs2237892 with *P*-value= 0.03 is slightly related to T2DM (26). In China, many studies have been performed on this polymorphism. In one study, an odds ratio of 1.53 with *P*-value= $5x10^{-16}$ for risk allele, and *P*-value= $9x10^{-16}$ for genotypes were found (33). An association was also found in Jiangsu province population (34). In a prospective study in South Korea, rs2237892 was showed to affect T2DM progress with *P*-value= 0.04, OR= 2.61 (35). In addition, the study of this polymorphism on three Malaysian, Chinese, and Indian populations in Singapore showed *P*-value= 0.002 and OR= 1.38 for the risk allele (14). This polymorphism was also examined on two Chinese and Malaysian populations in Malaysia which demonstrated a medium relationship between rs2237892 and T2DM (36). This polymorphism showed a subtle relationship with this disease in Tunisia (37). Investigation of allele distribution of rs2237892 in Lebanon led to high effectiveness with OR= 2.53 (2.09-3.5) and *P*-value= $1.8x10^{-18}$. Genotypes frequencies analysis also showed a significant difference with *P*-value= $2.3x10^{-9}$ (38). The relation of this polymorphism with T2DM has also been confirmed in the Netherlands (39) and Mexico (40). Two different studies on African American population have been done. Both showed a significant relation (31,32). As mentioned before, three studies recently have been investigated the rs2237892 polymorphism on Iranian populations. A study of this polymorphism on a population of the northwest of Iran showed no evidence of a significant relationship with type 2 diabetes (25). Also, another study of a different population of Iran using a more reliable method (Sanger sequencing) did not show any correlation (24). While these two studies showed similar results as ours, one study of a smaller population in Tehran province showed a significant relation of rs2237892 polymorphism (23). Therefore, it seems that regarding rs2237892 polymorphism there is different genetics effects on the progression of T2DM in Iranian population compare to the other populations.

We studied on Yazd population (central area of Iran). Differences in our result with some other Iranian studies may reflect genetic variation and different effects of polymorphisms on diabetes susceptibilities in various populations within Iran. So more investigation on different populations can better reveal these differences. Also, studies with larger cohorts using more reliable techniques such as sequencing would be recommended.

Conclusions

This study set out to determine the effect of two polymorphisms of the *KCNQ1* gene in T2DM patients and comparing them with

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healthy controls of an Iranian population. The results of this investigation showed that rs231362 polymorphism has a significant relation with T2DM. In general, it seems that the rs231362 polymorphism increases the susceptibility to T2DM in the Yazd population of Iran. However, further studies with a higher sample size can more precisely confirm our results.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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