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A STUDY ON ASSOCIATION OF POLYMORPHISMS IN *Calpain10* AND *TCF7L2* GENES WITH TYPE 2 DIABETES MELLITUS

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Polymorphisms of the *Calpain10* and *TCF7L2* genes were identified as possible type 2 diabetes susceptibility genetic markers. We conducted a case-control study to evaluate the relation between *SNP43* of calpain-10 and *rs12255372* and *rs7903146* in the *TCF7L2* with type2 diabetes in western–north of Iran. The role of these variants in Iranian population was less clear. A total of 202 patients and healthy controls were enrolled to analysis the frequency distribution of *Calpain10* and *TCF7L2* polymorphisms (*SNP43*, *rs12255372* and *rs7903146*) using polymerase chain reaction-restriction fragment length polymorphism (PCR – RFLP) method. The frequency of allele A in controls was significantly greater than that of diabetic patients (P=0.031), whereas the difference between distribution of *SNP43* genotypes (A/A, A/G, G/G) were non-significant in case and control groups. Non significant association was also observed between G/G, A/G or A/A genotypes and type 2 Diabetes. The frequency of the "T" allele of *rs12255372* (G/T) was significantly associated with type 2 diabetes (OR= 0.55, 95% confidence interval [CI], 1.11-1.51; P<0.001). No allelic association was found for *rs7903146*(C/T) polymorphism.The distribution of alleles in case and control groups are significantly different indicating the G allele is associated with type 2 diabetes. The *rs12255372* (G/T) may be associated with type 2 diabetes.

Key words: Calpain10, Eastern Azerbaijan, Iran, *SNP43*, Type 2 Diabetes

INTRODUCTION

Diabetes mellitus is a heterogeneous metabolic disorder that manifests itself by increased blood sugar. The disease is caused by deficiency, dysfunction or absence of insulin in affected

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individuals and may cause blindness, renal failure, heart failure, stroke, amputation of leg and decreased life expectancy in lack of efficient control (HORIKAWA *et al*. 2000). Diabetes mellitus is one of the most common disorders worldwide affecting more than 135 million of human population (LARIJANII *et al.* 2002; KING *et al.* 1998) and this will be increased to 300 million by 2025 according to the report of world Health Organization (WHO 1997). It is estimated that more than 7.7% of Iranian urban population are affected by diabetes (ESTEGHAMATI *et al.* 2008). The increase rate of affected individuals with diabetes is expected to be about 122% within years 1995-2025 (DELAVARY *et al.* 2004).

The cost of diabetes care is a major economical challenge in most countries. The budget needed for diabetes care in Iran has been reported to be about $10 billion per year (HOSSEIN-NEZHAD *et al*. 2002; AMINI *et al*. 2002). All of the above mentioned, indicate that prevention of T2DM is one of the most important health burden in most countries.

Previous linkage studies and candidate gene approaches have identified several genes associated with T2DM, such as *CAPN10*, *ENPP1*, *HNF4A*, *ACDC*, *PPARG*, *KCNJ11* and *SLC30A8* (WEEDON *et al*. 2003; HANIS *et al*. 1996; MOHADDES *et al*. 2012). Whole genome association studies (WGAS) has also showed *genetic variants in more than 15 genes/loci to be associated with T2D (ZEGGINI et al. 2007; SAXENA et* al. 2007; SCOTT *et al.* 2007).

Calpain10, a gene that encodes a nonlysosomal cysteine protease, has been recently proposed as a type 2 diabetes susceptibility gene in the non – insulin – dependent diabetes mellitus 1 region. An A to G variant in intron 3 (*SNP43*) of the *Calpain10* gene was identified as a possible type 2 diabetes susceptibility genetic marker (WEEDON *et al*. 2003).

Similar results had been reported for a number of ethnicities when the present study was started (STUMVOLL *et al.* 2001; BAIER *et al*. 2000; SREENAN *et al*. 2001; COX *et al*. 2004; LYNN *et al*. 2002), however no published data was present about the population investigated in our study.
Newly reported Transcription factor 7–like 2 (*TCF7L2*) gene is another susceptible gene that was strongly associated with type 2 diabetes mellitus. *TCF7L2* gene coded protein is a transcription factor that has an important role in the Wnt signaling pathway and may regulate levels of glucagon-like peptide 1, which influences insulin secretion from the β-cells of the pancreas, so *TCF7L2* may has a role in type 2 diabetes pathogenesis (GRANT *et al*. 2006).

Polymorphisms *rs12255372* and *rs7903146* in *TCF7L2* gene were first reported by Florez et al to be associated with an evaluated risk of type 2 diabetes mellitus (FLOREZ *et al*. 2006)
Although more and more T2D associated gene/loci are being identified, the replication study has played a critical role in confirming the reported T2D associated genes/loci, especially within different ethnic populations. Our team has initiated research in detection of risk factors in multifactorial disease from five years ago (GHARESOURAN *et al*. 2013; GHARESOURAN *et al*. 2014) and the present study was carried out to reveal the possible association of *SNP43* within *CAPN10* gene and the *rs12255372* and *rs7903146* polymorphisms of the *TCF7L2* gene with T2D in the population of eastern Azerbaijan of Iran.

MATERIALS AND METHODS

Study subjects

Our case-control study was included 202 unrelated individuals including: 101 T2DM patients and 101 non-diabetic controls, collected by simple random sampling method according to the ADA definitions and criteria. The sampling was performed between 2009 until 2011. All the participants were residents of eastern Azerbaijan of Iran. Both study groups were provided with an

informed consent form and the agreement of the ethics committee of Tabriz University of medical sciences was achieved for the study (agreement No: 5/4/7167). The sample size was calculated according to OD =1.4, the encounter incidence of about 70%, α = 0.05 and power of 80%. The patients received a standard questionnaire that contained questions regarding the sex, race, family history and other issues. Only patients with a clinical diagnosis of T2DM, with no insulin therapy and without attention to control level of blood sugar, consuming drug and side effect were recruited. The study individuals underwent a basic physical examination that included the measurement of height and weight.

The control group contained only individuals with normal fasting glucose, normal GTT and negative family history of T2DM among first degree relatives. Both the case and control groups consisted of individuals aged between 40-70 years old.

DNA extraction and polymorphisms Genotyping

DNA samples were isolated from peripheral blood lymphocytes using standard salting out protocol and used for the PCR reaction and RFLP. PCR primers were designed using online Primer3 software. The PCR reaction was prepared using 0.1 µg of template DNA, 0.01 µg each of forward (5´CACGCTTGCTGTAAGTAATGC3´) and reverse (5´CTCTGATTCCCATGGTCTGTAG3´) primers, 0.5 unit of Taq DNA polymerase, 0.2 mM each of dNTPs in 10 mM TrisC1 and 50 mM MgCl2. The volume was adjusted to 25 µl by dH2O. The cycling conditions were as follows: after an initial denaturation at 94º C for 4 min, 35 cycles of polymerization was carried out by denaturation at 94º C for 1 min, hybridization at 59º C for 30s, and extension at 72º C for 30s. A final extension was performed at 72º for 10 min.

The resulting 144 bp product was digested with restriction enzyme *NsiI* at 37º C for 12-16 hours followed by electrophoresis through a 2% agarose gel containing 250 nmol/L ethidium bromides. For *SNP43*, the presence of allele A, was associated with existence of a restriction site for *NsiI* enzyme and digestion of the 144 bp PCR product to 121 and 23 bp fragments; for the allele G, no *NsiI* restriction site was present.

Genotyping of *rs12255372* and *rs7903146* polymorphisms were performed by using PCR/RFLP with the following primers: forward, 5′-CCCAGGAATATCCAGGCAAGGAT-3′, reverse 5′-CAAATG GAGGCTGAATCTGGCA-3′ and forward, 5′-TTAGAGAGCTAAGCAACTTTTTAGTA-3′, reverse 5′-ACTAAGTTACTTGCCTTCCCTG-3′, respectively. The PCR reaction was prepared in a final reaction volume of 15 µL of polymerase chain reaction containing 100 ng genomic DNA, 1 Mmol of each primer, polymerase chain reaction buffer with 1 Mmol/L of MgCl2, 0.5 Mmol/L of each deoxynucleotide triphospate (dNTP), and 0.5 U of Taq polymerase. Thermal Cycler optimized with following conditions: 95°C for 5 minutes, followedby 31 cycles of 95°C for 2 minutes, 60°C for 1 minutes , 72° for 2 minutes, and a final extension of 72°C for 5 minutes. PCR products were digested using *BseGI* enzyme for the *rs12255372* (G/T) polymorphism and *RsaI* for the *rs7903146*(C/T) polymorphism. The resulting products were electrophoresed on a 2% agarose gel.

Statistical analysis:

The data analysis was performed using descriptive statistical method (frequency-percent), chi-square test, or Fisher's exact test. The independent samples t-test was employed to compare the frequency distribution and quantitative variables between the two study groups. Logistic regression models by SPSS package ver. 17 was used to estimate the Odds Ratio (OR) with 95%

accuracy. The P values lower than 5% were considered as significant in this study from statistical point of view.

RESULTS

In this study 101 diabetic cases and 101 healthy controls were evaluated. The case control groups were matching by age, sex and dwelling, however the mean of low density lipoprotein (LDL) and triglyceride (TG) were significantly different between the two groups (P<0.05, Table 1).

Table 1. Characteristics of participants (mean±sd) in case and control groups

Variables	Case(N=101)	Control(N=101)	*P*_value
Age(year)	55.51± 7.35	54.66± 8.64	0.26
Sex (Male %)	50.5	49.5	0.9
Education (lower than high school pass certificate %)	74.5	73.6	0.89
Income (Less than 2500000 Rials %)	26.5	33.3	0.081
BMI (kg/m^2)	27.7±4.4	31.55±6.9	0.24
Cholesterol (mmol/l)	225.12± 47.04	192.57± 44.88	0.16
LDL (mmol/l)	168.54±19.61	136.25±31.73	<0.001
HDL (mmol/l)	44.63±7.33	53.40±13.86	0.15
TG (mmol/l)	228.04±61.78	149.94±51.26	<0.001

Approximately 1.5 percent of participants were homozygous for allele A (A/A), 89.6 percent were Homozygous for allele G (G/G) and 8.9 percent were heterozygous (A/G). In diabetic and control groups the frequency of A/A was zero and 3 percent, G/G was 6.9 and 11 percent and A/G was 93.1 and 86 percent respectively. Because of the significant role of LDL and TG on developing diabetes, these variables were considered as co-variants and their effects were calculated on the allele frequencies.

Table 2 shows distribution of A and G alleles in diabetic and control groups by Hardy-Weinberg equilibrium. The results indicate that the frequency of allele A in controls is significantly greater than that of diabetic patients (P=0.03). Whereas the difference between distribution of *SNP43* genotypes (A/A, A/G, G/G) were non-significant in case and control groups (p=0.12, Table 3).

Table 2. Distribution of SNP43 alleles in calpain-10 gene in case and control groups, frequency

Groups	Alleles		P_value
	G	A	
Case (T2DM)	197 (96.6%)	7 (3.4%)	0.031
Control	183 (91.5%)	17 (8.5%)	

Table 3. Distribution of SNP43 genotypes in Calpain10 in case and control groups, frequency

Groups / Genotypes	Case	Control	OR (%95CI)	P_value
G/G	95 (93.1%)	86 (86%)		
A/G	7 (6.9%)	11 (11%)	1.73(0.64-4.67)	0.27
A/A	0 (0%)	3 (3%)	1.58(0.74-3.29)	0.17

Tables 4 and 5 show the genotype and allelic distribution of the *rs12255372* (G/T) and *rs7903146*(C/T) polymorphisms in cases and controls. The frequency of the "T" allele of *rs12255372* (G/T) was significantly associated with type 2 diabetes (OR= 0.55, 95% confidence interval [CI], 1.11-1.51; P<0.001). No allelic association was found for *rs7903146*(C/T) polymorphism.

Table 4. Allelic distribution of polymorphisms in TCF7L2 in cases and controls

Allele	AD patients *n*=117	Healthy controls *n*=117	P value	OR(95% CI)
rs7903146(C/T)				
C	65(55.31)	61(52.13)	P=0.58	0.91(0.67-1.24)
T	52(44.68)	56(47.76)		
rs12255372 (G/T)				
G	78(66.7)	62(52.8)	P<0.001	0.55(0.40-0.76)
T	39(33.3)	55(47.2)		

Table 5. Genotypic association analysis of polymorphisms in cases and controls

Allele	AD patients *n*=117	Healthy controls *n*=117	P value
rs7903146(C/T)			
CC	30(25.6)	77(65.8)	
TC	65(55.6)	36(30.8)	P=0.08
TT	22(18.8)	4(3.4)	
rs12255372 (G/T)			
G/G	28(23.9)	71(60.7)	
T/G	71(60.7)	40(34.2)	P<0.001
T/T	18(15.4)	6(5.1)	

DISCUSSION

Calpain10 was the first susceptible gene to type 2 diabetes which, was recognized by linkage analysis in Hispanic population (HANIS *et al.* 1996). The linkage of *SNP43* within *Calpain10* gene had previously been identified by several studies, but these studies were mainly performed in other populations. Horikawa *et al*. 2000 and Garant *et al*. 2002 in two unrelated studies showed that in both Mexican-American and African-American populations, existence of G

allele was correlated with increased risk of T2DM. Similar results were reported by other investigators for different populations (BAIER *et al*. 2000; CASSELL *et al.* 2002).

We replicated previous findings of association for *SNP43* in Azeri population of Iran suggesting that the selected SNP is also associated with the disease in our population. We analyzed *SNP43* within *Calpain10* gene in a type 2 diabetes case-control cohort comprising 201 Azeri individuals. The distribution of alleles in case and control groups was significantly different (*P*=0.03; Table2). On the other hand, we showed that the G allele is associated with type 2 diabetes (OR=2.61; %95 CI: 1.06-6.45, *P*=0.03).

The result obtained from the present study is similar to that reported by Horikawa et al. 2000, but disagrees with the results reported for population of UK (EVANS *et al*. 2001), Oji-Cree (HEGELE *et al.* 2001) and Caucasians (ELBEIN *et al.* 2002). This may be explained by different environmental risk profiles between our population, body composition and genetic backgrounds.

Similar to the results reported by MALECKI *et al*. (2002) no association was observed between the genotypes and T2D. We also found no correlation between genotypes and related characteristics of T2D (*P*>0.05) which is in agreement with report of GARANT *et al*.(2002) in this instance. A greater frequency of G allele were detected in the Azeri population compared to the 5 other countries by cluster analysis, while the frequency was similar to those of Biak (0.91), Druzel (0.90) and Nasioi (0.90) populations.

Similar several studies in different populations, we observed a significant association between the T allele of the *rs12255372* (G/T) and type 2 diabetes mellitus in Azeri population, but unlike these studies there was no allelic association between *rs7903146*(C/T) and T2DM.

In several studies which have been done in British (GROVES *et al*. 2006), US (ZHANG *et al.* 2006), northern Swedish (MAYANS *et al.* 2007), and Indian (BODHINI *et al.* 2007) populations association of *rs12255372* (G/T) and *rs7903146*(C/T) polymorphisms with type 2 diabetes mellitus was confirmed. Horikoshi and et al. demonstrated only association of the *rs7903146* polymorphism with type 2 diabetes mellitus in Japanese population (HORIKOSHI *et al.* 2007).

WANG *et al.* (2013) in a meta-analysis study suggest that the *rs7903146* SNP of the *TCF7L2* gene is associated with increased susceptibility to T2DM in the Chinese population. Unlike their report we found no association between the *rs7903146* and T2DM in our population.

Recently another study performed by JYOTHI *et al.* (2013) they found significant association between allelic frequency of these two SNPs of *TCF7L2* and T2DM susceptibility, but we just found association only with *rs12255372* (G/T) SNP.

In contrast with AMOLI *et al.* (2010)eport that confirms the association between the *rs7903146* T allele and T2DM in an Iranian population we don't found any relation between this SNP and T2DM. Another study performed in the province of Isfahan, Iran show that *rs7903146* of *TCF7L2* gene is associated with susceptibility for T2DM (PALIZBAN *et al*. 2012).

The allelic distribution of these 3 SNPs in different populations and the related stratification of G and A alleles have been demonstrated in tables 6 and 7 respectively.

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ISPITIVANJA ASOCIJACIJE POLIMORFIZMA GENA *Calpain10* I *TCF7L2* SA TIPOM 2 *Diabetes mellitus*

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Izvod

Polimorfizam gena *Calpain10* i *TCF7L2* su identifikovani kao mogući genetički markeri tipa 2 diabetesa. Izvršena je evaluacija odnosa između *SNP43* calpain-10 , *rs12255372* i *rs7903146* u *TCF7L2* slučajevima tipa 2 diabetes u severozapadnom Iranu. Vršena su ispitivanja distribucije *Calpain10* i *TCF7L2* polymorfizma (*SNP43*, *rs12255372* I *rs7903146*) kod ukupno 202 pacijenta uključujući i zdrave pacijente kao kontrole, korišćenjem PCR – RFLP metoda. Utvrđeno je da je razlika distribucije alela kod obolelih i kontrolnih pacijenata statistički značajna ukazujući da je G alel vezan sa tipom 2 diabetesa kao i mogućnost asosijacije *rs12255372* (G/T) sa tipom 2 diabetesa

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