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ORIGINAL ARTICLE

Association of rs11558471 in SLC30A8 Gene with Interleukin 17 Serum Levels and Insulin Resistance in Iranian Patients with Type 2 Diabetes

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ABSTRACT

Background: According to genome wide association studies, SLC30A8 is among the loci containing SNPs associated with type 2 diabetes (T2D) risk. This gene encodes an islet zinc transporter (ZnT8). **Objective:** To provide new information on the association of the SNP rs11558471 in SLC30A8 gene with IL-17 levels and insulin resistance in an Iranian population with T2D. **Methods:** A total of 133 patients with T2D and 128 control subjects were included in this study. Insulin and IL-17 concentrations were determined using ELISA. Insulin and fasting blood glucose levels were employed to determine homeostasis model assessment for insulin resistance (HOMAIR). PCR-based restriction fragment length polymorphism was performed to determine rs11558471 polymorphism. **Results:** The risk allele frequency of rs11558471 in studied population was among the highest frequencies in different populations. In T2D patients, compared with the GG genotypes, IL-17 concentrations were significantly higher in the GA+AA group (p=0.042). According to the genotypes of this SNP, IL-17 concentrations, fasting blood glucose and HOMA-IR increased with the following order: GG<GA<AA. A multiple regression revealed that following an adjustment for age and gender, rs11558471 as an independent variable was significantly associated with IL-17 (p=0.039), fasting blood glucose (p=0.003) and HOMA-IR (p=0.042) as the dependent variables. **Conclusion:** The present study demonstrated for the first time, the genetic association of rs11558471 with IL-17 and glycemic traits in Iranian patients with T2D. The association of rs11558471 with glycemic traits showed that it might be useful for the identification of individuals who are at high risk for the development of T2D.

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Keywords: IL-17, Insulin Resistance, rs11558471, SLC30A8, Type 2 Diabetes

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INTRODUCTION

The growing prevalence of type 2 diabetes (T2D) has raised serious concerns in several countries. T2D is believed to be a complex disease characterized by insulin resistance and impaired pancreatic beta cell. This dangerous disease could get even more important when you realize that it can affect younger people, particularly in Asian populations. In Iran, the prevalence of diabetes has been estimated to be 7.7% although it may be as high as 14% in crowded cities. Despite the fact that its increasing prevalence is mainly due to negative changes in lifestyle, genetic determinants widely contribute to the susceptibility to this disease (1-4). Based on genome wide association studies, SLC30A8 is among the loci containing variants associated with type 2 diabetes risk (5,6).

SLC30A8 gene is located in chromosome 8q24.11 and encodes a 369 amino acid islet zinc transporter (ZnT8), which is responsible for zinc transportation into beta-cell insulin-secretory granules (7-9). ZnT8 is expressed almost exclusively in pancreatic alpha- and beta-cells (10). This transporter might be contributed to the autoimmunity of T1D, and as a result the related antibody and T cells are distinguished in the initiation and development of T1D (11). Altered ZnT8 expression is involved in the modulation of insulin secretion (12). Reduced glucose-stimulated insulin secretion has been observed in pancreatic beta cells with ZnT8 knockdown (13). According to Hardy *et al.* (14) a global loss of ZnT8 gene in mice exacerbates obesity, glucose intolerance and insulin resistance, during high-fat diet feeding. Interleukin (IL)-17 is a pro-inflammatory cytokine, which is mainly secreted by T-helper type 17 (Th17) cells and macrophages. Evidence has illustrated that IL-17 is plays a role in inflammation, type 1 diabetes (T1D), insulin resistance, and T2D (15,16). T cell-derived IL-17 has been indicated to promote the production of pro-inflammatory cytokines and directly cause damage of beta cells (17-19). This interleukin might be contributed to nitric oxide-dependent damage of beta cells (19).

Variation in SLC30A8 gene could be one of the causes of impaired beta cell function (20). The single nucleotide polymorphism (SNP) rs13266634, as an important genetic variant in SLC30A8, has been associated with the production of a less active ZnT8 resulting in less efficiency of insulin crystallization (14). It is suggested that rs13266634 influence the synthesis, storage, and/or secretion of insulin from beta-cells (10). Polymorphism rs11558471, another important SNP in SLC30A8, has been also found to be associated with the risk susceptibility in T2D (12, 21). This polymorphism is in strong linkage disequilibrium with the rs13266634, which is one of the most confirmed genetic markers of T2D in Europeans and East Asians (12, 22).

ZnT8, known as a major islet-cell autoantigen and, can trigger immune responses. Autoantibodies against ZnT8 as well as T-cell responses against this transporter are produced in the patients with autoimmune diabetes (13). Functional SNPs in ZnT8 may be key determinants of humoral autoreactivity to ZnT8. Therefore, ZnT8 and its functional SNPs could play a pivotal role in genetic susceptibility for beta-cell dysfunction, which in turn may contribute to the accelerated progression of beta-cell destruction (23,24). On the other hand, IL-17 can have a role in the T-cell-mediated beta-cell dysfunction (18,19). This beta-cell dysfunction is in turn affected by functional variants in ZnT8, such as rs11558471 (25,26). Hence, the present work is expected to provide new information on the association of rs11558471 as an important SNP in ZnT8 gene with IL-17 levels and insulin resistance, and also compare its risk allele frequency in an Iranian population, a high-risk population for T2D (27) compared with other populations.

MATERIALS AND METHODS

Study Population. This study comprised 133 patients with T2D diagnosed based on the WHO criteria (age range 51.84 ± 6.27 years) and 128 control subjects (age range 46.29 ± 7.39 years). The required information about demographic parameters, medical history, personal habits, and medication use was obtained from all the participants utilizing a questionnaire. All the patients had no type 1 diabetes, no myocardial infarction or stroke, no renal failure, no liver diseases, and no chronic diseases, and were not pregnant. Some of them were taking metformin, a sulfonylurea and/or a statin. The research protocol was approved by the Local Ethics Committee of the university and was conducted in accordance with the principles of the Helsinki Declaration. The written informed consent was obtained from the participants before enrollment.

Genotyping. The primer was designed using the program BatchPrimer3. The SLC30A8 gene information and rs11558471 was extracted based on the database of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The specificity of the designed primer was predicted by the Primer-BLAST on the NCBI. The primer sequences were: F 5´-CATTTTGGCAATAAATCCCTCT -3´ and R 5´-CCCATTGACATAGGATTTCCA -3´. Initially, 5 mL venous blood samples were collected in EDTA tubes from each participant and genomic DNA was extracted with a phenol-chloroform method. Subsequently, DNA concentration was measured utilizing a Picodrop spectrophotometer (Picodrop, Saffron Walden, UK). Standard PCR protocol, followed by restriction fragment length polymorphism (RFLP) was used to screen the study polymorphism. The 25 µL PCR reaction was employed containing 200-400 ng genomic DNA, 300 nmol/L of each primer, 200 µmol/L of each dNTP, 1.5 mmol/L $MgCl_2$ and 1 unit of Taq polymerase. Afterwards, DNA was amplified with an initial melting temperature of 95°C for 3 min, followed by 35 cycles. Each cycle consisted of denaturation at 95°C for 40 s, an annealing temperature at 52°C for 40 s and extension at 72°C for 40 s, followed by a final extension of 5 min at 72°C. Electrophoresis of PCR products were then performed on agarose gel (1%) and were visualized with green viewer staining. Amplification products (439 bp) were digested overnight with the restriction enzyme *PstI*. The digested products (210 and 229 bp) were ultimately separated by electrophoresis on 3% agarose gel.

Laboratory Analysis. Venous blood was obtained from the participants following an overnight fast. Insulin concentrations were measured using ELISA kit (Diametra Corporation, Milan, Italy) with a sensitivity of 0.25 µU/mL. IL-17 levels, which were assayed employing an ELISA kit (Hangzhou Eastbiopharm Co. Ltd., Hangzhou, China). Fasting glucose, TG, LDL-C, and HDL-C were measured with the commercial kits (Greiner Diagnostics GmbH, Bahlingen, Germany). The HbA1c levels were measured with Pars Azmoon kit (Pars Azmoon Co., Tehran, Iran). Fasting glucose and insulin concentrations were used to determine homeostasis model assessment for insulin resistance (HOMA-IR) using the following equation (28):

HOMA-IR = fasting glucose (mmol/L) × fasting insulin (µU/mL)/22.5

Statistical Analysis. The normal distribution of the data was determined applying the Lilliefors test. Mann–Whitney U test was performed for nonparametric variables and parametric variables were analyzed with t-test or ANOVA. The association of categorical variables was tested with Fisher exact and chi-square. The association of the study SNP with glycemic traits was assessed with multiple linear regression. Deviations from Hardy Weinberg Equilibrium expectations were analyzed employing *chi*-square

test. The data analyses were performed using the software SPSS (version 16.0) and R (version 3.0.1). The statistical significance was accepted at p less than 0.05 (Two-tailed).

RESULTS

The values of the study parameters in the patients and controls were compared and are represented in Table 1. Fasting glucose, HOMA-IR and HbA1c were observed to be significantly higher in patients with T2D compared with controls. No significant difference was found between controls and the patients with regard to gender, smoking, HDL-C, and total cholesterol.

Table 1. The comparison of values of the study parameters in patients and controls.

Parameter	Patients (n=133)	Controls (n=128)	p-value
Women (%)	69.2	61.4	**0.189**
Current smoking (%)	8.6	6	**0.423**
Diastolic blood pressure (mmHg)	70.68 ± 10.21	70.48 ± 10.38	**0.311**
Systolic blood pressure (mmHg)	120.76 ± 20.19	110.73 ± 10.94	**<0.001**
Insulin (µU/mL)	12.39 ±8.73	10.41 ±7.66	**0.057**
HbA1c (%)	8.04 ± 1.61	5.33 ± 0.28	**<0.001**
Fasting glucose (mmol/L)	9.45 ± 3.74	4.76 ± 0.41	**<0.001**
HOMA-IR	5.14 ± 4.28	2.22 ± 1.74	**<0.001**
Total cholesterol (mmol/L)	4.87 ± 1.25	4.98 ± 1.07	**0.362**
Triglyceride (mmol/L)	2.22 ± 1.31	1.76 ± 0.86	**0.004**
HDL-C (mmol/L)	1.17 ± 0.3	1.23 ± 0.28	**0.143**
LDL-C (mmol/L)	2.56 ± 0.82	2.83 ± 0.77	**0.010**

The results of PCR-RFLP are indicated in Figure 1. The products of the PCR amplification (439 bp) were subjected to restriction analysis resulting in 439, 229 and 210 bp fragments in the presence of GA genotypes, and 229 and 210 bp fragments in the presence of GG genotypes. AA genotype corresponded to a non-digested fragment of 439 bp. The genotype distribution and allele frequencies of rs11558471 are shown in Table 2. These genotypes were in Hardy-Weinberg equilibrium in both patient population and controls. The allele frequencies were 0.199 G and 0.801A in T2D patients and 0.223 G and 0.777 A in controls. Genotype distributions and allele frequencies of the patients with T2D revealed no significant differences compared with those of controls. However, the prevalence of the AA genotype and frequency of the risky A allele all increased in T2D patients compared with that in controls (OR= 1.44, 95%CI 0.47-4.65, p=0.584 for AA genotype versus GG genotype; OR= 1.15, 95%CI 0.75-1.76, p=0.521 for A allele versus G allele).
As could be seen in Figure 2, in T2D patients, compared with the GG genotypes, IL-17 concentrations were significantly higher in the GA + AA group (p=0.042).

Table 2. Genotype distributions, allele frequencies and Hardy–Weinberg equilibrium test of the variant rs11558471 in patients with type 2 diabetes and controls.

	Patients (n=133)	Controls (n=128)	OR	95% CI	p-value	HWE for patients χ^2 (p-value)	HWE for controls χ^2 (p-value)
Genotype, n (%)							
GG	6 (4.51)	8 (6.25)	1				
GA	41 (30.83)	41 (32.03)	1.32	0.41-4.44	0.774	0.153 (0.695)	0.713 (0.398)
AA	86 (64.66)	79 (61.72)	1.44	0.47-4.65	0.584		
Allele frequency							
G	0.199	0.223	1				
A	0.801	0.777	1.15	0.75-1.76	0.521		

The genotype of GG as well as the allele of G were considered as references. OR: odds ratio; CI: confidence interval; HWE: Hardy–Weinberg equilibrium.

Both fasting glucose levels (Figure 3A) and HOMA-IR (Figure 3B) were higher in the GA + AA group compared with GG homozygotes (p=0.029 and p=0.058, respectively).

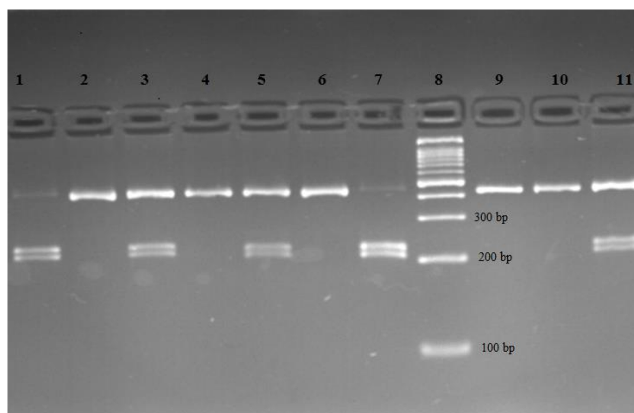

Figure 1. PCR-based restriction fragment length polymorphism (RFLP) for the rs11558471 polymorphism. GG genotypes were identified in the presence of 229 and 210 bp fragments (Lanes 1 and 7). The presence of 439, 229 and 210 bp fragments (Lanes 3, 5 and 11) corresponded to GA genotypes and the presence of an undigested fragment (Lanes 2, 4, 6, 9 and 10) was for AA genotypes. Lane 8 is DNA ladder (100-1000 bp).

Fasting glucose, HOMA-IR and IL-17 levels increased in the patients with the following order: GG<GA<AA. No such pattern was observed for these factors in the control group. The correlation analysis was conducted to study the association of IL-17 with the atherogenic index log (TG/HDL-C) according to the genotypes of rs11558471.

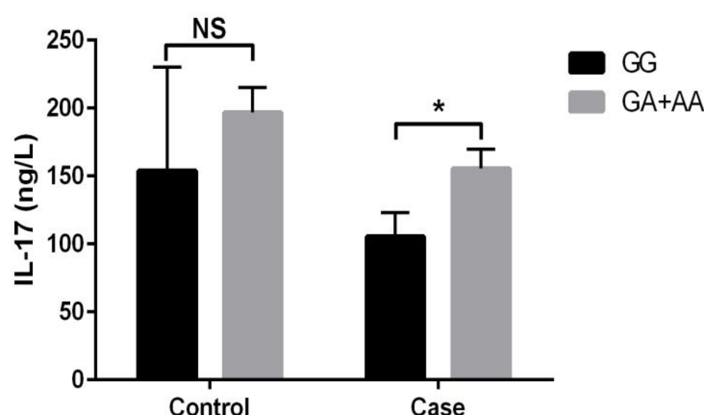

Figure 2. Change in IL-17 concentrations according to the genotypes of the polymorphism rs11558471 [GG genotypes vs. GA + AA (risk allele-containing genotypes)] in the patients with type 2 diabetes and control subjects. NS $p>0.05$, * $p<0.05$

As shown in Figure 4, there was a positive and significant correlation (r=0.178, p=0.049) between IL-17 and log (TG/HDL-C) in the GA + AA group (risk allele-containing genotypes); however, the correlation was not statistically significant in the GG genotypes. Three different linear multiple regressions were conducted to estimate the independent association of the study polymorphism with IL-17, fasting glucose and HOMA-IR, separately. After an adjustment for age and gender in the multiple analysis, considering IL-17, fasting glucose and HOMA-IR as the separated dependent variables, the polymorphism rs11558471 (as independent variable) was found to be significantly associated with IL-17 (β= -4.69, p=0.039), fasting glucose (β= -1.48, p=0.003) and HOMA-IR (β= -1.26, p=0.042).

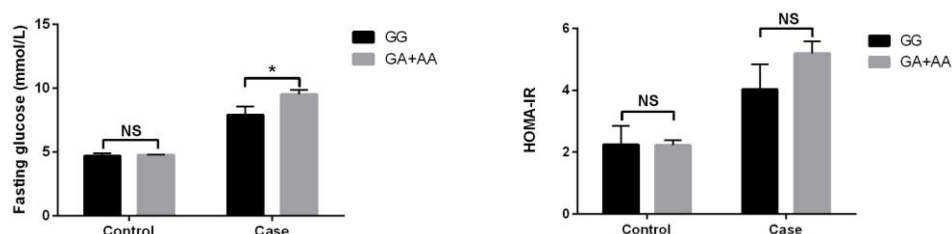

Figure 3. (A) Change in fasting glucose levels according to the genotypes of the polymorphism rs11558471 [GG genotypes vs. GA + AA (risk allele-containing genotypes)] in the patients with type 2 diabetes and control subjects. (B) Change in HOMA-IR according to the genotypes of the polymorphism rs11558471 [GG genotypes vs. GA + AA (risk allele-containing genotypes)] in the patients with type 2 diabetes and control subjects. NS $p>0.05$, * $p<0.05$

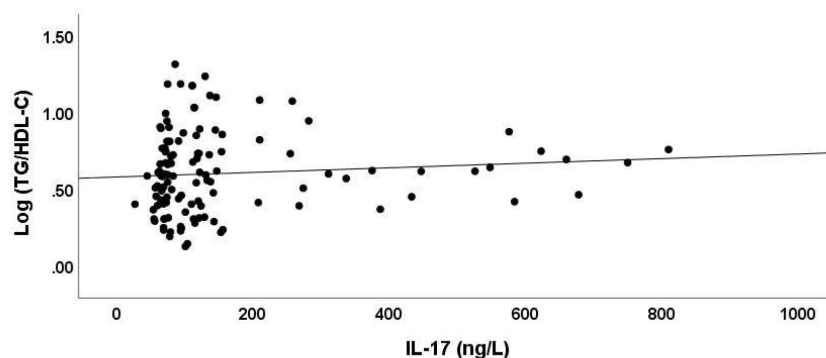

Figure 4. Correlation between IL-17 and the atherogenic index log (TG/HDL-C) in type 2 diabetic patients with risk allele-containing genotypes (r=0.178, p=0.049).

DISCUSSION

The present research indicated that rs11558471 was associated with IL-17 levels and glycemic traits in an Iranian population, in which based on reports, the prevalence of T2D seems to be among the highest in developing countries (27). The importance of rs11558471 in the ZnT8 gene becomes more apparent when you realize that this polymorphism is in strong linkage disequilibrium with the rs13266634, which according to genome wide association studies is one of the most confirmed genetic markers for T2D (12, 22).

Herein, in T2D patients, the risk allele (A allele) frequencies increased compared to control subjects. Our findings were consistent with the results of Rees et al. (29) in a UK Asian Diabetes Study (UKADS) and Diabetes Genetics in Pakistan (DGP). In our study, the frequency of A allele in the patients with T2D was found to be 0.801. Fesinmeyer *et al.* (30) reported the frequency of A allele as 0.91 in an African American population and as 0.68 in a European American population. Kim *et al.* reported the frequency of this risk allele in a Korean population with impaired fasting glucose or newly diagnosed T2D as 0.627 (21). Yang *et al.* (31) also reported the frequency of A allele as 0.69 in a non-Hispanic white population, 0.887 in a non-Hispanic black population, and as 0.729 in a Mexican American population. Overall, these findings imply that the risk allele frequency of rs11558471, as an important SNP in the ZnT 8 gene in this Iranian population, is among the highest in different populations.

ZnT8 has been presented as a major autoantigen in human T1D (32) and autoantibodies against this transporter, which can provide an independent marker of disease susceptibility in prediabetic people (33). Functional SNPs in ZnT8, rs11558471 for instance, may be contributed to genetic susceptibility for beta-cell dysfunction (23,34). Our findings revealed that rs11558471 associates with IL-17 levels in T2D patients; accordingly, based on the genotypes of this polymorphism, IL-17 concentrations increased with the following order: GG<GA<AA. It was indicated that the increased IL-17 concentrations could be attributed to beta cell damage. Moreover, anti-IL-17 antibody can play a role in reducing T-cell infiltration of islets and in reducing autoantibody levels in NOD mice (35). Furthermore, according to other studies, IL-17 is involved in T1D, insulin resistance, T2D, and damage of beta cells (15,18,19).

Therefore, T cell-derived IL-17 can be contributed to beta-cell dysfunction, which in turn is affected by rs11558471 as well.

The analyses based on the genotypes of rs11558471 showed that in T2D patients, both fasting glucose and HOMA-IR increased with the following order: GG<GA<AA. The analyses using a multiple regression revealed that the polymorphism rs11558471 significantly influences fasting glucose and HOMA-IR. Accordingly, after an adjustment for age and gender, our analyses about the association of rs11558471 with fasting glucose showed that this SNP may be contributed to fasting glucose in this Iranian population, supported by the following findings. Kim *et al.* (21) reported that rs11558471 was nominally associated with impaired fasting glucose in a Korean population. Based on the findings of Rees *et al.* (29), among 16 SNPs only the rs11558471 polymorphism was nominally associated with fasting glucose concentrations in Pakistanis. The association of this SNP with fasting glucose was observed by Ramos *et al.* (34) in a cohort of African-Americans. Furthermore, the rs11558471 ZnT8 variant was associated with total zinc intake in a meta analysis by Kanoni *et al.* (12), so the people carrying the glucose-raising A allele showed a stronger inverse association with fasting glucose concentrations compared with individuals carrying the G allele. Therefore, our findings are in line with the results of the studies indicating that the A allele of rs11558471 is an allele involved in increased glucose.

Based on our study, in addition to fasting glucose, the rs11558471 polymorphism may affect HOMA-IR-estimated insulin resistance. It is noteworthy to mention that racial/ethnic differences could influence the association of this SNP with glycemic traits. For example, according to the findings by Fesinmeyer *et al.* (30), rs11558471 was significantly associated with fasting glucose levels among European Americans, but not in African Americans. The study by Strawbridge *et al.* (36) indicated that this SNP was significantly related to fasting glucose, but not HOMA-IR in nondiabetic adults of European ancestry. Of note, based on the study by Yang *et al.* (31), the allele frequencies of glycemic traits-associated SNPs, such as rs11558471, varied significantly in terms of race/ethnicity.

In the current study, IL-17 was found to have a positive and significant correlation with the atherogenic index log (TG/HDL-C). This may be of great importance in atherosclerotic events in patients with T2D. It should be mentioned that there is a positive correlation between log (TG/HDL-C) and the fractional esterification rate of HDL (FERHDL) and it can be an alternative marker of plasma atherogenicity (37,38). Our findings were in agreement with those of Ribeiro et al. (39), who found that IL-17 had a negative correlation with HDL and a positive relation with total cholesterol, LDL, and TG. Additionally, in a study, Zeng and colleagues (40) reported that the levels of Th17 cells in the patients with T2D were negatively related to plasma HDL. Therefore, this study offered the evidence for the relationship of dyslipidemia with the alterations of IL-17 levels in T2D patients.

In conclusion, herein, we demonstrated for the first time the genetic association of rs11558471 as an important SNP in ZnT8 gene toIL-17 concentrations and glycemic traits in an Iranian population, which is known to be a high-risk population for T2D. According to our results, rs11558471 was associated with IL-17, which in turn may influence beta-cell dysfunction. Of note, the association of rs11558471 with insulin resistance and fasting glucose showed that the variant can be useful for the identification of individuals who are at high risk for the development of T2D. However,

further studies are required to better understand how this SNP is contributed to glycemic traits, particularly in high-risk populations for T2D, such as Iranian population.

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