Journal of Genetics, Vol. 97, No. 5, December 2018, pp. 1407–1412
https://doi.org/10.1007/s12041-018-1032-7

RESEARCH ARTICLE

The genetic variants of solute carrier family 11 member 2 gene and risk of developing type-2 diabetes

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Received 20 January 2018; revised 18 July 2018; accepted 14 August 2018; published online 29 November 2018

Abstract. Type-2 diabetes (T2DM) is a metabolic disorder characterized by long-term insulin resistance, impaired insulin secretion from β-cells, and loss of beta cell mass and function. Inflammation and oxidative stress play a key role in the development of diabetes and are associated with insulin resistance. Notably, recent studies have demonstrated an association between body iron stores, insulin resistance and T2DM. Free iron, a powerful pro-oxidant molecule, is involved in oxidative stress, lipid peroxidation and endothelial dysfunction via its ability to generate free radicals. Specifically, the accumulation of iron in beta cells triggers oxidative stress and DNA damage, which have been reported to be associated with β-cell death and apoptosis. Solute carrier family-11 member-2 (SLC11A2) functions to transport ferrous iron and some divalent metal ions throughout the plasma membrane and across endosomal membranes. Functional polymorphisms in the *SLC11A2* gene have been reported to cause excess storage of iron, resulting in iron overload. In this study, we evaluated the association between T2DM and *SLC11A2* gene variants IVS4+44C/A, 1303C/A and 1254T/C by performing PCR-RFLP analysis on 100 T2DM patients and 100 healthy subjects. PCR products were digested with *Mnl*I, *Mbo*I and *Sfa*NI restriction endonucleases and the products were then separated by 3% agarose gel electrophoresis. The genotype frequencies of the 1254T/C and 1303C/A *SLC11A2* gene variants did not differ between healthy controls and T2DM patients ($P > 0.05$). But, in recessive model ($P = 0.037$) and homozygous CC genotype ($P = 0.030$) for IVS4+44C/A showed significant correlation with T2DM risk. It is thought that presence of C allele of IVS4+44C/A plays pathological roles.

Keywords. type-2 diabetes; IVS4+44C/A variant; 1254T/C variant; 1303C/A variant; *SLC11A2* gene.

Introduction

Type-2 diabetes (T2DM) is a chronic metabolic disorder characterized by insulin resistance that affects many tissues such as adipose tissue, skeletal muscles and the liver, defects in insulin secretion and also β-cell dysfunction. The incidence of the disease is increasing every day and has become a worldwide health problem (Donath 2014; Esser *et al.* 2014; Guariguata *et al.* 2014).

It has been reported that combinations of genetic and environmental factors lead to T2DM by affecting insulin sensitivity and β-cell death (Cnop *et al.* 2005).

Although the reason for the impaired insulin sensitivity and β-cell death are not fully understood, some studies indicate that increased risk of T2DM is associated with excessive levels of circulating ferritin which is a marker of body iron stores. Further, the serum ferritin concentration has been suggested as a component of the peripheral and hepatic insulin resistance, circulating insulin and glucose levels, hypertension, dyslipidaemia and obesity (Fernández-Real *et al.* 2002; Rajpathak *et al.* 2009).

Iron is a transitional metal and has vital importance for all living organisms because of the essential for a wide variety of metabolic processes including DNA synthesis, oxygen and electron transport (Conrad and Umbreit 2000; Rajpathak *et al.* 2009). However, due to the potent pro-oxidant feature and capability of inducing the

production of reactive oxygen species (ROS), excess iron is hazardous (Conrad and Umbreit 2000; Fernández-Real *et al.* 2002).

Iron overload is associated with the overproduction of superoxide and hydroxide that inducing lipid peroxidation, and inflammation and accumulation of iron leads to cellular toxicity, tissue injury and organ dysfunction by affecting the cells of various organs such as the pancreas, liver and heart (Fernández-Real *et al.* 2002; Gurzau *et al.* 2003; Ramm and Ruddell 2005).

Iron toxicity is found to be associated with many pathophysiological conditions such as liver, heart and lung diseases, as well as diabetes mellitus, hormonal abnormalities and a dysfunctional immune system (Gurzau *et al.* 2003).

Solute carrier family 11 member 2 (SLC11A2) is a transmembrane protein involved in dietary iron uptake in duodenal enterocytes and transferring the iron from the transferrin receptor endosomal cycle into the cytosol in erythroid cells (Iolascon *et al.* 2006). It has been suggested that increased expression SLC11A2 leads to increased iron absorption in primary iron overload and some mutation alter the expression of SLC11A2 (Zoller *et al.* 2001).

SLC11A2 has an affinity for divalent cations including iron, lead, nickel, cadmium, manganese, cobalt, copper, zinc and consequently it was thought that mutations or polymorphisms of *SLC11A2* gene can impair the metal trafficking. As evidence, in a recent study, SLC11A2 IVS4+44 CC genotype was found to be associated with a risk of increased blood iron, lead and cadmium levels (Kayaaltı *et al.* 2015).

Five gene variants are identified within the *SLC11A2* gene; 1303C/A mutation, 1254T/C, IVS2 + 11A/G, IVS4 + 44C/A and IVS6 + 538G/G del polymorphisms (He *et al.* 2011). Three of them, the 1254T/C and IVS4+44C/A polymorphism and 1303C/A mutation are commonly studied genetic alterations of the *SLC11A2* gene, and they have been evaluated in many pathophysiological processes including Parkinson's disease (PD) (He *et al.* 2011; Saadat *et al.* 2015), lead-related hypertension (Kim *et al.* 2013), hereditary haemochromatosis (Kelleher *et al.* 2004) and inflammatory bowel disease (Stokkers *et al.* 2000).

The variation of 1254T/C is a single-nucleotide polymorphism (SNP), and occurs in the coding region of SLC11A2 but does not cause an amino acid change. The IVS4 + 44C/A polymorphism occurs +44 nucleotides in intron 4 of the *SLC11A2* gene. The 1303C/A mutation also occurs in the coding region of SLC11A2 and results in an amino acid change from leucine to isoleucine (Lee *et al.* 1998; He *et al.* 2011).

According to these data, the purpose of this study was to determine the role of the 1254T/C and IVS4+44C/A polymorphisms and 1303C/A mutation of *SLC11A2* gene in T2DM.

Methods and materials

Patient selection and sample collection

Peripheral blood samples were obtained from T2DM patients and healthy controls without T2DM that were enrolled at the Department of Endocrinology, Eskisehir Osmangazi University, Medical Faculty in Turkey. This study was approved by the local ethics committee (Medical Faculty of the Eskisehir Osmangazi University, Turkey. According to the Helsinki Declaration, informed consent was obtained from all patients prior to study inclusion.

Demographic and clinical variables were documented for all cases, and personal files were generated to record clinical data. The study group included 100 T2DM patients (47 men and 53 women) and control groups ($n = 100$) were included 49 men and 51 women ranging in age from 40 to 70 years. The mean ages of participants were 48 ± 7 years in controls and 58 ± 8 in T2DM patients. Moreover, controls and patients were mainly matched on sex and ethnicity.

The definition and diagnosis of T2DM were based on the criteria as follows; fasting plasma glucose (FPG) of $\geq$126 mg/dL, haemoglobin A1C (HbA1C) of $\geq$6.5%, oral glucose tolerance test (OGTT) (with 75 gr glucose) with 2 h (120 min) blood glucose of $\geq$ 200 mg/dL.

One hundred control subjects were selected from the outpatients according to the following criteria; FPG levels below 100 mg/dL, HbA1C levels below 5.7%, OGTT level below 140 mg/dL.

Genotyping analysis

Genomic DNA was extracted from peripheral blood using a PureLink Genomic DNA Mini kit (Invitrogen Corporation, Carlsbad, USA) according to the manufactures instructions.

The IVS4+44C/A (rs224589), 1254T/C (rs1048230) and 1303C/A (rs144863268) variants of *SLC11A2* gene were analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Polymerase chain reactions were performed in a total volume of 25 μL using OneTaq Quick-Load 2x master mix with standard buffer (New England Biolabs, Ipswich, USA) according to the manufacturer's instructions. Briefly, the 25-μL reaction volume consisted of 2 μL of genomic DNA, 0.5 μL of each primer (5 μM), 12.5 μL of master mix, and 9.5 μL of nuclease-free water.

PCR reactions were performed by using Bio-Rad Thermal Cycler (T100, Foster city, USA). Sequences of oligonucleotide primers obtained from Alpha DNA (Montreal, Canada) the reaction conditions and restriction endonucleases (New England Biolabs, Beverly, USA) are shown in table 1. The yields were analysed by electrophoresis in a 3% agarose gel stained with RedSafe nucleic acid staining solution (Intron Biotechnology, Seoul, Korea)

Table 1. PCR amplification primers, reaction conditions and restriction endonucleases.

Variation	Oligonucleotide primer sequences	Enzyme	PCR reaction conditions	
IVS4+44C/A	F: 5′-AGGCTACTATCCAACATGCAG-3′	*Mnl*I	First denaturation: 94°C for 30 s	
rs224589	R: 5′-CTTCCCACTGAGCAGGTTG-3′	(37°C, 15 min)	Denaturation: 94°C for 30 s	
1254T/C	F: 5′-CTTTGCCCGAGTGGTTCTGACTCGCTCGAT-3′	*Mbo*I	Annealing:59 for 1 min	30 cycle
rs1048230	R: 5′-TTCCTCTCAATATCCCCC-3′	(37°C, 15 min)	Extension: 68°C for 1 min	
1303C/A	F: 5′-ATTCTTCTGAGGTCTCTCCTG-3′	*Sfa*NI	Final elongation: 68°C for 5 min	
rs144863268	R: 5′-AGACCACAACCATGCCTCTG-3′	(37°C, 1 h)	Final hold: 4°C for ∞	

and visualized by GeneGenius Gel Light Imaging System (Syngene, Cambridge, UK). To confirm the genotyping results, we randomly selected 10% of cases and controls, and the DNA samples were genotyped again.

Statistical analysis

A sample size of 194 achieves 90% power to detect an effect size (W) of 0.2555 using a 2 degrees of freedom chi-square test with a significance level (alpha) of 0.05000 by PASS 11 Software according to Saadat *et al.* (2015) report. In this direction, we included 200 individuals in the study and aimed to reach over 90% of the power.

The comparisons of genotypes among the groups and odds ratios were evaluated with chi-square analysis (Pearson and exact chi-square tests) using IBM SPSS Statistics 21 software. Allele frequencies and Hardy–Weinberg equilibrium (HWE) were evaluated by chi-square test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) and $P < 0.05$ were considered significant.

Results

The 352 bp amplification product of IVS4+44C/A site yielded 217/100/35 bp when the A allele is present. When the C allele is present, the products length are 183/100/35/34 bp. The 34 and 35 bp fragments are not visible on the 3% agarose gel (figure 1).

The 229 bp amplification product of 1254T/C site yielded 198 and 29 bp when the C allele is present. When the T allele is present, the product length is 229 bp. The 29 bp fragment is not visible on the 3% agarose gel (figure 2).

Mutation 1303C/A destroys a SfaNI site. The 362 bp PCR product is digested to a 197 and 165 bp fragments when the normal 1303C is present (figure 3).

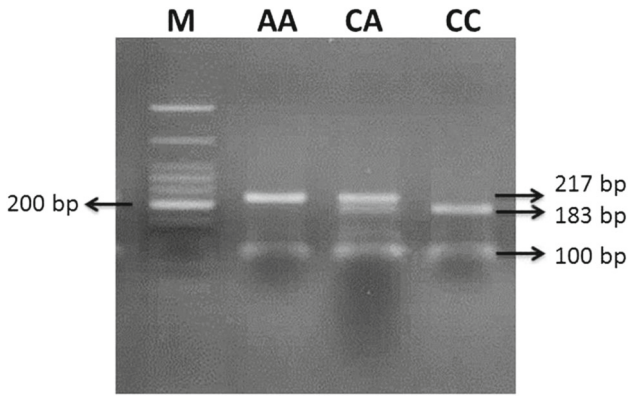

Figure 1. Gel electrophoresis of PCR fragments on 3% agarose gel to determine the *SLC11A2* gene IVS4+44C/A genotypes.

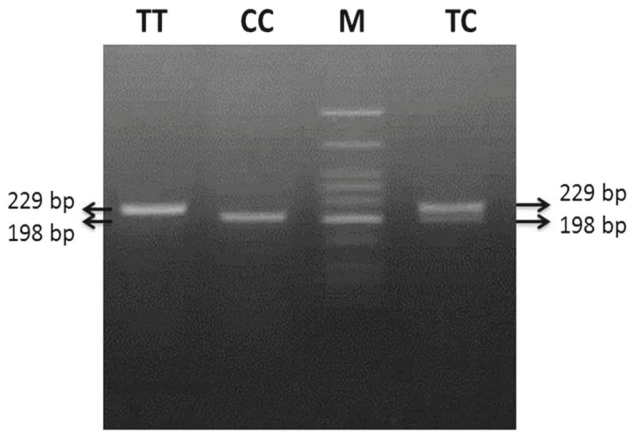

Figure 2. Gel electrophoresis of PCR fragments on 3% agarose gel to determine the *SLC11A2* gene 1254T/C genotypes.

All of the studied groups were in Hardy–Weinberg equilibrium ($P > 0.05$), and comparisons of genotype and allele frequencies showed no significant differences

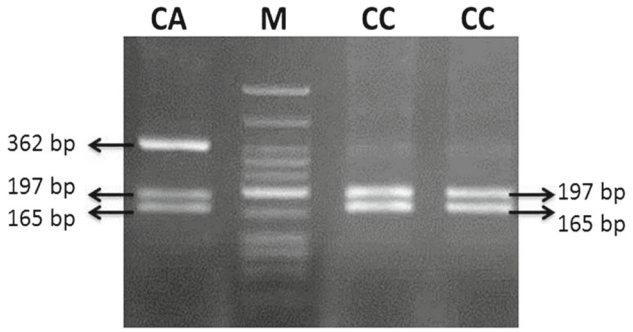

Figure 3. Gel electrophoresis of PCR fragments on 3% agarose gel to determine the *SLC11A2* gene 1303C/A genotypes.

among the groups for IVS4+44C/A, 1254T/C and 1303C/A variants.

The genotype and allele frequencies of the SLC11A2 IVS4+44C/A variant are presented in table 2. A total of 94 (47.0%) individuals had the CC genotype, 90 (45.0%) had CA, and 16 (8.0%) had AA genotype. There were no significant differences in genotype frequencies between controls and T2DM in the IVS4+44C/A polymorphisms ($P = 0.96$). The allele frequencies of the IVS4+44 C and A allele in the studied population were 278 (69.5%) and 122 (30.5%), respectively, and comparisons of alleles (C vs A) showed no significant differences ($P = 0.082$).

The homozygous CC genotype was found to be associated with T2DM ($P = 0.030$, OR = 0.281, 95% CI (0.084–1.265), $\chi^2 = 4.68$) and no association with T2DM was found for the heterozygous genotype: $P = 0.56$, OR = 0.843, 95% CI (0.472–1.505), $\chi^2 = 0.33$. There was no significant difference in dominant model (CC vs CA+AA) of IVS4+44C/A variant but distribution of IVS4+44C/A in recessive model (CA+CC vs AA) showed a significant difference ($P = 0.037$).

Table 3 shows genotype frequencies of the SLC11A2 1254T/C variant and there were no significant differences in the frequencies of 1254T/C genotypes in the patient compared to the control ($P = 0.603$). The most common genotype in both the patients (77.0%) and in healthy individuals (81.0%) was TT. The comparisons alleles (T vs C) showed no significant differences in the studied population ($P = 0.419$). The distribution of IVS4+44C/A in dominant (TT vs TC+CC), recessive (TC+TT vs CC), homozygous (TT vs CC) and heterozygous (TT vs TC) models showed no significant differences also ($P > 0.05$).

The 1303C/A mutation were seen at heterozygote state in only two of control individuals and one of T2DM patients ($P = 1.000$). The most common genotype in both the

Table 2. Genotype frequencies of the SLC11A2 IVS4+44C/A variant.

Group	Allele		*P*	OR (95% CI)	Genotype			Statistic	HWE
	C *n* (%)	A *n* (%)			CC *n* (%)	CA *n* (%)	AA *n* (%)		
Control	131 (65.5)	69 (34.5)	0.082	0.685 (0.446–1.051)	43 (43)	45 (45)	12 (12)	$P = 0.096$	0.96
T2DM	147 (73.5)	53 (26.5)			51 (51)	45 (45)	4 (4)		0.12

Test for association OR (95% CI) (Risk allele A)				
Heterozygous CC vs CA	Homozygous CC vs AA	Dominant AA+CA vs CC	Recessive AA vs CC+CA	Armitage's trend test
0.843 (0.472–1.505) $P = 0.563$	0.281 (0.084–0.935) ***P* = 0.030**	0.725 (0.415–1.265) $P = 0.257$	3.273 (1.018–10.523) ***P*= 0.037**	Common OR: 0.629 $P = 0.072$

OR, odds ratio; CI, confidence interval. Significant results are represented in bold.

Table 3. Genotype frequencies of the SLC11A2 1254T/C variant.

Group	Allele		*P*	OR (95% CI)	Genotype			Statistic	HWE
	T *n* (%)	C *n* (%)			TT *n* (%)	TC *n* (%)	CC *n* (%)		
Control	181 (90.5)	19 (9.5)	0.419	1.299 (0.687–2.455)	81 (81)	19 (19)	0 (0)	$P = 0.603$	0.29
T2DM	176 (88)	24 (12)			77 (77)	22 (22)	1 (1)		0.67

Test for association OR (95% CI) (risk allele C)				
Heterozygous TT vs TC	Homozygous TT vs CC	Dominant CC+TC vs CC	Recessive CC vs TT+TC	Armitage's trend test
1.218 (0.612–2.425) $P = 0.574$	3.155 (0.127–78.619) $P = 0.306$	1.273 (0.643–2.521) $P = 0.487$	0.330 (0.013–8.199) $P = 0.316$	Common OR: 1.557 $P = 0.403$

OR, odds ratio; CI, confidence interval.

Table 4. Genotype frequencies of the *SLC11A2* 1303C/A variant.

		DMT1 1303C/A genotypes					
		CC		CA		AA	
Group	*n*	*n*	%	*n*	%	*n*	%
Control	100	98	98.0	2	2.0	0	0.0
T2DM	100	99	99.0	1	2.0	0	0.0
Total	200	197	98.5	3	1.5	0	0.0
Statistic		$P = 1.000$					

patients (99.0%) and in healthy individuals (98.0%) was CC (table 4). The genotype distributions of 1303C/A were nonpolymorphic in our population.

Discussion

Iron is a powerful pro-oxidant because it catalyse the production of hydroxyl radicals. It has been demonstrated that iron metabolism can affect the insulin sensitivity and lead to development of T2DM (Jiang *et al.* 2004). The main mediator of iron transfer is SLC11A2 and iron is absorbed through the apical transporter SLC11A2 in intestinal epithelial cells and macrophages (Ganz 2003).

Therefore, we hypothesized that gene variation of SLC11A2 can affect the metal trafficking and cause the cytotoxic effects on pancreatic cells and lead to development of T2DM.

There is only one study in the literature that investigated the relationship between diabetes and *SLC11A2*. In this study, Hansen and colleagues reported that due to the increased β-cell iron content, the *SLC11A2* expression induced by proinflammatory cytokine IL-1β. Suppression of *SLC11A2* expression by genetic knockdown and iron chelation leads to reduction of cytokine-induced ROS formation and cell death. In SLC11A2 knockout islets glucose-stimulated insulin secretion is defective. Therefore, *SLC11A2* knockout mice are protected against multiple low-dose streptozotocin induced type-1 diabetes and high-fat diet-induced glucose intolerance in T2DM (Hansen *et al.* 2012).

The 1254T/C and IVS4+44C/A variation of *SLC11A2* gene were investigated in PD and found that frequency of TT genotype for the 1254T/C polymorphism is high in PD patients whereas there was no association between the IVS4+44C/A polymorphism and PD (Saadat *et al.* 2015). He *et al.* (2011) also investigated one mutation (1303C/A) and two SNPs (1254T/C and IVS4 + 44C/A) in *SLC11A2* gene and found that CC haplotype of 1254T/C and IVS4 + 44C/A variants is a possible risk factor for PD. But researchers did not find an association among genotype in 1303C/A, 1254T/C and IVS4 + 44C/A in *SLC11A2* gene and PD.

Kim *et al.* (2013) evaluated the association of *SLC11A2* with clinical variables in a lead-exposed workers and investigated 1303C/A, 1254T/C and IVS4+44C/A variation of *SLC11A2*. They found significant association between IVS4+44C/A variation and increased risk of lead-related hypertension.

The IVS4+44C/A variant of *SLC11A2* gene also investigated in our population without any pathology and no statistically significant association was found in relation to age and gender (Kayaalti *et al.* 2011).

In another study performed by Kayaalti and co-workers, the association of *SLC11A2* IVS4+44 C/A polymorphism with blood iron, lead and cadmium levels was evaluated and it was indicated that *SLC11A2* IVS4+44 CC genotype found to be associated with risk for increased blood iron, lead and cadmium levels (Kayaaltı *et al.* 2015).

According to previous studies (He *et al.* 2011; Kayaaltı *et al.* 2015), the presence of CC genotype and C allele of IVS4+44C/A was considered to be pathological.

The present study is first report that investigates the association of the IVS4+44C/A, 1254T/C and 1303C/A variants of *SLC11A2* gene with T2DM.

The CA genotypes IVS4+44C/A variant were evenly distributed in both of control and patients. The 1254T/C variation was present only in a heterozygous state in one of 100 T2DM patients. The 1303C/A mutation was present only in a heterozygous state in one of 100 T2DM patients and in two of 100 control individuals. The homozygote AA genotype was not detected in all individuals.

We did not find a statistically significant difference between the control and patient groups in terms of the genotypes of 1254T/C and 1303C/A variants of *SLC11A2*. But, in recessive model (AA vs CA+CC) for IVS4+44C/A genotypes showed significant correlation with T2DM risk.

In conclusion, the results of the present study do not support the hypothesis that variation in the *SLC11A2* gene locus has an influence on development of T2DM. This may be related to sample sizes and study population. The 1254T/C and 1303C/A variations in the *SLC11A2* gene are not associated with T2DM risk in patients of Turkish origin and IVS4+44C/A polymorphism is associated the T2DM risk at recessive state.

Acknowledgement

We thank all consultants, staff and interviewees who participated in the study. No outside funding or grants received for this study.

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Corresponding editor: INDRAJIT NANDA