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THE ABCC8 R1273R (G/A) AND PPARγ2 Pro12Ala (C/G) GENETIC MARKERS ARE NOT ASSOCIATED WITH TYPE 2 DIABETES; A STUDY CONDUCTED IN IN RAFHA CITY OF THE NORTHERN BORDER REGION, SAUDI ARABIA

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ABSTRACT

Type 2 diabetes is characterized by impaired insulin secretion and insulin resistance. Genes encoding proteins having role in insulin secretion are particularly accused for the pathogenesis of the disease. ABCC8, the ATP-binding cassette, sub-family C gene encodes for the sulfonylurea receptor 1 (SUR1) protein. This protein is one part (subunit) of the ATP-sensitive potassium (KATP) channels in β cells regulating insulin secretion. The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor family and has a role in the maturation of adipocytes, regulation of lipid metabolism and enhancing insulin sensitivity.

Different studies have shown that ABCC8 R1273R (G/A) and PPARγ2 Pro12Ala (C/G) genetic polymorphism are associated with the onset of T2D. We studied this association among the type 2 diabetic patients in Rafha city of Saudi Arabia. Genomic DNA was extracted from peripheral blood samples collected from 100 patients and 40 controls. The respective DNA fragments spanning the polymorphic regions were amplified using specific oligonucleotide primers and sequenced. The ABCC8 R1273R (G/A) polymorphism was found to be almost equally distributed among patients and controls. In case of PPARγ2 Pro12Ala (C/G) genetic polymorphism, the C allele was seen in all the controls and 98% of the patients. Only two patients had the G allele which was obviously statistically insignificant. In

conclusion, we report that the ABCC8 R1273R (G/A) and PPARγ2 Pro12Ala (C/G) polymorphisms are not associated with type 2 diabetes in this part of the world.

Keywords: Type 2 diabetes mellitus, genetic polymorphism, insulin secretion, pathogenesis, Polymerase Chain Reaction, DNA sequencing

INTRODUCTION

Type 2 diabetes (T2D) is associated with reduced insulin secretion and insulin resistance; both of these conditions contribute to the pathogenesis of the disease. The genes encoding proteins associated with insulin secretion and other functions of pancreatic B-cell can be associated with T2D disease susceptibility.

ATP-sensitive potassium (KATP) channels in β cells regulate insulin secretion through coupling the metabolic state of the cell to membrane potential. Increased glucose concentrations reduce permeability of KATP channels that leads to increase in Ca2+ influx into the cell and hence stimulates insulin secretion (Seino and Miki, 2003). ABCC8 is "ATP-binding cassette, sub-family C, member 8." This gene is located on the short (p) arm of chromosome 11 at position 15.1. It encodes for the sulfonylurea receptor 1 (SUR1) protein which is one part (subunit) of the KATP (Aguilar-Bryan and Bryan, 1999; Smith*et al.,* 2007). The channel is an octamer, consisting of four inwardly rectifying potassium channels (Kir) encoded by KCNJ11 gene that make the pore, and four sulfonylurea receptor SUR1 subunits encoded by ABCC8 gene that have regulatory function and are a target of sulfonylurea drugs (Aguilar-Bryan and Bryan, 1999; Smith *et al.,* 2007; Nichols *et al*., 2007). In humans, more than 300 genetic variations of ABCC8 genes have been reported and have been widely studied for association with defective insulin secretion and glucose intolerance but most could not provide appreciable results (van Dam *et al*., 2005; Doliba *et al*., 2004). The R1273R, a silent A-to-G nucleotide substitution at codon 1273 in exon 31 (rs1799859), has been found to be associated with T2D (Reis *et al*., 2000; Laukkanen *et al.*, 2004).

The peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor family, and consists of three subtypes namely alpha, beta, and gamma. In humans, alternative splicing of PPARγ results in three different isoforms: PPARγ1, PPARγ2 and PPARγ3. The PPARγ2 is almost exclusively expressed in white and brown adipose tissue (Ackert-Bicknell and Rosen 2006; Fajas *et al*., 1997;

Douglas *et al*., 2001). It has a role in the maturation of adipocytes, regulation of lipid metabolism and enhancing insulin sensitivity. The thiazolidinediones (TZDs), PPARγ2 agonists, inhibit the expression of many immune factors including tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), interleukin-6 (IL-6) (Calnek *et al*., 2003; Law *et al*., 2000). Recently, it has been observed that Pro12Ala polymorphism PPARγ2 gene is associated with type 2 diabetes mellitus (DM) (Soskić *et al.,* 2010).Many studies have shown the association of Pro12 allele with the onset of the disease (Douglas *et al*., 2001; Memisoglu *et al.,* 2003; Mori *et al.,* 2001; Ghoussaini *et al*., 2005). Here we report the absence of any association of ABCC8 R1273R and Pro12Ala polymorphism PPARγ2 markers with T2D in Saudi patients in Rafha city of the Northern Border Region.

METHODS

Ethics statement

The present study was approved by the Ethics Committee of Northern Border University, ArAr, Kingdom of Saudi Arabia (Approval No. 3-14-1436-5). The subjects were recruited in the Rafha Central Hospital. Informed consents from all participants were taken prior to sample and data collection. The samples were collected strictly according to the recommendations of the committee.

The research site

The study was carried out at faculty of pharmacy, Northern Border University Rafha branch. A total of 100 T2D patients and 40 normal controls were included in this study. The patients were selected according to the approved criteria (HbA1c > 6.4 and plasma glucose concentration > 7.8 mmol/l) under the supervision of the consultant endocrinologist. The controls had no past history of glucose intolerance and HbA1c level of <6.4%. Blood (1 to 2mL) samples collected in appropriate containers. The demographic data was recorded on prescribed forms.

DNA extraction from blood

The genomic DNA from blood samples were extracted using the solution based blood DNA preparation kit (Jena Biosciences, Germany, Cat# PP205S) according to the manufacturers' recommendations. Briefly 300ul of blood was mixed with 900ul RBC Lysis Solution in 1.5 tubes and centrifuged at 8000rpm for 1 minute. After discarding supernatant, Cell Lysis Solution (300ul) was mixed with WBCs and proteins were precipitated with 100ul Protein Precipitation Solution. The supernatant was taken to a new tube after centrifugation. An equal volume of

2-propanol was added for DNA precipitation followed by centrifugation and washing the DNA pellet with 500ul Washing Buffer. The DNA was dissolved in 70ul of DNA Hydration Solution and stored at -20 ^{0}C.

PCR Amplification

In ABCC8 PCR, a 217 base pair fragment covering *ABCC8 gene* exon 31 was amplified in a total PCR volume of 20µl containing 1X PCR buffer (Thermos Scientific), 1.5mM $MgCl_2$, 0.2mM of each dNTPs, 0.5µM each primer (ABCC8-1273F and ABCC8-1273R, Table 1), 0.5 units Taq polymerase (Thermos Scientific) and 1µL of extracted DNA (around 40nmol). Cycling conditions for PCR were: Initial denaturation step at 95°C for 5 minutes and then 40 cycles each of 95°C for 10 seconds, 56°C for 20 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. A known negative control contained sterile water. Five microliters of each PCR product were run on a 2% agarose gel in 1X TBE buffer to confirm the presence of PCR product.

In PPARγ PCR, a 266 base pair fragment covering PPARγ *gene* was amplified in a total PCR volume of 20µl containing 1X PCR buffer (Thermos Scientific) , 1.5mM $MgCl_2$, 0.2mM of each dNTPs, 0.5µM each primer (PPARγ-F and PPARγ-R, Table 1), 0.5 units Taq polymerase (Thermos Scientific) and 1µL of extracted DNA (around 30nmol). Cycling conditions for PCR were: Initial denaturation step at 95°C for 5 minutes and then 40 cycles each of 95°C for 20 seconds, 50°C for 30 seconds and 72°C for 40 seconds, followed by a final extension at 72°C for 5 minutes. A known negative control contained sterile water. Five microliters of each PCR product were run on a 2% agarose gel in 1X TBE buffer to confirm the presence of PCR product.

All the amplified fragments of both the genes from patients and controls were sent to BGI Tech Solutions, a commercial lab in Hong Kong for DNA sequencing.

Statistical analysis

Data were recorded, validated and analyzed for average and percentage prevalence between groups. Chi square test were used as tests of significance at 5 % level of significance. The *P* values were calculated online at following website http://www.quantpsy.org/chisq/chisq.html

Table 1: Oligonucleotide primers used for PCR amplications

	Primer sequence	Product size
ABCC8 gene exon 31		
ABCC8-1273F	5'-ccgcactgtccctctggcatcagat-3'	217 bp
ABCC8-1273R	5'-agcggtgacctccatctccaactcc-3'	
PPARγ gene		
PPARγ-F	5'-ccaattcaagcccagtcctttc-3'	266 bp
PPARγ-R	5'-gatatgtttgcagacagtgtatcagtgaaggaatcgctttccg-3'	

RESULTS

The clinical features of patients enrolled in the current study are summarized in Table 2. The Fasting plasma glucose, HbA1c and systolic blood pressure were higher in the patients than that of controls.

Due to certain limitation of the resources, genetic analysis was restricted to 100 patients and 40 controls only. The representative DNA sequences of the polymorphism for both the genes are shown in Figure 1 and 2. In case of ABCC8 R1273R, the allele A did not show any association with T2D. The genotypes A/A, A/G and G/G were equally distributed between patients and controls (Table 3). Although slightly higher percentage of allele A was seen among patients vs controls (48.5 vs 44%), this was statistically insignificant (p = 0.66). The PPAR gamma Pro12Ala gave surprisingly unexpected results. The Pro/Pro genotype was observed in almost all the patients and controls except two patients which had Ala/Ala genotype. The Pro/Ala genotype was not seen in any case (Table 3).

Table 2: Demographic data of the patients and controls

Characteristics	T2D (N=100)	Controls (50)	*p*
Age (years)	35 ± 7.2	36 ± 7.6	0.32
Sex (Male/female)	41/59	18/22	0.54
Disease duration	9.2 ± 6.9	--	--
Body mass index (Kg/m^2)	28.7 ± 5.9	26.9 ± 5.3	0.290
HbA1c (%)	8.9 ± 1.48	5.5± 0.64	0.0024
Fasting plasma glucose (mmol/l)	9.4 ± 2.1	5.4 ± 0.8	0.0009
Systolic blood pressure (mmHg)	126 ± 11	110 ± 9.5	0.021
Diastolic blood pressure (mmHg)	92 ± 12.6	83 ± 10	0.061

Table 3.Genotypes and allele frequency found in the study

	Frequency, N (%)		*P*
	T2D (N=100)	Control (N=40)	
A/G R1273R ABCC8			
A/A	27 (27)	8 (20)	0.50
A/G	43 (43)	19 (47.5)	0.76
G/G	30 (30)	13 (32.5)	0.83
Allele G	103 (51.5)	45 (56)	0.69
Allele A	97 (48.5)	35 (44)	0.66
Pro12Ala PPAR gamma			
Pro/Pro	98 (98)	40 (100)	0.91
Pro/Ala	0	0	--
Ala/Ala	2	0	0.37
Allele Pro	196 (98)	80 (100)	0.91
Allele Ala	4 (2)	0	0.21

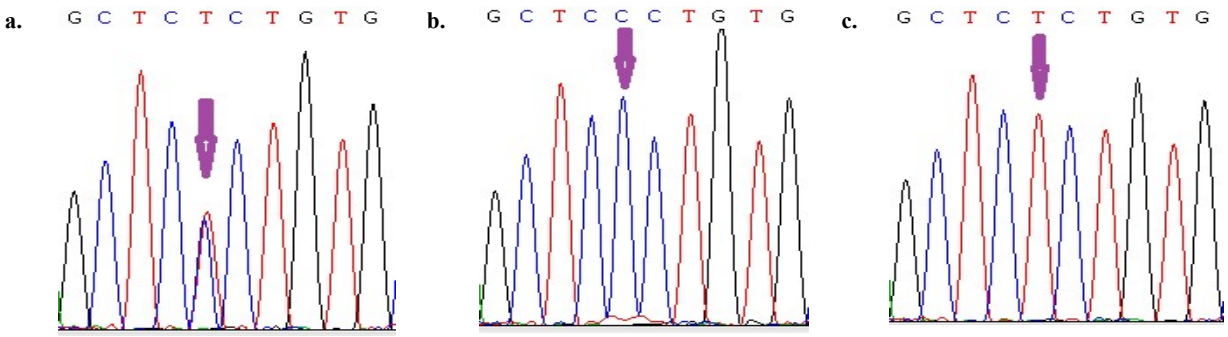

Figure 1. DNA sequencing results of ABCC8 R1273R (G/A) genetic polymorphism with reverse primer; a) heterozygous C/T, b) homozygous C, c) homozygous T

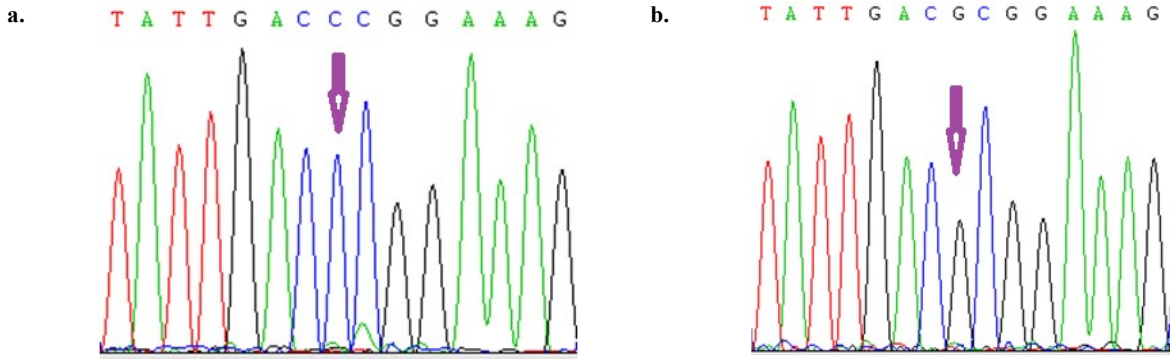

Figure 2. DNA sequencing results of PPARγ2 Pro12Ala (C/G) genetic polymorphism; a) homozygous C, b) homozygous G

DISCUSSION

People with a family history of T2DM are three times more likely to develop the diabetes (D. Sluik, *et al.,* 2014; Dorman and Bunker, 2000). Multiple genes have been reported to be involved in the onset of DM. some of these which have got more attention are ABCC8 (ATP-binding cassette transporter subfamily member 8) gene; the KCNJ11 gene; and the PPARG (peroxisome proliferator-activated receptor-gamma) gene. These are involved in the insulin release/action and/or glucose metabolism (Schwenk, *et al*., 2013).

We studied the ABCC8 R1273R (G/A)and PPARγ2 Pro12Ala (C/G) genetic polymorphism as a risk factor for the onset ofT2DM in the citizen of Rafha city of Saudi Arabia. No association between ABCC8 R1273R (G/A) and PPARγ2 Pro12Ala (C/G) genetic polymorphism and T2DM wasfound in the diabetic patients of this region. For the carriers of allele A in case of ABCC8 R1273R (G/A), a non-significant increase in the risk for T2DM onset was seen.

Different studies involving ABCC8 R1273R (G/A) and/or PPARγ2 Pro12Ala (C/G) genetic polymorphism show a wide variety of results in different population. Same is the

case when studying the association of above polymorphisms with T2D (Ludovico *et al*., 2007). Goksel *et al.,*1998,reported that the allele A ofthe exon 31 in ABCC8 gene was significantly associated with hyper-insulinemia in Mexican Americans, a population with higher prevalence of T2D. Similar results were observed in both fasting state and following an oral glucose load. Chistiakov *et al.,* 2008, showed that the allele A of ABCC8 R1273R (G/A) exon 31 polymorphism was associated with T2D. They also showed its association with altered b-cellhomeostasis in glucose-tolerant individuals.

Many studies have shown the association of PPARγ2 Pro12Ala polymorphism with the onset of T2DM and claimed that the Pro12 allele is a risk factor for the disease (Mori *et al*., 2001; Douglas *et al.,* 2001; Memisoglu *et al.,* 2003; Ghoussaini *et al.,* 2005). Ringel *et al*., 1999 and Mancini*et al.,* 1999 on the other hand have reported no such association in Germany and Italy respectively.

Some other studies have reported a trend of increased frequency of 12Ala allele rather than the Pro12 allele in the T2DM patients but they could not provide a statistically significant correlation (Lindi, *et al.,* 2002; Malecki *et al*., 2003; Evans, *et al*., 2001; Sramkova*et al*., 2002).Berhouma*et al*., 2012 reported an absence of association of PPARg with T2D in Tunisian population.

In the current study, we report that the 12Ala allele is rare whereas the Pro12 is almost equally prevalent among T2D patients and controls in this part of the world. We therefore report no significant or insignificant association between the PPARγ2 Pro12Ala polymorphism and the onset of T2DM.

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