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Lack of association between *UBE2E2* gene polymorphism (rs7612463) and type 2 diabetes mellitus in a Saudi population

Khalid Khalaf Alharbi[1], Imran Ali Khan[1]✉, Yazeed A. Al-Sheikh[1], Fawiziah Khalaf Alharbi[2], Fahad Khalaf Alharbi[3] and May Salem Al-Nbaheen[4,5]

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Kingdom of Saudi Arabia; [2]Department of Biology Science, College of Science and Arts, Al-Qassim University, Buraidah, Kingdom of Saudi Arabia; [3]Saudi Programme of Internal Medicine, King Fahad Specialist Hospital-Al-Qassim, Kingdom of Saudi Arabia; [4]Stem Cell Units, Anatomy Department, College of Medicine, King Khalid University Hospital, Kingdom of Saudi Arabia; [5]Preparatory Year-Saudi Electronic University, Riyadh, Kingdom of Saudi Arabia

The ubiquitin-conjugating enzyme E2E 2 *(UBE2E2*) gene plays an important role in insulin synthesis and secretion under conditions in which stress to the endoplasmic reticulum is increased in β-cells. In this case-control study, we have selected rs7612462 polymorphism within *UBE2E2* gene to identify in a Saudi population the type 2 diabetes mellitus (T2DM) subjects. In total, 376 subjects with T2DM and 380 controls were enrolled in this study. We have collected 5 mL of peripheral blood from each participant for biochemical and molecular analyses. PCR-RFLP was used to generate genotypes at rs7612462 in all of the study subjects. Clinical data and anthropometric measurements of the patients were significantly different from those of the controls ($p<0.05$). All of the subjects used in this study were non-obese (25<BMI<30). None of the alleles or genotypes of rs7612462 were associated with T2DM (OR=1.251, 95% CI=0.7703–2.034; $p=0.3641$). Our data suggest that rs7612462 polymorphism in *UBE2E2* does not contribute to T2DM susceptibility in the Saudi population.

Key words: T2DM, *UBE2E2*, PCR-RFLP, Saudi population

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INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) affects nearly 300 million individuals worldwide, and its prevalence (23.1%) continues to increase in many countries, including Kingdom of Saudi Arabia (Al-Daghri *et al.,* 2011). Environmental factors such as increased calorie intake, physical inactivity, and obesity are known to contribute to the recent diabetes epidemic (Park *et al.,* 2011). Although the precise mechanisms underlying the development and progression of T2DM have not been elucidated, a combination of multiple genetic and/or environmental factors contribute to the pathogenesis of the disease (Iwata *et al.,* 2012). Impaired insulin secretion and insulin resistance, the two main pathophysiological mechanisms leading to T2DM, have a significant genetic component (Stancakova *et al.,* 2009). Clinical and epidemiological studies have indicated that obesity is a major risk factor for T2DM, as obesity is associated with an increased risk of developing insulin resistance and impaired glucose tolerance (Yamakawa-Kobayashi *et al.,* 2012).

Ubiquitin-conjugating enzyme E2E 2 (*UBE2E2*) gene on chromosome 3p24.2 encodes the ubiquitin-conjugating enzyme E2E, which plays an important role in insulin synthesis and secretion under conditions where endoplasmic reticulum stress is increased in β-cells. A single nucleotide polymorphism (SNP; rs7612463) in *UBE2E2* was identified as a T2DM susceptibility locus by a genome-wide association study (GWAS) conducted in a Japanese population (Yamauchi *et al.,* 2010). The silent C91227A substitution in intron 3 of *UBE2E2* was not significant for T2DM disease. In the present study, we analyzed the relationship between rs7612463 and T2DM susceptibility in a Saudi population.

MATERIAL AND METHODS

Selection of subjects. 376 T2DM patients and 380 healthy controls used in this study were recruited from King Khalid University Hospital (KKUH), Riyadh, Saudi Arabia. All T2DM subjects had developed the disease more than 5 years prior to their enrollment, and all had a fasting glucose level of 126 mg/dL or >7.0 mmol/L, following standards established by the World Health Organization. Individuals with a history of other metabolic disorders apart from T2DM were excluded from the study. Healthy volunteers ($n=380$) had normal glucose levels, although some had a family history of T2DM. Cases and controls were properly matched for age and gender. All participants gave informed consent and the study was approved by a local ethics committee from KKUH.

Sample collection. A total of 5 mL of venous blood was collected from each participant; 3 mL of serum was used for biochemical analysis and 2 mL was stored with EDTA and used for molecular analysis.

Anthropometric measurements. Participants' weight, height, waist and hip circumference, and blood pressure were measured as previously described (Alharbi *et al.,* 2012). Hypertension was defined as blood pressure measurements > 140/90 mmHg with

✉e-mail: imkhan@ksu.edu.sa

Abbreviations: T2DM, type 2 diabetes mellitus; UBE2E2, ubiquitin-conjugating enzyme E2E 2; GWAS, genome-wide association study; BMI, body mass index; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

or without antihypertensives. Body Mass Index (BMI) was calculated as weight (in kg) divided by height (in m^2) squared (kg/m^2).

Biochemical parameters. Fasting blood biochemical parameters: HDL-C, triglycerides, total cholesterol, and plasma glucose were measured using an automated clinical chemistry analyzer (Kit provided by KoneLab, Espoo, Finland). Concentrations of LDL-C were calculated using Friedwald's formula. Insulin was quantitated by immunoassay (Medgenix INS-ELISA, Biosource, Belgium). Insulin resistance was measured by the homeostasis model assessment (HOMA-IR), using the formula: Insulin resistance = insulin (μU/ml) × glucose (mmol/l)/22.5. Dyslipidemia (low levels of HDL-C) was defined as HDL-C levels < 1.03 mmol/l for men and < 1.29 mmol/l for women (Alharbi *et al.,* 2012).

DNA extraction and UBE2E2 genotyping. Genomic DNA was extracted from peripheral blood leukocytes using a Norgen DNA extraction kit (Norgen Biotek Corp, Canada). DNA samples were stored at –80°C. Genotyping of rs7612463 was performed by Polymerase Chain Reaction (PCR) using the Norgen 2X master mix, followed by the application of the Restriction Fragment Length Polymorphism (RFLP) method and agarose gel electrophoresis. Specific primers were designed using the primer 3 plus software: forward primer, 5'-TCAAGACGTGGCTCATCT-GT-3'; reverse primer, 5'-ATGTCACTTGCAGCC-CTCTT -3'. Primers were synthesized by Bioserve Biotechnology, (Hyderabad, India). For PCR reactions, DNA was denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 45 sec, and a final extension step at 72°C for 5 minutes. For RFLP analysis, 15 μL of the PCR product was incubated with 1.0 μL of AluI [AG↓CT] (10U/μL, New England BioLabs, USA) in a final volume of 20 μL for 2 hours at 65°C. Digested products were run on 2% agarose for 60 minutes at 250 V. Bands were analyzed using a transilluminator.

Statistical analysis. All values are expressed as mean ± S.D. Results were analyzed by *t*-test, with a *p* value threshold of 0.05. Genotypic and allelic frequencies were compared between T2DM cases and control subjects. Allele frequencies were estimated by the gene counting method, and the Chi square test was used to identify departures from Hardy–Weinberg equilibrium. Statistical significance was examined by two-sided tests, and statistical analyses were performed with SPSS version 16.0 software.

RESULTS

Patient Characteristics

Clinical and anthropometric data for control and T2DM patients are shown in Table 1. Results revealed that Anthropometric measurements, including hip circumference were also significantly higher in T2DM patients were significantly older than controls ($p<0.05$). T2DM subjects appear to have higher levels of fasting glucose and lipids (TG, TC, HDL-C and LDL-C), as well as higher systolic and diastolic blood pressures but not HDL-C ($p<0.05$). BMI and waist circumference were not different between cases and controls ($p>0.05$).

Allele and genotype frequencies at rs7612463 in cases and controls

All three potential genotypes, AA, AC, and CC, were detected at rs7612463 in *UBE2E2* gene. Their frequencies in T2DM cases and controls are shown in Table 2. The distribution of genotypes at rs7612463 deviates Hardy–Weinberg equilibrium and was not significantly different between cases and controls ($p>0.05$; Table 3). rs7612463 AA genotype and A allele (CA+AA *vs.* CC) were not associated with increased risk of T2DM (OR = 1.249, 95% CI = 0.7056–2.21; $p = 0.4448$). Likewise, under the additive model, the A allele was not significantly associated with an increased risk of T2DM (OR = 1.251, 95% CI = 0.7703–2.034; $p = 0.3641$). In addition, there was no evidence for an association between rs7612463 and risk of T2DM under the recessive model (AA *vs.* CA + CC) or the co-dominant model (CA *vs.* CC+AA) (OR = 1.223, 95% CI = 0.6072–2.465; $p = 0.5720$).

Table 1. Demographic characteristics of the study population.

	T2DM (*n*=376)	Controls (*n*=380)	*p*
Age (Years)	50.63±10.39	46.06±7.66	<0.0001
Body mass index (kg/m^2)	29.51±5.92	29.19±5.53	0.18
Sex: Male/Female	(59.8%)/ (40.2%)	(53.2%)/ (46.8%)	0.003
SBP (mmHg)	123.94±11.74	114.96±7.84	0.0001
DBP (mmHg)	78.21±7.43	75.78±20.26	0.01
Waist (cms)	94.3±22.36	91.2±20.26	0.055
Hip (cms)	104.83±21.44	94.46±7.80	<0.0001
FBS (mmol/L)	9.89±5.22	5.25±0.60	<0.0001
Triglycerides (mmol/L)	2.23±1.65	1.61±0.86	<0.0001
Cholesterol (mmol/L)	5.63±1.26	5.05±0.97	0.001
HDL-cholesterol (mmol/L)	0.93±0.75	0.63±0.23	<0.0001
LDL-cholesterol (mmol/L)	3.79±1.07	3.68±0.85	0.0008
Glucose (mmol/L)	9.4±1.5	8.69±1.82	0.0001
Insulin (μU/ml)	16.2±2.2	12.3±1.7	0.0006
HOMA-IR	7.1±2.4	3.15±1.9	<0.0001

Table 2. Genotype and allele frequencies at rs7612463.

rs7612463	T2DM Cases (*n*=376)	Controls (*n*=380)	OR	95% CI	χ^2	p value
CC	348 (92.6%)	357 (94%)	Reference			
CA	18 (4.8%)	15 (3.9%)	1.231	(0.61–2.48)	0.33	0.56
AA	10 (2.6%)	8 (2.1%)	1.281	(0.50–3.28)	0.26	0.60
CA+AA	28 (7.4%)	23 (6)	1.249	(0.70–2.21)	0.58	0.44
C	714 (0.95)	729 (0.96)	Reference			
A	38 (0.05)	31 (0.04)	1.252	(0.77–2.03)	0.82	0.36

DISCUSSION

Availability of molecular studies by PCR-RFLP, DNA sequencing and *UBE2E2* gene allows accurate diagnosis and characterization in T2DM subjects. In this case-control study, we genotyped in Saudi T2DM subjects at rs7612463 in *UBE2E2* gene. T2DM is a complex disease that is categorized by insulin resistance and impaired β-cell function. Prevalence of T2DM is increasing at an alarming rate, in parallel with expanding obesity rates worldwide, making it a major public health concern. Search for genetic determinants of T2DM has changed dramatically. Linkage analysis and small-scale candidate gene studies were highly successful in the identification of risk genes such as *PPARG* and *KCNJ11*, which are strongly implicated in T2DM susceptibility. Large scale association analysis, including GWAS, was the first completion of the human genome sequence, which in turn led to the detailed maps of common single nucleotide polymorphism (SNPs) and patterns of linkage disequilibrium (Wheeler *et al.,* 2011).

GWASs conducted in Japanese T2DM cohorts have identified *KCNQ1, UBE2E2, C2CD4A-C2CD4B*, and *ANK1* as susceptibility loci (Yamauchi *et al*., 2010; Unoki *et al*., 2008; Yasuda *et al*., 2008; Imamura *et al*., 2012), all of which, except for *UBE2E2*, have also been associated with T2DM in European populations (Yamauchi *et al*., 2010; Unoki *et al*., 2008; Yasuda *et al*., 2008; Imamura *et al*., 2012). This illustrates the importance of carrying out GWASs in multiple ethnic populations to identify the risk loci that are either ethnicity-specific or common to many populations (Fukuda *et al.,* 2012).

UBE2E2 gene has been reported to be expressed in human pancreas, liver, muscle, and adipose tissues, as well as in a cultured insulin secreting cell line. It has been reported that the ubiquitin proteasome system plays a pivotal role in maintaining normal insulin biosynthesis, secretion, and signaling, especially under conditions that increase the stress in the endoplasmic reticulum of pancreatic β-cells (Hartley *et al.,* 2009).

Earlier studies showed that proteasome inhibition by pharmacological inhibitors reduced proinsulin biosynthesis (Kitiphongspattana *et al.,* 2005), the molecules involved in insulin secretion (Kawaguchi *et al.,* 2006), and glucose stimulated insulin secretion (Lopez-Avalos *et al*., 2006; Matthews *et al*., 1985), whereas other investigators reported that proteasome inhibitors enhanced acute glucose induced insulin secretion in isolated rat islets (Warton *et al.,* 2004). Both results suggest that the ubiquitin proteasome system plays an important role in insulin secretion.

Yamauchi and coworkers (2010) showed that in *UBE2E2* gene Fasting plasma glucose and Insulin levels of CC+CA genotypes (*n*=846) showed a significantly lower Homa-β (Homeostasis model assessment of β-cell function) than those with the risk of AA genotypes (*n*=26).

In this study, we investigated the potential association between rs7612463 in *UBE2E2* gene and the risk of T2DM in a Saudi population. Our study on rs7612463 polymorphism was carried out among the Saudi population, although this polymorphism was identified from the GWAS studies in Japanese population. Consistent with our results, rs7612463 was not previously associated with T2DM in a Japanese population (Yamauchi *et al.,* 2010). We observed the distribution of the three genotypes to be 92.6%, 4.8% and 2.6% in the T2DM cases and 94%, 3.9% and 2.1% in the controls. A number of studies have investigated the association between rs7612463 and T2DM risk (Yamakawa-Kobayashi *et al*., 2012; Yamauchi *et al*., 2010). However, results from these studies are conflicting. This is the first study to carry out an association study of rs7612463 and T2DM in a Saudi population as well as in Arab countries. A limitation of the current study includes the selection of a single specific SNP. Moreover, we conducted the current study with a single rs number and did not consider the interactions between the gene and its protein, what would require further studies.

In conclusion, our results revealed that rs7612463 was not associated with T2DM which were non-obese. Additional studies of rs7612463 in T2DM patient cohorts from other ethnic backgrounds should be conducted to further validate our results. Functional studies remain to be performed to establish the precise roles of these variants and pathways.

Table 3. Statistical analysis conducted for rs7612463.

Genotypes	Odds ratio and pvalue with 95%CI
AA Vs CA+CC	OR = 1.27, 95%CI = 0.4959–3.255; p = 0.6172
CA+AA Vs CC	OR = 1.249, 95%CI = 0.7056–2.21; p = 0.4448
CA Vs CC +AA	OR = 1.223, 95%CI = 0.6072–2.465; p = 0.5720
A Vs C	OR = 1.251, 95%CI = 0.7703–2.034; p = 0.3641

Conflict of Interests

We declare that there is no conflict of interest.

Authors Contribution

Design of the work: AKK, and IAK. Sample collection and genotyping: IAK, AYA and AMS. Statistical analysis: AFF and AFF. Manuscript written, and edited: AKK and IAK.

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