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ABSTRACT This study aimed to estimate the frequency of the SNPs (+45T>G and +276G>T) genotypes and investigate the association between the two SNPs and adiponectin concentration, metabolic parameters and risk of T2DM in the Bahraini population. We genotyped the two ADIPOQ SNPs in 140 unrelated T2DM patients and 66 nondiabetic controls using the polymerase chain reaction-restriction fragment length polymorphism assay. Lipid profile was measured by enzymatic methods. Total serum adiponectin levels were measured by immunoassay. T2DM patients had reduced adiponectin levels compared with controls. +45T>G was more prevalent in patients than controls. The rare G allele of +45T>G occurred more frequently than the common T allele in T2DM patients compared with controls, and was associated with lower serum adiponectin levels. There was no significant difference in allele and genotype frequencies of +276G>T between type T2DM patients and controls. There was no association between both SNPs and metabolic parameters.

Associations entre les polymorphismes mononucléotidiques du gène ADIPOQ, l'adiponectine sérique et le risque accru de diabète de type 2 dans la population bahreïnienne

RÉSUMÉ La présente étude avait pour objectif de mesurer la fréquence des polymorphismes mononucléotidiques (+45T>G et +276G>T) des génotypes et d'évaluer l'association entre ces deux polymorphismes et la concentration d'adiponectine, les paramètres métaboliques et le risque de diabète non insulino-dépendant (DNID) dans la population bahreïnienne. Nous avons génotypé les deux polymorphismes mononucléotidiques du gène ADIPOQ chez 140 patients atteints de DNID sans lien de parenté et 66 témoins non diabétiques en recourant à l'analyse du polymorphisme de longueur des fragments de restriction par réaction en chaîne de polymérase. Le profil lipidique a été mesuré au moyen de méthodes enzymatiques. Les concentrations d'adiponectine totale sérique ont été mesurées par immunodosage. Les patients atteints de DNDI affichaient des concentrations d'adiponectine réduites par rapport aux témoins. Le polymorphisme +45T>G avait une prévalence plus élevée chez les patients que chez les témoins. L'allèle rare G du polymorphisme +45T>G apparaissait plus fréquemment que l'allèle commun T chez les patients atteints de DNID que chez les témoins, et était associé à des concentrations d'adiponectine sérique plus faibles. Il n'existait pas de différence significative entre les fréquences des allèles et des génotypes du polymorphisme +276G>T entre les patients atteints de DNID et les témoins. Aucune association entre les deux polymorphismes et les paramètres métaboliques n'a été notée.

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Introduction

T2DM is a major public health problem worldwide. Five GCC countries are among the world's top 10 highest for diabetes prevalence: Kuwait, Oman, Saudi Arabia, United Arab Emirates and Bahrain, which is ranked third (1,2). The International Diabetes Federation estimated that the prevalence of diabetes in the Bahraini population aged 20 years was 20% in 2011 (3). Statistics from the World Health Organization predict that the number of patients with diabetes in Bahrain will triple by the year 2025.

Recently, adipose tissue has become increasingly seen as a metabolically active endocrine and paracrine organ. It secretes several mediators, known as adipokines, which participate in diverse metabolic processes (4). There is great interest in clarifying the role of adipokines as possible mediators between obesity, insulin sensitivity and diabetes. Adiponectin is a protein secreted mainly from adipocytes, which plays an important role in regulation of lipid and glucose metabolism (5). Several studies have demonstrated that serum adiponectin concentrations are decreased in individuals with obesity (2), insulin resistance, dyslipidaemia (6) and T2DM (7). Thus, adiponectin may have a causal role in the pathogenesis of T2DM and insulin resistance. The ADIPOQ gene has been identified and located on human chromosome 3q27 (8). Saito et al. determined that ADIPOQ spans 16kb and contains 3 exons and 2 introns (8). Genome-wide scans in humans have suggested that this chromosomal region is a susceptibility locus for T2DM and metabolic syndrome (9).

Two SNPs at the ADIPOQ locus have been extensively studied: a silent T to G substitution in exon 2 (+45T>G, rs2241766) and a G to T substitution in intron 2 (+276G>T, rs1501299). However, association studies of these two SNPs, either independently or as a haplotype, with T2DM, adiponectin levels and insulin sensitivity have given conflicting results in different populations and sample types (10,11,12,13, 14). It has been seen that the association between these SNPs and T2DM differs among populations suggesting that a genetic factor might be involved. Further studies are needed to elucidate the effect of genetic background on the role of adiponectin.

In the present study, we estimated the frequency of SNPs (+45T>G and +276G>T) genotypes and investigated the association between these two SNPs with adiponectin concentration and metabolic syndrome parameters, in a representative sample from Bahrain. To the best of our knowledge, this is the first study to investigate the association between variants of ADIPOQ and serum adiponectin level and its relation to T2DM and metabolic syndrome in a Bahraini population.

Methods

Study participants

We enrolled using convenience sampling 140 unrelated patients with T2DM (age 47 years; 74 women and 66 men) attending the Diabetic Clinic at the Bahrain Defence Force (BDF) Hospital for routine follow-up, and 66 unrelated healthy control individuals without diabetes (age 46 years; 6 women and 60 men) during August 2009 to April 2011. The presence or absence of T2DM was established according to the American Diabetes Association criteria (15), in which diabetes is defined as fasting blood glucose (FBG) ≥126 mg/dL (7 mmol/L). Patients with T1DM and women with gestational diabetes were excluded. The controls were from the same

geographic areas as the patients and were individuals who visited the blood bank of the BDF hospital for blood donations. The following exclusion criteria were applied to the controls: fever (temperature ≥38 °C), hospitalization in the previous 2 weeks, known diabetes, arterial hypertension [systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg], dyslipidemia [total cholesterol (TC) ≥6.21 mmol/L (240 mg/dL), triglyceride 1.7 mmol/L (150 mg/dL), high-density lipoprotein-cholesterol (HDL-C) ≤1.03 mmol/L (40 mg/dL)], coronary heart disease, congestive heart failure, stroke, chronic renal disease, active hepatic disease. Individuals were also excluded if they were taking any medication known to affect metabolic profile.

The study protocol was approved by the ethics committees of the Royal College of Surgeons in Ireland – Medical University of Bahrain (RCSI-Bahrain) and the BDF Hospital. Written or verbal informed consent was obtained from the study participants in accordance with the Helsinki Declaration.

Anthropometric and blood pressure measurements

The study participants were asked to complete a self-administered questionnaire on general health. Where patients were unable to complete the questionnaire by themselves, because of illiteracy, assistance was offered. Obesity was defined as body mass index (BMI) ≥30 kg/m2. For all participants, SBP, DBP, body weight and height were measured, and BMI was calculated as weight divided by height squared.

Blood collection

Venous blood samples (3 mL) were drawn from all participants after an overnight fast for at least 12 hours. The serum and red blood cells were separated immediately by centrifugation at 3,000 g for 10 minutes at room temperature and serum was stored at –80 °C until biochemical analysis. Separate 3 mL venous blood samples were collected in EDTA-coated tubes and stored at –80 °C until DNA extraction for SNP analysis.

Biochemical measurements

Blood samples were analysed for biochemical variables. Haemoglobin A1c was assayed using the latex agglutination reaction on a Roche Cobas c111 automatic analyser (Roche Diagnostics, Indianapolis, IN, USA). FBG, TC, triglyceride, HDL-C and LDL-C were assayed using the Roche Cobas 6000 automatic analyser. FBG was measured using the hexokinase/glucose-6-phosphate dehydrogenase method (16), and serum levels of triglyceride,

TC and HDL-C and LDL-C were measured by enzymatic colorimetric test (17).

Total adiponectin levels were measured by quantitative sandwich enzyme immunoassay technique (Quantikine Human Adiponectin ELISA kit; R&D Systems, UK). Adiponectin and biochemical measurements were not performed in 29 samples (11 diabetic and 18 controls) because the participants presented in a nonfasting state.

Genetic analysis

Genomic DNA was isolated from whole blood samples using the phenol–chloroform DNA extraction method (18). Polymerase chain reaction (PCR) amplification was carried out using specific primers for human ADIPOQ, as previously reported by Mackevics et al. (19). For the SNP +45T>G, a 305-bp DNA fragment containing the polymorphic site was amplified by PCR in a DNA Engine Peltier Thermal Cycler (PTC-0200; Bio-Rad, Hercules, CA, USA) using forward primer 5′-TGTGTGTGTGGGGTCTGTCT-3′ and reverse primer 5′-TGTGATGAAAGAGGCCAGAA-3′. For the SNP +276G>T, A 110-bp DNA fragment was amplified by PCR using forward primer 5′-CTACACTGATATAAACTATATGGAG-3′ and reverse primer 5′-CCCCAAATCACTTCAGGTTG-3′. PCR was carried using 100 ng DNA in 50 µL containing 5 µL 10× Biotherm PCR buffer (Genecraft, Germany) with a final concentration of 1.5 mM magnesium chloride, 0.2 µM each primer, 200 µM dNTPs and 2.5 U Biotherm Taq Polymerase (Genecraft). Thirty-five cycles of amplification were performed and each cycle consisted of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72°C for 30 seconds, with additional steps of initial denaturation at 95 °C for 5 minutes and final extension at 72 °C for 7 minutes (19).

The PCR product (10 □L) of SNP +45T>G (305 bp) was digested with 10 U Eco88I (AvaI) in the recommended 10× Tang Buffer (ER0381; MBI Fermentas, Thermo Fisher Scientific, USA) at 37 °C overnight. The PCR product (10 □L) of SNP +276G>T (110 bp) was digested with 10 U HinfI in the recommended 10× Buffer R (ER0801; MBI Fermentas, Thermo Fisher Scientific) at 37 °C overnight. The fragments were separated on 12% native polyacrylamide gel. The DNA fragments were stained with ethidium bromide and visualized by UV illumination.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). Genotype and allele frequency distributions were compared using the $\chi 2$ test. Hardy–Weinberg equilibrium (HWE) was calculated to the expected genotype distribution. Comparisons between means were performed using Student's independent t test (two-tailed) for normally distributed data or the Mann–Whitney U test for parameters presenting with non-normal distribution. Categorical variables were analysed by the chi-squared test. Univariate

general linear model analysis was carried out to investigate the effect of SNPs+45T>G and +276G>T on serum adiponectin concentrations and the various metabolic parameters. All analyses were adjusted for gender, age and BMI. P

Results

Association between SNP +45T>G of ADIPOQ and T2DM

To assess whether SNPs +45T>G and +276G>T of ADIPOQ contribute to T2DM, we genotyped 140 T2DM patients and 66 control individuals by PCR-restriction fragment length polymorphism (RFLP). After polyacrylamide gel electrophoresis, genotypes were determined by examining the length of the restriction fragments for SNP +45T>G (Figure 1). For SNP +45T>G, fragments of 305 bp for the TT wild-type homozygote (absence of AvaI cutting site) and 105 and 200 bp for the GG mutant homozygote (presence of AvaI cutting site) were produced. The heterozygote TG included 3 fragments of 105, 200 and 305 bp.

Genotype frequencies were found to be in agreement with HWE, which is described by the binominal distribution: p2+2pq+q2=1.0, thus fulfilling this requirement for the present study population. Testing for HWE was done using the $\chi 2$ test ($\chi 2 = 5.36$, $P = 0.02$ in T2DM patients and $\chi 2 = 0.07$, $P = 0.50$ in controls). In the T2DM group, 100 (71.4%) patients were homozygous for the wild-type allele (TT), 32 (22.9%) were heterozygous (TG) and only 8 (5.7%) were homozygous for the mutation (GG) (Table 1). In the non-diabetic control group, 56 (84.8%) individuals were homozygous for the wild-type allele (TT), 10 (15.2%) were heterozygous (TG) and none was homozygous for the mutation (GG). The frequencies of TG and GG genotypes were significantly higher in the T2DM group than the control group. In contrast, the frequency of the TT genotype was significantly higher in the control group. Individuals with the GG genotype were at increased risk for T2DM compared with those having the TT genotype (relative risk = 2.3, confidence interval = 95%, $P = 0.0137$).

The T allele frequency was significantly higher in the control group (92.4%) than the T2DM group (83%) ($P = 0.009$) (Table 1). In contrast, the G allele frequency was significantly higher in the T2DM group (17%) than the control group (7.6%) ($P = 0.009$).

Association between SNP +276G>T of ADIPOQ and T2DM

After polyacrylamide gel electrophoresis, genotypes were determined by analysing the length of the restriction fragments for SNP +276G>T (Figure 2). For SNP +276G>T, fragments of 110 bp for the GG wild-type homozygote (absence of HinfI cutting site) and 26 and 84 bp for the TT mutant homozygote (presence of HinfI cutting site) were produced. The heterozygote GT included 3 fragments of 26, 84 and 110 bp.

Genotype frequencies were found to be in agreement with the HWE. Testing for HWE was done using the $\chi 2$ test ($\chi 2 = 12.7$, $P = 0.0003$ in T2DM patients and $\chi 2 = 8.1$, $P = 0.004$ in controls). In the T2DM group, 130 (92.9%) patients were homozygous for the wild-type allele (GG), 8 (5.7%) were heterozygous (TG) and only 2 (1.4%) were homozygous for the mutation (TT) (Table 2). In the nondiabetic control group, 58 (87.9%) individuals were homozygous for the wild-type allele (GG), 6 (9.1%) were heterozygous (TG) and only 2 (3%) were homozygous for the mutation (TT). There were no significant differences in genotype distribution between the T2DM and control groups.

The G allele frequency was 95.7% in the T2DM group and 92.4% in the control group (Table 2) . The T allele frequency was 4.3% in the T2DM group and 7.6% in the control group. Neither of these differences was significant.

Relationship between SNPs +45T>G and +276G>T and serum adiponectin levels

Table 3 shows the mean serum adiponectin concentrations according to SNP +45T>G and +276G>T genotypes in the T2DM and control groups. The mean values were divided via a general linear model adjusting for age, gender and BMI. Twenty-nine samples (11 T2DM and 18 controls) without measurement of adiponectin were excluded from the analysis. Serum adiponectin levels were significantly lower in the T2DM than in the control group (P G. There was a clear multiplicative trend of decreasing adiponectin levels per copy of the rare G allele. The G allele was associated with lower serum adiponectin levels compared with the T allele carriers (6.5 ± 4.1 µg/mL for T/T genotype, 3.7 ± 3.4 µg/mL for the T/G genotype and 2.1 ± 2.2 µg/mL for the G/G genotype, P

For SNP +276G>T, there was no significant difference in the mean serum adiponectin levels between the three genotype subgroups of SNP +276G>T (Table 3).

Effects of serum adiponectin levels and SNPs+45T>G and +276G>T on features of metabolic syndrome

To assess whether serum adiponectin levels and SNPs+45T>G and +276G>T had any effect on metabolic syndrome, we compared anthropometric and metabolic characteristics among genotype subgroups. A series of metabolic and clinical parameters (age, BMI, SBP and DBP, HbA1c, TC, HDL-C, LDL-C, triglyceride, FBG and insulin resistance estimated by triglyceride/HDL-C index) were analysed with respect to their association with serum adiponectin levels and SNP +45T>G and +276G>T genotypes in T2DM patients. As a result of incomplete clinical data for the control sample, the correlation was only evaluated for T2DM patients. Considering all T2DM patients, serum adiponectin level showed significant inverse correlations with HbA1c and triglycerides ($P = 0.039$ and $P = 0.020$ respectively), and a positive correlation with HDL-C ($P = 0.036$). No association was found with the remaining metabolic parameters ($P > 0.05$). No significant difference in the metabolic parameters among G/G, T/G

and T/T carriers of SNPs+45T>G and +276G>T was observed in both genders (P > 0.5).

Discussion

In the present study, we observed that adiponectin SNP +45T>G was associated with risk of T2DM and adiponectin levels in a Bahraini population, but no significant association was seen with SNP +276G>T. We also observed that the G allele of SNP +45T>G occurred more frequently than the T allele in Bahraini T2DM patients compared to controls. Moreover, the G allele were associated with lower serum adiponectin levels and increased risk for T2DM. However, the data did not show an association of either of these two SNPs with features of metabolic resistance.

Several susceptibility loci for T2DM have been identified by genome-wide linkage scans (20). Two common SNPs in ADIPOQ, +45T>G in exon 2, and +276G>T in intron 2, have been widely studied in several populations including Europeans, Asians and Americans (21,22). Although there is a lack of consistency among studies, the results indicate that variation in ADIPOQ is associated with insulin resistance and T2DM (23,24). Ramya et al. found that SNP +276G>T of ADIPOQ was strongly associated with increased risk of development of T2DM, obesity and hypoadiponectinaemia in 1100 unrelated Indian patients with T2DM (23). Also, in a Spanish population, Gonzalez-Sanchez et al. found that both SNPs +45T>G and +276G>T were associated with decreased serum adiponectin levels and risk of impaired glucose tolerance in 747 unrelated Spanish individuals (12).

However, similar associations were not revealed in a Korean (13) or Chinese (25) population. In the Korean study, plasma levels of adiponectin did not differ significantly according to SNPs+45T>G and +276G>T in either control individuals or T2DM patients. The genotype distribution of these SNPs had no association with the risk of T2DM or metabolic syndrome. In the Chinese study however, only variation at SNP +45T>G was associated with T2DM.

Our results are in agreement with the study of Zacharova et al. who found that the G allele of SNP +45T>G was associated with lower serum adiponectin levels and increased risk for T2DM in the STOP-NIDDM trial (which was conducted in Canada, Germany, Austria, Norway, Denmark, Sweden, Finland, Israel and Spain) (11). Similar results were observed by Mohammadzadeh and Zarghami in an Iranian population (26).

Cross-sectional studies have yielded inconsistent findings concerning the association of SNP +45T>G with T2DM. These conflicting findings could be due to true differences in allelic

association with the disease phenotype in various ethnic populations, which may be the result of different environmental and genetic influences in those populations (27). Our data suggest that this SNP may play a direct role in altering ADIPOQ expression in Bahraini population.

Our observations that the G allele of SNP +45T>G is associated with T2DM and with serum adiponectin levels might be explained by several mechanisms that have yet to be demonstrated conclusively. T2DM is characterized by lower levels of serum adiponectin compared to those in individuals without diabetes. Therefore, the role of the G/G genotype of SNP +45T>G in increasing the risk of T2DM in some populations is consistent with its role in decreasing serum adiponectin levels. This could also be due to the effect of adiponectin on both glucose and lipid metabolism (5). Therefore, deficiencies in adiponectin could presumably trigger development of T2DM.

The mechanism by which these ADIPOQ variants affect adiponectin levels and diabetes in different populations remains unclear. Although SNP +45T>G does not cause an amino acid change (GGT to GGG, Gly15Gly), Yang et al. suggested that the silent +45T>G mutation alters RNA splicing or stability, resulting in allele-specific differential expression of adiponectin (27).

It is also reported that SNP +45T>G is in strong linkage disequilibrium with other SNPs of ADIPOQ or other nearby genes that may affect adiponectin expression, secretion, structure or action, such as SNPs –11377 and –11391 in the 5′ promoter region (28).

Although we observed an association between variations in ADIPOQ and adiponectin levels, we did not detect an association between either SNP and the anthropometric and metabolic parameters related to insulin resistance. It is reasonable to infer that the genetic effects of SNP +45T>G on T2DM are mediated through altered expression of ADIPOQ that eventually affects some of the metabolic parameters. Nevertheless, statistical analysis did not reveal any significant association of either SNP with the majority of the clinical and metabolic parameters. This discrepancy has also been shown in other studies in which both SNPs were related to adiponectin concentration but not to the metabolic parameters (19).

Several circumstances might have contributed to this observation. The causal association between metabolic syndrome parameters and T2DM is not entirely clear, and different sets of genes might be involved in the pathogenesis of the two conditions. The metabolic parameters of insulin resistance might be determined by serum adiponectin levels or ADIPOQ expression, as well as other multiple genetic and environmental factors or lifestyle interventions in different

populations and sample sizes (29).

In conclusion, this study showed that T2DM is associated with low serum adiponectin concentrations. We also showed that ADIPOQ SNP +45T>G was associated with risk of T2DM and low serum adiponectin levels. The current study provides evidence of potential involvement of ADIPOQ as a risk factor for T2DM in the Bahraini population, independent of other known risk factors such as obesity and insulin resistance.

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