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Regular paper

Association of *RETN* +299(G>A) polymorphism with type two *diabetes mellitus*

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The global prevalence of type-two *diabetes mellitus* (T2DM) makes it a disease of public health concern. T2DM is strongly linked with insulin resistance caused by increased levels of visceral fat. Visceral fat secretes several adipocytokines that regulate body metabolism. Resistin is one of these adipocytokines which is encoded by the *RETN* gene. Herein, we tested the association of the *RETN* +299(G>A) and −420(C>G) single nucleotide polymorphisms (SNPs) with T2DM. T2DM patients (n=282) and healthy subjects (n=125) were included in the study. Subjects with metabolic syndromes other than diabetes were excluded. Genotyping of subjects was performed using PCR-RFLP. The +299(G>A) SNP was associated with T2DM (*P*=0.038). The AA genotype was higher in T2DM (17%) compared to controls (8%) with an odd ratio of 2.16 and 95% CI of 1.34 to 4.56. With respect to −420(C>G) SNP, no significant association was found with the risk of T2DM (*P*=0.128). The haplotype analysis of the examined SNPs indicated that the CA haplotype of the −420 and +299 SNPs in *RETN* was associated with T2DM risk (*P*=0.004; odd ration 4.0, 95% CI: 1.56–10.0). In conclusion, the present findings suggest a role of the *RETN* locus in modulating the risk of T2DM.

Keywords: resistin, type two diabetes mellitus, single nucleotide polymorphisms, haplotype, risk, +299 SNP, −420 SNP

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Abbreviations: ADA, American diabetes association; BF, Body fat; BMI, Body mass index; BW, body weight; DM, diabetes mellitus; FBG, Fasting blood glucose; HOMA, Homeostatic model assessment; LBM, Lean body mass; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; SNP, Single nucleotide polymorphism; T2DM, Type-two diabetes mellitus

INTRODUCTION

Diabetes mellitus (DM) is a debilitating metabolic disease linked with atherosclerosis, hypertension and kidney failure (Schmidt, 2018). In 2015, more than 400 million people were diagnosed with DM, with the majority of the patients living in low to middle income countries. The disease is currently recognized as a heterogeneous group of diseases manifested by chronic hyperglycemia. The most common form of DM is type two (T2DM), which is caused by insulin resistance in target cells in the body (Schmidt, 2018). T2DM is strongly linked with abdominal obesity; a major cause of insulin resistance (Kahn *et al.*, 2006). Interestingly, some individuals appear to be genetically more predisposed than others to developing T2DM. Indeed, genetic variations in several loci has been found to modulate the risk of T2DM in the human population (Ingelsson & McCarthy, 2018).

Abdominal obesity is associated with visceral fat accumulation and insulin resistance. Visceral fat has been shown to secrete several hormones, known as adipocytokines, which appear to modify the tissues' response to insulin secretion (Ouchi, 2016). Resistin, a cysteine rich protein, is an example of one of these hormones. In a case-control study performed on T2DM patients attending a tertiary hospital in Jordan, Gharibeh and colleagues reported that resistin levels were higher in T2DM compared to non-diabetic subjects (Gharibeh *et al.*, 2010). Interestingly, in the case of T2DM, positive correlations were reported between the serum resistin concentration and the HOMA (Homeostatic Model Assessment) index, which reflects insulin resistance in patients (Gharibeh *et al.*, 2010).

Resistin is encoded by the *RETN* gene (Hu *et al.*, 2015). Genetic variants in the *RETN* gene may affect resistin expression and consequently its levels (Hivert *et al.*, 2009). Given the association between the resistin protein and T2DM, we hypothesized that *RETN* gene variants may modulate the risk of T2DM.

The two commonly investigated SNPs in *RETN* gene are −420(C>G) and +299(G>A) (Amal *et al.*, 2013; Wen *et al.*, 2013). The −420(C>G) is found in the promoter region and was shown to affect *RETN* transcription and the subsequent resistin level (Azuma *et al.*, 2004, Osawa *et al.*, 2004). The +299(G>A) SNP is located in the 2nd intron and was also shown to be associated with resistin level. Previous literature has shown that +299 SNP was associated with T2DM in different populations (Chung *et al.*, 2014, de Luis *et al.*, 2020, Thammakun *et al.*, 2017, Zhang *et al.*, 2013). Similarly, –420 SNP has been shown to be associated with T2DM among Pakistani population (Nadeem *et al.*, 2018). Herein, we tested the association of the −420(C>G) and +299(G>A) SNPs in *RETN* gene with T2DM among Jordanians.

MATERIALS AND METHODS

Design and participants

The study design included a retrospective case-control. Institutional Review Boards of the Jordan University of Science and Technology (JUST) and King Abdullah

Table 1. Primers and enzymes used for RFLP analysis of single nucleotide polymorphisms of the *RETN* gene.

SNP	Primer	Sequence 5′ to ′3	Amplicon size (bps)	Restriction enzyme
+299(G>A)	Forward Reverse	GAGAGGATCCAGGAGGTC GTGAGACCAAACGGTCCCTG	533	BpiI
−420(C>G)	Forward Reverse	TGTCATTCTCACCCAGAGACA TGGGCTCAGCTAACCAAATC	243	AluI

University Hospital (KAUH) approved the study. Study participants were required to sign a consent form prior to their enrollment. Subjects were recruited at the outpatient clinics of KAUH, a tertiary hospital affiliated with JUST.

A total of four hundred and seven subjects were enrolled in this study. Two hundred and eighty-two participants were T2DM patients diagnosed by endocrinologists according to the American Diabetes Association (ADA) criteria that include fasting blood sugar greater than or equal to 126 mg/dl and HbA1c ≥ 6.5. Subjects with diabetes were patients actively treated for T2DM at the Endocrinology Clinics of KAUH. A total of 125 control non-diabetes subjects were recruited during their visit to other KAUH clinics. Based on a short interview, it was determined that the control subjects did not complain of any of the usual symptoms associated with T2DM at the time of their recruitment. Moreover, control subjects were requested to measure their 12-hour fasting blood glucose (FBG) and HbA1c levels at two separate times to confirm the absence of T2DM. Control group participants with repeated FBG of 100 to 125 mg/dL or a HgbA1c between (5.7–6.4)% were excluded. Subjects with metabolic syndromes other than diabetes, Cushing's syndrome, and thyroid diseases were also excluded from the study. All recruited participants were of Jordanian descent and patients from other nationalities were excluded.

Anthropometric measurements

During the subject's visit to KAUH clinics the height (H, in cm) and body weight (BW, in Kg) of the subjects were recorded. The height and BW were then used to compute the body mass index (BMI) according to the mathematical formula: BMI=BW (kg)/H^2 (m^2). Lean body mass (LBM) was calculated in men using the formula: LBM (kg) =0.32810×BW (kg) +0.33929×H (cm) –29.5336, and in women using the formula: LBM (kg) =0.29569×BW (kg) +0.41893×H (cm) – 43.2933. Body fat (BF) was calculated from the body weight (BW) and LBM using the following equation: BF=BW–LBM.

Blood sampling

The blood was collected from the subjects following a 12-hour fast.4 mL was collected into an evacuated EDTA tube, and 5 mL into a plain tube (AFCO, Amman, Jordan). EDTA blood was used to measure HbA1c levels and the remainder was used for DNA extraction as described below. Plain blood was centrifuged at 4500×*g* for 5 min to obtain the serum for the measurement of FBG, triglycerides, and lipid profile (total cholesterol, HDL, and LDL).

Biochemical measurements

Serum samples collected from the subjects were submitted to the laboratories of KAUH to measure glucose and lipid profile using an automated biochemical analyzer obtained from Roche Diagnostics (Germany). HbA1c was measured in EDTA collected blood using the above system as well.

Molecular analysis

DNA was extracted from whole blood using QIAamp DNA extraction Kit purchased from Qiagen (Germany). The concentration and the purity of the extracted DNA was assessed at 260 OD and 260/280 OD ratio respectively using a Nanodrop machine obtained from Thermo Fisher (MA, USA). Analysis of the −420(C>G) or +299(G>A) polymorphisms in the *RETN* gene was achieved using polymerase chain reaction followed by restriction fragment length polymorphism analysis. All PCR reactions were performed in 20 μL final volume using ready to use master mix obtained from Promega Company (WI, USA), 10 ng of DNA and .2 μM of each of each primer. The sequences of the used primers, PCR amplicon size, PCR cycling parameters and the restriction enzyme conditions are listed in Table 1. All restriction enzymes were purchased from NEB (New England Biolabs, MA). The undigested PCR products and the fragments that resulted from the restriction enzyme digestion were separated using 2.5% agarose gel electrophoresis stained with ethidium bromide. The sizes of restricted DNA fragments were estimated under UV light and a proper DNA ladder.

Statistical analysis

Demographic characteristics and biochemical parameters (HbA1c, blood glucose, triglycerides, and lipid profile), BMI, and BF were compared between the control group and T2DM patients using the Student's *t*-test. The frequencies of the different genotype categories and alleles of each SNP were compared between the control group and T2DM patients using the SNPStats software tool that is widely used in genetic association studies (http://bioinfo.iconcologia.net/SNPstats). Haplotype analysis of *RETN* polymorphisms was performed using the above tool as well. The power analysis was carried out using G. Power version 3.0.10 (Franz Faul, Universität Kiel, Kiel, Germany). In all tests, the power was more than 60.

RESULTS

Table 2 presents the characteristics of patients and controls. T2DM patients were similar in mean age to the controls (52.90 *vs.* 52.02; $P>0.05$). Male participants represented 51.2% of the control group and 47.5% of the patient group. Significant differences in FBG ($P<0.001$), HbA1c ($P<0.001$), triglyceride ($P<0.05$), and BMI ($P<0.05$) were found between the control and patient groups. No significant differences between the compared groups were found in total cholesterol, LDL, HDL, and percent BF..

We next tested the association of *RETN* −420(C>G) or +299(G>A) SNPs with T2DM. Geno-

Table 2. Baseline characteristics of the control and patient groups

Parameter *	Control group	Patient group	*P* value
Mean age: year	51.95 (11.40)	53.94 (9.26)	0.063
Gender N (%)			0.470
Male	64 (51.2)	134 (47.5)	
Female	61 (48.8)	148 (52.5)	
Fasting blood sugar	5.50 (.57)	9.65 (5.47)	<0.001
HbA1c (%):	5.79 (.51)	7.36 (1.76)	<0.001
Total cholesterol (mmol/L)	4.87 (1.02)	5.00 (1.42)	0.536
Triglyceride (mmol/L)	1.90 (1.27)	2.39 (2.03)	0.013
HDL (mmol/L)	0.97 (.28)	1.01 (.34)	0.249
LDL (mmol/L)	3.26 (.90)	3.22 (1.15)	0.471
BMI (Kg/m^2)	29.8 (4.81)	31.14 (5.30)	0.015
Body fat	30.6 (9.0)	32.29 (10.8)	0.164

*All parameters were expressed as mean (SD) except gender as N(%)

Table 3. Association between RETN SNPs and the risk of diabetes mellitus

RETN SNP	Cases N (%)	Controls N (%)	Odd Ratio (95% CI)	*P* value
+299				
GG	142 (50)	64 (51)	1.00	
GA	92 (33)	51 (41)	1.23 (0.78-1.93)	0.038
AA	48 (17)	10 (8)	2.16 (1.34-4.56)	
Allele G	376 (67)	179 (72)		0.163
Allele A	188 (33)	71 (28)		
-420	N (%)	N (%)		
CC	95 (34)	35 (28)	1.00	
GC	161 (57)	73 (58)	0.81 (0.50-1.31)	0.128
GG	26 (9)	17 (14)	0.56 (0.27-1.16)	
Allele C	351 (62)	143 (57)		0.174
Allele G	213 (38)	107 (43)		

type and allele frequencies of each SNP in the control or patient groups are presented in Table 3. Our findings indicated a significant association between +299(G>A) genotypes and T2DM ($P<0.05$). Specifically, the AA genotype was more common in the T2DM group (17%) compared to the control group (8%). Accordingly, AA was associated with an increased risk of T2DM than other genotypes. The frequency of the A allele was higher in the patient group compared to the controls but without statistical significance ($P=0.163$). With respect to −420(C>G) SNP, no significant association was found between the genotypes or alleles of this SNP and the risk of T2DM (Table 3). With respect to Hardy-Weinberg test, the 299(G>A) SNP was in equilibrium ($P=0.971$), whereas the *RETN* −420(C>G) SNP was not in equilibrium ($P=0.031$).

Table 4 presents the results of haplotype analysis of *RETN* gene polymorphisms examined in this investigation. Our findings demonstrated that the CA haplotype was significantly ($P<0.01$) enriched in the patients (12.5%) compared to the controls (2.5%). It can thus be concluded that the CA haplotype of RETN −420 and +299 SNPs increased the risk of T2DM in this population. No significant association between the three other haplotypes (i.e.. GG, GA, or CG) and T2DM was detected in our population ($P>0.05$). Linkage disequilibrium analysis of the examined SNPs was $D=0.1055$, $D'=0.5618$, and $r=0.4674$.

Table 4. Analysis of the association of RETN haplotypes with the risk of T2DM

	Cases Frequency	Controls Frequency	OR (95% CI)	*P*-value
CG	50.2	54.7	1.00	------
GA	21.4	25.9	0.83 (0.56-1.22)	0.350
GG	16.4	16.9	0.94 (0.59-1.5)	0.820
CA	12.0	2.5	4.0 (1.56-10.0)	0.004

Finally, we performed cross tabulation analysis between the genotypes of examined SNPs and the biochemical parameters presented in Table 2. None of these parameters were found to be associated with SNPs genotypes ($P>0.05$, data not shown).

DISCUSSION

T2DM is strongly linked with insulin resistance and abdominal obesity. The exact mechanism that explains the relationship between abdominal obesity and T2DM is currently unknown, but could be related to changes in the secretory profile of visceral fat, which is a type of fat that surrounds internal organs and most often increases in abdominal obesity.

Several hormones secreted from visceral fat tissue, known as adipocytokines, appear to modify the response of tissues to insulin activity (Alfaqih *et al.*, 2018). Resistin is an adipocytokine secreted by visceral fat cells (Fain *et al.*, 2003). A previous report demonstrated an association between resistin protein and an increased risk of T2DM (Gharibeh *et al.*, 2010). Genetic variants of the gene that codes for resistin, called *RETN*, were shown to be linked with altered levels of resistin protein in a number of populations (Chen *et al.*, 2010; Zayani *et al.*, 2018). Given these reports, we tested the association of two single nucleotide polymorphisms in *RETN*, −420(C>G) and +299(G>A), with T2DM.

We demonstrated in this study that the +299(G>A) SNP was associated with T2DM. Specifically, we found that the AA genotype of the +299(G>A) SNP was more frequently observed in T2DM patients than in controls. Given this result, it may be presumed that the relatively infrequent AA genotype of this SNP may be associated with an increased risk of T2DM as well. Although our findings indicated a higher frequency of the A allele in the patients compared to the controls, our results did not reach statistical significance. A larger sample size with greater statistical power may be needed to demonstrate that the A allele of the +299(G>A) SNP in *RETN* is associated with an increased risk of T2DM. The role of *RETN* SNPs in modulating the risk of T2DM was demonstrated in previous studies conducted in other populations (Chung *et al.*, 2014; de Luis *et al.*, 2020; Thammakun *et al.*, 2017; Zhang *et al.*, 2013). In agreement with the present finding, +299(G>A) SNP was found to be associated with T2DM among Chinese population (Chung *et al.*, 2014; Zhang *et al.*, 2013). In a recent meta-analysis, a significant association was observed between +299(G>A) SNP and T2DM, with a pooled odds ratio of 1.45 (95% CI 1.10–1.92) (Kumar *et al.*, 2020). In addition, The SNPs 3'UTR C/T and +62 G>A of *RETN* gene were found to be associated with T2DM in Spaniards and Thais, respectively (de Luis *et al.*, 2020, Thammakun *et al.*, 2017). Moreover, a study that was conducted in Pakistan showed an association between the G allele of *RETN* –420 SNP and the risk of T2DM (Nadeem *et al.*, 2018). The *RETN* SNPs were also found to be associated with obesity and metabolic syndrome parameters that are related to diabetes (Boumaiza *et al.*, 2012; Hivert *et al.*, 2009; Menzaghi *et al.*, 2006). These findings collectively suggest a role for *RETN* SNPs in the risk of T2DM in the different populations.

The +299 (G>A) SNP is located in the second intron of the *RETN* gene (Suriyaprom *et al.*, 2009). Several explanations may explain the association of this SNP with T2DM (El-Shal *et al.*, 2013; Li *et al.*, 2007; Rode *et al.*, 2019; Suriyaprom *et al.*, 2009). For example, this SNP may be in linkage disequilibrium with another SNP that modulates level of gene expression of resistin, similar to that reported between –358 and +420 *RETN* SNPs (Onuma *et al.*, 2010). Another explanation would be that this SNP could be part of a sequence motif or a regulatory element that mediates the binding of a coregulatory protein involved in the regulation of *RETN* gene expression, similar to that reported for other *RETN* SNPs (Osawa *et al.*, 2004). Finally, the SNP could be part of a micro-RNA transcript (Li *et al.*, 2007), which regulates the stability of a gene transcript involved in the pathophysiology of T2DM. The confirmation of any of the above hypothesis requires further experiments performed on relevant cell and/or animal models and are beyond the scope of the current investigation.

Association studies based on haplotype analysis of several SNPs are believed to have more statistical power than those based on individual SNPs (Bashir *et al.*, 2019; Bader, 2001). Herein, we carried haplotype analysis of both SNPs in the *RETN* gene that were also examined individually for their association with T2DM. We found that one of the haplotypes, CA, was significantly more frequent in the T2DM cases than in controls, which indicates that this haplotype increases the risk of T2DM. This result is in line with our observation that the AA genotype of the +299(G>A) SNP is significantly more frequent in T2DM patients.

In this investigation, data related to the presence of cardiomyopathy and/or other diabetic complications were not collected by the research team. This is a limitation of this study as there are a number of reports that indicated dysregulation in resistin levels in diabetic patients suffering from cardiomyopathy (Lebeche, 2015). It could thus be assumed that genetic variants in the *RETN* locus could modulate the risk of cardiomyopathy or other complications in T2DM patients (Hussain *et al.*, 2010). However, the above hypothesis was not tested in this investigation and could be a subject for future investigations. Another limitation was that the sample represented a single tertiary institution from one geographic area in Jordan. Further validation of the findings presented in this study is thus required using data extracted from patients from several geographic regions.

CONCLUSIONS

This study reported an association between the +299 AA genotype in the *RETN* gene and T2DM. This result provides further support to the role of the *RETN* locus in mediating the risk of T2DM. The findings of this report, however, require further testing in a larger population.

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