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A single nucleotide polymorphism in *BCAT1* gene is associated with type 2 diabetes mellitus

Mahmoud A. Alfaqih[1]✉, Zaina E. Abu-Khdair[1], Omar Khabour[2], Khalid A. Kheirallah[3] and Mariam Khanfar[1]

[1]Department of Physiology and Biochemistry, Jordan University of Science and Technology, Irbid 22110, Jordan; [2]Department of Medical Laboratory Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan; [3]Department of Public Health and Community Medicine, Jordan University of Science and Technology, Irbid 22110, Jordan

The level of circulatory branched chain amino acids (BCAAs) is often increased in type 2 diabetes mellitus (T2DM). Catabolism of BCAAs involves a transamination reaction mediated by the branched chain amino acid aminotransferase (BCAT1) enzyme. Differences in the level of BCAT1 were found to be linked with hypertension, obesity, and cancer. Herein, using a case control design, we tested the association of rs9668920 and rs12321766 polymorphisms in *BCAT1* gene with T2DM. Three hundred subjects were recruited in the study. Genotyping of the indicated polymorphisms was achieved using restriction fragment length polymorphism technique after amplification of the target sequences. The results showed that, under a recessive inheritance model, the GG genotype of rs9668920 increased the risk of T2DM (*P*=0.026; OR 2.60; 95% CI 1.119–6.048). This effect was independent of the age, body mass index, waist circumference, serum glucose, cholesterol, triglycerides, and BCAAs (*P*>0.05). In conclusion, The GG genotype of *BCAT1* rs9668920 SNP might be a risk factor of T2DM. More studies are required to confirm this finding.

Keywords: BCAT1, Branched chain amino acids, Type 2 diabetes mellitus

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✉e-mail: maalfaqih@just.edu.jo
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Abbreviations: branched chain amino acids, BCAAs; T2DM, type 2 diabetes mellitus; BCAT, branched chain amino acid aminotransferase; DM, diabetes mellitus; MENA, Middle East and North Africa; ADA, American diabetes association; JUST, Jordan University of Science and Technology; Kauh, King Abdullah University Hospital; FBG, fasting blood glucose; BMI, body mass index; WC, waist circumference; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; IR, insulin resistance

INTRODUCTION

Diabetes mellitus (DM) is currently considered a global health problem with a particularly high prevalence in low-middle income nations (Correia *et al*., 2019) such as countries of the Middle East and North Africa (MENA) regions. In 2019, 463 million adults (20–79 years) were living with DM worldwide (IDF diabetes atlas, 2019). The economic burden of DM is among the highest in the MENA region as it reached 17.1 billion USD in the year 2015 and is expected to double by the year 2040 (Ogurtsova *et al*., 2017). Thus, there is an urgent need for a better understanding of the factors that increase the risk of DM worldwide to search for interventions that can mitigate the effects DM has on health and the economy (Ali *et al*., 2020).

DM has long been recognized to be a disease caused by a dysregulation in the metabolism of carbohydrate, fat and protein (Goyal *et al*., 2020). For example, changes in hundreds of metabolites including amino acids were implicated in the etiology of DM (Hameed *et al*., 2020). Although differences in the levels of circulating amino acids in DM were indicated as early as 1970 (Felig, 1975), the role of these changes in determining disease risk or pathogenesis has not received attention until recently (Hameed *et al*., 2020). Of the many amino acids that may contribute to DM pathogenesis, the role of the branched chain amino acids (BCAAs) has been extensively studied over the last few years (Nawaz & Siddiqui, 2020; Siddik & Shin, 2019). A growing body of literature has shown elevated BCAAs levels in the serum of DM patients (Floegel *et al*., 2013; Isanejad *et al*., 2017). BCAAs (leucine, isoleucine, and valine) are grouped together because of similarities in their structure, absorption, metabolism, and function. For instance, BCAAs have a branch in their aliphatic side chain and are absorbed through a common carrier across the epithelial wall of the gut (Zhou *et al*., 2018). Moreover, BCAAs reach the systemic circulation without any regulation of their plasma levels by the liver (Nawaz & Siddiqui, 2020). An essential step in BCAA metabolism is catalysed by BCAT1 enzyme and involves an aminotransferase reaction where the amino group of the BCAA is transferred to α-ketoglutarate (Adeva-Andany *et al*., 2017). This is followed by an oxidative decarboxylation step which is catalysed by the branched chain α-keto acid dehydrogenase complex enzyme (Adeva-Andany *et al*., 2017). Following this step, the catabolic pathway of each BCAA diverges with each of the acids producing its corresponding acyl-CoA product which eventually enters the citric acid cycle (Yoon, 2016).

Noteworthy, mammalian tissues have two different *BCAT* genes located on two different chromosomes; a cytosolic BCAT isoform encoded by the *BCAT1* gene locus on chromosome 12 (Duarte *et al*., 2007), and a mitochondrial BCAT isoform encoded by the *BCAT2* gene locus on chromosome 19 (Bledsoe *et al*., 1997). A number of studies found that polymorphisms in the *BCAT1* gene are associated with chronic diseases such as hypertension (Rhee *et al*., 2011), obesity (Chen *et al*., 2013) and cancer (Wang *et al*., 2015). However, the association between SNPs in *BCAT1* and the risk of T2DM has not been investigated in any country of the MENA region before. Herein, two SNPs in *BCAT1* (rs9668920

and rs12321766) were tested for their association with T2DM.

MATERIALS AND METHODS

Study design

The study is case-control in design. The study was conducted accordingly to the ethics committee (249/2016) of the Jordan University of Science and Technology (JUST). Study participants were interviewed and were asked to fill an informed consent prior to their enrolment. This included a full explanation of the study objectives and procedures. Enrolment of subjects was during the year 2017 at King Abdullah University Hospital (KAUH) located in Irbid, Jordan. The minimum sample size required for this study, based on an assumed odds ratio of 3, alpha level of 0.05, power of 0.8, and 1 to 1 case to control ratio, was a total of 166 cases and control (83 cases and 83 controls). For post hoc power calculation revealed that the power to detect a difference as that under investigation was 90%, assuming alpha of 0.05, 150 cases, 150 controls, with case to control ratio of 1:1, and an estimated odds ratio of 2.6.

Subject description

A total of three hundred subjects (age 35–70 years) were enrolled in the study. One hundred and fifty T2DM patients diagnosed according to the American Diabetes Association (ADA) guidelines were included as cases (HbA1c≥6.5%, fasting blood glucose (FBG) ≥126 mg/dL). The diabetic subjects were patients actively treated for T2DM at the Endocrinology clinics of KAUH and had no history of macro or microangiopathies at the time of recruitment. A total of 150 healthy and non-obese subjects, with no chronic illness at the time of the study, were recruited during their visit to other KAUH clinics. Following a short interview, it was determined that the non-diabetic subjects did not complain about any of the usual symptoms associated with T2DM at the time of their recruitment. Moreover, non-diabetic subjects were requested to give a venous blood sample collected in a plain tube to assess their FBG levels on two separate occasions to confirm the absence of T2DM. Exclusion criteria include pre-diabetic individuals (FBG: 100–125 mg/dL), Cushing's syndrome, polycystic ovarian syndrome, thyroid dysfunction, or hyperprolactinemia.

Anthropometric measurements

During the subject's visit to KAUH clinics, the height (cm) and the weight (Kgs) of the subjects were measured and recorded to calculate body mass index (BMI) as previously described (Alomari *et al.*, 2020). During the same visit, the waist circumference (WC) was obtained in centimetres using a tape measure.

Blood sampling

Following a 12-hour fast, 3 mLs of blood were collected into an evacuated EDTA tube (AFCO, Amman, Jordan) and 5 mLs of blood were collected in a plain tube obtained from AFCO (Amman, Jordan). Plain blood samples were centrifuged at 4500×*g* for 5 min and the resulting serum was pipetted into 1.5 mL tubes and stored at –80°C until its use for the measurement of FBG, cholesterol, triglycerides and BCAAs.

Biochemical measurements

Biochemical parameters were measured in the laboratories of KAUH using an automated biochemical analyser system obtained from Roche Diagnostics/Germany. BCAAs were measured using a commercially available colorimetric assay (catalogue number: ab83374; Abcam, Cambridge, UK) as previously described (Alfaqih *et al.*, 2018).

Determination of *BCAT1* genotypes

DNA was purified from whole blood samples using a kit obtained from Qiagen (Germany) as explained previously (Alfaqih *et al.*, 2018). The quality and quantity of extracted DNA were assayed on a Nanodrop instrument (Thermo-Scientific, MA, USA). Genotyping of rs9668920 and rs12321766 was performed using polymerase chain reaction (PCR) followed by digestion with a restriction enzyme and then agarose gel electrophoresis. All amplification reactions were carried out in 25 µl reaction volume using ready to use master mix (Promega, Madison, USA), 10 Nano gram of DNA and 0.5 µM of each primer. The sequences of the primers, the corresponding fragment sizes and restriction enzymes are listed in Table 1. All restriction enzymes were purchased from NEB (New England, MA). The undigested PCR products and the fragments that resulted from the restriction enzyme digestion were electrophoresed on a 3% agarose gel stained with ethidium bromide. The products were then visualized under UV light. Genotyping strategy of each SNP is illustrated in Fig. 1. To validate the genotyping procedure, 10 samples of each genotype were randomly selected for sequencing at Princess Haya Centre for Biotechnology (Irbid, Jordan).

Statistical analysis

The student's t-test was used for two group comparisons of means. The frequencies of the different genotype categories of each SNP were compared between the two groups using Pearson Chi-square. The SNPStats software tool (http://bioinfo.iconcologia.net/SNPstats) was used to test if allele and genotype frequencies deviated from Hardy Weinberg equilibrium and to test the association of each SNP with T2DM in different inheritance models. Binary logistic regression analysis was used to assess the adjusted effects of the independent

Table 1. Single nucleotide polymorphisms of *BCAT1*

rs number	Primer	Sequence 5′ to ′3	Amplicon size (bps)[a]	Restriction enzyme
rs9668920	Forward	CATAGCAAGGTCAGGCTTAC	383	HpyCH4III
	Reverse	ACAATTGCCTAATCCAAGGTC		
rs12321766	Forward	ACGACATATGGTTGCACAGTG	289	HpyCh4V
	Reverse	ACTTGCTGATGCCTCTGCC		

All single nucleotide polymorphisms information were obtained from the NCBI dbSNP database. [a]bps: base pairs.

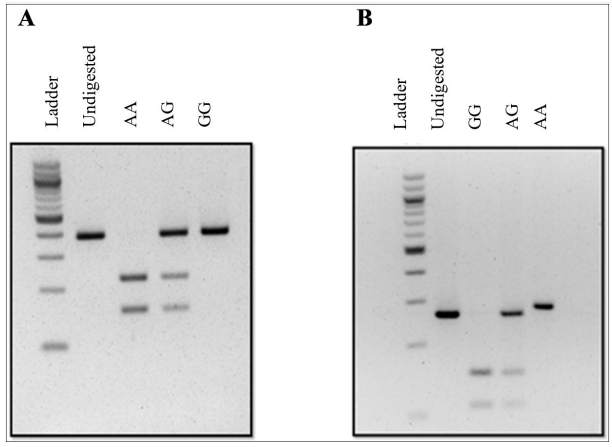

Figure 1. PCR-RFLP genotyping strategy of *BCAT1* SNPs rs9668920 and rs12321766.
A 3% agarose gel image of the different genotype classes of (**A**) the rs9668920 and (**B**) rs12321766 SNPs of *BCAT1* observed in the study subjects following PCR-RFLP strategy (look at materials and methods for more details).

variables on diabetes; a backward selection method was utilized. Variables identified in the model were presented in terms of adjusted odds ratio (OR), 95% confidence interval (CI) and *P* value. The null hypothesis was rejected if *P*-value<0.05.

RESULTS

Subject description and biochemical profile

One hundred and fifty diabetic and one hundred and fifty non-diabetic subjects agreed to enrol in the study. Diabetic subjects were different from non-diabetic subjects in age, WC, FBG, total cholesterol, triglycerides, and BCAAs (*P*<0.01, Table 2).

Association of rs9668920 with T2DM

Two SNPs in *BCAT1* gene (rs9668920 and rs12321766) were tested for their association with T2DM. Both SNPs were in the Hardy-Weinberg equilibrium (*P*>0.05) and were genotyped using a PCR- restriction fragment length polymorphism (RFLP) based strategy (Fig. 1A and 1B). The genotype and allele frequencies of rs9668920 and rs12321766 are displayed in Table 3. Statistical analysis showed that rs9668920 is associated with T2DM under a recessive inheritance model (Table 4). In this model, the GG genotype of rs9668920

Table 2. Baseline variables of study subjects. Data presented as mean ± S.D.

Variable	Non-diabetic (n=150)	Diabetic (n=150)	*P*-value
Gender			
Males	57 (38%)	72 (48%)	0.051
Females	93 (62%)	78 (52%)	
Age years	52.47±10.04	58.28±10.07	<0.001
BMI (Kg/m²)	29.54±5.09	30.66±4.87	0.053
WC (cm)	101.31±11.53	109.21±1.98	<0.001
Glucose (mg/dL)	86.65±10.84	167.67±82.59	<0.001
Cholesterol (mg/dL)	182.65±39.41	207.61±68.46	<0.001
Triglycerides (mg/dL)	131.51±87.96	165.58±99.35	0.002
BCAAs (µmol/L)	408.01±93.07	461.73±99.63	<0.001

Table 3. Genotype and allele frequencies of rs9668920 and rs12321766

SNP ID	Group	Genotype Frequency			*P*-value*	Allele Frequency		*P*-value*
		AA (n) (%)	AG (n) (%)	GG (n) (%)		A (n) (%)	G (n) (%)	
rs9668920	Non-diabetic	68 (45%)	69 (46%)	13 (9%)		205 (68%)	95 (32%)	
	Diabetic	66 (44%)	58 (39%)	26 (17%)	0.070	190 (63%)	110 (37%)	0.200
rs12321766	Non-diabetic	28 (19%)	58 (39%)	64 (43%)		114 (38%)	186 (62%)	
	Diabetic	19 (13%)	71 (47%)	60 (40%)	0.200	109 (36%)	191 (64%)	0.700

*P-values were calculated using Chi-square test.

Table 4. Association of rs9668920 with the risk of T2DM under different models of inheritance

Model	Genotype	Non-diabetic (n) (%)	Diabetic (n) (%)	OR (95% CI)	*P*-value
Codominant	AA	68 (45%)	66 (44%)	1	
	AG	69 (46%)	58 (39%)	0.87 (0.53- 1.4)	0.070
	GG	13 (9%)	26 (17%)	2.04 (0.98- 4.35)	
Dominant	AA	68 (45.3%)	66 (44%)	1	
	AG-GG	82 (54.7%)	84 (56%)	0.95 (0.60 -1.49)	0.820
Recessive	AA-AG	137 (91%)	124 (83%)	1	
	GG	13 (9%)	26 (17%)	2.20 (1.09- 4.49)	0.028
Over-dominant	AA-GG	81 (54%)	92 (61%)	1	
	AG	69 (46%)	58 (40%)	1.35 (0.85- 2.14)	0.200

Table 5. Association of rs12321766 with the risk of T2DM under different models of inheritance

Model	Genotype	Non-diabetic (n) (%)	Diabetic (n) (%)	OR (95% CI)	*P*-value
Codominant	GG	64 (43%)	60 (40%)	1	
	AG	58 (39%)	71 (47%)	0.77 (0.47–1.26)	0.200
	AA	28 (19%)	19 (13%)	1.38 (0.70–2.73)	
Dominant	GG	64 (43%)	60 (40%)	1	
	AG-AA	86 (57%)	90 (60%)	0.90 (0.57–1.42)	0.640
Recessive	GG-AG	122 (81%)	131 (87%)	1	
	AA	28 (19%)	19 (13%)	1.58 (0.84–2.98)	0.150
Over-dominant	GG-AA	92 (61%)	79 (52%)	1	
	AG	58 (39%)	71 (47%)	0.70 (0.44–1.11)	0.130

Table 6. Binomial regression analysis of study participants with gender, age, BMI, waist circumference, BCAA, cholesterol and triglycerides as variables in the model

Variable	Genotype	OR (95% CI)	*P*-value
Age (years)	–	1.06 (1.03–1.09)	<0.001
WC (cm)	–	1.05 (1.025–1.077)	<0.001
BCAAs (μmol/L)	–	1.01 (1.001–1.007)	0.004
Cholesterol (mg/dL)		1.01 (1.005–1.015)	<0.001
rs9668920	AA-AG		0.026
	GG	2.60 (1.119–6.048)	

significantly increased the risk of T2DM by twofold (P=0.028; OR 2.20; 95% CI 1.09–4.49). On the other hand, rs12321766 was not associated with T2DM under any of the tested inheritance models (Table 5).

In binomial regression analysis, the GG genotype of rs9668920 remained associated with T2DM and increased its risk relative to the (AA-AG) genotype category (P=0.026; OR 2.60; 95% CI 1.119–6.048) (Table 6) after adjustment for gender, age, BMI, WC, BCAAs, cholesterol, and triglycerides.

DISCUSSION

In this investigation, two SNPs (rs9668920 and rs12321766) located in different regions of the *BCAT1* gene were tested for their association with T2DM. The results demonstrated that rs9668920 SNP was associated with T2DM under a recessive model of inheritance. In this model, subjects with the GG genotype were at an increased risk of T2DM relative to individuals with the AA-AG genotypes. In multivariate analysis, the effect of rs9668920 SNP in modulating the risk of T2DM was independent of age, BMI, WC, serum glucose, cholesterol, triglycerides, and BCAAs levels.

BCAT1 gene encodes branched-chain amino acid transaminase enzyme located in the cytosol. The function of this enzyme is to catalyse the transamination of branched-chain α-keto acids to branched-chain L-amino acids. Such a reaction is essential for homeostasis within cells. Genetic variations in *BCAT1* gene have been shown to be associated with several conditions. For example, The *BCAT1* rs7961152 SNP has been shown to be associated with salt sensitivity after adjusting for confounding factors among the Korean population (Rhee *et al.*, 2011). In addition, rs2242400 has been shown to be associated with T2DM (Rampersaud *et al.*, 2007). Finally, genome wide association scans have identified *BCAT1* as a candidate gene for T2DM and resting heart rate (Eijgelsheim *et al.*, 2010; Zemunik *et al.*, 2009; Zhou *et al.*, 2018). In the present study, an association between rs9668920 SNP and T2DM was reported. Since this is the first study that examined rs9668920 and rs12321766 *BCAT1* SNPs in T2DM, there is no literature available to compare the present findings.

The mechanism by which rs9668920 modifies T2DM risk remains to be determined. Rs9668920 is an upstream variant of the *BCAT1* gene that could be part of an enhancer sequence element (Zerbino *et al.*, 2018). Accordingly, rs9668920 might modulate the expression of the *BCAT1* gene. *BCAT1* gene codes for an enzyme that catalyses the first step in the catabolism of BCAAs (Suryawan *et al.*, 1998). A study found that the skeletal muscle tissues of individuals with T2DM have lower levels of BCAAs catabolic products relative to those recovered from individuals with normal glucose tolerance (Lerin *et al.*, 2016). Taken together, a model of BCAAs metabolism in T2DM patients can be deduced from the above data. In this model the expression/activity of enzymes involved in BCAAs catabolism in skeletal muscles or other tissues, including BCAT1, is lower in individuals with T2DM (Lerin *et al.*, 2016). It is then proposed that downregulation of BCAAs catabolism in extrahepatic tissues results in an elevation in serum BCAAs levels as observed in a number of reports (Wang *et al.*, 2011; Xu *et al.*, 2013). An elevation in serum BCAAs could contribute to the development of insulin resistance (IR) *via* an impairment of the insulin signalling cascade (Yoon, 2016). In line with the above assumption, Newgard *et al.* reported a decrease in BCAAs catabolism in obese individuals (Newgard *et al.*, 2009). However, it still remains to be determined whether rs9668920 indeed affects expression/activity levels of BCAT1 in the different tissues of T2DM patients.

Rs12321766 was not associated with T2DM under any of the tested models of inheritance. This might be explained by a lack of linkage disequilibrium between rs9668920 and rs12321766 or the presence of other SNPs in the vicinity of this chromosomal region which might be modifying the association of rs12321766 with T2DM. More investigations are required to confirm the current findings.

Among the limitations of the current study is that only two SNPs in *BCAT1* gene were examined. Another SNP in this gene called rs2242400 has been shown to be associated with T2DM using genome-wide association approach (Rampersaud *et al.*, 2007). Thus, inclusion of more SNPs in the *BCAT1* gene is strongly recommended in future investigations to uncover the role of this gene in the etiology of diabetes.

CONCLUSION

In conclusion, rs9668920 in *BCAT1* gene is associated with the risk of T2DM in a Jordanian population. Previous reports found that variations in *BCAT1* gene were also found to be associated with obesity and hypertension. Taken together, our findings and those of others, highlight that BCAAs and/or their metabolites could be a common factor implicated in the etiology of several chronic diseases. The results presented in this report require further validation in other populations.

Conflicts of Interest

Authors have nothing to declare.

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