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Original article

Evaluation of the role of *CDKN2B* gene in type 2 diabetes mellitus and hypertension in ethnic Saudi Arabs

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ABSTRACT

Background: Coronary heart disease (CAD) is a multiple with several contributory risk traits, including type 2 diabetes and hypertension, which may share common genetic risk variants with the disease. Genome-wide association studies (GWASs) have yielded a wealth of information suggesting that CAD, the extent of contributory variants may differ according to genetic locus. The present study aimed at verifying whether the cyclin-dependent kinase 4 inhibitor B (*CDKN2B*) genomic region strongly associated with coronary artery disease (CAD)/myocardial infarction (MI) may also constitute risk for its risk factors type 2 diabetes mellitus (T2DM) and hypertension (HTN) in ethnic Saudi Arabs.

Methodology: We genotyped eight *CDKN2B* SNPs for cardiovascular risk in a total of 4650 Saudi Arabs, (3049 male and 1601 female) by Taqman assay. Of these individuals, 3732 had primary hypertension and 2576 had type 2 diabetes mellitus.

Results: Out of the eight studied SNPs, two, rs10757274_A [0.915 (0.840–1.00); p = 0.042], rs1333045_T [0.92(0.84–1.00); p = 0.048] were initially associated with type 2 diabetes but lost the association after multivariate adjustments for CAD, hypertension and MI, while rs10757274_A showed borderline association with hypertension.

Conclusions: Our finding does not support the notion of a critical role for the *CDKN2B* gene locus as a HTN or T2DM cardiovascular risk in ethnic Arabs. The study also demonstrates the importance of replication studies in ascertaining the role of a genomic sequence in disease.

1. Introduction

Coronary artery disease is a multifactorial cardiovascular disease whose manifestation is often influenced by the presence of several risk traits including hypertension (HTN) and type 2 diabetes mellitus (T2DM) and dyslipidaemic disorders. Several studies have indicated that CAD may share common predisposing genetic variants with these risk traits particularly HTN and T2DM. One of the loci that has been linked with CAD thus far is the cyclin-dependent kinase (CDK) 4 inhibitor B, also known as multiple suppressor 2 or p15, a protein that is encoded by the cyclin-dependent kinase 2B (*CDKN2B*) gene in humans (Tsubari et al., 1997). This protein is a cell growth regulator that inhibits the cell cycle G1 progression by forming a complex with CDK4 or CDK6 thereby prevent the activation of the CDKs by cyclin D (Hannon and Beach, 1994). The *CDKN2B* gene itself is located within the p.14- p.16 gene cluster at 9p21 locus. Apart from CAD, this gene cluster harbours several genes implicated in various other disorders, such as, coronary artery disease, changes in lipid levels, coronary microvascular dysfunction, diabetes, cancer and periodontitis in different ethnic populations (Wakil et al., 2016a; Zhou et al., 2012; Hannou et al., 2015; Mafi Golchin et al., 2017; Wahlstrand et al., 2009; Yang et al., 2009; Johnson et al., 2013; Wakil et al., 2016b; Yoshino et al., 2014; Aarabi et al., 2017; Almontashiri et al., 2015; Congrains et al., 2012; Ghanbari et al., 2015; Guo et al., 2013; Helgadottir et al., 2007; Holdt and Teupser, 2013; Jeemon et al., 2011; Landman et al., 2012; Matsuoka et al., 2015; Motterle et al., 2012; Pilbrow et al., 2012; Sousa et al., 2011; Tajbakhsh et al., 2016; Visel et al., 2010; Saade et al., 2011). Specifically, a recent genome-wise study by Wakil et al. (2016a) has illustrated through a genome-wide association study (GWAS) that several variants at the *CDKN2B* locus that were implicated in CAD/MI, but also suggestive of constituting similar risk for HTN or T2DM in ethnic Saudi Arabs (Wakil et al., 2016a). While some studies have associated this gene with T2DM (Peng et al., 2013; Cugino

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et al., 2012), other have failed to verify the observations, pointing to the fact that the role of the *CDKN2B* gene locus in the cardiovascular disease traits a still not well defined (Bao et al., 2012; Duesing et al., 2008). Hence, their actual impact of these traits on the latter requires to be validated through replication studies. Besides, since most of the identified variants are believed to constitute simply representatives for other functional ones, the actual causative entities remain to be fully characterized. In the present study, we elected to verify the possibility of the *CDKN2B* genomic region being a risk factor for T2DM or HTN in this ethnic population.

2. Methodology

2.1. Study population

The study population comprised a total of 4650 Saudi individuals (Table 1). Among these, 2576 (1728 male; 848 female) individuals had type 2 diabetes mellitus (T2DM) (formerly called non-insulin-dependent diabetes mellitus or adult onset diabetes) compared 2074 (1316 male; 758 female) individuals free of the disease (Table 1). The USA National Diabetes Data Group and the second World Health Organization (WHO) Expert Committee on Diabetes Mellitus (1998) defines type 2 diabetes mellitus as a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (Alberti and Zimmet, 1998). The second subset of interest comprised 3732 (2451 male; 1281 female) candidates with primary (essential) hypertension (HTN) compared with 918 (593 male; 325 female) non-hypertensive controls (Table 2). Hypertension was defined and classified as ≥140 Hg systolic blood pressure and ≥90 Hg diastolic pressure based on criteria of The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VI). Accordingly, essential, primary, or idiopathic hypertension is defined as high blood pressure (BP) in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or Mendelian forms (monogenic) are not present (Carretero and Oparil, 2000). The study population was derived from a national CAD registry cohort, hence some of the individuals may have carried the disease. Individuals were excluded from the study population if they were diagnosed with major cardiac rhythm disturbances, incapacitating or life-threatening illness, major psychiatric illness or substance abuse, history of cerebral vascular disease, neurological disorder, and administration of psychotropic medication. The control groups (CON) consisted of angiographed individuals undergoing surgery for heart valvular diseases and those who may have reported with chest pain but were established to have no significant coronary stenosis by angiography. Furthermore, these individuals were free of the traits (HTN or T2DM) under consideration. Exclusion criteria for this group were, among others, diseases such as cancer, autoimmune disease, or any other disorders likely to interfere with variables under investigation. This study was performed in accordance with the regulations laid down by the Hospital Ethics Committee and in accordance with the principles of the Declaration of Helsinki as well as Title 45, Part 46 of the USA code of Federal Regulation on Protection of Human Subjects. All participants signed an informed consent.

Table 1
Demographics of the type 2 diabetes study population.

	Control			Cases		
	All	Male	Female	All	Male	Female
T2D	2074	1316 (0.63)	758 (0.37)	2576	1728 (67.1)	848 (32.9)
Age	50.3 ± 16.7	51.9 ± 16.3	47.5 ± 17.1	59.6 ± 11.4	59.5 ± 11.4	59.7 ± 11.4
BMI	28.0 ± 6.1	27.3 ± 5.5	29.3 ± 6.8	30.0 ± 5.8	28.8 ± 5.1	32.4 ± 6.5
CAD	771	632 (0.82)	139 (0.18)	1653	1227 (0.74)	426 (0.26)
MI	1069	832 (0.78)	237 (0.22)	2004	1443 (0.72)	561 (0.28)
HTN	1400	912 (0.65)	488 (0.35)	2332	1539 (0.66)	793 (0.34)
lHDL	730	570 (0.78)	160 (0.22)	1225	937 (0.76)	288 (0.24)
hTG	372	293 (0.79)	79 (0.21)	813	550 (0.68)	263 (0.32)
hChol	595	412 (0.69)	183 (0.31)	1108	714 (0.64)	394 (0.36)
OBS	690	366 (0.53)	324 (0.47)	1127	621 (0.55)	506 (0.45)
Smokers	771	737 (0.96)	34 (0.04)	1003	964 (0.96)	39 (0.04)

Numbers in brackets represent percentages within a group. CAD, coronary artery disease; BMI, body mass index; FH, family history; OBS, obesity; hChol, hypercholesterolaemia; hTG, hypertriglyceridemia; HTN, hypertension; lHDL, low high density lipoprotein; MI, myocardial infarction; T2D, type 2 diabetes mellitus.

Table 2
Demographics of the primary hypertension study population.

	Control			Cases		
	All	Male	Female	All	Male	Female
HTN	918	593 (0.65)	325 (0.35)	3732	2451 (0.66)	1281 (0.34)
Age	45.2 ± 16.5	46.9 ± 16.0	42.0 ± 17.1	57.9 ± 13.1	58.4 ± 12.8	57.0 ± 13.7
BMI	27.6 ± 6.1	27.1 ± 5.8	28.5 ± 6.5	29.5 ± 5.9	28.4 ± 5.2	31.5 ± 6.7
CAD	280	239 (0.85)	41 (0.15)	2144	1620 (0.76)	524 (0.24)
MI	401	330 (0.82)	71 (0.18)	2672	1945 (0.73)	727 (0.27)
T2D	244	189 (0.77)	55 (0.23)	2332	1539 (0.66)	793 (0.34)
IHDL	333	266 (0.80)	67 (0.20)	1622	1241 (0.77)	381 (0.23)
hTG	173	140 (0.81)	33 (0.19)	1012	703 (0.69)	309 (0.31)
hChol	206	152 (0.74)	54 (0.26)	1497	974 (0.65)	523 (0.35)
OBS	284	159 (0.56)	125 (0.44)	1533	828 (0.54)	705 (0.46)
Smokers	356	342 (0.96)	14 (0.04)	1418	1359 (0.96)	59 (0.04)

Numbers in brackets represent percentages within a group. CAD, coronary artery disease; BMI, body mass index; FH, family history; OBS, obesity; hChol, hypercholesterolaemia; hTG, hypertriglyceridemia; HTN, hypertension; lHDL, low high density lipoprotein; MI, myocardial infarction; T2D, type 2 diabetes mellitus.

2.2. Association studies

In all, eight SNPs (1) rs10738607, (2) rs564398, (3) rs1412829, (4) rs10120688, (5) rs4977756, (6) rs10757274, (7) rs4977574 and (8) rs1333045 at the *CDKN2B* gene locus were selected for the association study. Selection of the SNPs of interest was based on the findings of the previous GWAS study in which several of these variants had shown either significant or borderline association with CAD/MI (Wakil et al., 2016a). In the present study, their association with disease was evaluated using the Applied Biosystems Real-time PCR system (ABI Inc. CA, USA). Genotyping was accomplished by Taqman chemistry on the ABI Prism 7900HT Sequence Detection System. Primers and the TaqMan fluorogenic probes bearing a suitable reporter dye on the 5′-end and a quencher dye on the 3′-end were designed using the Primer Express software V2.0 (ABI Inc., Foster City, CA, USA) and procured from Applied Biosystems (ABI, Warrington, UK). One probe (allele 1) was labeled with VIC dye and the other (allele 2) with FAM dye at the 5′-end, and serial dilutions were run to determine the optimal working concentration. For each reaction, a 25 μl reaction was prepared by mixing 5 μl containing 50 ng DNA, 12.5 μl of 2x Universal mix (Eurogentec, Liege Science Park, 4102 Seraing, Belgium), 1.25 μl of 20x probe Assay mix and 6.25 μl DNase-free distilled water. Three no-template controls were included in each plate for normalization of emission signal. The thermal profile for the first cycle amplification was set at 50 °C for 2 min, and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 30 sec. The plates were then scanned for FRET signal using the 7900HT sequence detection system and data analyzed using SDS 2.0 software (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical analysis

Comparison of genotypes and alleles between the cases and controls for continuous dependent variables was achieved by Analysis of Variance (ANOVA) or Student's test as appropriate. Categorical variables were analyzed by Chi-Square test, and logistic regression analysis was used to compute odds ratios and their 95% confidence intervals. All other statistical analyses were performed using the SPSS software version 14 (SPSS Inc., Chicago, USA), and data are expressed as mean ± SEM. Associations with a two-tailed $p < 0.05$ was considered statistically significant.

3. Results

In all, a total of 8 SNPs (1) rs10738607_A>G, (2) rs564398_A>G, (3) rs1412829_C>TG, (4) rs10120688_A>G (5) rs4977756_A>G, (6) rs10757274, (7) rs4977574_A>G and (8) rs1333045_C>T at the *CDKN2B* locus were investigated for T2DM and HTN. The first analysis was performed in 2576 T2DM versus 2074 controls (Table 1). Multiple regression analysis for HTN demonstrated that two of these SNPs, the rs10757274_A [0.92 (0.84–1.00; p = 0.042], rs1333045_T [0.92 (0.84–1.00); p = 0.048] were initially significantly associated T2DM. However, both lost this association after multivariate adjustments for CAD, HTN and MI. The second analysis was done in 3732 HTN versus 918 controls (Table 2). None of the studied variable was significantly associated with the disease, except the rs10757274_A [0.91(0.82–1.01; p = 0.075] showed only borderline association in the univariate analysis. These analyses are summarized in Table 3.

4. Discussion

The present study tested the likelihood that the *CDKN2B* gene on chromosome p9.21 constitutes a risk for type 2 diabetes and hypertension, two important risk traits for CAD/MI. Eight SNPs were selected for the study. These SNPs had been linked with CAD/MI in the same population in a recent GWAS by Wakil et al. (2016a). In that study, among the most significantly associated SNPs were the two variants, the rs10757274 and rs1333045. Interestingly, in the present study these two were only weakly associated with T2DM, while the former showed borderline activity with HTN. Nonetheless, although multivariate analysis indicated lack of an independent association with these two diseases, it cannot be ruled out that the genomic region might indeed be involved in their manifestation. However, in light of our present findings, the fact that these variants show weak associations in our population may also be a reflection of ethnic specificity in the way genomic changes may influence disease in general.

To begin with, the genomic locus of the *CDKN2A/B* gene on chromosome 9p21 has been associated not only with CAD/MI, but other cardiovascular risk traits such as higher coronary artery calcium levels hypertension, ischemic stroke, T2DM visceral and subcutenous fat (Guo et al., 2013; Helgadottir et al., 2007; Matsuoka et al., 2015; Pilbrow et al., 2012; Saade et al., 2011; Mafi Golchin et al., 2017; Wahlstrand et al., 2009; Yang et al., 2009; Johnson et al., 2013; Wakil et al., 2016b; Yoshino et al., 2014; Nawaz et al., 2015; McPherson et al., 2007; Lettre et al., 2011; Hu et al., 2009; Hotta et al., 2012; Carty et al., 2015; Bayoglu et al., 2016; Akinyemi et al., 2017; Nakaoka et al., 2010; Shanker et al., 2014; Shen et al., 2008a, 2008b; Zhao et al., 2016). Notably also while, in general the gene has been associated with T2DM, these observations have been partly refuted by other studies (Kong et al., 2018; Peng et al., 2013; Cugino et al., 2012; Bao et al., 2012; Duesing et al., 2008). Besides, the rs10757274 has also been linked to diseases, such as acute coronary syndrome, hypertension, stroke in hypertensive patients, metabolic syndrome, T2DM, ischemic stroke in different ethnic groups (Wahlstrand et al., 2009; Hu et al., 2009;

Table 3
CDKN2B polymorphism in type 2 diabetes mellitus and hypertension.

Type 2 diabetes (Cases = 2576; controls 2074)							
SNP ID	Genotype	Cases	Controls	Univariate analysis		Multivariate analysis	
				OR(95%C.I.)	P-value	OR(95%C.I.)	P-value
rs10757274_G>A	G	64.4	62.3	0.915 (0.840–0.997)	0.042	1.022 (0.932–1.121)	0.638
	A	35.6	37.7				
rs1333045_C>T	C	54.8	52.6	0.918 (0.843–0.999)	0.048	0.962 (0.878–1.055)	0.410
	T	45.2	47.4				
Hypertension (Cases = 3732; controls = 918)							
rs10757274_G>A	G	63.9	61.6	0.908 (0.816–1.010)	0.075	1.073 (0.957–1.202)	0.227
	A	36.1	38.4				

The table displays the variations that were linked with type 2 diabetes mellitus and hypertension; Each SNP was entered into multivariate analysis including coronary heart disease and myocardial infarction hypertension (for type 2 diabetes) and vice versa. OR, odds ratio; C.I., confidence interval.

Zhao et al., 2016; Zhang et al., 2012, 2014; Taheri et al., 2017; Kunnas et al., 2018). The suggestions that it may be linked to T2DM or stoke in hypertensive individuals seems to indicate that indeed it may influence pathways leading to these diseases. Similarly, although the rs1333045 was only weakly linked with T2DM, a number of studies have implicated in CAD (Wakil et al., 2016a); aneurysmal subarachnoid haemorrhage (Olsson et al., 2011) and some subtypes of intercranial aneurysma (Nakaoka et al., 2010). These observations furnish support for the notion that this *CDKN2B* genomic region harbours gene/gene variants that are involved in disease manifestation of various cardiovascular risk traits. Besides, several SNPs that predict CAD events appear to involve pathways not currently indexed by the known or emerging risk factors, as well as those involved changes in blood lipids, for example. Thus, this overlapping association of SNPs with multiple risk factors at this locus points the existence of shared points of regulation for these phenotypes. One suggestion has been that that several cardiovascular disease-associated SNPs at this locus affect the expression of ANRIL, which, in turn modulate cell growth, possibly via CDKN2A/B regulation (Congrains et al., 2012); macrovascular complications (Sousa et al., 2011) influences vascular smooth muscle proliferation (Motterle et al., 2012) and heart failure (Yamagishi et al., 2009). Similarly, the weak association found in this study seems to suggest that these associations may be influence by other factor sharing these pathways. The calls for replication of such studies to exploit further the possible mechanism underlying these regulatory junctions.

In summary, the present study detected some weak link of variants on the CDKN2B locus with two important risk factors for CAD in Saudi Arabs, possibly suggesting that this locus may not directly pause risk for T2DM or HTN at least in the same way as it does to CAD/MI manifestation.

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