Association between the rs1805081 polymorphism of Niemann-Pick type C1 gene and cardiovascular disease in a sample of an Iranian population

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Received March 21, 2016; Accepted September 6, 2016

DOI: 10.3892/br.2016.802

Abstract. The aim of the present study was to investigate the association between a genetic variation, A+644G, in the Niemann-Pick type C1 (NPC1) gene and the risk of cardiovascular disease (CVD) in a Southeast Iranian population. A total of 320 individuals, including 200 patients with CVD and 120 healthy individuals, were involved in the present study. The polymorphism was determined using a polymerase chain reaction-restriction fragment length polymorphism method. The results indicated that the frequency of the GG genotype was markedly lower in patients with CVD compared with the control group (7 vs. 16.7%), and that the NPC1 rs1805081 polymorphism was associated with reduced risk of CVD [odds ratio (OR)=0.110; 95% confidence interval (CI)=0.017-0.715; P=0.021]. In addition, the prevalence of the minor allele (G) in patients with CVD differed from that of the control group with the frequency of 25.5 and 33.4% for the former and latter, respectively, and this difference reached statistical significance (OR=0.658; 95% CI=0.482-0.971; P=0.037). Furthermore, analysis of clinical characteristics of the individuals according to the NPC1 genotypes revealed an association between the lipid profile and NPC1 gene polymorphism. These findings demonstrated that the NPC1 A+644G variant was associated with reduced risk of CVD and serves a protective role against susceptibility to CVD in the Iranian population.

Introduction

Despite substantial progress in medical care over the past 25 years, cardiovascular disease (CVD) remains a serious public health challenge. Numerous risk factors have been characterized for CVD; however, obesity, hypertension, insulin resistance and dyslipidemia are considered to be the most crucial factors associated with higher incidence of major cardiovascular events (1,2). Dyslipidemia is diagnosed when patients exhibit abnormal serum level of lipids, which includes both hypertriglyceridemia and hypercholesterolemia (3). Mounting evidence has demonstrated that hypercholesterolemia, hypertriglyceridemia, high low-density lipoprotein cholesterol (LDL-C) and low high-density lipoprotein cholesterol (HDL-C) are the major risk factors associated with atherosclerotic CVD, heart disease, heart attack and stroke (3-5).

Cholesterol is an essential structural component of mammalian cell membranes and is required to establish proper membrane permeability and fluidity. Although cholesterol is important for numerous biological systems, immoderate cholesterol, termed hypercholesterolemia, has been linked to damage to arteries and CVD (6), coronary heart disease (CHD) (7), Alzheimer's disease (AD) (8), renal dysfunction (9) and cerebral vascular disease (8,10). In the blood, cholesterol is transported primarily by LDL and transport by the LDL receptor is the predominant mechanism by which cholesterol is taken up by cells. LDLs are brought into the cell via receptor-mediated endocytosis and are delivered to the late endosome (LE)/lysosome (L), where cholesterol esters (CE) within the core of the LDL particle are hydrolyzed by acid lipase (11). Unesterified cholesterol/phospholipid complexes leave the LE/L, through a Niemann-Pick type C1/C2 (NPC1/NPC2)-dependent mechanism and are dispersed to the plasma membrane as well as the endoplasmic reticulum (ER) (12-14).

The NPC1 gene is mapped on chromosome 18 (18q11-q12) (15). It spans >47 kb and has 25 exons that encode a NPC1 protein of 1,278 amino acids with a molecular weight of 142 kDa (16). The NPC1 protein is a transmembrane protein containing a sterol-sensing domain with key roles as cholesterol transporter and cholesterol sensor (12,17). The most striking biochemical characteristic of NPC1-deficient cells is an immoderate accumulation of unesterified cholesterol, as well as sphingomyelin, glycosphingolipids, including

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Key words: NPC1, cardiovascular disease, genetic polymorphism

glucosylceramide, lactosylceramide, complex gangliosides (predominantly GM2 and GM3) and sphingosine (11,13,18). NPC1-mutant cells, due to sequestration of cholesterol, exhibit lysosomal accumulation of LDL-C, delayed downregulation of the LDL receptor, *de novo* cholesterol biosynthesis (19) and impaired ABCA1-mediated cholesterol efflux (20). ABCA1, an efficient exporter of cholesterol from macrophages and other cells, mediates transport of cellular cholesterol and phospholipids to HDL apolipoproteins (20). HDL-C, a complex of Apo-A lipoproteins, has antioxidative, antiproliferative, antithrombotic and anti-inflammatory properties, and its protective roles against CVD has been clearly demonstrated (4). NPC1-deficient subjects exhibit decreased plasma HDL-C cholesterol levels that are consistently associated with increased risk for all forms of atherosclerotic disease (21) and its clinical sequelae (22), including myocardial infarction (23), stroke (24) and sudden mortality (5,25).

Accumulation of lipoprotein-derived cholesterol in NPC1-deficient macrophages also promotes differentiation into cholesteryl ester-laden macrophages, or foam cells, and eventually cell death through apoptotic pathways (26). Foam cells are present at all stages of atherogenesis and are hypothesized to serve a critical role in the pathogenesis of atherosclerosis through formation of atherosclerotic plaque. It is well established that foam cell-originated atherosclerotic plaque is the hallmark event leading to cardiovascular events (27).

To date, a small number of previous studies have examined genetic polymorphisms of the NPC1 gene; rs1805081 (+644A>G) is the most studied polymorphism that has been reported to be associated with CHD (28), AD (29), overweight, obesity and morbid obesity (30); however, the results are controversial. Ma *et al* (28) reported that the G allele of the +644 locus is correlated with the reduced risk of CHD, whereas Rodriguez-Rodriguez *et al* (29) have demonstrated that the minor allele of rs1805081 is associated with increased risk of developing AD. NPC1 has been shown to be associated with cardiovascular-associated risk factors; however, to the best of our knowledge, no study has reported any association between genetic polymorphisms of NPC1 gene and risk of CVD. Therefore, the present study aimed to evaluate the association of the NPC1 rs1805081 (+644A>G) genetic polymorphism in patients with CVD and compare with healthy individuals.

Materials and methods

Patients and clinical data collection. A total of 320 individuals, including 200 patients with CVD and 120 healthy individuals, were recruited for the genotyping of the NPC1 rs1805081 (+644A>G) polymorphism.

The control individuals were selected from the normal population who, based on their stated information, had no disease or family history for CVD. The patients with CVD were characterized by the existence of recognized myocardial infarction, coronary bypass surgery or the presence of angiographically determined narrowing of coronary vessels by ≥50%. Blood samples were collected after a 12 h overnight fasting prior to cardiovascular procedures. At the time of enrollment, participants completed questionnaires on race/ethnic status, demographics, history of cigarette smoking, medical history and medications. In addition, use of lipid-lowering drugs (LLD), family history of hypertension, diabetes mellitus (DM), serum levels of total plasma cholesterol (TC), total triglycerides (TG), LDL-C and HDL-C were recorded (Table I). Hypertension was determined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg or therapy for hypertension. DM was diagnosed as a fasting blood glucose level >126 mg/dl or current use of hypoglycemic agents (19). TC, TG, LDL-C and HDL-C levels were measured as mg/dl using a standard enzymatic kit (Pars Azmoon Inc., Tehran, Iran). The present study was approved by the institutional review board of cardiovascular institute and the participating hospitals.

DNA preparation, polymerase chain reaction (PCR), and PCR-based allele genotyping. Blood samples were collected in EDTA-containing tubes and genomic DNA was isolated from peripheral blood leukocytes using a salting-out method, as described previously (31). Genotyping was performed by PCR-restriction fragment length polymorphism analysis. Primers were designed using Fast-PCR software and are shown as rs1805081, forward: 5'-GTTGCCTTGGTATG TGGTGTAC-3' and rs1805081, reverse: 5'-GATGACATA CATGGCGTCCAAG-3'. PCR was performed using commercially available PCR premix (AccuPower PCR PreMix; Bioneer, Daejeon, Korea), according to the manufacturer's protocol. A 0.2 ml PCR tube containing the AccuPower PCR PreMix, 1 μl template DNA (100 ng/μl), 1 μl of each primer (10 μM) and 17 μl DNase-free water were added. The reaction mixture was subjected to denaturation at 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec, then by a final extension at 72˚C for 5 min. The resultant PCR products were digested with *Nco*I (Fermentas, Vilnius, Lithuania), and the resulting fragments were separated by gel electrophoresis on a 2.5% agarose gel.

Statistical analysis. Statistical analysis was performed using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA). The allele frequencies were calculated and performed a Hardy-Weinberg equilibrium (HWE) test using the Fisher probability test statistic. Odds ratios (ORs) and 95% confidence intervals (CIs) were computed using binary logistic regression analyses. Adjusted ORs were stratified by age, gender, LLD usage, ethnicity, history of CVD, hypertension and DM. Statistical analysis of continuous variables of demographic data was evaluated using two-sided Student's t-test or Mann-Whitney U test. Categorical data were analyzed by the χ^2 test. Analysis of variance, followed by Sheffe's test was used for analysis of the serum lipid levels according to each genotype. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The present results demonstrated that a number of clinical characteristics of individuals, including gender, LLD usage, ethnicity, history of CVD, hypertension, DM, serum levels of TG, TC, LDL-C and HDL-C were significantly different between CVD patients and healthy controls (Table I). Since only a fraction of CVD patients took LLD, compared with control, their serum TG, TC and LDL-C levels were

Table I. Clinical and biochemical data in the CVD cases and control individuals.

Parameter	CVD cases	Control	P-value
No. of patients	200	120	-
Age, years	60.84±10.85	60.41±9.16	0.718
No. of males (%)	129 (64.5)	53 (44.2)	<0.001
No. of LLD users (%)	74 (37)	6 (5)	<0.001
Ethnicity, n (%)			<0.001
Fars	112 (56.0)	77 (64.2)	
Balouch	79 (39.5)	18 (15.0)	
Afqan	2 (1.0)	25 (20.8)	
Current smoking, n (%)	34 (17)	9 (7.5)	0.006
History of CVD, n (%)	66 (33)	7 (5.8)	<0.001
History of hypertension, n (%)	78 (39)	7 (5.8)	<0.001
History of diabetes, n (%)	77 (38.5)	3 (2.5)	<0.001
TG level (mg/dl)	214.02±40.56	119.21±44.53	<0.001
Cholesterol level (mg/dl)	209.99±39.27	175.28±28.73	<0.001
Cholesterol LDL (mg/dl)	130.39±29.99	112.20±34.89	<0.001
Cholesterol HDL (mg/dl)	37.57±5.85	49.56±8.75	<0.001

Data are presented as the mean ± standard deviation. CVD, cardiovascular disease; LLD, lipid-lowering drugs; TG, total triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table II. Distribution of NPC1 genotypes in patients with CVD and healthy individuals.

Genotype	CVD, n (%)	Control, n (%)	OR (95% CI)	P-value	Adjusted OR[a] (95% CI)	Adjusted P-value
AA	112 (56.0)	60 (50.0)	-	Ref.	-	Ref.
AG	74 (37.0)	40 (33.3)	0.991 (0.603-1.628)	0.972	1.326 (0.592-2.969)	0.493
GG	14 (7.0)	20 (16.7)	0.375 (0.177-0.795)	0.011	0.110 (0.017-0.715)	0.021
Allele A	298 (74.5)	160 (66.6)	-	Ref.	-	Ref.
Allele G	102 (25.5)	80 (33.4)	0.685 (0.482-0.971)	0.037	0.685 (0.482-0.971)	0.037

[a]Adjusted ORs were stratified by age, gender, LLD usage, ethnicity, history of CVD, hypertension and DM. ORs and 95% CIs were computed using binary logistic regression analyses. NPC1, Niemann-Pick type C1; CVD, cardiovascular disease; OR, odds ratio; CI, confidence intervals; LLD, lipid-lowering drugs; DM, diabetes mellitus; -, not applicable; Ref., reference.

significantly higher and HDL-C level was significantly lower in the patients with CVD (P<0.01). Regarding the single nucleotide polymorphism rs1805081, the observed genotype frequency in the cases was in agreement with HWE (P=0.711); however, the genotype distribution of NPC1 rs1805081 in controls deviated from HWE (P=0.006).

The PCR products (437 bp) following digestion yielded 2 DNA fragments of 268 and 169 bp for the A allele and remained undigested for the G allele, as presented in Fig. 1.

The frequency of the NPC1 rs1805081 (+644A>G) genotypes was compared between 200 cases of CVD and 120 controls (Table II). The GG genotype was more prevalent in the control group compared with that in patients with CVD (16.7 vs. 7.0%), and was associated with reduced risk of CVD (OR=0.110; 95% CI=0.017-0.715; P=0.021). In addition, the allelic frequency of this polymorphism was revealed to

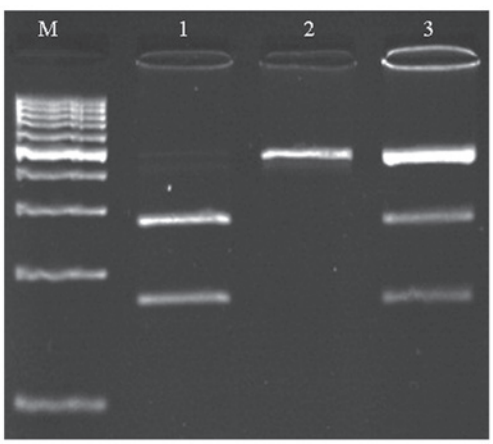


Figure 1. Gel patterns of A+644G polymorphism. M, 100 bp ladder; lane 1, homozygous AA genotype; lane 2, homozygous GG genotype; lane 3, heterozygous AG genotype.

Table III. Demographic characteristics of individuals according to NPC1 genotypes.

Parameter	NPC1 rs1805081 (+644A>G) genotype			P-value
	AA	AG	GG	
Age, years	60.98	60.64	59.22	0.673
No. of males (%)	96 (56.5)	64 (57.7)	22 (66.7)	0.553
No. drug users (%)	42 (41.2)	37 (44.0)	1 (3.8)	0.001
Ethnicity				0.025
Fars	92 (55.1)	72 (63.7)	25 (75.8)	
Balouch	61 (36.5)	33 (29.2)	3 (9.1)	
Afqan	14 (8.4)	8 (7.1)	5 (15.2)	
Current smoking, n (%)	22 (14.5)	15 (13.6)	6 (18.8)	0.769
History of CVD, n (%)	38 (23.3)	31 (27.7)	4 (12.1)	0.259
History of hypertension, n (%)	49 (36.8)	27 (26.5)	9 (27.3)	0.201
History of diabetes, n (%)	46 (29.5)	29 (27.9)	5 (17.2)	0.399
TG level (mg/dl)	187.7±56.9	179.0±62.67	131.3±68.53	0.0001
Cholesterol level (mg/dl)	203.3±36.71	192.5±43.32	185.7±36.37	0.030
Cholesterol LDL (mg/dl)	129.4±31.18	117.2±34.44	116.2±32.50	0.009
Cholesterol HDL (mg/dl)	40.3±7.95	41.7±9.07	50.1±10.56	0.0001

Data are presented as the mean ± standard deviation. CVD, cardiovascular disease; LLD, lipid-lowering drugs; TG, total triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

be different between the two groups, with the frequency of 33.4 and 25.5% in the controls and cases, respectively, and this difference reached statistical significance (OR=0.658; 95% CI=0.482-0.971; P=0.037). The association remained after adjustment for age, gender, LLD usage, ethnicity, history of CVD, hypertension and DM. Furthermore, Table III represents demographic characteristics of individuals, according to NPC1 genotypes. It can be inferred that the status of ethnicity and serum levels of TG, TC, LDL-C and HDL-C were different among three NPC1 genotypes. The majority of patients with the GG genotype were of Fars ethnicity and the lowest rate of LLD usage. Additionally, compared with the individuals with the other two genotypes, the GG carriers were from Fars ethnicity and compared with individuals with the other two genotypes, exhibited elevated levels of HLD-C and lower serum TC, TG and LDL-C levels, which suggested a protective role of the G allele for CVD.

Discussion

In the present study, it was revealed that the allelic and genotypic distributions of the NPC1 rs1805081 polymorphism were different between patients with CVD and healthy individuals. Since the prevalence of the G variant (both G allele and GG genotype) was elevated in the control group, it can be interpreted that the minor allele of the rs1805081 of NPC1 serves a protective role for CVD. In agreement with the present results, Ma *et al* (28) demonstrated that compared with the wild allele, the G allele of the rs1805081 locus is associated with the reduced risk of CHD in a Chinese population, suggesting the potential protective role of this variant for CHD. By contrast, in studies on AD, Rodriguez-Rodriguez *et al* (29) and Erickson *et al* (32) have reported that individuals with the GG genotype for NPC1 were at a higher risk of developing AD compared with individuals without these risk genotypes.

NPC1, a crucial protein in the cellular lipid trafficking, serves a protective role against intracellular cholesterol accumulation (19). In NPC1-deficient cells, despite increased cell cholesterol content, ABCA1 protein level is reduced (33). ABCA1 mediates the efflux of free cholesterol or unesterified cholesterol/phospholipid complexes from peripheral cells, including macrophage-derived foam cells, to apolipoprotein (apo)A-I and is responsible for nascent HDL particle formation (6,21,26). In the absence of ABCA1, HDL-cholesterol levels are extremely low, thus cholesterol efflux through HDL-mediated reverse cholesterol transport (RCT) is weakened (20). RCT is a key process through which peripheral cells unload their unwanted, surplus cholesterol by returning it to the liver for eventual disposal. The NPC1/ABCA1/HDL-mediated efflux process for the elimination of lipids from arterial wall cells and macrophages in the peripheral tissues is critical for the maintenance of lipid homeostasis and protection against foam cells-derived atherosclerotic CVD (34), diabetes mellitus type 2 (T2DM) and metabolic syndrome (20,33,35,36). In support of these reports, the present findings certified the function of NPC1 in the maintenance of lipid homeostasis, since individuals with the GG genotype exhibited higher levels of serum HDL-C, and lower levels of TG, TC and LDL-C, likely due to increased function of NPC1 in these individuals (Table III). By contrast, Sandholt *et al* (30) demonstrated that the NPC1 rs1805081 variant is associated with overweight, obesity and increased levels of TC; however, Wu *et al* (37) found no statistically

significant association between this variant and BMI/obesity in Chinese children.

Additionally, impairment of ABCA1-mediated cholesterol efflux in the NPC1-deficient cells is associated with initiation of atherosclerosis, since in NPC1-defect individuals, excessive storage of cholesterol within peripheral macrophages (38,39), accumulation of modified LDL such as oxidized (ox)LDL, and recruitment of monocytes in the arterial subendothelial matrix are key initiators of atherosclerosis. Additionally, monocyte-derived macrophages take up oxLDL through the scavenger receptor (SR) pathways and finally become foam cells. As sterol uptake mediated by SRs is not under negative feedback control, macrophages accumulate massive quantities of lipoprotein-derived lipids and become foam cells, the hallmark for early events of atherosclerosis (40-42).

Cholesterol crystals are another form of atherosclerotic lipid deposits in NPC-lacking individuals (43). Damaged trafficking of cholesterol in NPC1-deficient macrophages or mice hepatocytes have been shown to result in excessive uptake of cholesteryl ester lipid droplets and accumulation of unesterified cholesterol by the cells that induce the formation of cholesterol crystals (43). These cholesterol crystals may be outside or inside cells, appearing within cellular vacuoles or lysosomes. Cholesterol crystals act as an endogenous danger signal that initiates local inflammatory response. Local inflammation is critical in formation and expansion of the necrotic core that can cause plaque rupture and/or erosion by expanding sharp-tipped cholesterol crystals that lead to the initiation of systemic inflammation. A systemic process can influence the local milieu by activating the macrophages to become foam cells and phagocytic (25). In addition, crystals in the macrophage-derived foam cells induce apoptosis, leading to further attraction of macrophages that form an extracellular lipid pool and vulnerable plaque within the arterial wall (27,44,45).

Another important metabolic event in NPC1-deficient macrophages is dysregulation of cholesterol homeostasis (46). The ER serves as a cholesterol sensor, allowing the cell to regulate cholesterol synthesis and uptake via the sterol regulatory element-binding protein pathway (47). In NPC cells, LDL-cholesterol transport to the cell surface and ER is also greatly delayed, and the rising cholesterol level in the cell is not sensed by the ER, so homeostatic mechanisms are not initiated and it fails to downregulate cholesterol synthesis. Therefore, the LE/E continues to accumulate cholesterol and become highly enlarged leading to the formation of lipid droplet rich foam cells (20,46). In accordance with these facts, the present results demonstrated that individuals with a GG genotype for the NPC1 exhibited elevated levels of HDL-C and lower levels of TG, TC and LDL-C, suggesting the protective function of NPC1 against rising of serum lipids and thus susceptibility to CVD (Table III).

The variant rs1805081 has been located on exon 6 of the NPC1 gene (A, histidine; G, arginine at amino acid 215, a non-conserved amino acid), however no study has examined the functionality of this variation and it has not been fully determined whether the G variant is associated with increased/reduced activity of NPC1. From the present results as well as other findings it can be proposed that the G allele of the rs1805081 variant is correlated with raised activity of the NPC1 protein, since the G variant has shown protective characteristics against CVD and CHD (28,32).

The deviation from HWE in controls may be associated with the sampling bias or a relatively small number of individuals. Besides, the control group in the present study contained subjects with three ethnicities (Fars, Balouch and Afqan) that were not equal in numbers and may not have accurately represented the general population; therefore, the genotype distribution of NPC1 rs1805081 deviated from HWE. This is the limitation of the present study; therefore, the results must be interpreted with care.

In conclusion, the present findings demonstrated that NPC1 rs1805081 (A+644G) genetic variant is linked with a lower risk of CVD, and may contribute to protection against CVD. To the best of our knowledge, this is the first report regarding the association of the NPC1 genetic variation and susceptibility to CVD. The present results require replicating in other nationalities and require confirmation with larger samples size. The mechanisms are required to be entirely elucidated.

Acknowledgements

The present study was financially supported by a dissertation grant (M.Sc. thesis of MA) from Zahedan University of Medical Sciences. The authors are grateful to all individuals who willingly participated in the study.

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