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Screening of common genetic variants in the APOB gene related to familial hypercholesterolemia in a Saudi population

A case–control study

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Abstract

Familial hypercholesterolemia (FH) is a monogenic dominant inherited disorder of lipid metabolism characterized by elevated low-density lipoprotein levels, and is mainly attributable to mutations in low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), and proportein convertase subtilisin/kexin type 9 (*PCSK9*) genes. Next-generation and exome sequencing studies have primarily involved genome-wide association analyses, and meta-analyses and next-generation studies examined a few single-nucleotide polymorphisms (rs151009667 and Val2095Glu) in the ApoB gene. The present study was conducted to investigate the association of *APOB* and patients with FH in a Saudi population.

We genotyped 100 patients with FH and 100 controls for 2 polymorphisms in *APOB* using polymerase chain reaction-restriction fragment length polymorphism, followed by 3% agarose gel electrophoresis. The strength of the association between the genotype and allele frequencies with the risk of developing FH was evaluated. Clinical details and genotype analysis results were recorded.

For the rs151009667 polymorphism, 18% of the CT genotypes were observed only in patients with FH. There was a positive association between CT and CC (odds ratio [OR] 45.07 [95% conflict of interest (CI), 2.67–759.1]; $P = .0001$) and between T and C (OR 87.8 [95% CI, 5.34–144.2]; $P < .0001$). However, no Val2095Glu mutations were found in patients with FH or controls. There was also no correlation between clinical characteristics and the rs151009667 polymorphism.

In conclusion, we confirmed the association between the rs151009667 polymorphism and FH in a Saudi population. The Val2095Glu novel variant did not appear in either patients with FH or controls. Similar studies should be performed in different ethnic populations to rule out the role of this polymorphism in FH.

Abbreviations: ApoB = apolipoprotein B, BMI = body mass index, CI = conflict of interest, FH = familial hypercholesterolemia, Glu = glutamine, HDLC = high-density lipoprotein receptor, LDLR = low-density lipoprotein receptor, NGS = next-generation sequencing, PCSK9 = pro-protein convertase subtilisin-Kexin type 9, SD= standard deviation, TC = total cholesterol, TG = triglyceride, Val = valine.

Keywords: ApoB gene, familial hypercholesterolemia, rs151009667, Saudi population, Val2095Glu

1. Introduction

Familial hypercholesterolemia (FH; OMIM# 143890) is defined as a common genetic condition categorized by increased plasma levels of low-density lipoprotein-cholesterol (LDL-C) and premature atherosclerotic cardiovascular disease.[1] The disease FH was initially discovered in 1920 by Beeson,[2] and is typically considered to be a single-gene disorder.[3] The FH inheritance pattern was initially described by Khachadurian[4] in Lebanon, before the genes contributing to the disease were identified. FH is defined as an autosomal dominant disease with a clinical presentation based on the phenotype severity of homozygous and heterozygous forms, and with serum LDL-C levels that are 2- to 4-fold the normal level, respectively.[5] In FH, the frequency varies between heterozygous (1/500) and homozygous (1/1,000,000) FH. However, a recent population analysis estimated the prevalence to be as high as 1/250.[6] FH develops between the ages of 30 and 50 years in men and 40 and 60 years in women. Patients with FH are not always diagnosed properly, leading to inappropriate treatment strategies. FH-associated complications are common because of premature diagnosis and therapeutic interventions.[7] FH may be caused by a gain-of-function mutation in the LDL receptor (*LDLR*), apolipoprotein B (*APOB*), and pro-protein convertase subtilisin-Kexin type 9

Editor: Nikhil Jain.

This project was supported by the College of Medicine Research Centre, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia.

The authors have no conflicts of interest to disclose.

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Medicine (2019) 98:4(e14247)

Received: 5 September 2018 / Received in final form: 21 December 2018 / Accepted: 3 January 2019

http://dx.doi.org/10.1097/MD.0000000000014247

(*PCSK9*) genes. *LDLR* may suppress protein synthesis, which is translocated to the cell surface in monogenic mutations and by an additional mechanism that involves mutation affecting *APOB*, which encodes a key structural component of LDL and very low-density lipoprotein (VLDL). In addition, ApoE and LDLR adaptor protein 1 are required for LDLR and FH.[8,9] FH-affecting mutations were present in 60% to 80% of patients with a clinical diagnosis of pure FH, and 20% to 30% of the affecting mutations may appear in conceivable FH.[10] Genetic variations in *LDLR* are loss of function mutations, whereas *APOB* and *PCSK9* show similar lipid profile homeostasis functional defects. To date, >1000 genetic variations in *LDLR*, *APOB*, and *PCSK9* have been reported in the British Heart Foundation and other databases.[11] The FH diagnostic criteria are based on the Simon Broome criteria (UK); (Dutch Lipid Clinic Network Criteria (Netherland); and MedPed criteria (USA).[12] Various proteins, cholesterol internalization, and cellular metabolism have been connected to FH (e.g., ApoB-100, PCSK9, and LDLR). Genetic variations originating in the proteins include large rearrangements of intronic regions, coding, synonymous, non-synonymous substitutions, and mutations in regulatory regions or splicing sites. Missense mutations were the most frequent mutation type and were identified using second-generation sequencing techniques such as exome and next-generation sequencing (NGS) technologies within the exon coding region.[13] Radovica-Spalvina et al[3] previously performed NGS, and confirmed novel and documented variants in their cohort subjects. Our study was conducted to investigate the novel mutation Val2095Glu and familial variant rs151009667 in *APOB* in a case–control study of patients with FH in a Saudi population.

2. Materials and methods

2.1. FH participants

The Institutional Review Board of the College of Medicine at the King Saud University (KSU) provided ethics approval (E-12-829) for this study. All subjects who participated in study including patients with FH and control subjects signed an informed consent form. This study was performed according to the principles of the Declaration of Helsinki. As described in our prior publications,[7,14,15] 100 patients with FH were recruited from King Khalid University Hospital (KKUH) at KSU. Inclusion criteria were as follows: FH diagnosis made according to the Dutch group criteria[7]; male or female; subjects who underwent regular checkups; and subjects with no endocrine, metabolic, chronic, and other diseases. Exclusion criteria included the following: subjects with abnormal body mass index (BMI); subjects with diabetes; subjects with liver, renal, or thyroid disease or any other type of diseases; and subjects recruited outside the KKUH. Sex-matched controls (n = 100) were recruited from contract-based KKUH staff who may or may not have been outpatients.

2.2. Blood collection

From each patient, 5 mL of the peripheral blood was collected into 2 tubes (plain and EDTA tubes) by an experienced nurse. A 3-mL sample was used for biochemical analysis of the lipid profile, such as triglycerides (TGs), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and LDL-C. The remaining 2 mL was used for molecular analysis and was collected into an EDTA tube. Biochemical indications were analyzed using an automated clinical chemistry analyzer (KoneLab, Espoo, Finland).[14]

2.3. Molecular genotyping

Genomic DNA was extracted from the EDTA blood using a commercial human DNA kit as described by Alharbi et al.[7] To quantify genomic DNA, a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA) was used and primers were designed for the selected *ApoB* variants based on Radovica-Spalvina et al.[3] The complete details of *ApoB* variants are listed in Table 1. The primers were designed using Primer 3 software. For the Val2095Glu and rs151009667 variants, genotyping was performed by polymerase chain reaction with a 25-μL sample consisting of nuclease-free water, buffer, 2.5 μL $MgCl_2$, 0.5 μL dNTPs, 0.5 μL Taq DNA polymerase, 100 pmol of both sense and antisense primers, and 50 ng quantified genomic DNA. Initial denaturation was carried out at 95°C for 5 min followed by 35 cycles of 30 s at 64°C (for both variants), and 95°C for 45 min for initial elongation. The final elongation step was carried out at 72°C for 5 min. For both variants, restriction fragment length polymorphism analysis was conducted. Both the *RsaI* ($GT^{\downarrow}AC$) and *FNU4HI* ($GC^{\downarrow}GGC$) restriction enzymes (NEB England BioLabs, Ipswich, MA) were used to digest the samples for 18 h at 37°C. The digestion products were separated on a 3% agarose gel, which was stained with Ethidium bromide and visualized using ultraviolet light.

2.4. Statistical analysis

Clinical data between patients with FH and controls were evaluated using SAS software (version 9.3, SAS Institute, Cary, NC). Hardy-Weinberg equilibrium was tested between patients and controls. Descriptive characteristics of categorical variables are presented as the count (%), and continuous variables are presented as the mean and standard deviation. Between patients with FH and controls, the association was tested using 2 independent sample *t* tests.[7] Genotype and allele frequencies between patients with FH and controls were assessed using Openepi software (version 2.3.1) to determine the odds ratios, χ^2,

Table 1

Genetic details of the *APOB* gene evaluated in this study.

Gene	rs number	Nucleotide change	Amino acid change	Region	Mutation type	Forward primer	Reverse primer	PCR product size	Enzyme	Band size
APOB	—	C>T	Val-2095-Glu	—	Missense	GAGAGATGC CGTTGAGAAGC	AGCTTGCTGTGGGAGTTTTC	470 bp	RsaI	C-241/147/82 bp T-388/82 bp
APOB	rs151009667	C>T	Arg-1689-His	c.5066G>A	Missense	TTTGCATTGTG TCCCTGAAG	GCTCACAAGGCGACACTAAG	169 bp	FNU4H/	A-1696 bp G-149/20 bp

APOB = apolipoprotein B, Arg = arginine, bp = base pair, Glu = glutamine, His = histidine, Val = valine.

Table 2

Clinical features of familial hypercholesterolemia (FH) patients and controls.

No.	Features	FH patients (n = 100)	Controls (n = 100)	*P*
1	Age, y	51.66 (9.92)	44.02 (6.29)	.0001
2	Sex (male:female)	37:63	40:60	.62
3	Weight, kg	74.1 (9.40)	NA	NA
4	Height, cm	165.7 (7.53)	NA	NA
5	BMI, kg/m^2	27.1 (1.91)	NA	NA
6	TC, mM	5.4 (1.1)	4.8 (0.73)	.003
7	TG, mM	2.2 (1.2)	1.6 (0.99)	.009
8	HDL-C, mM	0.7 (0.2)	0.6 (0.27)	.71
9	LDL-C, mM	4.5 (0.9)	3.7 (0.72)	.003

BMI = body mass index, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol, NA = not analyzed/not applicable, TC = total cholesterol, TG = triglycerides.

and *P* values with and without Yates correction. Analysis of variance was also performed between the FH genotypes for rs151009667 and clinical characteristics.

3. Results

3.1. Clinical analysis

The results of anthropometric and biochemical measurements of patients with FH and controls are presented in Table 2. The mean ages of patients with FH and controls were 51.66 ± 9.92 and 44.02 ± 6.29 years, respectively, which was significantly different ($P = .0001$). Patients with FH and controls had a nearly equal sex ratio with no significant difference between groups ($P > .05$). We measured the height (165.7 ± 7.53 cm) and weight (74.1 ± 9.40 kg) only of patients with FH, and the overall BMI was 27.1 ± 1.91 kg/m^2 for this group. Lipid profile analysis revealed a positive association with TC, TG, and LDL-C ($P < .05$), whereas HDL-C showed a negative association when comparing patients with FH and controls ($P = .71$).

3.2. Genetic analysis of the rs151009667 polymorphism

No deviation from Hardy–Weinberg equilibrium was detected in the control group for both variants. Allele and genotype analyses for FH patients and controls are presented in Table 3. All alleles and genotypes in both variants were adjusted by Yates correction. The rs151009667 genotype frequencies for CC and CT were 82% and 18%, respectively, for patients with FH patients, whereas the genotypes in the control group were all CC. However, a strongly significant association was observed only for CT versus CC (odds ratio [OR] 45.07 [95% conflict of interest (CI), 2.67–759.1]; $P = .0001$) and *T* vs *C*: (OR 87.8 [95% CI, 5.34–144.2]; $P < .0001$). No mutations were detected for CT genotypes within control subjects. No homozygous variants appeared in either patients with FH or control subjects for rs151009667.

3.3. Val2095Glu allele and genotype frequencies

A 470 bp polymerase chain reaction product encompassing the novel variant Val2095Glu was digested using the FNU4H1 restriction enzyme, which yielded a 241/147/82 bp fragment, confirming the presence of the C allele. Only the CC genotype was detected in patients with FH and controls. No significant association was observed between the alleles and genotypes for CT vs CC (OR 1.00 [95% CI, 0.01–50.88]; $P = .99$) and *T* vs *C* (OR 1.00 [95% CI, 0.01–50.63]; $P = .99$). Thus, this locus was not further analyzed. Sanger sequencing was performed on 30 FH samples, which showed the same results.

3.4. Analysis of variance

Anthropometric measurements and the lipid profiles of patients with FH and controls with the CC, CT, and TT genotypes of the rs151009667 polymorphism were compared (Table 4). No FH patient characteristics showed a positive association between the rs151009667 polymorphism and age ($P = .57$), BMI ($P = .41$), TC ($P = .31$), TG ($P = .48$), HDL-C ($P = .47$), and LDL-C ($P = .95$).

4. Discussion

No case–control studies have investigated rs151009667 and the novel Val2095Glu variant in the global population. This is the first study to analyze these factors in a Saudi population and the world. We investigated the association between 2 genetic variants

Table 3

Yates correction for allele and genotype frequencies of APOB polymorphisms in patients with familial hypercholesterolemia (FH) and controls.

Mutation	Genotypes	FH cases (n = 100)	Controls (n = 100)	χ^2	OR (95% CI)	*P*
rs151009667	CC	82 (82%)	100 (100%)	R	R	R
	CT	18 (18%)	0 (0%)	16.79	OR 45.07 (2.67–759.1)	.0001
	TT	0 (0%)	0 (0%)	0.05	OR 0.02 (0.0002–3.35)	.03
	CT+TT vs CC	18 (18%)	0 (0%)	0.23	OR 0.22 (0.004–11.67)	.41
	CT vs CC+TT	18 (18%)	0 (0%)	0.0009	OR 201 (1.64–245.0)	.00005
	TT vs CC+CT	0 (0%)	0 (0%)	0.0009	OR 201 (1.64–245.0)	.00005
	C	164 (82%)	200 (100%)	R	R	R
	T	36 (18%)	0 (0%)	37.99	OR 87.8 (5.34–144.2)	<.0001
Mutation	Genotypes	FH cases (n = 100)	Controls (n = 100)	X^2	OR (95% CI)	*P*
Val2095Glu	CC	100 (100%)	100 (100%)	R	R	R
	CT	0 (0%)	0 (0%)	1.005	OR 1 (0.01–50.88)	.99
	TT	0 (0%)	0 (0%)	—	—	—
	CT+TT vs CC	0 (0%)	0 (0%)	—	—	—
	CT vs CC+TT	0 (0%)	0 (0%)	—	—	—
	TT vs CC+CT	0 (0%)	0 (0%)	—	—	—
	C	200 (100%)	200 (100%)	R	R	R
	T	0 (0%)	0 (0%)	1.002	OR 1 (0.01–50.63)	.99

CI = conflict of interest, R = reference.

Table 4

ANOVA analysis performed between the rs151009667 polymorphism of APOB and nongenetic variables.

Variables	CC (n = 82)	CT (n = 18)	TT (n = 00)	*P*
Age, y	51.73 ± 10.14	51.33 ± 9.10	0.00 ± 0.00	.57
BMI, kg/m^2	27.00 ± 2.40	26.43 ± 2.04	0.00 ± 0.00	.41
TC, mM	5.33 ± 1.03	5.89 ± 1.24	0.00 ± 0.00	.31
TG, mM	2.08 ± 1.32	2.25 ± 1.15	0.00 ± 0.00	.48
HDL-C, mM	0.70 ± 0.21	0.65 ± 0.24	0.00 ± 0.00	.47
LDL-C, mM	3.76 ± 0.86	3.60 ± 0.85	0.00 ± 0.00	.95

BMI = body mass index, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein cholesterol, TC = total cholesterol, TG = triglycerides.

and patients with FH in a Saudi population. To confirm the findings of this study, we screened the Val2095Glu and Arg1689His (rs151009667) variants in patients with FH and controls. The rs151009667 SNP was found to be associated in patients with FH compared to controls ($P < .05$). However, Val2095Glu showed no heterozygous (CT) and homozygous variant (TT) genotypes in patients with FH or controls ($P > .05$). These 2 variants were screened by exome sequencing analysis, as used in a previous study of a Latvian population of patients with FH. Generally, heterozygous variants detected in FH disease assists with detection using a sequence base as the prime methodology, and NGS/ES techniques have made these methods easier to use for diagnostic purposes.[3]

Alharbi et al[7] defined FH as a well-known autosomal dominant disorder with an earlier increased risk of coronary heart disease. FH is the only disease diagnosed based on cascade screening of genetic mutations such as *LDLR*, *APOB*, and *PCSK9* and based on high cholesterol levels and a family history. It is recommended to diagnose FH as soon as possible to prevent morbidity and mortality. However, the latest statins in pharmacotherapy have helped to lower LDL-C levels, decreasing morbidity and mortality related to cardiovascular disease.[16] A recent study by Ghaleb et al[17] confirmed that *ApoE* is an additional causative gene that contributes to screening for FH. Genetic variants in *LDLR*, *APOB*, *APOE*, and *PCSK9* are present in 80% of patients with FH. Genetic risk profiling improved after genome-wide screening was introduced and can simultaneously screen millions of polymorphisms for any complex disease.[18] In France, genetic cascade screening is currently used to help diagnose FH. NGS techniques enable examination of the monogenic and polygenic origins of elevated LDL-C levels observed in patients with FH.[19] There are a few common misconceptions in case control studies, such as matching in these studies does not always eliminate confounding factors; and matching analysis may not always be required. A previous study by Pearce[20] clarifies the misconceptions in case–control studies. In this study, we selected sex-based samples in the recruited subjects. Patients with FH included 37% male and 63% female subjects, whereas controls included 40% male and 60% female subjects. However, age-matched controls were not recruited in this study because FH can develop at any age.

APOB is one of the major genes used for the molecular diagnosis of FH. The chromosomal region is located in the 2p24.1 region.[21]*APOB* was initially discovered in 1987 in phenotypic patients with FH, which revealed that FH is not related to mutations in *LDLR*.[22]*ApoB* has a specific role in FH disease, and variants in *APOB* typically occur in the exons. Patients with FH with a familial deficiency in *APOB* may have a milder form of the disease, which can affect *LDLR* mutations.[23]*APOB* is known to be associated with FH and it very important in the diagnosis of FH disease. Patients with homozygous FH may be documented in a proband with the appearance of biallelic pathogenic variants.[24] This gene encodes 2 proteins (ApoB-48 and ApoB-100), which play a major role in disease diagnosis. ApoB-48 is present in the intestine and ApoB-100 is in the liver. FH disease with *APOB* is confirmed by documenting 5 known mutations in ApoB-100. Each mutation blocks cholesterol in the walls of coronary arteries, increasing cholesterol levels and thus the risk of heart attack. Each mutation modifies a single protein building block in a critical region of ApoB-100. LDLs are removed from the blood by the modified proteins, leading to increased cholesterol levels. This is the major relationship between FH and *APOB*.[25] In any population, patients with mutations in Val2095Glu may have a greater risk of developing FH disease. The 2 SNPs (rs151009667 and Val2095Glu) examined in the present study may promote FH disease development because of the appearance of homozygous and heterozygous variants.

The role of the rs151009667 polymorphism is mostly related to FH and hypertriglyceridemia,[26] whereas for the novel variant Val2095Glu, its genetic role in any disease is unknown. More studies enrolling patients of different ethnicities should be performed to understand the connection with the novel variant in FH. The in vivo relationship between the molecular mechanisms of *APOB* variation and FH disease is well documented.

Few genetic studies have been performed in Saudi populations with FH and no NGS/ES studies have been performed in the Saudi population.[27]*APOB* is commonly used to diagnose all patients with FH; however, in this study, we identified novel SNPs and examined their association with FH. Few studies have used meta-analysis to examine FH disease.[28–31] Currently, only one ES study was performed in the global population,[32] whereas a limited number of NGS studies have been performed worldwide.[3,33–37] Radovica-Spalvina et al[3] identified 111 variants as synonymous, nonsynonymous, close proximity to the intron-exon boundary region, and other using NGS techniques in Latvian patients with FH. A meta-analysis of GWAS data can expand the associated data documented in earlier studies using imputed genotype data. However, preferred data selection should consider that the GWAS and meta-analysis results are combined with a unique number of genetic associations that have strong statistical support. GWAS and meta-analysis have shown that convincing size effects are always moderate when using the current platforms and the sample sizes can explain most of the large genetic risks common to most diseases.[38]

The present study had several limitations. We did not collect smoking status, exercise, diet, or clinical and medication details from the enrolled patients with FH. Only 100 FH cases and 100 control subjects were evaluated and thus larger samples sizes are needed to confirm our results. However, we enrolled all subjects from a native Saudi population, including both patients with FH and controls. Some unknown SNPs were screened in this study, and one novel variant was identified as well as another SNP reported in only a few studies. Most sex-matched subjects were recruited for both patients with FH and controls, which was another limitation of our study. In conclusion, we confirmed the association between the rs151009667 polymorphism and FH in a Saudi population. The Val2095Glu novel variant did not appear in either patients with FH or controls. These similar SNPs should be screened in different ethnic populations with FH disease to document their role in FH diagnosis. Accurate meta-analysis

results will reveal the genetic role of the diagnostic markers. Future studies should also include NGS/ES studies in the Saudi population. Large-scale studies would be required from different areas and races to screen such a polymorphism.

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