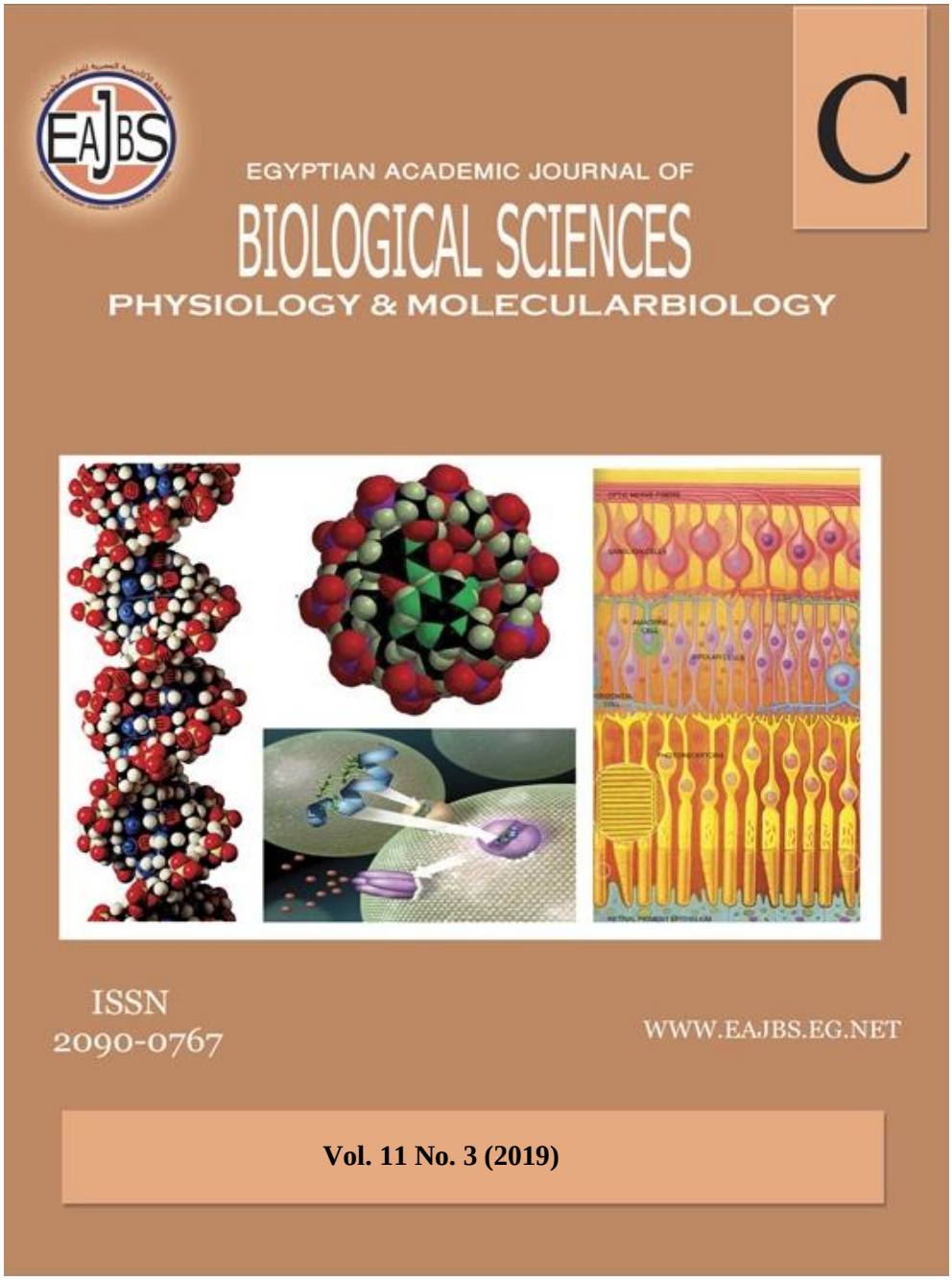

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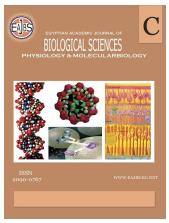

A Homozygous Missense Variant in the *APOB* gene in Patients from Hypercholesterolemia Families

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal codominant, life-threatening inherited condition. FH is characterized by an increased blood level of low-density lipoprotein cholesterol (LDL-C). Patients with FH are at serious risk of developing premature atherosclerotic cardiovascular disease. Association of FH with genetic variants in three genes (*APOB, LDLR*, and *PCSK9*) is well established, however, the data related to mutation spectrum and prevalence of FH in Saudi population is largely missing. Here, we studied two Saudi families segregating FH in an autosomal dominant manner. All exons and intro-exons junctions of three candidate genes (*APOB, LDLR*, and *PCSK9*) were sequenced using Sanger approach. Data analysis identified variants in exon 14 (c.1853C>T; p.Ala618Val) and exon 29 (c.13013G>A; p.Ser4338Asn) of the APOB gene in both families. Both variants perfectly segregating with FH phenotype in families. The variant (c.13013G>A) is located in the well-established active site of apolipoprotein B, thus, it might influence the enzyme activity. In conclusion, we found homozygosity for variant in APOB in families segregating FH. This study expanded the mutational spectrum of APOB in FH. In addition, the present study provided additional evidence that supports the important involvement of apolipoprotein B dysregulation in Saudi FH patients.

INTRODUCTION

Familial hypercholesterolemia (FH) is known to segregate in an autosomal dominant manner in families. FH has a global prevalence of approximately 1:300-500 (Campbell et al., 2017). FH is characterized by a high level of low-density lipoprotein cholesterol in blood that may lead to increased risk of cardiovascular disease later in life (Chiou and Charng, 2016, Lee, 2017, Xiang et al., 2017). FH patients may encounter premature coronary artery disease as well (Nohara, 2016). FH patients carrying heterozygous mutations have cholesterol level between 250 and 300 mg/dL

and males develop disease between the age of 30 - 50 years while females develop the disease between the age of 40 - 60 years (Kayıkçıoğlu and Tokgözoğlu, 2017).

Most of the FH cases have not been appropriately diagnosed thus resulting in treatment mismanagement. Early genetic diagnosis and therapeutic intervention are necessary for the prevention of FH related complications. Genetic analysis using DNA from isolated hypercholesterolemia patients as well as patients from FH families have identified disease-causing mutations in *APOB, LDLR* and *PCSK9* genes (Fairoozy et al., 2017, Rashidi et al., 2017). Although studies have been carried out in samples from Saudi population (Alharbi et al., 2015, Al-Allaf et al., 2017, Al-Allaf et al., 2016, Nuglozeh, 2017). However, mutation spectrum and FH disease prevalence is largely unknown in Kingdom of Saudi Arabia. In addition, most of the reports on Saudi patients describe mutations in *LDLR* gene. Moreover, to the best of our knowledge, this is the first report of *APOB* mutations from Saudi population.

MATERIALS AND METHODS

Description of Families:

Two large Saudi families with FH were enrolled in this study. In both families, consanguineous marriages were observed and FH was found to be transmitted vertically. In Family A (figure 1a), twenty-two DNAs were available, including seven affected individuals (III:2, III:3, III:5, III:6, IV:1, IV:5, IV:7). In Family B (figure 1b), samples from four affected (II:2; II:4, III:6, III:9) and eight unaffected individuals were analysed. Both families originated from different regions of Saudi Arabia. Approval to conduct the study was obtained from the Ethical Review Committee of the College of Medicine, Taibah University Almadinah. Members of both families provided written informed consent.

Biochemical Analysis of Blood:

Fasting venous blood samples (in EDTA anticoagulant) collected from all participants was used to measure a lipid profile. Individuals were asked to fast for at least 9 hours before blood was drawn. A simplified Canadian definition was used to evaluate patient status. An individual was considered a definite FH, if, he or she has an elevated LDL-C ($\geq$ 4.2 mmol/L). Total cholesterol, triglyceride, and LDL were measured enzymatically using Dimension RxL (Siemens Healthcare Diagnostics, Eschborn, Germany).

Normal range for cholesterol: 2.08-5.2 mmol/L, triglycerides: $\leq$ 1.69 mmol/L, and VLDL: $\leq$ 0.34 mmol/L.

Genomic DNA Extraction and Sanger Sequencing:

The manufacturer performed DNA extraction using QIA quick genomic DNA extraction kit according to the provided protocol. In Family A, DNA of two affected individuals were (III:3, IV:1; figure 1a) used for sequence the entire APOB coding region, LDLR and PCSK9 genes. In family B, these genes were sequenced in the DNA from two affected individuals (II:2, III:9; figure 1b). Experimentation was performed at the Center for Genetics and Inherited Diseases (CGID), Taibah University Almadinah, Saudi Arabia.

Genomic sequences of APOB (ENSG00000084674), LDLR (ENSG00000130164), and PCSK9 (ENSG00000169174) were taken from the ensemble genome browser (www.ensembl.org). Online tool, Primer3, was used to design primers. Primers were optimized using control DNA sample and all exons of three genes were PCR amplified using DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Waltham,

Massachusetts, US). PCR products resolved on 2% agarose gel. Amplified products were purified and labelled with fluorescent dideoxynucleotide dyes using BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, Massachusetts, US). Labelled products were sequenced using ABI 3500 genetic analyser (Applied Biosystems Inc., 850 Lincoln Centre Drive, Foster City, CA 94404, USA). BioEdit sequence alignment tool (Ibis Therapeutics, Carlsbad, CA) used to align the patient sequence recorded with the reference genomic sequence.

RESULTS

Table 1 shows the lipid profiles of individuals studied of family (a), family (a) include 27 individuals enrolled in this survey, Out of them, just 7 individuals were known hypercholesterolemia. One of them (II:2) has died. Two individuals (IV:5, IV:7) showed chest wounds and xanthomas. In addition, clinical evaluation identified bilateral carotid artery stenosis attributed to severe familial hyperlipidemia, as well, in some subjects (IV:3, III:4, III:5, III:7). For instance, an individual (III:5) lipid profile showed increased level of cholesterol (11.9 mmol/L), (normal value 2.08-5.2 mmol/L), very high triglyceride level (9.4 mmol/L), (normal value ≤ 1.69 mmol/L) and a very high VLDL level (1.9 mmol/L), (normal range ≤ 0.34 mmol/L) (Table 1).

On the other hand; table 2 describes the lipid profiles status in family (b); it includes 24 subjects, 6 of them were known hypercholesterolemia. Also one individual (I:1) their grandfather died. Interestingly, the lipid profile of two individuals (III:6 and III:9) characterized by very high cholesterol and Triglyceride levels.

Sequencing Data Analysis Identified Variants in *APOB* gene :

Sequencing reads of *APOB, LDLR,* and *PCSK9* were aligned to the reference genomic sequence using BioEdit sequence alignment tool. In severely affected individuals from family a and b (Fig. 1), homozygous missense variants in exon 14 (c.1853C>T) and exon 29 (c.13013G>A) of the APOB gene were detected, respectively (Figs. 1c and 1d). Segregation analysis identified multiple homozygotes for these variants in both pedigrees.

The variant in exon 14 (c.1853C>T) changes alanine amino acid at position 618 with valine (p.Ala618Val). Similarly, the variant in exon 29 (c.13013G>A) changes neutral amino acid serine at position 4338 to a hydrophilic amino acid asparagine (p.Ser4338Asn).

Table 1: Lipid profile of available individuals from family (a).

Member ID	Gender	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	VLDL (mmol/L)	LDL-cholesterol (mmol/L)	HDL-cholesterol (mmol/L)
I:1	F	4.7	2.2	0.44	3.5	0.75
I:2	M	3.6	1.2	0.24	2.4	0.95
II:1	F	4.2	1.9	0.38	3	0.83
II:2#	M					
II:3	F	4.4	2.3	0.46	3.2	0.74
II:4	M	3.9	2.1	0.42	2.7	0.8
II:5	M	4.1	1.7	0.34	2.9	0.8
II:6	F	3.9	1.6	0.32	2.4	1.2
III:1	M	4.8	1.1	0.22	3.6	0.98
III:2	F	9.6*	9.0	1.8	7.4	0.4
III:3	F	7.1*	5.6	1.12	5.2	0.78
III:4	F	4.2	1.9	0.38	2.9	0.92
III:5	F	11.9*	9.4	1.88	9.8	0.22
III:6	F	9.5*	7.9	1.58	7.2	0.72
III:7	M	5.1	2.4	0.48	3.9	0.72
III:8	M	3.8	0.9	0.18	2.8	0.82
III:9	F	4.6	1.2	0.24	3.4	0.96
IV:1	M	7*	2.5	0.5	5.8	0.7
IV:2	F	5.3	2.3	0.46	4	0.84
IV:3	M	3.8	1.4	0.28	2.6	0.92
IV:4	F	3.6	1.1	0.22	2.4	0.98
IV:5	M	7.4*	2.1	0.42	5.8	1.18
IV:6	M	4.6	2.2	0.44	3.3	0.86
IV:7	F	8.7*	1.7	0.34	7.5	0.86
IV:8	M	5.2	2.5	0.5	3.9	0.8
IV:9	F	4.6	2.3	0.46	3.5	0.64
V:1	F	4.4	1.7	0.34	3.3	0.76

LDL: Low Density Lipoprotein, HDL: High Density Lipoprotein.
*: Hypercholesterolemia. #: Died member.

Table 2: Lipid profile of available individuals from family (b).

Member ID	Gender	Total cholesterol (mmol/L)	Triglyceride (mmol/L)	VLDL (mmol/L)	LDL-cholesterol (mmol/L)	HDL-cholesterol (mmol/L)
I:1#	M					
I:2	F	3.98	0.93	0.186	2.7	1.1
II:1	M	4.43	1.48	0.296	3.2	0.9
II:2	F	8.8*	4.8	0.96	7.4	0.44
II:3	F	4.5	2.54	0.508	3.3	0.69
II:4	F	9.7*	9	1.8	7.2	0.66
II:5	M	5.1	5.63	1.126	3.4	0.55
II:6	M	5.9	3.34	0.668	4.6	0.6
II:7	M	5.9	2.17	0.434	4.6	0.9
II:8	M	5.4	0.54	0.108	4.1	1.2
II:9	M	10.2*	8.6	1.72	8.1	0.38
II:10	F	4.8	0.7	0.14	3.6	1.1
III:1	F	3.7	1.23	0.246	2.4	1.1
III:2	M	3.9	1.57	0.314	2.6	0.9
III:3	M	4.6	0.47	0.094	3.3	1.2
III:4	M	4.8	2.7	0.54	3.5	0.76
III:5	F	4.8	1.25	0.25	3.54	1.05
III:6	M	11.2*	3.7	0.74	7.3	0.56
III:7	F	3.4	1.9	0.38	2.2	0.8
III:8	F	4.1	2.3	0.46	2.8	0.8
III:9	F	10.9*	7.2	1.44	7.7	0.5
III:10	M	5.4	2.9	0.58	4.1	0.7
III:11	M	3.8	1.8	0.36	2.5	0.9
III:12	M	4.2	2.1	0.42	2.9	0.9

LDL: Low-Density Lipoprotein, HDL: High-Density Lipoprotein.
*: Hypercholesterolemia. #: Died member.

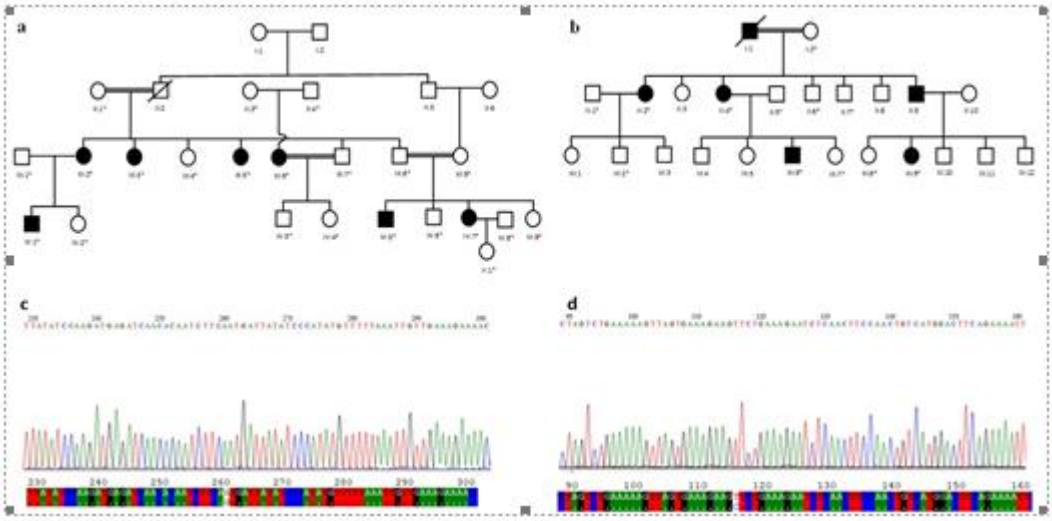

Fig. 1: Pedigrees of the two Saudi families with familial hypercholesterolemia (FH). Open squares and circles represent unaffected males and females, respectively. Filled squares and circles represent affected individuals. Double lines between symbols are representative of consanguineous unions. Individuals marked with an asterisk are available for genetic analysis (a and b). Partial sequence chromatograms of APOB gene represent the nucleotide sequences in all affected individuals (c and d).

DISCUSSION

Lipoproteins play significant roles in cellular function and are involved in regulating specific metabolic pathways. The apoB100 is the main protein for functional and structural constituents of the HDL and triglyceride-rich lipoproteins (Phillips et al., 2011). ApoB100 is the transporter of cholesterol and phospholipids thus providing constant supply of cholesterol to peripheral tissues and cells (Oram and Heinecke, 2005). The plasma level of APOB may reflect LDL concentration (Pischon et al., 2005). Few mutations in *APOB* gene been shown to cause familial hypercholesterolemia (FH) (Pullinger et al., 1999, Alves et al., 2014). Although, majority of FH patients have mutations in *LDLR* gene (Soutar and Naoumova, 2007).

Individuals with FH show increasing levels of plasma cholesterol and, thus, they are at high risk to develop cardiac complications. Early diagnosis can help in proper treatment. Consequently, atherosclerosis can be avoided and FH individuals can have a usual life. Mutations in one or more genes (*LDLR, APOB, PCSK9*) are considered as the molecular basis of the FH disease. However, some evidence suggesting that FH was misdiagnosed and inadequately treated in all populations. Specifically, their prevalence is largely unknown in the Kingdom of Saudi Arabia. A high rate of consanguinity, increased incidence of diabetes mellitus and obesity in Saudi Arabia further increases the risk and burden of FH cases.

Here, we screened complete coding regions of the known FH genes in 2 large Saudi families segregating FH. We identified homozygous missense variants in the *APOB* gene. Both variants have been described as population polymorphisms and are included in the dbSNP database. The variant in exon 14 (c.1853C>T) has an ID in dbSNP database rs679899, however, a PolyPhen software predicted this variant as a damaging.

Likewise variant in exon 29 (c.13013G>A) has an ID rs1042034 but this variant has been associated with the risk of ischemic stroke (Xiao et al., 2017, Zhou et al., 2018) and hyperlipidemia (Gu et al., 2017). Moreover, this variant is present in the well-established domain (Armadillo-type fold) of the apolipoprotein B enzyme, thus, it might influence the enzyme activity. Heterozygous variant in *APOB* gene in patients from Saudi family segregating hypercholesterolemia has been reported earlier (Muiya et al., 2009).

In silico studies of variant identified in this study and structural analysis of APOB mutant protein may provide very information to understand their role in FH disease.

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Author contributions:

All authors participate in all stages of this research including, collection of data, analysis, writing and reviewing the manuscript.

Conflict of Interest: None declared.

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