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Compound heterozygous LDLR variant in severely affected familial hypercholesterolemia patient

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Familial hypercholesterolemia (FH) is most commonly caused by mutations in the LDL receptor (LDLR), which is responsible for hepatic clearance of LDL from the blood circulation. We described a severely affected FH proband and their first-degree blood relatives; the proband was resistant to statin therapy and was managed on an LDL apheresis program. In order to find the causative genetic variant in this family, direct exon sequencing of the *LDLR*, *APOB* and *PCSK9* genes was performed. We identified a compound heterozygous mutation in the proband with missense p.(W577C) and frameshift p.(G676Afs*33) variants at exons 12 and 14 of the *LDLR* gene respectively. DNA sequencing of *LDLR* gene from the parents demonstrated that the missense variant was inherited from the mother and frameshift variant was inherited from the father. The frameshift variant resulted in a stop signal 33 codons downstream of the deletion, which most likely led to a truncated protein that lacks important functional domains, including the trans-membrane domain and the cytoplasmic tail domain. The missense variant is also predicted to be likely pathogenic and affect EGF-precursor homology domain of the LDLR protein. The segregation pattern of the variants was consistent with the lipid profile, suggesting a more severe FH phenotype when the variants are in the compound heterozygous state. The finding of a compound heterozygous mutation causing severe FH phenotype is important for the genotype-phenotype correlation and also enlarges the spectrum of FH-causative *LDLR* variants in the Arab population, including the Saudi population.

Key words: familial hypercholesterolemia (FH), low-density lipoprotein receptor (LDLR), compound heterozygous, missense variant, frameshift variant, sequencing, Arab, coronary artery disease (CAD), cholesterol, genetics

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Abbreviations: CAD, Coronary artery disease; CHD, Coronary heart diseases; FH, Familial hypercholesterolemia; LDLR, low-density lipoprotein receptor; APOB, apolipoprotein B; PCSK9, Pro-protein convertase subtilisin/kexin type 9; DNA, Deoxyribonucleic acid; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein-cholesterol; HDL, High-density lipoprotein; HDL-C, High-density-lipoprotein-cholesterol; TC, Total cholesterol; TG, Triglycerides; EDTA, Ethylenediaminetetraacetic acid; PCR, Polymerase chain reaction; EGF, Epidermal growth factor.

INTRODUCTION

Coronary heart diseases (CHD) inflict heavy economical and social cost on most populations including Saudis and contribute significantly to their morbidity and mortality rates. Familial hypercholesterolemia (FH) is hereditary in an autosomal dominant manner and is a major risk factor for the development of CHD (Cuchel *et al.,* 2014). If untreated, persistent hypercholesterolemia may produce tendon xanthomata (Achilles, extensor tendons of hands and feet), cutaneous planar, corneal arcus and premature cardiovascular disease (Al-Allaf *et al.,* 2015). Because the disease is initially asymptomatic and painless, the majority of patients may not be aware of their illness until a severe myocardial infarction occurrs in the fourth or fifth decade of life due to instant atheroma, which often leads to sudden cardiac death or other severe cardiovascular events (Al-Allaf *et al.,* 2010). FH is most commonly caused by mutations in the LDL receptor (LDLR), which is responsible for hepatic clearance of low-density lipoprotein (LDL) from the blood circulation. FH can also be caused by certain mutations in the apolipoprotein B (*APOB*) gene, which encodes the ligand for *LDLR* (Yang *et al.,* 2007). The pro-protein convertase subtilisin/kexin type 9 (*PCSK9*) gene was considered the third gene with pathogenic mutations accounting for some FH cases (Abifadel *et al.,* 2003; Timms *et al.,* 2004).

More than 1700 different variants of the *LDLR* gene were found to cause familial hypercholesterolemia (www.ucl.ac.uk/ldlr), making genetic screening very laborious. Mutations in the *LDLR* gene were divided into five classes based on biochemical and functional studies on *LDLR* variants; such that Class 1 mutations include null alleles that lack any *LDLR* protein product. Class 2 mutations encode LDL receptor (LDLR) proteins that are abnormally transported from the endoplasmic reticulum to the Golgi apparatus. In Class 3 mutations, LDLR protein shows defective binding of the LDL ligand, and Class 4 mutation genes encode LDLR with disrupted internalization/endocytosis of LDL. Class 5 mutations in the *LDLR* gene encode LDLR which displays defective recycling (Jeon *et al.,* 2005).

To date there is no cure for FH. The primary goal of clinical management in severely affected patients is to control hypercholesterolemia in order to decrease the risk of atherosclerosis and to prevent CHD. The most common cholesterol-lowering agents are statin and its derivatives. Treatment with non-statin cholesterol lower-

ing agents, for example bile acid resin (Davidson *et al.,* 1999), niacin (Berge & Canner, 1991), fibrate (Rubins *et al.,* 1999) or cholesterol absorption inhibitor (Sudhop *et al.,* 2002), is also recommended for certain cases. Several therapeutic approaches were developed recently to lower LDL-C, either as monotherapy or in combination with statins (El Harchaoui *et al.,* 2008) including: squalene synthase inhibitors, microsomal triglyceride transfer protein inhibitors (Cuchel *et al.,* 2007), siRNA for PCSK9 (Frank-Kamenetsky *et al.,* 2008) or for apolipoprotein B-100 (Soutschek *et al.,* 2004) silencing, antisense PCSK9 (Gupta *et al.,* 2010), and antisense apolipoprotein B-100 (Raal *et al.,* 2010). However, even after treatment with a combination therapy, the majority of severely affected FH patients may still have extremely raised LDL-C serum levels (Gagne *et al.,* 2002) and their risk of CHD remains unacceptably high. The preferred treatment for these patients is weekly or fortnightly plasma apheresis or LDL apheresis, a physical procedure in which LDL is selectively removed from the blood by running the plasma through columns that bind the LDL. LDL apheresis may lower LDL-C levels by about 55% and delay the onset and progression of atherosclerosis (Thompson *et al.,* 2010; Al-Allaf *et al.,* 2010).

In the majority of studied populations, the heterozygote form occurs in less than 1:500 and the homozygous form is one in a million (Heath *et al.,* 2001; Soutar & Naoumova, 2007). Some populations, such as Lebanese, French Canadians, Dutch Afrikaners and Ashkenazi Jews, are at a higher risk for FH due to the increased prevalence of heterozygous FH-associated mutations in the *LDLR* gene (Austin *et al.,* 2004; Cuchel *et al.,* 2014). However, recent studies in general populations suggested that the incidence of heterozygous FH based on the Dutch Lipid Clinic Network criteria may be as high as 1 in 200 (Nordestgaard *et al.,* 2013) or, for molecularly defined heterozygous FH, 1 in 244 (Sjouke *et al.,* 2015). Subsequently, homozygous FH may affect as many as 1 in 160 000–300 000 persons (Nordestgaard *et al.,* 2013; Cuchel *et al.,* 2014). The highest frequency of heterozygosity with the prevalence of less than 1:80 was found in the Afrikaner population in South Africa (Steyn *et al.,* 1996). Studies on the French Canadian population where five common mutations were reported showed a frequency of 1:270 (Leitersdorf *et al.,* 1990; Hobbs *et al.,* 1992; Zetterstrom *et al.,* 2011). This unusual high frequency is due to founder effects and consanguinity and no heterozygote advantage was identified. The prevalence of FH in Saudi Arabia is not known and there are limited reports on the molecular characteristics of FH. The disease may have a higher prevalence in Saudi Arabia than in other neighboring countries because of consanguineous marriages exceeding 50% (Jaber *et al.,* 1998); and due to the lack of national registries and genetic screening for FH, the disease is underdiagnosed and underestimated.

Recently we reported two novel mutations in the *LDLR* gene causing FH in severely affected Saudi patients. The first was a novel nonsense (Al-Allaf *et al.,* 2014) and the second was a novel frameshift variant (Al-Allaf *et al.,* 2015; Al-Allaf *et al.,*2016). In continuation to our further mutation screening for FH patients, herein, we describe a compound heterozygous mutation with missense and frameshift variants identified in the *LDLR* gene in severely affected FH patient who was resistant to statin therapy and was managed on an LDL apheresis program.

MATERIALS AND METHODS

Subjects. Recently we analyzed and reported two novel variants in the 23 subjects including 12 probands and 11 first-degree blood relatives from 12 unrelated Saudi families with FH (Al-Allaf *et al.,* 2014; Al-Allaf *et al.,* 2015; Al-Allaf *et al.,* 2016). In this study the analysis was performed on five subjects including one proband and their four first-degree blood relatives of a family that originates from a tribe that lives in the Northern region of Saudi Arabia. The proband was clinically diagnosed with homozygous FH. The proband was resistant to statin therapy and was managed on an LDL apheresis program. The sample collection and study were performed

Table 1. Description and Lipid profile of the studied family with the missense and frameshift variants of the LDLR gene.

Family members	Father	Mother	Proband	Brother	Sister
Missense variant in LDLR exon 12	–	c.1731G>T, p.(W577C), Htz	c.1731G>T, p.(W577C), Htz	c.1731G>T, p.(W577C), Htz	–
Frameshift variant in LDLR exon 14	c.2027delG, p.(G676Afs*33), Htz	–	c.2027delG, p.(G676Afs*33), Htz	–	–
Silent variant in LDLR exon 12	–	c.1725C>T, p.(=), Htz	c.1725C>T, p.(=), Htz	c.1725C>T, p.(=), Htz	c.1725C>T, p.(=), Htz
Sex (M/F)	M	F	M	M	F
Age (Year)	41	38	6	13	9
Total Cholesterol (mmol/l)	11.3	7.6	21.3	7.4	4.6
Triglyceride (mmol/l)	3.1	1.3	2.5	1.2	0.6
LDL-C (mmol/l)	8.8	5.5	18	5.9	3
HDL-C (mmol/l)	0.93	1.39	0.87	0.98	1.37
Symptoms	No	No	Tendon xanthomas, corneal arcus	No	No
Family history of CAD	Yes	Yes	Yes	Yes	Yes

Lipid profile represented here are untreated values. Normal range for lipid profile was described in our recent publication (Al-Allaf *et al.,* 2014). Abbreviations: M, male; F, female; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; LDLR, low-density lipoprotein receptor; CAD, coronary artery disease.

in accordance with the Research Ethics Committees regulations at King Faisal Specialist Hospital and Research Center (KFSHRC), Riyadh, Saudi Arabia and all subjects gave informed consent. The enrollment criteria for the patients' genetic screening were based on Simon Broome register (Scientific Steering Committee, 1991).

DNA sequencing. Genomic DNA was isolated from EDTA-treated whole blood using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the *LDLR* gene (including the 18 coding exons and flanking intron regions), *APOB* gene (exon 26 of the *APOB* gene containing codons 3475–3592, which harbors three known pathogenic variant sites, R3500Q, R3500W and R3527Q) and *PCSK9* gene (the 12 exons and flanking intron regions) was performed. Description of the primers used for amplifying and sequencing fragments was provided in Supplementary Table 1 (at www.actabp.pl). PCR was performed with 100 ng genomic DNA using the HotStarTaq Plus DNA Polymerase Kit (Qiagen, Hilden, Germany) as follows: *Taq* polymerase was activated at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 61–64°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. The amplified products were also separated on agarose gel to ensure the proper size and quality of the band (representative gel images are shown in Supplemental Material; Appendix B at www.actbp.pl). The PCR products were purified with magnetic beads method using Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). The purified products were used as templates for direct sequencing with a BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The sequencing reaction products were purified with BigDye X-terminator purification kit (Applied Biosystems) followed by capillary electrophoresis in an ABI 3500 Genetic analyzer (Applied Biosystems). The final analysis was performed using the Sequence Analysis Software v5.4 (Applied Biosystems). The LDLR transcript used for coding and protein position was NM_000527.The nomenclature of the identified variants was described as per the Mutalyzer program.

RESULTS

Two heterozygous variants c.1731G>T, p.(W577C) and c.2027delG, p.(G676Afs*33) were found in exons 12 and 14 of the *LDLR* gene respectively in a proband of a Saudi family from a tribe which lives in the Northern region of Saudi Arabia. The pedigree structure of the family is shown in Fig. 1. Both variants were identified in proband, while his father carried one heterozygous variant (c.2027delG) and mother carried another heterozygous variant (c.1731G>T). The brother of proband was found to be carrying one heterozygous (c.1731G>T) variant. However, the sibling (sister) did not show any mutation in any of the three candidate (*LDLR, APOB* and *PCSK9*) genes. In addition to this compound heterozygous variants, one known silent variant c.1725C>T, p.(=), rs1799898, NM_001195798.1 was also identified in exon 12 of the *LDLR* gene (Table 1). There were no variants detected at exons 12 and 14 of the *LDLR* gene in 10 healthy individuals studied. Figure 2 shows results of the DNA sequence analysis of exon 12 and 14 of the *LDLR* gene. The lipid profile of all the studied subjects is presented in the Table 1. The total and LDL cholesterol levels with values reaching 21.3 and 18.0 mmol/l respectively in proband are considered very high compared with the optimal level, which is <2.59 mmol/l (LDL cholesterol). In addition to the high levels of lipid cholesterol, the proband also displayed tendon xanthoma and corneal arcus, was resistant to statin therapy and managed on an LDL apheresis program. There was CAD history present in the family (Table 1). This confirmed the loss of LDLR function as a result of compound heterozygous variant presence in the DNA sequence. Compound heterozygous missense and frameshift variants may be associated with the severity of the disease.

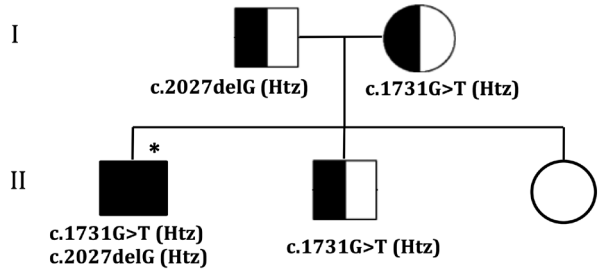

Figure 1. Pedigree of the patient family.
Filled symbols indicate compound heterozygous, half filled indicate heterozygous (Htz) and unfilled indicate unaffected individuals. *Proband

DISCUSSION

Familial hypercholesterolemia (FH) is a lipid metabolism disorder that is genetically inherited in an autosomal dominant manner, which clinically results in high concentrations of plasma cholesterol bound to LDL. Ap-

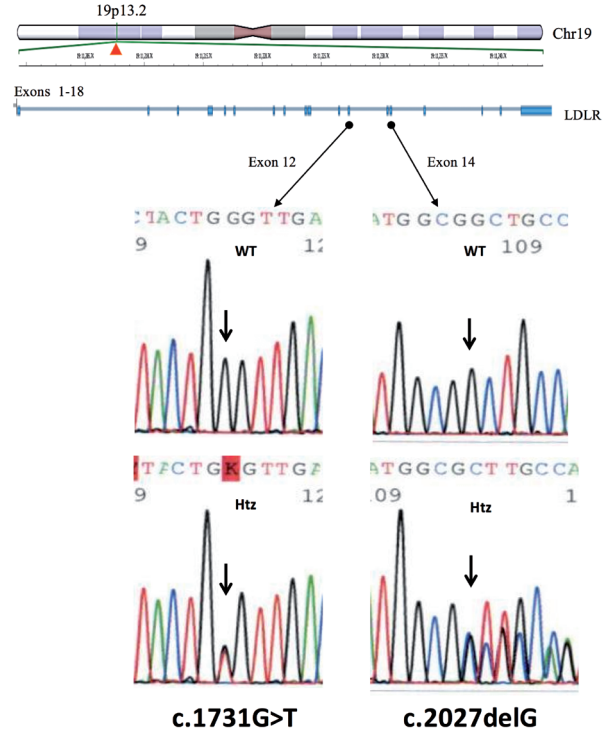

Figure 2. Structure and location of the *LDLR* gene on chromosome 19p13.2.
The position of the two variants, c.1731G>T and c.2027delG is indicated in exons 12 and 14, respectively. Representative DNA sequence from the control individuals (Wild type; WT) and heterozygous (Htz) of the family.

proximately, half of the heterozygous men with familial hypercholesterolemia, if untreated, develops clinically evident CHD by the age of 55 years. Affected heterozygous women from the same families typically develop CHD about 9 years later than their affected male relatives (Lindgren *et al.* 1985). Therefore, our aim was to describe a compound heterozygous variant with known missense and frameshift variants identified in the *LDLR* gene in severely affected FH patient who was resistant to statin therapy and was managed on LDL apheresis program.

The segregation pattern of the variant was compatible with the lipid profile (Fig. 1, Table 1). The severity of FH was higher in compound heterozygous proband (Table 1), due to the missense variant at maternal allele and frameshift variant at paternal allele that is predicted to cause abnormal LDLR function; regular heterozygous individuals on the other hand showed a reduced level of FH phenotype as the presence of one normal allele provides residual LDLR protein function. It was reported that patients homozygous or compound heterozygous for *LDLR* mutations or double heterozygous for *LDLR* and *APOB* mutations have higher LDL-C levels, more extensive xanthomatosis and more severe premature CAD than simple heterozygotes for mutations in either of these genes or for missense mutations in *PCSK9* gene (Muiya *et al.,* 2009). To the best of our knowledge this is the first report of such a mutation in the *LDLR* gene in the Arab population, including the Saudi population.

Approximately 54% of all *LDLR* gene mutations that result in FH occur in the EGF-precursor homology domain (Al-Allaf *et al.,* 2016), which means that this domain plays an important role in the function of the LDLR. The EGF-precursor homology domain controls lipoprotein release in low pH environments and the recycling of the receptor back to the cell surface (Rudenko & Deisenhofer, 2003). The missense p.(W577C) and frameshift p.(G676Afs*33) variants reported here are also located in the EGF-precursor homology domain of the LDLR protein. Hattori *et al* (2002) described the missense variant p.(W577C) as predicted to be likely pathogenic and affect EGF-precursor homology domain of the LDLR protein. The LDLR protein and LDL uptake were each decreased to 64% of controls by this variant (Hattori *et al.,* 2002).

Interestingly, the deletion mutation reported here, encodes a stop signal 33 codons downstream of the deletion, which could probably lead to either mRNA degradation or a truncated protein that lacks important functional domains. Furthermore, this truncated protein may be degraded and affect LDLR protein levels in mutation carriers; however, in case when a truncated protein survives in the cell, it could interfere with the normal assembly of the receptor protein complex. Similarly, we reported a novel nonsense variant in severely affected FH patients from a Saudi family who were also resistant to statin therapy and were managed on an LDL apheresis program (Al-Allaf *et al.,* 2014). Furthermore, more recently, we also reported the novel frameshift mutation p.(G676Afs*33) as a highly recurrent mutation in *LDLR* gene observed among individuals belonging to a selected group of Saudi children who were also subjected to the LDL apheresis program (Al-Allaf *et al.,* 2015; 2016).

In conclusion, the segregation pattern of the variants is consistent with the lipid profile, suggesting a more severe FH phenotype when the variants are in the compound heterozygous state. The finding of this study could be useful in developing critical genetic screening for severely affected FH patients. These results also enlarge the spectrum of FH-causative *LDLR* mutations in the Arab population, including the Saudi population.

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