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1 Abstract

2 ¹⁵ **Background:** Familial hypercholesterolemia (FH) is a common inherited metabolic disease that causes
3 premature atherosclerosis, cardiovascular disease, and even death at a young age. Approximately 95%
4 of FH-causing genetic variants that have been identified are in the *LDLR* gene. However, ⁶ only 10% of
5 the FH population worldwide has been diagnosed and adequately treated, due to the existence of
6 numerous unidentified variants, uncertainties in the pathogenicity scoring of many variants, and a
7 substantial number of individuals lacking access to genetic testing.

8 **Objective:** ¹⁴ The aim of this study was to identify a novel variant in the *LDLR* gene that causes FH in a
9 Chinese family, thereby expanding the spectrum of FH-causing variants.

10 **Methods:** ⁷ Patients were recruited from Beijing Anzhen Hospital, Capital Medical University. FH
11 ¹³ diagnosis was made according to the Dutch Lipid Clinical Network (DLCN) criteria. Whole-exome
12 sequencing (WES) was conducted to identify the FH-causing variant in the proband, and amplicon
13 sequencing was used to verify the variant in his family members.

14 **Results:** A three-generation Chinese family was recruited, and two FH patients were clinically
15 diagnosed, both without known FH-causing variants. These two FH patients and another possible
16 patient carried a novel variant, NC_000019.9(NM_000527.5):c.89_92dup
17 (NP_000518.1:p.Phe32Argfs*21), in ¹² the ligand-binding domain of the low-density lipoprotein (LDL)
18 receptor that led to a frameshift. The FH adults in the family showed severe clinical symptoms and
19 statin therapy resistance.

20 **Conclusion:** This study identified a novel pathogenic *LDLR* variant, c.89_92dup, associated with
21 severe FH clinical manifestations and statin therapy resistance.

22

23 ³ **Keywords:** familial hypercholesterolemia; low-density lipoprotein receptor; c.89_92dup variant;
24 ligand-binding domain; whole-exome sequencing

25

26 ⁸ **Background**

27 Familial hypercholesterolemia (FH) is an autosomal dominant metabolic disorder that is characterized
28 by high serum levels of low-density lipoprotein (LDL) cholesterol [1]. The prevalence of FH is
29 approximately 1 in 250~500 worldwide [2, 3], which is higher than that of most other inherited
30 diseases. In patients carrying heterozygous variants, serum LDL cholesterol levels are typically above
31 5.0 mmol/L, which is 2 times greater than those in unaffected individuals within the same family [4]. In
32 patients carrying homozygous variants, serum LDL cholesterol levels frequently exceed 13.0 mmol/L
33 [5]. Long-term hypercholesterolemia can manifest as tendon xanthomas and arcus cornealis [1]. When
34 left untreated, FH patients often suffer from premature atherosclerosis, cardiovascular disease (CVD),
35 and even death at a very young age [6, 7]. In China, individuals with FH have a 15-fold increased ²¹ risk
36 of developing CVD compared to those without FH [8].

37 Known FH-causing variants are found in several genes that are associated with the removal of LDL
38 particles from the circulation by hepatocytes, such as ⁹ *LDL receptor* (*LDLR*, >95%), *apolipoprotein B*
39 (*APOB*, 2~11%), and *proprotein convertase subtilisin/kexin type 9* (*PCSK9*, <1%) [1, ¹¹ 9]. The *LDLR*
40 gene is located at 19p13.2 and comprises 18 exons spanning 45 kilobases (kb). It encodes an 860 amino
41 acid protein that consists of ¹ five functional domains, including a ligand-binding domain, an epidermal
42 growth factor (EGF) precursor homology domain, an O-linked polysaccharide domain, a
43 transmembrane domain, and an intracellular domain [10]. According to the statistical data from the
44 ClinVar ¹⁹ database [11] (<http://www.ncbi.nlm.nih.gov/clinvar/>), more than 40% of FH-causing variants

are located in the EGF precursor homology domain, which impairs lipoprotein release and receptor recycling [12]. Another 40% of variants are located in the ligand-binding domain and therefore influence the binding of lipoproteins to LDL and VLDL (very low-density lipoprotein) [13]. No more than 20% of variants are scattered across the other three domains and introns [14].

According to the Familial Hypercholesterolemia Foundation and the World Heart Federation, only 10% of the FH population has been diagnosed and adequately treated [15]. Diagnoses are missed partially due to the existence of numerous unidentified variants, uncertainties in the pathogenicity scoring of many variants, and a substantial number of individuals lacking access to genetic testing. In this study, a three-generation Chinese FH family was diagnosed using the Dutch Lipid Clinical Network (DLCN) criteria [16]. A novel frameshift variant, c.89_92dup, was identified in the ligand-binding domain of the LDLR protein via whole-exome sequencing (WES). This variant broadens the spectrum of FH-causing variants, facilitating the diagnosis of individuals with FH.

57

Materials and Methods

Study Subjects

The subjects were recruited from an FH cohort at Beijing Anzhen Hospital, Capital Medical University. The proband was hospitalized and clinically diagnosed with FH according to the DLCN diagnostic criteria. Then, cascade screening was conducted for his family members. Six participants were included in the study, and all the subjects underwent a detailed physical examination. This study has been granted approval by the Ethics Committee of Beijing Anzhen Hospital (2022038X). All participants willingly took part in the research and provided their signatures on informed consent forms.

67 **5 Isolation of peripheral blood mononuclear cells (PBMCs)**

68 Peripheral blood was collected from the proband and his family members into Vacutainer tubes
69 containing EDTA-2K and processed for PBMC isolation in a short time. Following plasma extraction
70 by centrifugation (2000 rpm, 10 min), the precipitate was resuspended in **5** an equal volume of
71 phosphate-buffered saline (PBS). The sample was then layered onto Ficoll-Paque (GE Healthcare,
72 Marlborough, MA, USA) and subsequently processed **22** in accordance with the manufacturer's
73 instructions. DNA was extracted from approximately 1×10^6 PBMCs for genetic analysis.

74 **Biochemical analysis**

75 A Roche COBAS 701 analyzer was used to assess **2** the serum total cholesterol (TC), total triglyceride
76 (TG), high-density lipoprotein (HDL) cholesterol, non-HDL cholesterol, low-density lipoprotein (LDL)
77 cholesterol, small dense LDL cholesterol, lipoprotein (a), apolipoprotein A1, and apolipoprotein B
78 levels. The LDL cholesterol level was measured using **7** a direct assay (LDL-Cholesterol Gen.3
79 (LDLC3), Roche Diagnostics).

80 **WES library preparation and sequencing**

81 A Qiagen DNeasy kit (Qiagen, USA) was used to extract genomic DNA from the peripheral blood cells
82 of the proband. After sonication, the DNA fragments were subjected to end repair and were
83 supplemented with adapters at both ends. Following library amplification, the fragments were captured
84 and enriched using an exon array. After washing to remove the nonenriched fragments, the remaining
85 DNA was amplified and subjected to whole-exome sequencing using the Illumina HiSeq 3000 platform
86 in paired-end sequencing mode (analysis performed by the Nantong KingMed Center for Clinical
87 Laboratory).

88 The raw reads were mapped to the reference human genome (hg19) using Bowtie2 [17]. After filtering
89 low-quality nucleotides (quality < 30) and reads with lengths less than 18 bp by Trimmomatic [18], the
90 qualified reads were further processed with SAMtools to call SNPs using mpileup [19]. SNPs detected
91 on all known genes were annotated with ANNOVAR software [20].

92 **Amplicon sequencing**

93 Primers I and II were designed according to the *LDLR* sequence² in the GenBank nucleotide sequence
94 database (<https://www.ncbi.nlm.nih.gov/nucleotide/>) and had the following sequences: Primer I, 5'-
95 TTTATTAATGTGCATGGAAGTTCT-3'; and Primer II, 5'- AGACCAGAAATTCAAGACCAGC-3'.

96 With polymerase chain reaction (PCR) technology, amplicons containing the variant were amplified
97 from equal amounts of DNA samples. Sanger sequencing was employed to validate the identified
98 variant in other members of the family. Amino acid conservation near the variant position was
99 examined using MEGA 11.0 software.

100 **Open reading frame prediction of the *LDLR* variant**

101 The NCBI ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder>)⁴ was used to identify changes in the
102 open reading frame (ORF) for the NM_000527.5 sequence with the c.89_92dup variant.

103

104 **Results**

105 **Clinical characteristics of the FH family**

106 The proband was a 32-year-old man who was hospitalized for "unstable angina" and clinically
107 diagnosed with FH according to the DLCN criteria (Table 1). The patient showed severe clinical
108 symptoms of FH, including arcus comealis and tendon xanthoma (Figure 1). Coronary angiography
109 revealed triple-vessel disease involving the right coronary artery (RCA), left circumflex artery (LCX),

110 and left anterior descending artery (LAD), with complete occlusion in the distal segment of the
111 circumflex artery. The patient was insensitive to statin therapy. Before treatment, his LDL cholesterol
112 level reached 8.12 mmol/L. After two years of treatment with 20 mg atorvastatin plus 10 mg ezetimibe,
113 his LDL cholesterol level remained high at 6.92 mmol/L. Furthermore, he suffered an acute myocardial
114 infarction while on the lipid-lowering regimen. Fortunately, the patient responded well to a PCSK9
115 inhibitor. After one month of treatment with evinacumab (140 mg every 2 weeks), his LDL cholesterol
116 level decreased to 0.85 mmol/L.

117 Upon investigation of the proband's family history, it was discovered that his father (I.1) also met the
118 clinical criteria for FH (Table 1), with an untreated LDL cholesterol level of 6.75 mmol/L. In his forties
119 and again in his fifties, the father underwent arterial stent implantation for acute coronary syndrome
120 (ACS). The elder daughter (III.1) of the proband was seven years old and exhibited significantly higher
121 levels of LDL cholesterol (4.29 mmol/L). Neither the proband's mother (I.2) nor the younger daughter
122 (III.2) exhibited FH-related clinical symptoms. Table 2 shows the family's lipid profile and clinical
123 manifestations.

124

125 **Variant identification in the FH family**

126 In the proband (II.1), a novel variant, c.89_92dup, was identified in exon 2 of the *LDLR* gene by WES
127 (Figure 2A). No variants were identified in other FH-causing genes or other exons of the *LDLR* gene.
128 This duplication variant was located in a phylogenetically conserved region of the ligand-binding
129 domain (Figure 2B) and produced a frameshift in the coding sequence. The frameshift ORF included
130 an arginine amino acid variant at the 32nd position, which replaced the wild-type phenylalanine, and
131 translation terminated at the 52nd amino acid (Figure 2C). By predicting potential ORFs longer than 50

132 codons in the *LDLR* variant, four other ORFs were discovered to include novel translation start codons
133 (TSCs, Figure 2C). The longest predicted protein was truncated at the 263rd amino acid and lacked the
134 first five repeats and a portion of the sixth repeat³ of the ligand-binding domain of *LDLR*.

135

136 Variant Segregation Analysis

137 Amplicon sequencing confirmed that the c.89_92dup variant was carried by the proband's father (I.1)¹⁸
138 and elder daughter (III.1), while the proband's mother (I.2) and younger daughter (III.2) had the wild-
139 type *LDLR* gene. Figure 3 displays the pedigree of this FH family, showing that dyslipidaemia was
140 correlated with the segregation arrangement of the frameshift *LDLR* variant (Table 1). Therefore, the
141 c.89_92dup variant was determined to be the pathogenic variant for this FH family.

142

143 Discussion

144 This study reported a family in which the proband and his father were clinically diagnosed with FH
145 according to the DLCN criteria, and the proband's elder daughter exhibited significantly higher levels
146 of LDL cholesterol than her contemporaries. WES and amplicon sequencing confirmed that these three
147 patients were found to carry the same variant, c.89_92dup, which was consistent with autosomal
148 dominant inheritance. This duplication variant was situated within the ligand-binding domain of¹
149 *LDLR*, which consists of seven repeats of 40 residues each and contains the binding sites for
150 apoproteins B-100 (apoB-100) and E (apoE) [21, 22]. These apoproteins are essential components of
151 LDL and VLDL particles. Since this frameshift variant is absent from control populations (Genome
152 Aggregation Database,²³ 1000 Genomes Project, and Human Gene Mutation Database) [23-25] and

153 segregated with a phenotype in 3 informative meioses in the proband's family, the c.89_92dup variant
 154 was classified as pathogenic (PVS1, PM2, PP1 and PP4) according to the ACMG guidelines [26, 27].
 155 Five ORFs in the *LDLR* gene with the c.89_92dup variant were found to be longer than 50 amino
 156 acids. Among them, ORF1 had the same TSC as the wild-type *LDLR* transcript. This ORF potentially
 157 encoded a truncated protein consisting of only 32 amino acids at the N-terminus of LDLR, along with
 158 20 novel amino acids from the frameshift. Since dozens of RNA splice sites are located downstream of
 159 the premature termination codons (PTCs) in ORF1, the unremoved exon-exon junction complexes
 160 (EJCs) binding to mRNA should trigger nonsense-mediated mRNA decay (NMD) to degrade the
 161 transcript. ORF2 was predicted to produce an N-terminal truncated protein, adopting an ATG codon at
 162 positions 794-796 of the wild-type *LDLR* coding sequence (CDS) as the TSC. This truncated protein
 163 lacked the first five repeats and a portion of the sixth repeat of the ligand-binding domain of LDLR.
 164 The fifth repeat is required to bind β -VLDL, and the second, third, and sixth repeats are required for
 165 maximal binding of LDL [13]; therefore, the encoded protein produced by ORF2 lacks most of its
 166 ligand-binding functions. Additionally, the amino acid sequences encoded by the other three potential
 167 ORFs (ORF3-5) do not resemble those of the wild-type LDLR protein. In summary, although the
 168 impact of the c.89_92dup variant on LDLR protein sequence and abundance still needs to be verified,
 169 this variant may severely impair LDLR function in patients.

170 Consistent with the ORF analyses, patients with the c.89_92dup variant had more severe clinical
 171 symptoms. In the reported FH family, the proband and his father had LDL cholesterol levels of 8.12
 172 mmol/L and 6.75 mmol/L, respectively. These findings were significantly greater than the median level
 173 of 5.43 mmol/L (IQR: 4.32-6.72 mmol/L) observed in adults with heterozygous FH who did not use
 174 lipid-lowering medications [4]. In addition, the proband suffered an acute myocardial infarction at the

175 early age of 32, while his father had a myocardial infarction in his forties and a recurrent episode of
176 ACS at the age of 55. Both patients met the diagnostic criteria for severe FH [28].

177 More importantly, patients with the c.89_92dup variant seemed to be insensitive to statin therapy.
178 Here, the proband's LDL cholesterol level decreased by only 15% after two years of treatment with 20
179 mg atorvastatin plus 10 mg ezetimibe. This observation further showed that the duplication variant
180 significantly impaired LDLR function, resulting in an ineffective reduction in circulating cholesterol
181 levels despite inhibiting cholesterol synthesis and absorption. However, the proband responded well to
182 a PCSK9 inhibitor. When taken with statins and ezetimibe, coadministration of a PCSK9 inhibitor
183 reduced the patient's LDL cholesterol level by up to 90%. Because a heterozygous FH individual has
184 the homologous wild-type *LDLR* gene, a PCSK9 inhibitor increases LDLR abundance on the surface of
185 hepatocytes, thus enhancing its ability to clear LDL cholesterol [29, 30]. Therefore, PCSK9 inhibitors
186 are recommended for lipid-lowering therapy in patients carrying the *LDLR* c.89_92dup variant at an
187 early stage.

188

189 **Strengths and limitations of the study**

190 Using WES and variant segregation analysis, a frameshift variant, c.89_92dup, was identified as a
191 novel pathogenic variant for severe FH with a poor response to statin therapy. This finding contributes
192 to the clinical diagnosis and treatment of FH patients. Further molecular testing of the residual activity
193 of this LDLR variant could provide valuable insights into its biological functions.

194

195 **Conclusions**

196 The current study identified a novel pathogenic *LDLR* variant, c.89_92dup, associated with severe FH

197 clinical manifestations and statin therapy resistance. This study expands the spectrum of FH-causing
198 variants and provides assistance for disease screening and individualized treatment. For optimal
199 management, FH patients with the c.89_92dup variant should receive PCSK9 inhibitors in a timely
200 manner.
201