Research Article

Genetically confirmed familial hypercholesterolemia in outpatients with hypercholesterolemia

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Abstract

Background Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism which can lead to premature coronary heart disease (pCHD). There are about 3.8 million potential FH patients in China, whereas the clinical and genetic data of FH are limited. Methods Dutch Lipid Clinic Network (DLCN) criteria was used to diagnose FH in outpatients with hypercholesterolemia. Resequencing chip analysis combined with Sanger sequencing validation were used to identify mutations in the definite FH patients according to DLCN criteria. In silico analysis was conducted in mutations with previously unknown pathogenicity. Then, the novel mutant receptors were transfected into human embryo kidney 293 (HEK-293) cells. The binding and the internalization activities of the mutant receptors were analyzed by flow cytometry. Results The prevalence of definite FH in outpatients with hypercholesterolemia in this study is 3.2%. Using genetic testing, one homozygous FH (HoFH), one heterozygous FH (HeFH) and three compound heterozygous FH patients were confirmed. Eight mutations in low-density lipoprotein receptor (LDLR) gene were identified, in which c.357delG was a novel mutation and co-segregated with the FH phenotype. Bioinformatic analysis confirmed that c.357delG was a pathogenic mutation. Furthermore, when compared with the wild-type LDLRs by flow cytometry analysis, the binding and internalization activities of c.357delG mutant LDLRs were reduced by 35% and 49%, respectively. Conclusions This study identified eight LDLR gene mutations in five patients with definite FH, in which c.357delG is a novel pathogenic mutation. These findings increase our understanding of the genetic spectrum of FH in China.

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Keywords: Familial hypercholesterolemia; Low-density lipoprotein receptor; Mutation

1 Introduction

Familial hypercholesterolemia (FH; MIM: #143890) is an autosomal dominant genetic disease characterized by high levels of low-density lipoprotein cholesterol (LDL-C), cutaneous xanthomas and premature coronary heart disease (pCHD), which has recently become a big concern in the world.[1] Four genes have been identified as being responsible for FH, namely, LDL receptor (LDLR), apolipoprotein B (ApoB), proprotein convertase subtilisin/kexin-9 (PCSK9) and LDLRAP1. Mutations in LDLR are the most common cause of FH followed by mutations in ApoB and PCSK9.[2]

The LDLR gene lies on the short arm of chromosome 19 at 19p13.1-p13.3, spans 45 kb and consists of 18 exons and 17 introns.[3] The LDLR gene encodes the LDLR protein, which contains 843 amino acids.[4] So far, over 1700 mutations in the LDLR gene have been described in FH (www.ucl.ac.uk/ldlr/LOVDv.1.1.0/ and https://grenada.lumc.nl/LOVD2/UCLHeart/home.php? select_db = LDLR), including large fragment deletions, small fragment deletions, insertions, point mutations and splicing mutations. Different kinds of mutations in the LDLR gene generate malfunction of the protein. As a result, clearance of LDL-rich particles from the circulation decreases, and the elevated blood serum

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LDL-C levels cause early onset of atherosclerosis and an increased risk of pCHD in patients with FH.[5]

It is largely believed that in the general population, the prevalence of homozygous FH is 1/1,000,000, while the prevalence of heterozygous FH is 1/500. However, the heterozygous FH frequency is even higher than 1/500 in some populations, and the elevated frequency is generally due to a founder effect.[6] FH is under-diagnosed and under-treated in the general population worldwide and no more than 1% FH patients are diagnosed in most countries.[7] Hitherto, only one study has estimated the prevalence and treatment of FH in China, which included 9324 subjects from the Jiangsu Nutrition Study. The researchers reported that the prevalence of probable/definite FH was 0.28% (1.4/500) based on the modified Dutch Lipid Clinic Network (DLCN) definition. Only 15.9% of the patients received drug treatment, and none of them achieved the LDL target value. This study suggested that FH is also under-diagnosed and undertreated in China.[8]

With the advances in genetic testing, direct detection of mutations in candidate genes is now available. In this study, we used DNA re-sequencing array technique combined with Sanger sequencing validation to identify mutations in clinical FH patients. We also evaluate the pathogenicity of the mutations found in this study.

2 Methods

2.1 Study subjects

We studied 156 hypercholesterolemia patients attending the lipid clinic of Anzhen Hospital in Beijing, China over two 6-month periods from April 2014 to June 2016. Patients diagnosed as definite FH were enrolled for genetic testing according to the Dutch Lipid Clinic Network (DLCN) Criteria.[7,9] Secondary hypercholesterolemia, such as hypothyroidism, renal or hepatic disease, were excluded by laboratory tests. DNA samples from patients with definite FH were processed using a DNA sequencing array and validation by Sanger sequencing. Some pro-bands were reported in previous studies.[10]

The study was reviewed and approved by the Ethics Committee of the Beijing Anzhen Hospital. Written informed consent was obtained from all research subjects and their legal guardians.

2.2 Measurement of serum lipids

Peripheral venous blood samples were drawn from the pro-bands and available family members after a 12-h fast. Plasma total cholesterol (TC), LDL-C, triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) were measured using routine commercial kits (Beckman Coulter, Brea, USA) and an automated biochemistry analyzer (Beckman AU 4500, Brea, USA).

2.3 Identification of LDLR mutations

A re-sequencing microarray chip was used to identify candidate gene mutations from DNA samples provided by the five probands. The resequencing array was designed on the basis of photolithography and solid-phase DNA synthesis by Vita Genomics, Inc. and manufactured by Affymetrix (Santa Clara, CA).[11] Each micro-array contained 12.6 kb of coding exon and flanking intron sequences (both sense and antisense) of the three most relevant FH causing genes, LDLR, ApoB, and PCSK9, in duplication.[11] The FH arrays were scanned using the Affymetrix GeneChip scanner 3000 7G creating CEL files for subsequent analysis. According to the results of the microarray analysis, we amplified several mutated DNA fragments that would result in amino acid changes. Sanger sequencing was conducted to verify these mutations. Family verification was conducted by sequencing the DNA of both parents at the target segment. Novel mutations were tested in 50 unrelated healthy individuals. To confirm the heterozygous deletion mutation, clone sequencing analysis was conducted. T4 DNA ligase was used to ligate the target fragment to T vector. Then the target fragment was transformed into Escherichia coli. Further the positive clonal were picked up and cultured. Plasmid extraction was conducted, and finally the desired fragment was amplified by PCR and sequenced.

2.4 Prediction of mutation effects

Novel mutations found in this study were evaluated by searching the PubMed and LOVD databases (www.ucl.ac.uk/ldlr/LOVDv.1.1.0/ and https://grenada.lumc.nl/LOVD2/UCLHeart/home.php?select_db = LDLR). Pathogenicity prediction of the new mutations found in this study was assessed in silico using the following publicly available software: PolyPhen2,[12] SIFT,[13] and Mutation Taster.[14] The reference sequence used for LDLR was NM_000527.3 (NCBI RefSeq). The online SWISS-MODEL was taken to predict structural changes of mutant proteins (http://swissmodel.expasy.org/).

2.5 Construction of eukaryotic expression vectors for LDLR

Full-length human wild-type LDLR cDNA was inserted into pReceiver-M29 to construct an LDLR recombinant eukaryotic expression vector with an enhanced green fluorescent protein (eGFP) tag. DNA encoding the c.357delG mutant of the LDLR was made using the QuickChange XL
system (Stratagene, USA) and verified by DNA sequencing. The primer sequences used to generate the 357delG mutant were: forward, 5'-TCGCTGCCACGATGGAAGTGCATC TCTCGG-3'; reverse, 5'-CCGAGAGATGCACTTCCAT CGTGCCAGCGA-3'. cDNA for the wild-type LDLR and c.357delG and W462X LDLR mutants was transfected into HEK-293 cells using Lipofectamine-2000 (Invitrogen, USA).

2.6 Flow cytometric analysis

Flow cytometry was used to detect the amount of cell surface LDLR binding and LDL internalization. The fluorescence of 10,000 events for each sample was acquired for data analysis. Forward scatter versus side scatter gates were set to exclude dead cells and debris. Experiments were repeated at least three times with triplicate samples for each cell line.

To measure LDLR binding and internalization activity, the cells were transfected with wild-type, W462X and c.357delG LDLR were incubated in serum-free medium containing 20 mg/mL Dil-LDL for 4 h at 37°C. After the LDL incubation, the medium was removed, and the cells were cultured to perform the functional studies. The amount of LDL binding and internalization was detected using a FACS Calibur cell analyzer (Beckman Coulter).

3 Results

3.1 Clinical features

According to the Dutch Lipid Clinic Network Criteria, five patients were diagnosed as definite FH. The prevalence of definite FH in patients with hypercholesterolemia in this study is 3.2%. Clinical and biochemical characteristics of the probands are shown in Table 1. All the pro-bands presented xanthoma and two of them had corneal arcus. TC and LDL-C were extremely elevated and TG was normal.

3.2 Identification of gene mutations

DNA samples were analyzed by a DNA resequencing array (Figure 1). One HoFH, one HeFH and three compound heterozygous FH patients were confirmed. Overall, eight different LDLR mutations were found in the five probands (Table S1). No mutations in ApoB and PCSK9 genes were found in any of the probands. Among the eight mutations detected, there were five missense mutations, two nonsense mutations, and one frameshift mutation.

### Table 1. The clinical features of five definite FH probands.

| Index | Proband | Proband | Proband | Proband | Proband |
|-------|---------|---------|---------|---------|---------|
| Sex   | Male    | Male    | Female  | Female  | Female  |
| Age, yrs | 7      | 8       | 8       | 10      | 22      |
| TC, mmol/L | 19.54  | 15.86   | 7.60    | 10.19   | 16.36   |
| LDL-C, mmol/L | 17.18  | 13.85   | 5.37    | 7.53    | 14.1    |
| TG, mmol/L | 1.09   | 0.85    | 0.55    | 1.48    | 0.60    |
| HDL-C, mmol/L | 2.10   | 1.74    | 1.53    | 1.23    | 1.94    |
| Xanthoma | Y      | Y       | Y       | Y       | Y       |
| Corneal arcus | Y      | Y       | N       | N       | N       |
| Ultrasound | MR2    | AR(1), | TR(1)   | MR2     | AR(1), |
|           |        | AC      |         |         | MR(1), |
|           |        |         |         |         | TR(1)   | AC      |

AC: aortic calcification; AR: aortic regurgitation; FH: familial hypercholesterolemia; HDL-C: high-density lipoprotein; LDL-C: low-density lipoprotein cholesterol; MR: mitral valve regurgitation; N: no; TC: total cholesterol; TG: triglyceride; TR: tricuspid valve regurgitation; Y: yes.

A novel heterozygous deletion mutation (c.357delG) in exon 4 of the LDLR gene was found in proband III. Consistent with the data from the micro-array analysis, Sanger sequencing analysis of proband III revealed a heterozygous single nucleotide deletion (c.357delG) in exon 4 (Figure 2). Clone sequencing analysis verified the single heterozygous nucleotide G deletion. The heterozygous mutation was also present in another six members of her family who had hypercholesterolemia (Figure 3), and healthy members do not carry the mutation. Therefore, this mutation co-segregates with the FH phenotype. We also genotyped 50 unrelated healthy individuals as controls and did not find the mutant allele in any of these individuals.

3.3 Pathogenic mutations found in the LDLR gene

The new mutation (c.357delG), which is absent from published controls, confers a frameshift mutation Gly119fs with the new reading frame ending in a stop codon at 86 amino acid sites after the deletion site. Thus, this frameshift will cause a genetic loss of function. According to the three software tools, in silico analysis predicted this mutation to be pathogenic (Table S2). The protein structure prediction of c.357delG mutation by Swiss model showed no protein structure occurred at 86 amino acid sites after the deletion site (Figure S1).

S565X(c.1757C>A, p.Ser586X)[15], W462X(c.1448G>A, p.Trp483X)[16] and Q12X(c.97C>T, p. Gln33X) [5,16-20] also introduce premature stop codons leading to truncation of the LDLR protein which can severely damage the expression and function of LDLR. A606T (c.1879G>A, p.Ala627Thr)[16], L561F(c.1744C>T, p.Leu582Phe)[10], C122Y(c.428G>,

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Figure 1. The flow chart of mutations screen. SNP: single nucleotide polymorphism; SNV: single nucleotide variants.

Figure 2. The DNA sequencing results of the proband 3 and his mother. (A): The LDLR gene sequencing chromatogram on exon 4 of the proband 3, the heterozygous nucleotide G deletion (shown by the arrow) leads to a frame-shift in one allele; (B): proband 3’s mother carries the same hereozygous nucleotide G deletion mutation; (C&D): clone sequencing analysis verifies the single heterozygous nucleotide G deletion. Graph D shows a DNA strand with the single nucleotide G deletion and graph C shows the normal complementary strand. LDLR: low-density lipoprotein receptor.
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reduced to 65% and 51%, of the control values, respectively
also showed lower binding and internalization activities,
a known mutation, were significantly reduced to 17% bin-
and internalization in HEK-
W462X
transfected with wild-

3.4 The c.357delG mutant of the LDLR shows defects in LDL binding and internalization
Flow cytometry was used to observe cell-surface LDLR binding and internalization in HEK-293 cells that were
transfected with wild-type or mutant LDLR (c.357delG and
W462X). In comparison with the wild-type control, both the
W462X and c.357delG mutants showed lower LDL binding and reduced ability to internalize (Figure 4). The binding
and internalization activities of the W462X mutant, which is a known mutation, were significantly reduced to 17% binding and 39% internalizing ability. The c.357delG mutant also showed lower binding and internalization activities, reduced to 65% and 51%, of the control values, respectively (Figure 3).

4 Discussion
FH is not rare in the Chinese population and remains un-
identified and under-treatment. Many FH patients are not diagnosed until their first coronary events occurred. Early diagnosis is critical for FH patients, because Cholesterol-lowering medication such as Statin treatment can reduced the risk of premature atherosclerosis and improved the prognosis for FH. The development of next-generation sequencing technology makes rapid molecular diagnosis possible and provides a basis for personalized treatment.

In this study, five patients were diagnosed as definite FH by Dutch Lipid Clinic Network Criteria. DNA resequencing array was conducted in these patients and eight mutations include one novel mutation were identified. To ensure that this new mutation was associated with FH, verification of the c.357delG mutation was carried out by mutation allele-specific amplification in all 14 members of the proband’s family. We found that c.357delG segregated with the FH phenotype in affected family members. Furthermore, the mutation was not observed in 50 random healthy human DNA samples, indicating that it is not a common polymorphism in Chinese population. This deletion mutation (c.357delG) causes a premature stop, resulting in a truncated protein and reduce the function of LDLR. Using the three software tools, in silico analysis predicted this mutation to be pathogenic. Functional characterization of this variant was also found to be pathogenic.

We identified eight mutations in the LDLR gene of five FH patients in this study. Mutations in LDLR can occur in the promoters, introns and exons. LDLR mutations of Chinese FH patients occur mostly in the fourth exon, accounting for 19.5% of all the LDLR mutations. It is generally known that high frequency LDLR gene mutations can be useful for FH screening. For instance, the S472RfsX44 mutation accounts for approximately 42% of the FH patients in Tunisia. Previous studies have suggested that there is no definite common mutation in Chinese populations due to a founder effect. However, a systematic review of Chinese FH patients found that the three most common mutations: A606T, W462X and D601Y mutations are responsible for 29.3% of probands in mainland China. We also identified W462X and A606T mutations in the subjects of our study.

In this study, the c.357delG LDLR mutants displayed reduced binding and internalization activities compared with the wild-type LDLR. The LDLR is a highly conserved integral membrane glycoprotein consisting of five domains: a ligand-binding domain (approximately 292 amino acids, exons 2 to 6), an epidermal growth factor (EGF) precursor homologous domain (approximately 400 amino acids, exons 7 to 14), a carbohydrate-rich domain (approximately 58 amino acids, exon 15), a transmembrane domain (approximately 22 amino acids, exon 16) and a cytoplasmic tail (ap-
proximately 50 amino acids). The c.357delG frameshift mutation is located in exon 4 of the LDLR gene within the precursor domain that encodes the ligand-binding domain. The mutation results in a frameshift with an expected premature stop codon and produces a truncated protein, which leads to the absence of all or part of the ligand-binding domain, including the EGF precursor homologous domain, the O-linked sugar domain, the membrane-spanning domain and the cytoplasmic tail of the LDLR. Therefore, this mutation can severely damage the function of LDLR. Two other mutations near this locus in the LDLR gene, 355del7 and 353delA, have been reported in Japanese and British patients with FH. These mutations create premature stop codons 85 and 95 bases downstream of the deletion sites, respectively.

In conclusion, we identified eight mutations in the LDLR gene of five FH patients, in which c.357delG is a novel mutation. The novel mutation c.357delG was found to be co-segregated with the FH phenotype. Bioinformatic analy-
sis and flow cytometry confirm c.357delG to be a pathogenic mutation. This study increases our understanding of the mutational spectrum of FH in the Chinese population.

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