Mutations in the LDL receptor gene in four Chinese homozygous familial hypercholesterolemia phenotype patients

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Received 13 April 2008; received in revised form 13 July 2008; accepted 14 July 2008

KEYWORDS
Familial hypercholesterolemia; Low-density lipoprotein receptor; Novel mutation; Chinese population; Homozygous phenotype

Abstract  Background and aims: Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism caused by mutations in the low-density lipoprotein receptor (LDL-R) gene, leading to elevated levels of cholesterol and an increased risk of coronary heart disease. In this article, from four homozygous FH phenotype probands we identified disease causing mutations and analyzed the relationship between genotype and phenotype.

Methods and results: DNA sequencing identified five LDL-R point mutations in four unrelated families. We found a novel homozygous mutation (C210R), a homozygous mutation at W462X, a compound heterozygous mutation of C122Y and T383I, and a G > A intron 3 splice site homozygous mutation. The functional alteration caused by the novel C210R mutation was confirmed by FACS analysis. Four probands have high low-density lipoprotein cholesterol (LDL-C) levels, ranging from 14.65 to 27.66 mmol/L. Their heterozygous parents had relatively low levels. B-mode ultrasound supplemented by Doppler was used to examine aortic/mitral valve structural alterations and carotid intima-media thickness (ITM) in all probands. The ITM values were between 1.2 and 2.3 mm, much higher than the normal value of <0.8 mm.

Abbreviations: FH, familial hypercholesterolemia; LDL-R, low-density lipoprotein receptor; LDL-C, LDL cholesterol; CHD, coronary heart disease; IMT, intima-media thickness; CFVR, coronary flow velocity reserve; GFP, green fluorescent protein; LPDS, lipoprotein deprived serum; PE, phycoerythrobilin; CABG, coronary artery bypass graft.

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Introduction

Familial hypercholesterolemia (FH) is an autosomal disorder associated with severe hypercholesterolemia leading to premature coronary heart disease (CHD). In rare cases hypercholesterolemia is inherited as an autosomal recessive disorder caused by autosomal recessive hypercholesterolemia (ARH) adaptor protein mutation, which interacts with the cytoplasmic tail of the LDL receptor (LDL-R). Adaptor protein defect restricts the uptake of LDL from the cell surface by LDL-R. Autosomal dominant hypercholesterolemia is caused by mutations in the LDLR gene, apolipoprotein B-100 or proprotein convertase subtilisin/kexin type9 (PCSK9) [1]. LDL-R is a cell surface trans-membrane protein that mediates the specific uptake of plasma LDL via apoB transportation. PCSK9 accelerates LDL-R degradation.

LDL-R mutation is the major form of FH (in 60–65% cases) [1]. Receptor defects markedly impair LDL catabolism, and FH patients typically show severe elevations of plasma LDL cholesterol (LDL-C), tendon xanthomas, and premature coronary atherosclerosis. The disease is inherited as a dominant trait and displays a gene dosage effect, in that homozygous FH patients have a greater than 5-fold increase in plasma LDL concentration. Currently, FH is a paradigm in the study of the genetics of atherosclerosis [2]. Research investigating LDL-R gene mutation is important to elucidate the role of LDL-R in atherogenesis. To date, more than 920 different LDL-R mutations have been described (http://www.ucl.ac.uk/ldlr), which are equally spread over the entire gene. In most Caucasian populations, the frequency of heterozygous FH is estimated to be 1:500 and that of homozygous FH is 1:1,000,000 [3]. While the prevalence of FH in Caucasians and Asians is estimated to be similar [4], mutations underlying FH in Chinese are largely unknown as there have been few published studies focused on this population [5–11].

The current study investigated four unrelated Chinese probands, with clinical diagnosis of homozygous FH, and their first relatives. Our findings in these patients identified one novel and four previously described LDL-R mutations and excluded mutations in apoB and PCSK9. In addition the FH phenotype was examined by carotid interior-media thickness (IMT) echocardiography.

Methods

Clinical characteristics of patients

The diagnosis of homozygous FH was based on the following criteria: (1) plasma or serum LDL-CH>10 mmol/L; (2) presence of tendon and cutaneous xanthomas at an early age; (3) an autosomal mode of inheritance of hypercholesterolemia in relatives; and (4) the presence of primary hypercholesterolemia in the proband’s parents [12]. In the current study, the four unrelated probands, born between 1972 and 1993, displayed plasma LDL cholesterol concentrations of between 14.45–27.67 mmol/L before lipid-lowering therapy. Additionally, they had extensive cutaneous xanthomata on the creases of their hands, blepharons and haunches. Tendon xanthomata also appeared in young age. Secondary causes of hypercholesterolemia were excluded. This study was approved by the ethics committees of Beijing Anzhen Hospital.

Intima-media thickness (IMT)

IMT was investigated utilizing a B-mode ultrasound protocol. The common carotid, carotid bulb, internal carotid, and the common femoral and superficial femoral arterial far wall segments were scanned bilaterally in fasting patients. Ultrasound instruments (Acuson, Mountain View, CA) equipped with 5–10 MHz linear array broadband L7 transducers and Extended Frequency software were used. A standard view of 2 × 2 cm was imaged [13].

Coronary flow velocity reserve (CFVR)

CFVR was assessed by transthoracic Doppler echocardiography and calculated as the ratio of maximal (intravenous adenosine, 140 μg/kg/min) to baseline coronary velocities. Patients underwent continuous heart rate and ECG monitoring. Blood pressure was recorded at baseline every minute for 10 min following adenosine infusion, and after recovery [14]; CFVR values greater than 3.0 were considered to be normal.

Lipid determination

Blood samples were drawn from patients and their parents after 12 h of fasting. Total serum cholesterol (TC) and triglycerides (TG) were determined by colorimetric enzymatic assay systems (Daiichi Pure Chemicals Co., Japan). Serum LDL-C was calculated as previously described (LDL-C = TC – HDL-C – TG/2.2) [15].

LDL-R coding region sequence analysis

Genomic DNA was extracted from EDTA-anticoagulated blood. The LDL-R coding regions, including promoter, and 18 exons with splice sites, were amplified using commercial kits (Promega, Madison WI), as previously described [16]. All amplified products were purified and the sequences determined by dyeoxy sequencing of both strands. When mutations were detected, the corresponding fragments from their
parents were also sequenced. All sequencing was repeated at least twice with different batches of PCR products. The novel disease causing mutations were further investigated by sequencing the same site in 100 normal lipid controls.

The expression of LDL-R mutant

We cloned the LDL-R wild-type gene from the hepatocyte cell line BEL 7402 in the OmicsLink (FulenGen Co., Guangdong, China) mammal cell expression vector, which has an N terminal GFP (green fluorescent protein) tag. The LDL-R mutations were generated from OmicsLink-LDL-R by site-directed mutagenesis using a QuikChange XL mutagenesis kit (Stratagene, Cedar Creek, TX), according to the manufacturer’s instructions. The integrity of all of the constructs were confirmed by DNA sequencing and by western blot analysis. The WT and mutant plasmids were transfected into HEK 293 cells, which do not have endogenous LDL-R expression, by lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the users’ manual. Transfection efficiency was estimated by counting the GFP positive cells under a fluorescent microscope. The transfection efficiency was about 50–65% in all experiments. Transfected cells were incubated in RPMI 1640 containing 10% LPDS (lipoprotein deprived serum) for 48 h. LDL-R function was then analyzed by flow cytometry.

Western blot analysis

Cells were lysed in a buffer containing 1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 20 mM Tris–HCl (pH 7.5). The proteins were then electrophoretically transferred to an Immun-Blot PVDF Membrane. Non-specific binding sites were blocked in 5% Blotting Grade Blocker non-fat dried milk for 1 h, and the membrane was then immunostained with the primary antibody the membranes were washed twice in Tris-buffered saline (TBS) containing 0.1% Tween-20 (Sigma–Aldrich Corp.) and then incubated for 1 h with sheep anti-rabbit IgG conjugated with horseradish peroxidase. After two more washing steps with TBS containing 0.1% Tween-20, bands were visualized with ECL (Pierce Biotechnology, Rockford, IL). After stripping (Pierce Biotechnology, Rockford, IL) for 15 min, anti β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to normalize band intensity. TotalLab 2.0 software was used for quantification of band intensities.

Flow cytometric analysis

For flow cytometric analysis; cells were harvested by trypsinization after transfection. After incubation in 1% BSA for 30 min, the cell suspension was divided into three parts: first part for LDL-R expression analysis; the second part for LDL binding ability analysis; the remaining part for analysis for LDL internalization. For expression analysis the cells were incubated with LDL-R antibody (1:200 rabbit polyclonal antibody, US Biological, Swampscott, MA) for 1 h at 4 °C. For analysis of LDL-R expression, PE labeled LDL-R antibody positive cells are plotted on the x-axis, while the fluorescence of individual GFP positive cells in arbitrary units is given on the y-axis. Dot plots in the upper-right areas represent GFP tagged protein and LDL-R antibody positive cells.

For analysis of LDL binding and LDL internalization, the activity of Dil-LDL binding, and of Dil-LDL internalization are given on the x-axis, while the fluorescence of individual GFP positive cells in arbitrary units is given on the y-axis. Dot plots in the upper-right areas represent GFP tagged protein and Dil-LDL positive cells. Data were collected and analyzed using SPSS version 11.0 software and a P-value < 0.05 was considered to be statistically significant.

Results

Clinical and biochemical features of FH probands and their first-degree relatives

The clinical data are presented in Table 1. The plasma levels of total cholesterol are uniformly very high in FH homozygotes and heterozygotes, irrespective of diet, medications, or lifestyle. The plasma levels of LDL-C in FH heterozygotes are lower (elevated two- to threefold) and much more dependent on other genetic and environmental factors than are those in FH homozygotes. Although the nature of the molecular defect has some impact on the severity of hypercholesterolemia, FH heterozygotes with the same LDLR mutation can have widely different plasma levels of LDL-C [1–3]. The mother in family 1 died of CHD in her thirties. However, the father of family 1 and the mother of family 3 have borderline to normal LDL-C. This may be because they have hypertriglyceridemia. Therefore, the calculated LDL-C is relatively low (LDL-C = TC – HDL-C – TG/2.2).

Standardized IMT measurements

The carotid and femoral IMT in the four FH probands was investigated. Our data suggested that the IMT of the carotid bifurcation and of the common carotid artery was increased in FH probands (from 1.8 mm to 2.5 mm) compared with the normal range (<0.8 mm, described in Table 2). Valve calcification was also common in FH patients, as well as moderate aortic stenosis and regurgitation.
<table>
<thead>
<tr>
<th>Family no.</th>
<th>Patient</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>TC (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>ApoB (g/L)</th>
<th>X</th>
<th>A</th>
<th>I</th>
<th>Family history</th>
<th>Mutations</th>
<th>Nucleotide change</th>
<th>Mutated site</th>
<th>Populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Proband 1</td>
<td>M</td>
<td>16</td>
<td>15.50</td>
<td>13.79</td>
<td>1.26</td>
<td>1.14</td>
<td>1.25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>X A I Family history</td>
<td>p.W642X c.G1448A</td>
<td>Exon 10</td>
<td>China, Canada, Austria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sister</td>
<td>F</td>
<td>13</td>
<td>20.29</td>
<td>18.44</td>
<td>1.22</td>
<td>1.27</td>
<td>1.68</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>p.W642X c.G1448A</td>
<td>Exon 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Father</td>
<td>M</td>
<td>43</td>
<td>6.0</td>
<td>2.90</td>
<td>3.58</td>
<td>1.37</td>
<td>1.17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.W642X c.G1448A</td>
<td>Exon 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Proband 2</td>
<td>M</td>
<td>13</td>
<td>28.83</td>
<td>27.66</td>
<td>1.04</td>
<td>0.99</td>
<td>0.78</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FH-Erverum</td>
<td>c. G 313 + 1A</td>
<td>Intron3</td>
<td>Norway, Austria, Belgium, Denmark, Germany, Italy, Spain, Korea, Netherlands, United Kingdom, Sweden, South Africa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brother</td>
<td>M</td>
<td>17</td>
<td>4.13</td>
<td>2.22</td>
<td>0.70</td>
<td>1.48</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Mother</td>
<td>F</td>
<td>42</td>
<td>7.29</td>
<td>4.90</td>
<td>0.89</td>
<td>1.74</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>FH-Erverum</td>
<td>c. G 313 + 1A</td>
<td>Intron3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Father</td>
<td>M</td>
<td>42</td>
<td>8.26</td>
<td>5.58</td>
<td>0.66</td>
<td>0.86</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>FH-Erverum</td>
<td>c. G 313 + 1A</td>
<td>Intron3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Proband 3</td>
<td>F</td>
<td>13</td>
<td>14.65</td>
<td>13.30</td>
<td>1.44</td>
<td>0.65</td>
<td>1.53</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>p.C122Y/p.T383I c.G428A/A4c. G313 + 1A</td>
<td>Exon 4/9</td>
<td>Germany, Russia, China/China</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mother</td>
<td>M</td>
<td>41</td>
<td>5.49</td>
<td>3.44</td>
<td>0.78</td>
<td>1.32</td>
<td>0.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.T383I c.T1211C</td>
<td>Exon 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Father</td>
<td>F</td>
<td>40</td>
<td>8.50</td>
<td>6.78</td>
<td>1.92</td>
<td>0.83</td>
<td>1.33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.C122Y c.G428A</td>
<td>Exon 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Proband 4</td>
<td>M</td>
<td>34</td>
<td>17.09</td>
<td>14.45</td>
<td>2.21</td>
<td>1.24</td>
<td>0.76</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.C210R c.T691C</td>
<td>Exon 4</td>
<td>Novel*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mother</td>
<td>M</td>
<td>53</td>
<td>7.55</td>
<td>5.79</td>
<td>1.31</td>
<td>1.72</td>
<td>0.59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.C210R c.T691C</td>
<td>Exon 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Father</td>
<td>F</td>
<td>56</td>
<td>7.79</td>
<td>5.65</td>
<td>0.79</td>
<td>1.77</td>
<td>0.52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>p.C210R c.T691C</td>
<td>Exon 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

X: xanthoma; A: Arcus; I: Ischemic ECG.

*No direct evidence for pathogenicity.
CFVR in FH probands

CFVR has been extensively used as a reliable marker to predict the presence of myocardial ischemia. Patient CFVR was assessed by transthoracic Doppler echocardiography was and calculated as the ratio of maximal (i.v. adenosine, 140 μg/kg/min) to baseline coronary velocities. As shown in Table 1, CFVR values were reduced to 1.5–2.73 in FH probands (normal value ≥3) (Table 2).

Identification of LDL-R mutations in Chinese FH patients

Genetic analysis revealed disease-causing mutations in all FH probands. Identified mutations in the LDL-R gene are shown in Table 1 and Figs. 1 and 2.

A nonsense mutation, caused by a homozygous G to A change in exon 10, was identified in Family 1, and resulted in replacement of the tryptophan codon TGG at position 1448 with a stop codon TAG (p.W642X), as shown in Fig. 2A. The proband’s father and sister were heterozygous for this mutation. Proband 1 and her brother carried the p.W642X homozygous mutation and were severely hypercholesterolemic at an early age and were given a diagnosis of homozygous FH. High levels of plasma LDL cholesterol (20.23 mmol/L and 16.21 mmol/L) were associated with extensive xanthomatosis, increased IMT and CHD. The mother of proband 1, with extensive xanthomatosis, died from myocardial infarction at the age of 32.

A homoygous mutation (splice site mutation) at the junction between intron 3 and exon 3 was identified in proband 2. The mutation was a G to A transition at position +1 from the end of exon 3. The same heterozygous mutation was found in the proband’s father and mother (Fig. 2B).

Proband 3 harbored two heterozygous missense mutations: a G>A substitution at base 428 in exon 4, causing a p.C122Y substitution and a C>T substitution at base 1211 in exon 9, leading to a p.T383I substitution. DNA sequence analysis suggested that the proband’s father had a heterozygous p.C122Y substitution in exon 4, and the mother had a heterozygous p.T383I mutation (Fig. 2C, D). The missense mutation C122Y resulted in the substitution a highly conserved cysteine to a tyrosine, in the fourth of the seven-tandem cysteine rich repeats, which is associated with the apoB ligand binding domain of the LDL-R [17]. The p.C122Y missense mutation has been reported in other populations, although there is no report of any functional consequence in vitro. We also identified a C>T substitution at base 1211 in exon 9, leading to a p.T383I substitution. Remarkably, the mutation T383I has been reported previously only in a Dutch FH patient [18]. Exon 9 of the LDL-R gene encodes the epidermal growth factor precursor homology domain. This domain is required for the acid-dependent dissociation

**Table 2** Genotype-phenotype comparisons in four severe FH subjects

<table>
<thead>
<tr>
<th>Proband</th>
<th>Type</th>
<th>Amino acid change</th>
<th>Clinical parameters</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carotid artery IMT (mm)</td>
<td>CFVR</td>
</tr>
<tr>
<td>1</td>
<td>Homozygous</td>
<td>p.W642X</td>
<td>2.3</td>
<td>1.53</td>
</tr>
<tr>
<td>2</td>
<td>Homozygous</td>
<td>c. 313 + 1 G&gt;A</td>
<td>2.3</td>
<td>1.73</td>
</tr>
<tr>
<td>3</td>
<td>Compound heterozygote</td>
<td>p.C122Y/p.T 383I</td>
<td>1.8</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>Homozygous</td>
<td>p.C210R</td>
<td>2.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

IMT normal value <0.8 mm; CFVR normal value ≥3.0 mm.
of the receptor from its ligand during binding and recycling. The FACS analysis confirmed the functional deficiency of this mutation in both LDL binding and internalization.

Proband 4 had a homozygous mutation in exon 4 at nucleotide 691, a T>C mutation, leading to a p.C210R substitution (Fig. 2E). The defective allele was inherited from his father and mother (Fig. 1). Exon 4 of the LDL-R gene encodes the ligand-binding motif and plays an important role in binding apoB. By searching FH patient databases (www.ucl.ac.uk/ldlr/Current/) [19], we found the potential disease-causing mutation was not previously registered, suggesting a novel FH mutation was identified. The same nucleotide mutation 691T>C was detected in Norwegian, South African and English populations. Screening of 100 healthy Chinese by SSCP did not reveal carriers of the novel mutation (p.C210R), suggesting it was more likely to be a disease-causing mutation, rather than a polymorphism. The different mutations carried by the four FH probands indicate that they are likely to have dissimilar clinical features (Table 1).

Novel mutation p.C210R has impaired LDL-R function

Western blot analysis of total cell lysates from cells transfected with wild-type and mutant LDL-R expression vectors was performed. We used p.W642X as a positive control, wild-type as a normal control and compared the functional impairment caused by p.C210R, p.C122Y and p.T383I. Compared with wild type (WT), single amino acid change mutants (p.C122Y, p.T383I, p.C210R) expressed the full length protein. In western blot and FACS analysis, we detected mutated LDL-R and wild-type protein with an antibody that recognizes an epitope between aa184-196, the linker region between repeats 4 and 5 of the extracellular domain. The LDL-R mutations did not affect the antibody-antigen reaction. All the mutants and the wild-type LDL-R were detected in bands of approximately 160 kDa. The p.W462X mutant produced a truncated protein of around 90 kDa.

LDL-R expression levels were quantified by FACS. Similar results were found in all transfected groups. GFP positive
cells expressed the same amount of LDL-R in p.W642X, p.C122Y, p.T383I, and p.C210R transfected cells (Fig. 4). The p.W462X mutation lacks the cytosolic domain (NPVY motif) that interacts with the traffic sorting adapter protein ARH. However, the LDL-R extracellular domain (YWSD motif) can interact with another sorting protein SorLA/LR11 [20,21]. We used HEK 293 embryonic kidney cells, without endogenous LDL-R, for functional analysis of the mutants. The sorting protein SorLA/LR11 is expressed in kidney and neural cells. We speculate that the expressed p.W642X mutant (Table 3) is transported to the cell membrane by SorLA/LR11, as an exceptional case, only in kidney but not in liver, the major cholesterol metabolic organ. The exact sorting mechanism remains to be further explored.

The p.C122Y, p.T383I and p.C210R mutations did not affect LDL-R production (Fig. 3). The characteristics of the novel mutation C210R are yet to be defined. Flow cytometry analysis showed that compared with wild-type control, like the other mutants, the C210R mutant has low LDL binding and internalization ability. In p.W642X transfected cells, LDL binds to the mutated receptor at very low levels. The majority of GFP positive cells cannot bind to LDL. This deficiency might be due to the deleted EGF precursor domain, transmembrane domain, or cytoplasmic domain. The LDL-R binding ability was defective in C122Y, T383I, and C210R mutated cells. Among the three single amino acid change mutations, T383I is located in EGF precursor domain, the other mutations are located in LDL binding domain.

Impairment of LDL binding is more severe in C122Y and C210R compared with T383I (Fig. 5). We found similar results in LDL-R internalization analysis. Compared with wild-type control, all mutants were deficient in LDL uptake to some extent (Fig. 6). The results are illustrated in Table 3.

**Discussion**

Although numerous LDL-R mutations have been identified in FH patients, genetic data on a Chinese population, particularly in China, are rare. In the present study, five point mutations were identified in the LDL-R gene (Table 1), one of which had not been previously described. Mutations were scattered over the LDL-R gene and changed amino acid sequences in different functional domains of the mature protein. All subjects with mutations had baseline total cholesterol levels ≥5.7 mmol/L, whereas relatives without mutations had total cholesterol levels <5.7 mmol/L. Three mutations (p.W642X, p.C122Y, and the intron 3 splicing site G>A) have been demonstrated to be disease-causing mutations in other populations [17,22,23]. The novel mutation (C210R) co-segregated with phenotypes, and was not present in the control population. Our *in vitro* experiment confirmed the functional deficiency of the novel mutation.

Proband 1 was found to be genetically homozygous for a nonsense mutation p.W642X. This mutation was located in exon 10 and resulted in a truncated protein (Fig. 3), lacking the EGF-precursor domain, sites for O-linked sugar chains,

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### Table 3

<table>
<thead>
<tr>
<th>Proband</th>
<th>Amino acid change</th>
<th>LDLR function analysis (fluorescent intensity GEO mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDLR expression (%)</td>
<td>LDL binding ability (%)</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>p. W462X</td>
<td>81 ± 19</td>
</tr>
<tr>
<td>2</td>
<td>p. C122Y</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>3</td>
<td>p. T383I</td>
<td>95 ± 14</td>
</tr>
<tr>
<td>4</td>
<td>p. C210R</td>
<td>89 ± 21</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD. Statistical significance between two groups was conducted by the Student t-test. *P < 0.05 was used as the criterion of statistical significance.
Figure 4  Characterization of LDL-R protein in transfected HEK 293 cells using flow cytometric measurements. Dot plots in the upper-right areas represent GFP and LDL-R double positive cells. There was no significant difference in the intensity of fluorescence among groups. The histogram displays the data as the percentage of mean fluorescence compared with wild-type, Bars and vertical lines indicate the mean of four independent measurements and standard deviation, respectively. HEK 293 cells: (A) transfected with wild-type LDLR; (B) transfected with LDL-R p.W642X mutation; (C) transfected with p.C122Y mutation; (D) transfected with p.T383I mutation; (E) transfected with p.C210R mutation. (F) Histogram of different samples.

Figure 5  Characterization of LDL-R binding functions in transfected HEK 293 cells using flow cytometric measurements. Dot plots in the upper-right areas represent GFP and Dil-LDL double positive cells. The activity of Dil-LDL binding is given on the x-axis, while the fluorescence of individual GFP positive cells in arbitrary units; is given on the y-axis. T histogram displays the data as the percentage of mean fluorescence compared with the wild-type, Bars and vertical lines indicate the mean of four independent measurements and standard deviation, respectively. HEK 293 cells: (A) transfected with wild-type LDL-R; (B) transfected with p.W642X mutation; (C) transfected with p.C122Y mutation; (D) transfected with p.T383I mutation; (E) transfected with p.C210R mutation. (F) Histogram of different samples. *P < 0.05 was used as the criterion of statistical significance.
the transmembrane domain and the cytoplasmic domains [24]. However, in kidney cells the truncated protein might be sorted to the cell surface by another sorting protein, such as SorLA/LR11 [20, 21].

Proband 2 was homozygous for a splicing defect c.G313+1A. in intron 3 of LDL-R. This single base substitution changed the highly conserved di-nucleotide GT, which forms part of the GT (A/G)AG recognition signal at the donor splice site, and which has been shown to result in incorrect splicing [25]. This mutation resulted in aberrant splicing of the mRNA, producing a mutant receptor protein that lacked the second disulfide-rich repeat of the binding domain [26]. Proband 2 exhibited very high levels of plasma LDL-C (27.58 mmol/L), which was associated with extensive xanthomatosis, increased carotid and femoral IMT and suffering from serious CHD. His parents were heterozygotes, carrying one copy of the same mutation, with cholesterol levels of 8.99 mmol/L and 7.64 mmol/L. Although both heterozygotes had deficient LDL-R, the differences between the two patients support the fact that other genes of lipid metabolism could exert an influence on the phenotypic expression of the LDL-R mutation.

Proband 3 was found to be two heterozygous for two mutations, p.C122Y and p.T383I. We confirmed a heterozygous mutation C122Y in the father’s exon 4 and a heterozygous mutation T383I in the mother’s exon 9.

Proband 4 was homozygous for a missense mutation at nucleotide 691 in exon 4. The proband had a high level of plasma LDL-C (14.45 mmol/L) and had undergone coronary artery bypass graft surgery when he was 33 years old. The in vitro data indicate the mutation results in receptor-binding deficiency (Table 2, Fig. 4).

Genotype-phenotype relationships were analyzed and Table 2 shows the correlation of clinical characteristics and the presence of LDL-R gene mutations. IMT and CFVR are valid surrogate markers for the progression of atherosclerotic disease and the prevalence of arterial calcification has also been shown to be directly related to LDL-C serum levels [27]. Proband 1 and her brother, carrying the W462X homozygous mutation, were severely hypercholesterolemic at an early age and were given a diagnosis of homozygous FH. High levels of plasma LDL cholesterol (20.23 mmol/L and 16.21 mmol/L) were associated with extensive xanthomatosis, increased IMT and CHD. The mother of proband 1, with extensive xanthomatosis, died from myocardial infarction at age 27. Proband 2 carried the splice site homozygous mutation and exhibited very high levels of plasma LDL-C (27.58 mmol/L). Although both homozygotes had deficient LDL-R, the differences in LDL-C levels, IMT, and CFVR between the two patients support the fact that other genes of lipid metabolism could exert an influence on the phenotypic expression of the LDL-R mutation.

Proband 3, who carried compound heterozygous mutations C122Y/T383I, exhibited a lower plasma LDL-C level (13.26 mmol/L) and had little xanthomatosis. Additionally, IMT and CFVR were less impaired, compared with the homozygous patients.

In summary, our data suggest that, in homozygous patients, nonsense and splice site mutations were associated...
with severe hypercholesterolemia, thicker carotid IMT and lower CFVR compared with patients with missense mutations. However it is obvious that our conclusions are limited because of the small number of patients examined. To address this, an age-matched, sex-matched FH patient study will be performed.

Acknowledgments

We thank the patients and their families for participation in this study. This work was supported by grants from the National Natural Science Foundation of China (No. 30470722), the Beijing Natural Science Foundation (Nos. 7032012, 7052021 and 7062010), the Beijing Science and Technology New Stars Foundation (No. 04B27 and No. 05A29) and the Basic-Clinical Medicine Cooperation Foundation of Capital University of Medical Science (No. 02JL19).

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