Novel compound heterozygous mutations in low density lipoprotein receptor gene causes a severe phenotype in a Chinese hypercholesterolemia family

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Abstract. Mutations in the low density lipoprotein receptor (*LDLR*) gene serve a causative role in the pathophysiology of familial hypercholesterolemia (FH), a common autosomal inherited disorder characterized by abnormal lipid metabolism. The aim of the present study was to investigate genetic defects in a Chinese family with FH. Clinical features and family histories were collected, as were the results of various laboratory tests, including determinations of serum lipid concentrations, ultrasonography and angiography results. Potential mutations in *LDLR* were screened using direct polymerase chain reaction (PCR) sequencing. Multiple sequence alignments, structure and hydrophobicity predictions were performed *in silico*. Novel compound heterozygote mutations in *LDLR* of the proband were identified, with a Trp577Term-bearing maternal allele and a Pro685Leu-bearing paternal allele. The proband, a 27-year-old male, had severe and diffuse coronary stenosis and non-ST segment elevation myocardial infarction, as well as multiple skin xanthomas and high serum lipid levels. The allele-dosage-dependent clinical features, including hypercholesterolemia and peripheral arterial atherosclerosis, were observed in the proband and the other heterozygous patients. Therefore, the coexistence of Pro685Leu and Trp577Term mutations in *LDLR* is a novel compound heterozygosis in Chinese patients and may lead to a severe FH phenotype. The explanation for the existence of compound heterozygous mutations instead of homozygous mutations in this particular family requires further study.

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Abbreviations: FH, familial hypercholesterolemia; LDLR, low density lipoprotein receptor; PCR, polymerase chain reaction; heFH, heterozygous FH; hoFH, homozygous FH; APOB, apolipoprotein B; LDL-C, LDL cholesterol; GLU, blood glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; EKG, electrocardiogram

Key words: coronary artery disease familial hypercholesterolemia, *LDLR* gene, atherosclerosis, xanthomas,

Introduction

Familial hypercholesterolemia (FH) is an inherited genetic disease characterized by hyperlipidemia, skin xanthomas on extensor tendons and/or corneal arcus, and early onset of all forms of atherosclerotic diseases including premature death secondary to lifelong pathogenic elevations of serum cholesterol (1). FH exhibits an autosomal dominant transmission pattern with ≥90% penetrance (1). It is typically divided into two main phenotypes: Heterozygous FH (heFH) and homozygous FH (hoFH). hoFH is less common than heFH and patients with hoFH often exhibit more severe symptomatic phenotypes with higher levels of serum cholesterol (2).

It has been reported that mutations in more than 3 genes, including low density lipoprotein receptor *(LDLR)*, apolipoprotein B-100 *(APOB)*, proprotein convertase subtilisin/kexin type 9 and LDL receptor adaptor protein 1, are involved in the pathogenesis of FH (3-6). Notably, *LDLR* mutations account for 85-90% of FH cases and >1,700 variants of *LDLR* have been identified (7,8). The human *LDLR* gene encodes an 860-amino-acid protein that serves a significant role in the uptake and degradation of LDL by the LDLR pathway (9). An *in vivo* study by Anderson indicated that mutations in the *LDLR* gene could cause dysfunction of the LDLR protein, leading to the destruction of the LDLR pathway *in vivo*, resulting in elevated serum cholesterol levels and premature coronary artery disease (10).

It has previously been reported that the incidence of *LDLR* mutations is ~70% in Chinese patients with FH. However, these patients remain largely unidentified, particularly in rural areas, due to patients and clinicians having little knowledge about this disease and limited access to genetic testing (11-13). A few cases of FH in China have been diagnosed based primarily on clinical symptoms, but the phenotype of FH

is highly variable (3). Different gene mutations and dosage effects of modifier genes may lead to discrepancies in clinical manifestations and responses to drugs (14-16). It is therefore important to investigate the association between FH genotype and phenotype. In the present study, a novel compound heterozygosis was identified by direct polymerase chain reaction (PCR) sequencing of *LDLR*. Laboratory tests and bioinformatics analysis were also conducted to investigate the possible role of this mutation.

Subjects and methods

Study subjects. A total of 10 individuals from a Chinese FH family were enrolled in the present study from December 2014 to May 2015 at Zhongnan Hospital of Wuhan University (Wuhan, China), including 4 males and 6 females, with an age range of 2-76 years (Fig. 1 and Table I). All individuals enrolled in our study were members of this FH family and consented to genetic and clinical examinations. Individuals in the family who refused examinations were excluded. The family history and basic physical information of subjects were collected. The present study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University. Prior informed consent was obtained from all subjects, including the parents of participants <16 years old. The young male proband (III1, 27-year-old) was the offspring of a consanguineous marriage (II1 and II2), as the grandfather of II1 and the grandmother of II2 were siblings.

Clinical examinations and biochemical tests. Blood samples were harvested following overnight fasting. Serum was separated by centrifugation at 2,200 x g at 4˚C and examined using an automatic biochemical analyzer (Abbot-AEROSET; Abbott Diagnostics, Santa Clara, CA, USA) for a series of biochemical indices in the clinical laboratory department of Zhongnan Hospital of Wuhan University: Blood glucose (GLU), total cholesterol (TC), LDL-C, high-density lipoprotein cholesterol (HDL-C), triglycerides, apolipoprotein (APO) A, APOB, lipoprotein a and C-reactive protein.

All participants received ultrasonography and electrocardiogram (EKG) examinations. A coronary contrast angiography was performed on the proband using the digital subtract angiographic system (Phillip Corp. FD 20; Phillips Healthcare, DA Best, The Netherlands). The number and severity of peripheral artery atherosclerotic plaques (PAS) of each individual was evaluated.

Genetic analysis. Genomic DNA was extracted from 1 ml peripheral blood of all subjects using the sodium dodecyl sulfate-proteinase K method as previously described (17). DNA quality was assessed using a NanoDrop 2000c spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All exons of *LDLR* were screened for mutations using PCR direct sequencing as previously described (18,19). Amplifications were performed in a Hema 9600 PCR thermocycler (Hema Medical Instrument Co. Ltd, Zhuhai, China) with a total volume of 25 μl, including 1x Taq DNA Polymerase (Thermo Fisher Scientific, Inc.), 1x Taq Buffer, 0.5 μM of forward and reverse primers (Table II) and 100 ng DNA template. The conditions of PCR amplification were 95˚C for 30 sec for denaturation, annealing temperature (Table II) for 45 sec for annealing, 72˚C for 45 sec for extension (38 cycles of amplification). PCR products were evaluated by 2% agarose gel electrophoresis, purified by an Axygen® PCR Clean-Up Kit (Axygen Biosciences, Inc.; Corning Incorporated, Corning, NY, USA) and directly sequenced using an ABI Genetic Analyzer 3730xl (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Multiple Sequence Alignments and prediction of LDLR mutations. The alignment of LDLR protein sequences in multiple species (obtained from the National Center for Biotechnology Information protein database, www.ncbi.nlm.nih.gov/protein) was performed using ClustalX2 software (Wellcome Trust Genome Campus, Hinxton, UK). The secondary structure and hydrophobicity of the mutant LDLR protein were predicted using ANTHEPROT 5.0 (PRABI-Lyon-Gerland, Lyon, France), and three-dimensional (3D) structure prediction was carried out using Swiss-Pdb Viewer 4.01 (Swiss Institute of Bioinformatics, Lausanne, Switzerland) (20-22).

Results

FH pedigree. The young male proband (III1, 27-year-old) was the offspring of a consanguineous marriage (II1 and II2), as the grandfather of II1 and the grandmother of II2 were siblings (Fig. 1). The proband was first diagnosed with hypercholesterolemia in May 2015 when a biochemical test was taken, due to aggravated angina. The proband presented with multiple skin xanthomas (Fig. 2A) of various sizes on his extensor tendons in the fingers, elbows, knees and Achilles. Notably, these xanthomas first appeared when he was 10 years old. Arcus corneas, white rings in the corneal margin, an indicator of lipid deposits, were found bilaterally (Fig. 2A). The proband frequently experienced (four times per day for two years) paroxysmal chest discomfort and pain of several minutes duration each time, with accompanying palpitations and breathlessness, which were heavier during physical exertion. The proband's father (II2) died as a result of lung cancer at the age of 33 and also had skin xanthomas according to descriptions by other family members. The proband's brother (III2, 22-year-old) exhibited moderate symptoms of partial xanthoma and sporadic angina. Abnormal symptoms did not manifest in other family members.

Results of clinical examinations and biochemical tests. The blood lipid levels of the subjects were shown in Table I. The proband exhibited elevated levels of TC (18.30 mmol/l), LDL-C (11.60 mmol/l) and APOB (3.81 g/l) compared with the normal range; the proband's mother, brother and aunt, heterozygous in this study, also had high TC (8.10-9.40 mmol/l), LDL-C (4.86-5.02 mmol/l) and APOB (1.65-1.86 g/l) levels (Table I). The rest of the subjects were found to have levels of TC, LDL-C and APOB within normal ranges (Table I). Furthermore, HDL-C level in the proband (0.60 mmol/l) was lower than the normal range, whereas it was within the normal range for all other subjects. These data are consistent with the diagnostic criteria for FH (23).

Clinical features and examination results are listed in Table III. For the proband, precordial auscultation revealed a

Table I. The serum lipid levels of the subjects.

Subjects	Sex	Age, years	TC mmol/l	TG mmol/l	HDL-C mmol/l	LDL-C mmol/l	APOA g/l	APOB g/l	LPa g/l	CRP mg/l	GLU mmol/l
Normal range	/	/	2.8-5.8	0.45-1.81	0.9-2	2.1-3.3	1.05-1.75	0.6-1.4	0-0.3	0-8	3.8-6.1
I1[a]	M	76	4.50	0.81	1.08	2.14	1.52	0.85	0.14	<0.5	3.90
II1[a]	F	52	8.10	1.14	1.04	5.02	1.37	1.72	0.48	<0.5	4.30
II5	F	48	5.10	0.75	1.45	2.38	1.89	0.89	0.09	<0.5	3.90
II7[a]	F	45	9.40	1.53	1.65	4.93	2.17	1.86	0.52	<0.5	3.90
III1[a]	M	27	18.30	2.67	0.60	11.60	1.11	3.81	0.14	<0.5	3.50
III2[a]	M	22	8.20	0.92	1.05	4.86	1.46	1.65	0.06	6.46	4.50
III3	F	22	3.90	0.61	1.34	1.61	1.77	0.61	0.13	<0.5	4.00
III4	F	27	4.50	0.33	1.58	1.84	1.85	0.66	0.27	<0.5	4.00
III6	F	22	3.90	0.74	1.13	1.69	1.64	0.68	0.48	<0.5	3.90
IV1	M	2	3.50	1.13	0.95	1.57	1.40	0.55	0.21	<0.5	5.00

Subjects marked with an [a]harbor a causative mutation. TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; APOA, apoprotein A; APOB, apoprotein B; Lpa, lipoprotein a; CRP, C reaction protein; GLU, glucose; M, male; F, female.

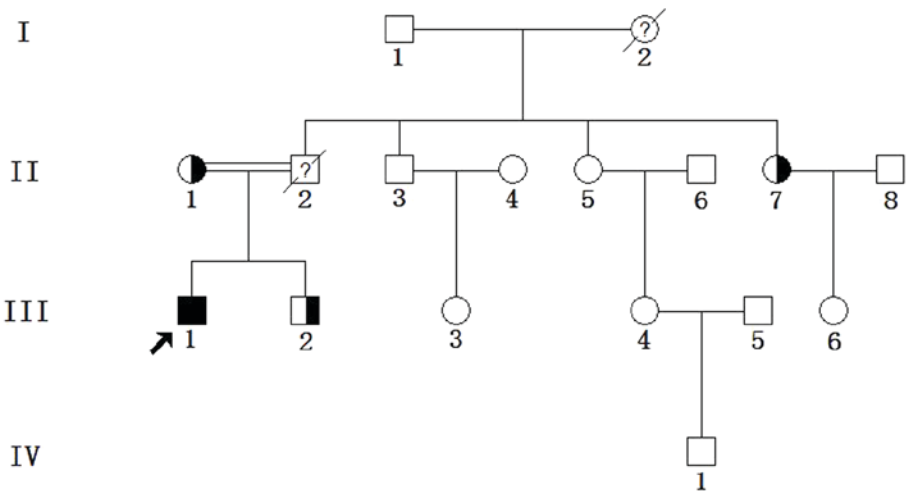

Figure 1. Pedigree chart for the familial hypercholesterolemia family in the present study. Black symbols indicate affected members who carry the Trp577Term or Pro685Leu mutation. Half black half white symbols indicate members heterozygous for *LDLR* mutation, fully black symbols indicate members homozygous for *LDLR* mutation, and fully white symbols indicate members without *LDLR* mutation. '?' indicate members with an unknown genotype. 'I', 'II', 'III' and 'IV' indicate the generations of this family. Squares indicate males, circles indicate females.

3-4/6 grade systolic murmur at the area of aortic valve. EKG found that the ST segment depressed in V4, V5 and V6 leads reaching to 0.5 mv. Angiography revealed diffuse and heavy coronary artery stenosis. The left main coronary trunk and the anterior descending branch narrowed at 80% and the proximal circumflex branch at 50%; the right coronary narrowed in the proximal segment at 85%, the middle at 85%, and the distal at 70% (Fig. 2C). Ultrasonography revealed that the aortic valve was calcified with mild stenosis and regurgitation, the mitral valve had mild regurgitation, and there was extensive and heavy peripheral arterial atherosclerosis (Fig. 2B). By contrast, other family members presented only subtle abnormalities or normal manifestations. Subjects with unique heterozygote mutations presented with mild abnormalities. Ultrasonography revealed fewer plaques (II1, II7, III2) and no cardiac structural malformation and dysfunction, and the EKGs found mild ST segment depression (II1) or normal state. The healthy members with no causative mutations (II5, III3, III4, III6, IV1) had normal cardiac and peripheral arterial results, with the exception of the proband's grandfather (I1) who had aortic valve calcification and mild regurgitation as well as inferior Q wave in the EKG. It was concluded that these characteristics were most likely due to age (Table III). Numerous peripheral artery atherosclerotic plaques were observed in the proband, and five, two and three plaques were observed in the proband's mother, brother and aunt, respectively. However, atherosclerotic plaques were not observed in non-FH members.

Trp557Term and Pro685Leu mutations in LDLR gene. The results from the genetic analysis are presented in Table III.

Table II. Primers for the *LDLR* gene.

Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon length (bp)	Annealing temperature (˚C)
5' near gene-Exon1	CTTCACCGGAGACCCAAATA	TTCCCTTAAATCCCTCAGACTC	592	58
Exon2	CAGACTGTTCCTGATCGGATG	AAGGGGTTAAGAATCGTGTCAC	422	60
Exon3	TGGGTCTTTCCTTTGAGTGAC	TAGCACCATCCCCACTTTGT	366	58
Exon4	TAGAATGGGCTGGTGTTGGG	TACTTTCTTGGCATGTTGTTGG	567	60
Exon5	AAGTAAGGTGGCACGATTATG	AGCAGCAAGGCACAGAGAAT	470	62
Exon6	AAGCAAACTGAGGCTCAGACAC	TGGAGTTCCCAAAACCCTACAG	272	62
Exon7	TGTAATGAGCCAAGGTTGGC	GTTTGGTTGCCATGTCAGGAA	261	58
Exon8	GCTGTTTCCTTGATTACATCTC	GATATGAGTCTGTGCAAAGTTC	367	60
Exon9-Exon10	CTTGGTTCCATCGACGGGTC	CATGCCCAGCCCACTAACCA	626	62
Exon11	GGTTCCCAGCAGGACTATTTC	GAAAGAGGGAAACCTTCAGG	358	60
Exon12	TGACCTCTCCTTATCCACTTGT	CTCCTAGTCACAACCAGTTTTC	272	60
Exon13-Exon14	GAGGGTGGCCTGTGTCTCAT	ATGAGTCCTTACAACGACCTTG	605	60
Exon15	GTCATTTGAGACTTTCGTCATTAG	AAGAGGGCAAGAACTGTTATTAGAC	457	60
Exon16	CTGCCTGCTCCATTTCTTGG	CTCCACATCCTCCATCTGACC	349	60
Exon17	TCAAGGTTATGGTACGATGCC	TTGCCCTGTCACCATCTGAT	485	62
Exon18-3' near gene	TTTCCTGAATGCTGGACTGAT	GAGAAACTCAAAACTTCCTGGAG	360	60

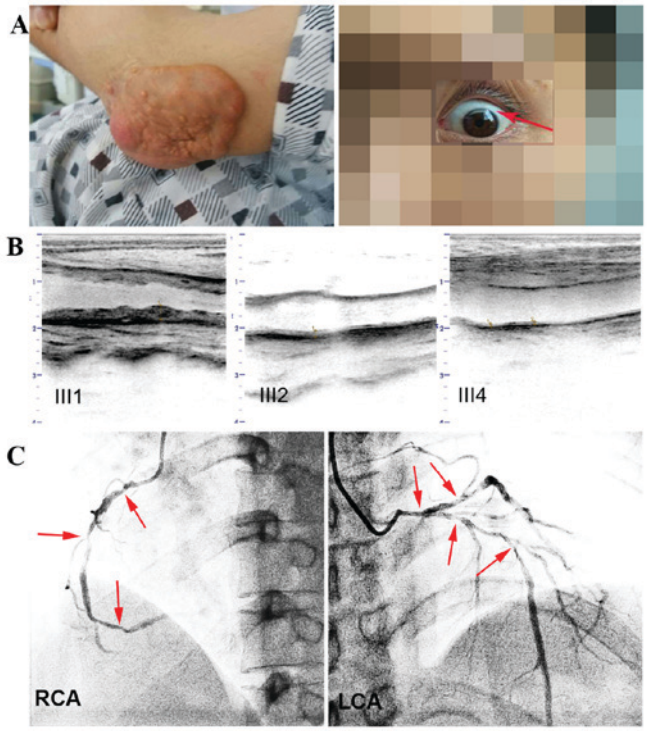

Figure 2. Clinical characteristics of III1 and photographic, ultrasonography results. (A) Elbow tendon xanthomata and the corneal arcus, which is a shadow-like white curve on the upper edge of the cornea. (B) Comparison of the distal carotid segment atherosclerosis between patients III1, III2 and III4. (C) Coronary angiogram from III1. Arrows indicate multiple stenosis of the RCA and LCA. III1, the proband. RCA, right coronary artery; LCA, left coronary artery. III1, the proband, the compound heFH subject. III2, the proband's brother, an heFH subject. III4, the proband's cousin, an unaffected subject.

Compound heterozygote mutations (Trp577Term and Pro685Leu) in *LDLR* were identified in the proband (Fig. 3A and B). The proband's mother (II1) and brother (III2) were heterozygous for the Trp577Term mutation and his aunt (II7) was heterozygous for the Pro685Leu mutation.

Multiple sequence alignments and prediction of LDLR mutations. Multiple sequence alignments revealed that these two amino acid alterations (Trp577Term and Pro685Leu) were located in the highly conserved region of *LDLR* in different species (Fig. 3C). The 3D ribbon model *in silico* prediction identified an obvious truncation in mutant LDLR protein caused by the Trp577Term mutation, which may lead to haplo-insufficiency (Fig. 4A and B). The secondary structure prediction revealed that the Pro685Leu mutant LDLR appeared to have more β-strands in its secondary structure than the wild type (Fig. 4C and D). Furthermore, the hydrophobicity of the Pro685Leu mutant protein was altered at the corresponding mutant region (Fig. 4E and F). Combined, these variations in the physicochemical properties of LDLR may be responsible for its functional abnormality.

Discussion

LDLR mutations are known to cause FH. In the present study, two novel compound heterozygous *LDLR* mutations, Trp577Term and Pro685Leu, were identified in the proband of a Chinese FH family. Furthermore, one of these two mutations was detected in the proband's mother, brother and aunt. According to pedigree analysis (Fig. 1), the Pro685Leu mutation was paternally inherited, whereas the Trp577Term

Table III. The clinical and molecular data of all subjects.

Subjects	Angina	Xanthoma	Mutation		EKG	PAS	EchoCG
			cDNA	Amino acid			
I1[a]	-	-	1773C>T;1959T>C	Asn591Asn;Val653Val	Inferior Q	3	AVC/AR
II1[b]	-	-	1731G>A	Trp577Term	STd	5	-
II5	-	-	-	-	STd	-	-
II7[b]	-	-	2054C>T	Pro685Leu	Normal	3	-
III1[b,c]	+	+	1731G>A;2054C>T	Trp577Term;Pro685Leu	STd heavy	Numerous	AVC/AS/AR/MR
III2[b]	-	+	1731G>A	Trp577Term	Normal	2	-
III3	-	-	-	-	Normal	-	-
III4	-	-	1773C>T;1959T>C	Asn591Asn;Val653Val	Normal	-	-
III6	-	-	-	-	Normal	-	-
IV1	-	-	-	-	Normal	-	-

Subjects marked with [a]smoke 1 pack/day for 9 years, [b]suffer from a causative mutation and [c]smoke 1-2 pack/day for 30 years. PAS, number of peripheral artery atherosclerotic plaques, referred as to IMT>1.5 mm or 50% neighbor thickness; EchoCG, echocardiography; Q, electrocardiographic Q wave; STd, ST segment depressed; AVC, aortic valvular calcification; AR, aortic valvular regurgitation; AS, aortic valvular stenosis; MR, mitral regurgitation.

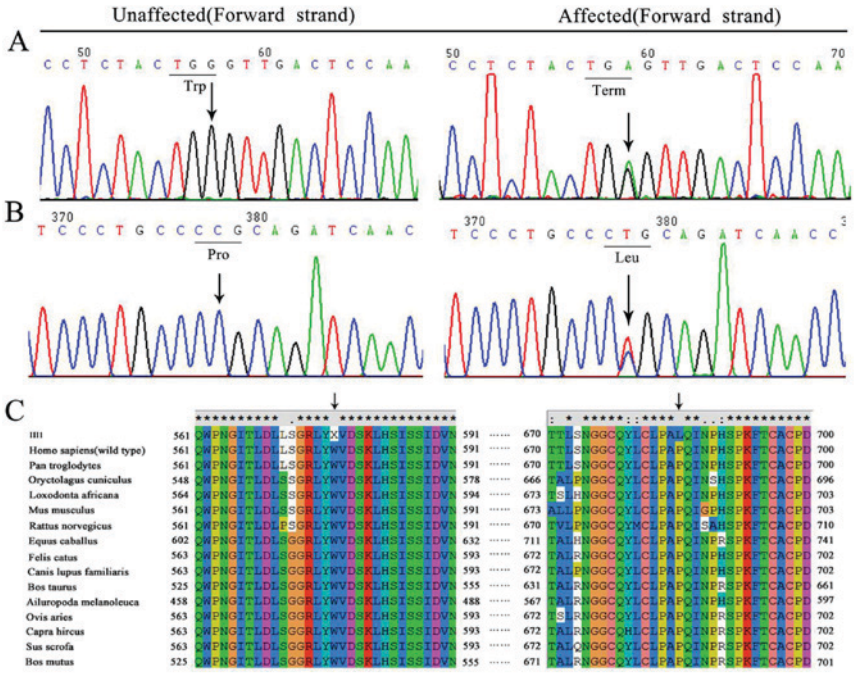

Figure 3. Sequencing results of the *LDLR* mutations and alignment of multiple LDLR protein sequences. (A) The sequence chromatogram of a wild-type allele with tryptophan (TGG) at codon 577, and the mutant allele with a heterozygous mutation of Trp577Term (c.1731G>A) in affected members. (B) The sequence chromatogram of a wild-type allele with proline (CCG) at codon 685, and the mutant allele with a heterozygous mutation of Pro685Leu (c.2054C>T) in affected members. (C) Multiple sequence alignments revealed that these two missense mutations affected amino acids located in the highly conserved amino acid region in different species. LDLR, low density lipoprotein receptor.

was maternally inherited. In this particular family, all mutation carriers were found to have elevated serum lipid levels, typical atherosclerotic plaques and coronary artery stenosis, all of which are indicators of early onset atherosclerosis. Notably, these symptoms were more severe in the compound heterozygote proband, suggesting that the *LDLR* mutation has a possible dosage effect in lipid metabolism. It was observed that the serum level of HDL-C was decreased in the proband and within the normal range for other family members, which suggests that elevated LDL-C may be responsible for low HDL-C levels, resulting in a vicious cycle effect in the proband. The proband also presented with bilateral corneal arcus and non-ST segment elevation myocardial infarction, and only he and his brother presented skin xanthomas. This indicates a sex-biased phenotype (II1 vs. III2). There was little difference observed in the phenotypes of two female suffers

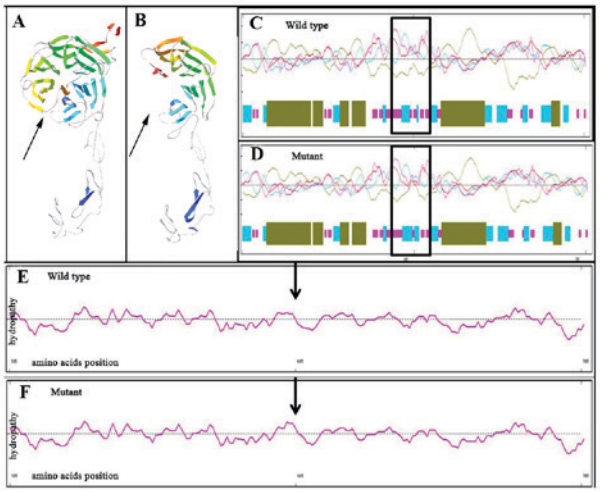

Figure 4. *In silico* predictions for LDLR mutations. The ribbon protein models of (A) wild-type and (B) Trp577Term mutant forms are displayed. The mutant protein exhibits an abnormally reduced amino acid chain. The predicted secondary structures of (C) the wild-type form and (D) the Pro685Leu mutant form. Black boxes indicate the mutated section. Hydropathy plot of (E) wild-type and (F) Pro685Leu mutant forms. Black arrows indicate the region of mutant site. LDLR, low density lipoprotein receptor.

(II1 and II7), which suggests that the two mutations have a comparable effect in FH. There was a step-wise severity in clinical symptoms from hoFH, heFH to unaffected subjects, including xanthoma, sporadic angina, hypercholesterolemia and peripheral artery atherosclerotic plaques and stenosis. These results suggest a genetic dosage-dependent clinical feature of FH.

The Trp577Term mutation is located in exon 12. The typical outcome of this type of mutation is premature termination of transcription, leading to a truncated protein lacking EGF, O-linked sugars, membrane spanning and cytoplasmic structure domains (9,24). The Pro685Leu mutation is located in exon 14, which is the highly conserved region of the EGF precursor domain in the LDLR protein (25). Previous studies have demonstrated that this alteration affects the flexibility of the LDLR polypeptide chain and the rigidity of the peptide bonds adjacent to proline (26,27).

Based on previous reports, each of the Trp577Term and Pro685Leu mutations in *LDLR* can cause FH independently (26,28). The Trp577Term mutation has only been documented in Chinese patients, and to the best of our knowledge, the compound heterozygous mutations have not been reported elsewhere. The present study indicated that compound heterozygous mutations resulted in a severe clinical manifestation of FH.

In the present study, the proband's parents were third degree relatives. It is well known that parents who both have common heterozygous mutations in a consanguineous marriage tend to give birth to homozygous children; however, in the present study the proband was a compound heterozygote instead. It is therefore worth studying the underlying association between incestuous family histories and the incidence of heterozygous mutations. Two missense mutations of Asn591Asn (c.1773C>T) and Val653Val (c.1959T>C) were also identified in this family, which are also able to increase LDL-C levels (29,30). However, the subjects carrying one or both of these variations in the present study had normal levels of TC and LDL-C. Since the study population of the above two studies were Canadians and Japanese, respectively, we speculate that the different outcome of these variations in our study may be influenced by the difference of environmental or ethnic factors.

There were certain limitations in the present study that must be considered. In the present study, the mechanism of destruction effects of Trp577Term and Pro685Leu mutations were only analyzed using bioinformatics tools. Further biological studies are required to confirm the underlying mechanism on mutant protein functions. Investigations involving more families and more mutation carriers will be helpful for the prevention and early intervention for Chinese FH populations.

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