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Two novel mutations 685del 1 and D129G in the low-density lipoprotein receptor gene in a compound heterozygote Chinese family with familial hypercholesterolemia

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Abstract

DNA sequencing analysis was used to scan the genes in a Chinese family with clinically diagnosed autosomal genetic hypercholesterolemia. Two mutations were identified in exon 4 of the low-density lipoprotein receptor gene, which is the possible molecular mechanism of etiology of the family. The proband's extremely high level of serum cholesterol and the related manifestations suggested that he was a familial hypercholesterolemia homozygote and that his parents were in a relatively milder condition. DNA sequencing revealed that the proband had an abnormal pattern of exon 4 of the low-density lipoprotein receptor gene due to a heterozygosity (A/G) at nucleotide 386 and a heterozygous single-base deletion (A) at 685. Nucleotide 386 is the second base of codon 129, and A→G mutation (D129G) changed this codon from Asp_{GAC} to Gly_{GGC}. The single-base deletion of A at 685 (685del 1) is a frameshift mutation. It changes the phase of triplets, so that all codons are misread after this site of mutation; consequently, the protein expressed by the gene must be abnormal in structure and function. DNA analysis of the other family members showed that the 2 mutations should be respectively located in different alleles of the proband. Both of the 2 mutations have not been reported previously.

1. Introduction

Generally, atherogenesis is considered a polygenic disease and numerous candidate genes are proposed [1]. In addition to environmental factors such as hypertension, diabetes, cigarette smoking, and obesity, gene mutations affecting any of the metabolic pathways involved in the development of atherosclerosis may contribute to the risk of coronary heart disease (CHD). Not all cardiovascular diseases are polygenic in nature, and among patients with CHD onset before the age of 55 about 5% of cases are attributable to monogenic origin. Premature onset of hypercholesterolemia often prompts that the genetic factors play more important roles. There are 4 main types of monogenic hypercholesterolemia that are closely related to CHD and result respectively from mutations in the low-density lipoprotein (LDL) receptor (LDLR) gene, the apolipoprotein (apo) B-100 gene, and the recently identified proprotein convertase subtilisin/kexin type 9 gene (PCSK9), and autosomal recessive hypercholesterolemia. These 4 types of hypercholesterolemia have a similar clinical phenotype that is characterized by increased plasma level of total cholesterol and LDL cholesterol, the formation of cutaneous and tendinous xanthomata, arcus corneae, and premature CHD. Among the 4 types of hypercholesterolemia, the first 3 are genetically autosomal dominant forms; there were no reports of homozygote with mutations of the PCSK9 gene. Clinically identified FH usually results from defects in the LDLR gene. Deficiency of LDLR results in accelerated cholesterol synthesis in cells and delayed clearance of LDL from the blood circulation [2]. Homozygous deficient patients with 2 abnormal LDLR genes, either identical or different mutant genes, which are rarely reported, typically exhibit life-threatening coronary atherosclerosis and subsequent myocardial infarction before age 30 [2]. In the present study we scanned the related gene of a clinically diagnosed autosomal genetic hypercholesterolemia family for the possible mutations and described

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Table 1
Serum lipid level of the main family members

Subjects	Age (y)	TC (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)	TG (mmol/L)	Lipoprotein(a) (mg/dL)
Proband 2-a	9	19.49	1.46	15.25	1.58	38.56
Father 1-a	41	10.64	1.32	8.75	1.37	–
Mother 1-b	40	8.11	1.42	6.33	1.52	–

2 different and novel mutations in the LDLR gene, which made the family a compound heterozygous family.

2. Subjects, materials, and methods

2.1. Subjects

The proband, a 9-year-old boy, was discovered with cutaneous vegetations on bilateral elbows, knees, and buttocks when he was 4 years old. The skin lesion spread slowly with no report of feeling of pain or pruritus. After admission, physical examination revealed 4 × 3-cm brownish-red skin lesions on the back of both elbows, 6 × 5-cm lesions on both knees, and 6 × 10-cm lesions on both hips. Vascular murmur could be heard in the carotid artery area. Doppler ultrasound revealed an obvious thickening of the intercellular layer of the carotid artery. The electrocardiogram was normal and we did not examine the coronary arteries although the coronarism was probably not optimistic. The parents are not in a consanguineous marriage. The outcome of the proband and his parents' serum lipid tests is shown in Table 1. According to the US MedPed Program diagnostic criteria for familial hypercholesterolemia (FH) [3], the proband (2-a) and his parents (1-a, 1-b) should be clinically diagnosed with FH. The proband has an extremely elevated serum cholesterol level (up to about 20 mmol/L), which makes probable the clinic diagnosis of the proband as a homozygote. The proband has a brother whose serum cholesterol is in the reference range. His mother has 2 brothers: one (1-d) had no hypercholesterolemia and the other (1-c) did and died at age 50 because of myocardial infarction. So we conjecture that 1-c is a heterozygote FH.

2.2. Amplification of the gene segments by polymerase chain reaction

About 4 mL of blood samples was drawn individually from 5 members of 2 generations of the family including the proband (2-a), his parents (1-a, 1-b), his brother (2-b), and his uncle (1-d) after fasting for 12 hours. The samples were centrifuged immediately. Genomic DNA was prepared from the peripheral white blood cells by phenol/chloroform extraction method [4]. The genome DNA prepared from the blood sample was then qualified by ultraviolet spectrophotometry and quantified by 1.2% agarose gel electrophoresis. Using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA), we designed all 19 primer pairs of promoter, exons 1 to 17 and the coding region of exon 18 of the LDLR gene, and the fragment of exon 26 of apo B including nucleotide 3500 and 3531 (Table 2). All the primers were synthesized by Bioasia Technology (Shanghai, China). Selective polymerase chain reaction (PCR) amplification of the apo B 3500 to 3531 fragment and of the promoter region and the entire coding sequence of the LDLR gene (18 exons) was performed on genomic DNA

Table 2
The primers of the gene fragments and amplification conditions of each PCR system

Amplified segments	Upstream primer (5′→3′)	Downstream primer (5′→3′)	Fragment length (bp)	PCR program (De, An, Ex)
Apo B-100 3500-3531	TGAGTCATCTACCAAAGG	GCTTGTATGTTCTCCGTT	280	94, 53, 74
LDLR promoter	CAGCTCTTCACCGGAGACC	ACCTGCTGTGTCCTAGCTGG	287	94, 58, 74
LDLR E1	CACATTGAAATGCTGTAAATGACG	CTATTCTGGCGCCTGGAGCAAGCC	215	94, 58, 74
LDLR E2	TTGAGAGACCCTTTCTCCTTTTCC	GCATAATCATGCCCAAAGGGG	183	94, 58, 74
LDLR E3	GCCTCAGTGGGTCTTTCC	GAGCAGGACCCCGTAGA	256	94, 55, 74
LDLR E4	ACACGGTGATGGTGGTCT	CCAGGGACAGGTGATAGG	469	94, 55, 74
LDLR E5	AGAAAATCAACACACTCTGTCCTG	GGAAAACCAGATGGCCAGCG	180	94, 58, 74
LDLR E6	TATGAATGAGTGCCAAGC	GAGTTCCCAAAACCCTAC	282	94, 53, 74
LDLR E7	GGCGAAGGGATGGGTGGGG	GTTGCCATGTCAGGAAGCGC	236	94, 58, 74
LDLR E8	CATTGGGGAAGAGCCTCCCC	GCCTGCAAGGGGTGATTCCG	220	94, 66, 74
LDLR E9	CCCCTGACCTCGCTCCCCGG	GCTGCAGGCAGGGGCGACGC	224	94, 66, 74
LDLR E10	ATGCCCTTCTCTCCTCCTGC	AGCCCTCAGCGTCGTGGATA	278	94, 58, 74
LDLR E11	TCCTCCCCCGCCCTCCAGCC	GCTGGGACGGCTGTCCTGCG	194	94, 64, 74
LDLR E12	ACTGGCATCAGCACGTGACC	CGTGTGTCTATCCGGCCACC	236	94, 58, 74
LDLR E13	GTCATCTTCCTTGCTGCCTG	TTCCACAAGGAGGTTTCAAGGTTGGGGGGG	329	94, 58, 74
LDLR E14	AAATTTCTGGAATCTTCTGG	GCAGAGAGAGGCTCAGGAGG	268	94, 55, 74
LDLR E15	AGAAGACGTTTATTTATTCTTTC	GTGTGGTGGCGGGCCCAGTCTTT	221	94, 55, 74
LDLR E16	CCTTCCTTTAGACCTGGGCC	CATAGCGGGAGGCTGTGACC	173	94, 58, 74
LDLR E17	GGGTCTCTGGTCTCGGGGGC	GGCTCTGGCTTTCTAGAGAGGG	242	94, 58, 74
LDLR E18	CCTCCAGCCGTGTTTCCT	GCAATGCTTTGGTCTTCTC	190	94, 53, 74

De indicates denaturation temperature (°C); An, annealing temperature (°C); Ex, extension temperature (°C).

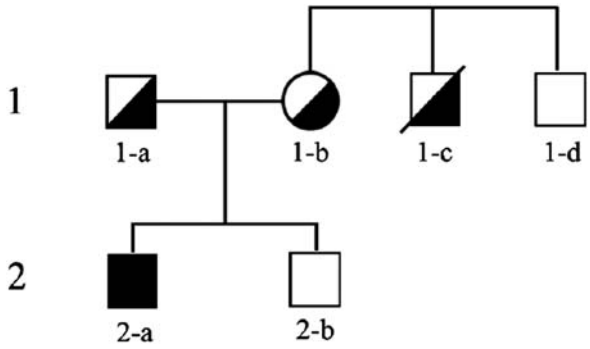

Fig. 1. Pedigree of the family.

from the proband and his family members, as previously described. The primers and cycle conditions for amplification are shown in Table 2. Each PCR was performed in a final volume of 60 μL containing 1 μL genomic DNA, 25 pmol each oligonucleotide primer, 30 pmol dNTP, 1 U *Taq* polymerase, and 6 μL 10× buffer.

2.3. Direct DNA sequencing and analysis

The PCR products of the apo B-100 and the LDLR gene fragments were purified, and then the proband's 20 PCR products were taken to Bioasia Technology for direct automated DNA sequencing on both sense and antisense strands. We downloaded the normal sequences of apo B-100 and LDLR gene from GenBank and used the DNA sequence analysis software DNAssist 2.5 (DNAssist, Los Alamos, NM) to identify the mutations in the 2 genes. When a mutation was detected, another sequencing was performed both on genomic DNA from the relatives and from a new PCR product from the proband. The resulting fragments were size-separated by electrophoresis on a 1.2% agarose gel.

3. Results

3.1. Pedigree analysis

The proband (2-a) was clinically diagnosed as homozygote because of the extremely high level of serum cholesterol, and his parents (1-a, 1-b) as heterozygote. His uncle (1-c) died of myocardial infarction at age 50, which suggests he was possibly another heterozygote. The family members 2-b and 1-d were considered to have normal genotype according to the normal serum lipid level. Results of the genotype study of the family confirm the inheritance pattern (Fig. 1).

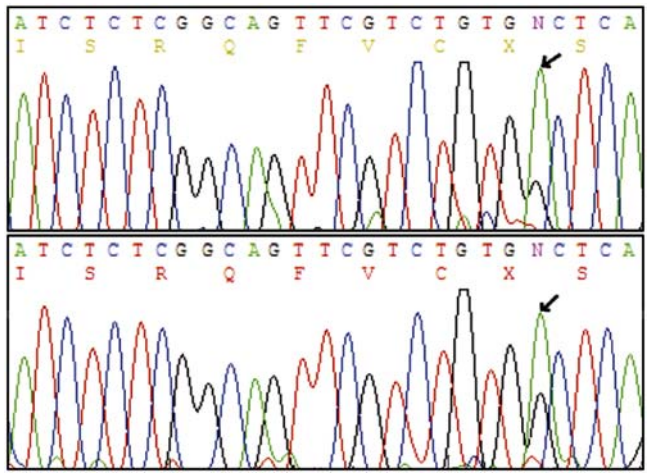

Fig. 2. The forepart of exon 4 of the proband and his mother's LDLR gene sequencing chromatogram. The chromatogram displays a heterozygous single-base mutation D118G (A→G 386). Correspondingly, the amino acid alters from aspartate to glutamate. Exon 4 codes a part of the ligand-binding domain, so the change in amino acid sequence can possible cut down its ability to bind LDL cholesterol.

3.2. Preclude familial defective apo B-100

Familial defective apo B-100 (FDB) was excluded, as no mutation was detected in the apo B-100 3500 to 3531 fragment according to the result of the DNA sequencing of the fragment.

3.3. LDLR gene sequencing and mutation analysis of the family

Direct DNA sequencing of the purified PCR products of the proband's LDLR gene followed by sequence analysis by DNAsist 2.5 reveals 2 missense mutations and 2 synonymous mutations (Table 3). As shown in Figs. 2 and 3, both of the 2 missense mutations of the proband's LDLR gene are located in exon 4 of the gene. One is D129G (A→G 386), the other is 685del 1. Both were heterozygous mutations. The mutation of D129G exists in the forepart of exon 4 for 2 different color curve peaks, which stand for A and G, respectively, and overlap in the DNA sequencing chromatogram (Fig. 2). The abnormal pattern of exon 4 of LDLR gene was due to a heterozygosity (A/G) at nucleotide 386. Nucleotide 386 is the second base of codon 129, and the alteration of the bases A→G induces the change of the corresponding amino acid from Asp_{GAC} to Gly_{GGC} (Table 3). In addition, as we see in Fig. 3, there are continuous double peaks followed by 2 A's. We analyzed the chromatogram

Table 3
Mutations in the LDLR gene of the family detected by DNA sequencing

Name	Nucleotide (location)	Predicted amino acid change	I2-a	1-a	1-b	2-b	1-d
W6W	T→C 80 (exon 2)	No	Homozygote	Homozygote	Heterozygote	Homozygote	Heterozygote
G450G	G→A 1413 (exon 10)	No	Heterozygote	Homozygote	–	Heterozygote	–
685del 1	Del A at 685 (exon 4)	Frameshift/stop	Heterozygote	Heterozygote	–	–	–
D129G	A→G 386 (exon 4)	Asp→Gly	Heterozygote	–	Heterozygote	–	–

Dashes indicate no mutation.

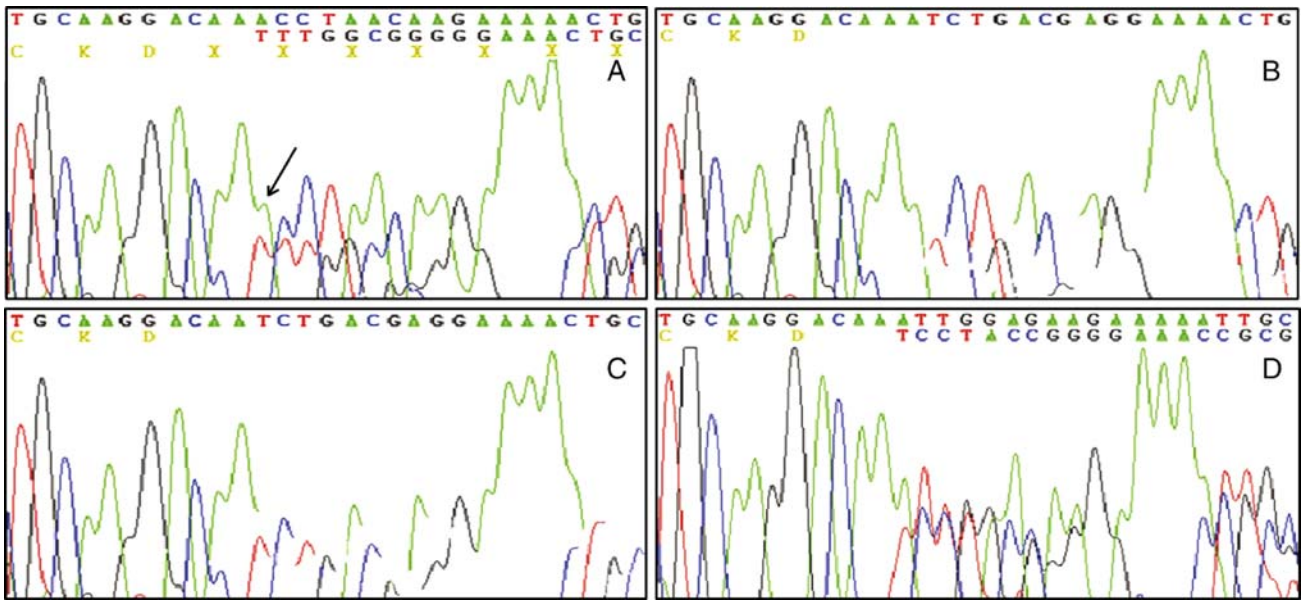

Fig. 3. The end part of exon 4 of the proband and his father's LDLR gene sequencing chromatogram. A, Sequencing pattern of exon 4 of the proband's LDLR gene. There are 2 A's followed by continuous double peaks. We isolated a normal sequence pattern (TGCAAGGACAAATCTGAGGAAACTG) from around the double-peak area (B). When we remove the normal sequence pattern in the double-peak area, we get another sequence (TGCAAGGACAATCTGAG-GAAACTG) (C). There is only one A disparity between the 2 sequences. D, Shows that the proband's father has a similar sequencing pattern as his son.

and isolated a normal sequence pattern (TGCAAGGA-CAAATCTGAGGAAACTG) from around the double-peak area. When we remove the normal sequence pattern in the double-peak area, we get another sequence (TGCAAGGA-CAATCTGAGGAAACTG). There is only one A disparity between the 2 sequences; thus, we predict that the mutation is a heterozygous single-base deletion, which is 685del 1. The single-base deletion of A at 685 (685del 1) is a frameshift mutation. It changes the phase of triplets, so that all codons are misread after this site of mutation, and the protein the gene expresses must be abnormal in structure and function. DNA sequencing analysis of the other family members shows that the 2 missense mutations are paternal (685del 1) and maternal (D129G) in origin. The proband's father is confirmed as an FH heterozygote with the mutation of 685del 1, whereas his mother is confirmed as an FH heterozygote with the mutation of D129G. Neither of the 2 mutations is found in the genome of 1-d and 2-b.

4. Discussion

The diagnosis of FH is generally made based on clinical features [5], but clinically diagnosed FH has been shown to result from mutations in some genes including apo B-100, PCSK9 [6,7], and LDLR. According to the inheriting pattern and the clinical feature of the proband and the other family members, we inferred that the hypercholesterolemia of this family might be caused by FDB or FH.

Apolipoprotein B, as ligand for LDLR, plays a central role in the catabolism of LDL [8-12]. To date, only 3 mutations of the apo B-100 gene have been described and associated with FDB: R3500Q, R3500W, and R3531C [13,14]. In the present study we preclude the FDB by amplifying followed by sequencing of the fragment including apo B 3500 and 3531 point mutations.

DNA sequencing of the proband revealed a heterozygosity (A/G) at nucleotide 386 and a heterozygous single-base deletion (A) at 685. The 2 mutations were confirmed to be novel mutations after searching the databases available for the collection of the reported mutations of LDLR gene on the Internet: http://www.ucl.ac.uk/fh [15,16]. With respect to the possible function of the mutations, nucleotide 386 is the second base of codon 129, and A→G mutation (D129G) changed this codon from Asp_{GAC} to Gly_{GGC}. The single-base deletion of A at 685 (685del 1) is a frameshift mutation. It changes the phase of triplets, so that all codons are misread after this site of mutation, and the protein the gene expresses must be abnormal in structure and function. Both of the 2 mutations have not been reported previously. Both of the 2 missense mutations existed in exon 4 of the LDLR gene. The 292-amino-acid ligand-binding domain at the amino-terminal end of the receptor encoded by exons 2 to 6 is assembled from 7 imperfect cysteine-rich tandem repeats of the 40 amino acids involved in apo B and apo E binding. It is possible that the mutant D129G affected the LDL cholesterol particle binding efficiency, whereas the frame shift mutation 685del 1, as described previously, caused an error in the reading frame behind the mutant point (exon 4 to exon 18) and severe change of the structure and the function of the LDLR gene. The area encoded by exons 5 to 18 plays an important role in proprotein translocation, recycling among the membranate structures and settling on the cell membrane. The serum cholesterol concentration of the proband and his parents verified the conjecture to some

extent. The mutations 685del 1 and D118G are paternal and maternal in origin, respectively, and the serum LDL cholesterol level of his father (with 685del 1 in LDLR gene) is higher than that of his mother (with D118G in LDLR gene).

After we detected the 2 missense mutations, we confirmed that the mutations were inherited from the parents. The 2 mutations were of paternal (685del 1) and maternal (D118G) origin and should be located in different alleles of the proband. This makes the proband a compound heterozygote [17]; compound heterozygotes are rarely reported.

The frequency of heterozygous FH is considerably higher than 1 in 500 in some populations, and there is an elevated frequency in some nationalities such as French Canadians [18] and South African Afrikaners [19], which is generally attributed to a founder effect. A founder effect occurs when a subpopulation is formed through the immigration of a small number of "founder" subjects, followed by a population expansion. If, by chance, some of the founders had FH, then genetic drift could lead to a high proportion of affected subjects who share specific mutations introduced by the founders. In these nations, the homozygote patients often have the same mutation in the alleles of the gene. In China, there is no confirmed founder effect in the population, and most of the homozygotes advent as the crossing of 2 FH families (ie, paternal family and maternal family) with different mutations, so it is feasible that the homozygotes here are more likely compound heterozygotes. The structure and function of the 2 mutations need to be studied further.

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