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Mutations in low-density lipoprotein receptor gene as a cause of hypercholesterolemia in Taiwan

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Abstract

Familial hypercholesterolemia (FH) is inherited as an autosomal dominant trait that has been associated with more than 920 different mutations in the low-density lipoprotein receptor (*LDLR*) gene. To characterize *LDLR* gene mutations in the Chinese of Han descent with FH, we isolated genomic DNA from peripheral blood samples of 20 affected subjects and 50 healthy subjects with no family history of hypercholesterolemia. We used polymerase chain reaction and long polymerase chain reaction to amplify the 18 coding exons and the minimal promoter of the *LDLR* gene, and subjected amplicons to direct sequence analysis. We identified 6 mutations in *LDLR* gene, including heterozygous missense mutations I420T (ATC→ACC), C660W (TGC→TGG), H562Y (CAC→TAC), and A606T (GCC→ACC), and a heterozygous and a homozygous mutation in codon P664L (CCG→CTG) as well as a homozygous large deletion of exons 6 to 8. The FH homozygotes manifested generalized xanthomatosis. One of the mutations we identified (C660W) was novel. In conclusion, we identified 5 missense mutations and 1 large deletion in *LDLR* gene, including 1 novel mutation in Han Chinese with FH in Taiwan.

1. Introduction

Familial hypercholesterolemia (FH) is caused by mutations in the gene encoding the low-density lipoprotein (LDL) receptor (LDLR) and is characterized by a marked elevation of the serum concentration of LDL cholesterol (LDL-C) [1-3]. The LDLR was first identified in 1973, and defective LDLR action was subsequently identified as an autosomal dominant cause of FH [1-3].

The *LDLR* gene was characterized in 1985. The human gene encoding LDLR is located on chromosome 19p13 and consists of 18 exons that span approximately 45 kilobases (kb). The human *LDLR* messenger RNA is 5.3 kb in length and encodes a protein of 860 amino acids [4,5]. The LDLR is a glycoprotein with an apparent molecular weight of 160 kd and is expressed on the surface of hepatocytes as well as most other cells [1,2]. The LDLR regulates plasma cholesterol by mediating the endocytosis of LDL, the major cholesterol transport protein in human plasma. Cells from individuals who are heterozygous for an *LDLR* gene mutation express half the normal number of functional receptors, and therefore, their cells bind, internalize, and degrade plasma LDL at half the normal rate [1,2].

The prevalence of *LDLR* gene mutations among patients with FH is highly variable among different populations. Heterozygous mutations are present in only 0.2% of the European and North American populations, but the incidences are 2- to 5-fold higher among French Canadians [4,5], Afrikaners in South Africa [6], and the Christian Lebanese [7]. This may reflect true genetic variation among populations, but it is more likely that ascertainment bias seems to play an important role. A wide variety of mutations, including insertions, deletions, nonsense, and missense mutations, has been described in patients with FH [8]. Familial hypercholesterolemia homozygotes, which are characterized by severe hypercholesterolemia and extensive xanthomatosis, affect about 1 per million people. Familial

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hypercholesterolemia has been reported in people of Mongoloid ethnicity, including Japanese [9,10], Korean [11,12], and the Chinese living in China [13,14], Taiwan [15], and Canada [16].

In the present study, we describe the identification of 6 mutations in the *LDLR* gene of the Han Chinese with FH living in Taiwan, including 1 unique mutation.

2. Materials and methods

2.1. Subjects

Twenty unrelated Chinese subjects of Han descent (6 men and 14 women; age range, 21-74 years) with FH, including 2 subjects with consanguineous parents, were entered into this study. A diagnosis of FH was made based on the following criteria: (1) plasma cholesterol and LDL-C concentrations higher than 7.77 mmol/L and 6.48 mmol/L, respectively, and (2) a positive family history of hypercholesterolemia or early coronary artery disease. Other secondary causes of hypercholesterolemia, including diabetes mellitus, hypothyroidism, and nephrotic syndrome, were excluded. We excluded patients with the familial defective apolipoprotein (apo) B mutation, an FH-like syndrome that occurs in 1 among every 40 patients with hypercholesterolemia in Taiwan [17]. Fifty unrelated healthy Han Chinese subjects with no family history of hypercholesterolemia were recruited as control subjects to determine whether any sequence changes might be a common polymorphism. None of them were taking any medications that may affect lipid metabolism. Demographic data and a detailed family history were collected for each patient. The study protocol was approved by the institutional review board of the hospital, and informed consent was obtained from each patient.

2.2. Biological investigations

Fasting plasma cholesterol and triglyceride (TG) levels were determined using a Hitachi 7600-310 AutoAnalyzer (Hitachi Instruments Corp, Tokyo, Japan). High-density lipoprotein cholesterol (HDL-C) was measured by the direct method using commercial kits from Kyowa (Tokyo, Japan). Low-density lipoprotein cholesterol was calculated according to the formula of Friedewald. The concentrations of apo A-I and apo B were measured by nephelometry (Behring Diagnostic, Marburg, Germany).

2.3. Polymerase chain reaction amplification

The 18 exons and the minimal promoter of the *LDLR* gene were amplified by polymerase chain reaction (PCR). Polymerase chain reaction was performed in a 50-μL volume containing 200 ng of genomic DNA prepared from EDTA whole blood (Gentra Systems, Inc, Minneapolis, Minn), 0.2 mmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.15 μmol/L of each primer, 10 mmol/L Tris-HCl (pH 7.4), 0.1% Triton X-100, 2 U of thermostable DNA polymerase (Protech Technology Co, Ltd, Taipei, Taiwan). The primers used in the present study have been previously described [8].

2.4. Long PCR amplification

To detect large deletion or insertion, a long PCR amplification was performed using the Expand Long Template PCR System (Roche, Mannheim, Germany). A 50-μL reaction contained 500-ng DNA, 0.3 μmol/L of each primer (Table 1), 0.5 mmol/L of each deoxynucleotide triphosphate, 2.75 mmol/L $MgCl_2$, and 4 U of enzyme mix. Amplification was set as denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, and extension at 68°C for 15 minutes. Deletion of a large fragment was verified by using reverse transcriptase–PCR and sequencing as previously described [15].

2.5. Direct sequencing

Polymerase chain reaction products were purified by using the GFX^{TM} PCR DNA purification kit (Amersham Pharmacia Biotech, Inc, Piscataway, NJ) and sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI Biosystems, Inc, Foster City, Calif) on the ABI PRISM 377 DNA sequencer (Perkin Elmer, Foster City, Calif).

2.6. Mutation analysis

Restriction endonuclease digestion of PCR amplicons was used to confirm and identify mutations detected by sequence analysis. The I420T and C660W mutations destroy restriction endonuclease sites for *Hin*fI and *Mnl*I, respectively. By contrast, P664L creates a restriction endonuclease site for *Pst*I.

2.7. Amplification-created restriction site analysis of mutation

To confirm the presence of H562Y and A606T mutations in the *LDLR* gene, we used modified PCR primers to introduce base substitutions adjacent to a codon of interest and thereby create an artificial restriction site on only 1 allelic form (wild type or mutant). We amplified exons 12 and 13 of the *LDLR* gene with modified primers to create a recognition site only if codons 562 and 606 carried the

Table 1
The primers used for the long PCR reaction

Amplified region	Forward primer	Reverse primer	Size (bp)
Exons 2-10	CCTTTCTCCTTTTCCTCTCTCTCAG	AGCCCTCAGCGTCGTGGATACGCAC	13595
Exons 6-13	TCCTTCCTCTCTCTGGCTCTCACAG	CTCTTGGCTGGGTGAGGTTGTGGAA	12854
Exons 12-18	TCTCCTTATCCACTTGTGTGTCTAG	GCTTTGGTCTTCTCTGTCTTTGAAT	14523

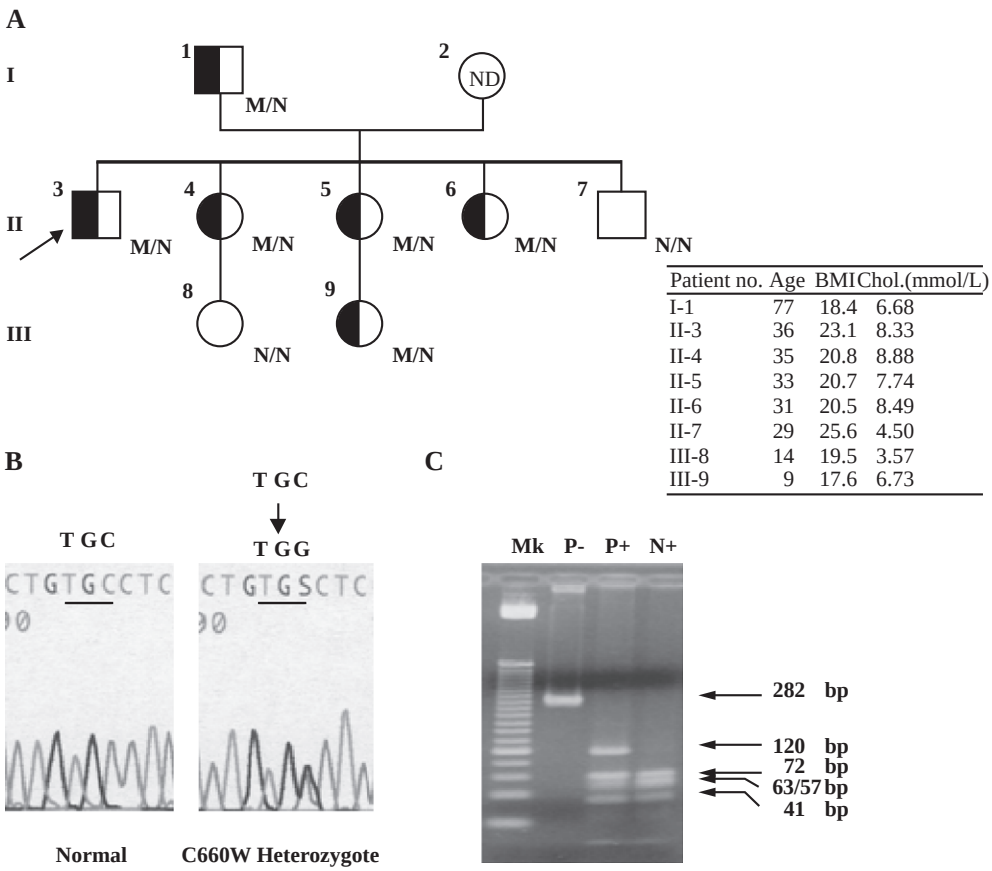
mutant type sequence. After the digestion of PCR products representing exons 12 and 13 with restriction endonuclease *Psp*1406I or *Mae*III, respectively, the samples were electrophoresed through on 3% agarose gels and stained with ethidium bromide.

2.8. Case report

A 36-year-old man had been medicated with niacin and gembrozil for 12 years after he was found to have hypercholesterolemia (cholesterol levels up to 10.34 mmol/L) during annual physical check-up. He first visited our hospital about 7 years before for the following values: total cholesterol of 8.33 mmol/L, HDL-C of 1.81 mmol/L, TG of 0.70 mmol/L, LDL-C of 6.20 mmol/L, and fasting plasma glucose of 4.66 mmol/L. The result of the electrocardiogram was normal. He had a family history of hypercholesterolemia. Physical examination showed a body height of 165 cm and a body weight of 63 kg as well as a body mass index of 23. Neither hepatosplenomegaly nor xanthomatosis was found. Laboratory evaluation showed the following values: fasting plasma glucose of 4.27 mmol/L, creatinine of 97 μmol/L, total bilirubin of 10.26 μmol/L, alanine aminotransferase of 36 mIU/mL, aspartate aminotransferase of 21 mIU/mL, apo A-I of 140 mg/dL, apo B of 96 mg/dL, cholesterol of 8.33 mmol/L, TG of 0.70 mmol/L, HDL-C of 1.34 mmol/L, and LDL-C of 6.67 mmol/L. The results of thyroid function and urinalysis were normal. When bezafibrate was shifted to lovastatin (40 mg/d), total cholesterol level dropped to 6.47 mmol/L, and LDL-C level to 5.06 mmol/L. The biochemical and clinical evaluation suggested a possible defect in LDLR, and thus we analyzed his *LDLR* gene. The result of the pedigree study is shown in Fig. 1.

3. Results

The demographic data for subjects with mutations are shown in Table 2. No familial defective apo B-100 R3500Q, R3500W, R3531C, and R3480W mutations were found in all subjects studied. Proband 1 had 1 heterozygous mutation in codon 660 of exon 14 (TGC→TGG), resulting in a substitution of cysteine by tryptophan (Fig. 1). This base change was not found in other siblings who were not hypercholesterolemic.



Patient no.	Age	BMI	Chol.(mmol/L)
I-1	77	18.4	6.68
II-3	36	23.1	8.33
II-4	35	20.8	8.88
II-5	33	20.7	7.74
II-6	31	20.5	8.49
II-7	29	25.6	4.50
III-8	14	19.5	3.57
III-9	9	17.6	6.73

Fig. 1. A, Pedigree of proband 1 with *LDLR* C660W mutation. The hypercholesterolemic patient is indicated by a black symbol. The normal cholesterolemic individuals are indicated by open symbols. The *LDLR* genotype of each individual is indicated below the symbol. N indicates normal; M, mutant; ND, not detected; arrow, the proband. B, The direct sequencing data show C660W mutation. C, Each PCR product of exon 14 was untreated or treated with restriction endonuclease *Mnl*I. *Mnl*I treatment would yield 2 fragments of 57 and 63 bp for wild type, whereas an undigested fragment of 120 bp for mutant type. Mk indicates 25-bp DNA ladder; N, normal; P, proband; minus sign, PCR product without *Mnl*I restriction enzyme; plus sign, PCR product after *Mnl*I digestion.

Table 2
Patient demographic data

Proband	Age (y)	Sex	Height (cm)	Weight (kg)	T-Chol (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	Apo A-I (mg/L)	Apo B (mg/L)	*LDLR* mutation
1	36	M	165	63	8.33	0.70	1.34	6.67	140	96	C660W[a]
2	45	M	168	78	9.46	2.02	1.06	7.47	192	225	I420T[a]
3	31	F	161	54	8.39	0.82	1.51	6.50	155	159	H562Y[a]
4	50	F	153	45	10.36	1.04	1.58	8.30	123	203	P664L[a]
5	23	F	157	52	17.90	1.48	1.14	16.08	142	175	P664L[b]
6	44	M	172	65	15.29	1.79	1.04	13.43	102	273	A606T[a]
7	40	F	157	72	15.10	0.88	0.70	14.00	69	261	Del e6-8[b]

T-Chol indicates total cholesterol; M, male; F, female.
[a] Heterozygous mutation.
[b] Homozygous mutation.

Proband 2 had 1 heterozygous mutation in codon 420 of exon 9 of *LDLR* gene (ATC→ACC), resulting in a substitution of isoleucine by threonine. This mutation has been described in the Netherlands [18]. Proband 3 had 1 heterozygous mutation in codon 562 of exon 12 (CAC→TAC), resulting in a substitution of histidine by tyrosine, similar to that previously described in China [13]. Probands 4 and 5 had 1 heterozygous and 1 homozygous (parental consanguinity) mutation, respectively, in codon 664 of exon 14 (CCG→CTG) that resulted in the substitution of proline by leucine. This mutation has been reported in diverse populations throughout the world, including Japanese, Asian Indian, Italian, English, and Norwegian [8,19,20]. Homozygous proband 5 had a serum total cholesterol level as high as 17.9 mmol/L and clinical xanthomatosis for both eyelids; both hands and elbows were observed.

Proband 6 had 1 heterozygous mutation in codon 606 of exon 13 (GCC→ACC), resulting in a substitution of alanine by threonine, as the one found in mainland China [13]. By using a long PCR, homozygous proband 7 (parental consanguinity) who had full-fledged xanthomatosis exhibited a deletion of exons 6 to 8 as previously described [15]. One mutation (C660W) was novel.

The I420T and C660W mutations destroy a restriction site for *Hin*fI and *Mnl*I, respectively, whereas P664L creates a new *Pst*I restriction site. In the amplification-created restriction site study, a new restriction site for *Psp*1406I was generated in alleles carrying the H562Y mutation, such that the digestion of the PCR product (153 base pairs [bp]) yields 2 fragments of 132 and 21 bp, whereas the normal one is not cleaved. With a similar approach, the mutant A606T PCR product (169 bp) is digested by *Mae*III to yield 2 fragments of 152 and 17 bp, whereas the normal allele is not cleaved. All restriction enzymes that distinguish between normal and mutant *LDLR* gene are shown in Table 3.

Table 3
List of restriction enzymes that distinguish between normal and mutant *LDLR* gene

Mutation	Position	Restriction enzyme	
		Natural	Created
ATC→ACC	Codon 420	*Hinf*I	–
TGC→TGG	Codon 660	*Mnl*I	–
CCG→CTG	Codon 664	*Pst*I	–
CAC→TAC	Codon 562	–	*Psp*1406I
GCC→ACC	Codon 606	–	*Mae*III

4. Discussion

Proband 1 manifested hypercholesterolemia during routine evaluation. He responded ill to fibrate therapy but moderately to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; nevertheless, cholesterol levels remained 6.47 to 7.24 mmol/L. Treatment of heterozygous FH patients with HMG-CoA reductase inhibitors can restore the capacity of the cells to clear LDL-C to about one-half normal levels [21]. In other reports, the response of heterozygous FH patients to HMG-CoA reductase inhibitors did not vary with different LDLR defects, whether the response was expressed as an absolute fall in LDL-C concentration or as a percentage of decrease from the initial value [22,23].

Several lines of evidence support the notion that C660W in proband 1 is related causally to FH: (1) this base was not found in 50 unrelated subjects, suggesting that it is not a common polymorphism; (2) no other mutation was found in the rest of the gene; (3) this base change was not found in other siblings who were not hypercholesterolemic; and (4) the C660 is evolutionally conserved in multiple animal species, suggesting that any substitution at this codon is not tolerated.

The C660W mutation is novel. The C660W is located in the region (exons 7-14) that encodes epidermal growth factor precursor homology domain, which is 400 amino acids in length [24,25]. It contains 3 repeats (A, B, and C) that are highly conserved, with each repeat containing 6 cysteine residues. The C660W mutation is in exon 14, which encodes the C repeat, and the missense mutation in codon 660 results in the substitution of tryptophan for 1 of the 6 cysteine residues. The effect of this mutation may block intracellular LDLR protein transport [26].

We found only 6 *LDLR* gene mutations in 7 families among the 20 cases of primary hypercholesterolemia we

studied. Our results support previous findings that FH is a heterogeneous complex disease caused by more than 920 mutations of the *LDLR* gene [27], which have been reported so far, and the 29 mutations of *LDLR* in Chinese patients with FH. It is not surprising to find 2 unrelated subjects (probands 3 and 6) with mutations that have been reported in other mainland Chinese [13], as these Taiwanese migrated from the Yangtze Valley of China 50 to 400 years ago.

In conclusion, we identified 5 missense and 1 large deletion mutations in *LDLR* gene, including a novel one in the Han Chinese with FH in Taiwan.

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