Identification and characterization of LDL receptor gene mutations in hyperlipidemic Chinese

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Abstract DNA screening for LDL receptor mutations was performed in 170 unrelated hyperlipidemic Chinese patients and two clinically diagnosed familial hypercholesterolemia patients. Two deletions (Del e3-5 and Del e6-8), eight point mutations (W-18X, D69N, R94H, E207K, C308Y, I402T, A410T, and A696G), and two polymorphisms (A370T and I602V) were identified. Of these mutations, C308Y and Del e6-8 were found in homozygosity, and D69N and C308Y were seen in unrelated patients. The effects of mutations on LDL receptor function were characterized in COS-7 cells. The LDL receptor level and activity were close to those of wild type in A696G transfected cells. A novel intermediate protein and reduction of LDL receptor activity were seen in D69N transfected cells. For R94H, E207K, C308Y, I402T, and A410T mutations, only ~20–64% of normal receptor activities were seen. Conversely, Del e3-5 and Del e6-8 lead to defective proteins with ~0–13% activity. Most of the mutant receptors were localized intracellularly, with a staining pattern resembling that of the endoplasmic reticulum and Golgi apparatus (D69N, R94H, E207K, C308Y, and I402T) or endosome/lysosome (A410T and Del e6-8). Molecular analysis of the LDL receptor gene will clearly identify the cause of the patient's hyperlipidemia and allow appropriate early treatment as well as antenatal and family studies.—Chang, J-H., J-P. Pan, D-Y. Tai, A-C. Huang, P-H. Li, H-L. Ho, H-L. Hsieh, S-C. Chou, W-L. Lin, E. Lo, C-Y. Chang, J. Tseng, M-T. Su, and G-J. Lee-Chen. **Identification and characterization of LDL receptor gene mutations in hyperlipidemic Chinese. *J. Lipid Res.* 2003.** 44: **1850–1858.**

Supplementary key words low density lipoprotein receptor mutation • cDNA expression • haplotype analysis

The concentration of plasma cholesterol is regulated mainly by the LDL receptor pathway, in which circulating LDL is taken into the cells by receptor-mediated endocytosis (1). A high LDL level due to defect in the LDL receptor is responsible for familial hypercholesterolemia (FH), an autosomal dominant disorder with estimated frequencies of one in 500 in most populations (2). In addition to a rise in the concentration of LDL cholesterol in blood, FH is frequently associated with tendon xanthomata and an increased risk of coronary artery disease (CAD). Due to a great biochemical and clinical variability among heterozygote carriers, measurements of plasma total cholesterol and LDL cholesterol do not allow a clear detection of individuals with FH (2). The homozygous FH occurs in about one in 1 million individuals and is more severely affected than the heterozygote carrier.

The LDL receptor gene consists of 18 exons spanning 45 kb on chromosome 19p13 (3). The 5.3 kb mRNA encodes a mature protein of 839 amino acids (4). Overall, more than 800 mutations, including gross deletions, minor deletions, insertions, point mutations, and splice-site mutations, scattered over the LDL receptor gene have been reported (5). Among these, deletions or insertions at the LDL receptor gene involve *Alu* sequences (6, 7). LDL receptor mutations can be studied by Western, flow cytometry, and fluorescence microscopy analyses of mutated cDNA-transfected COS cells (8–10). These mutations affect the synthesis (class 1), posttranslational processing (class 2), ligand binding activity (class 3), internalization (class 4), or recycling (class 5) of the LDL receptor. Defects of classes 2 and 4 can be either complete or partial. Heterogeneous mutations seem to have caused FH most in individual families (11), but in certain populations, a few mutations still prevail (12–14). Mutations in the LDL receptor gene are known to exist in Chinese FH patients in China, Hong Kong, Canada, and Southeast Asia (15–

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Abbreviations: CAD, coronary artery disease; FH, familial hypercholesterolemia; SSCP, single-strand conformation polymorphism.

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19). There is no definite common mutation in Chinese populations due to a founder effect.

In this study, 170 unrelated hyperlipidemic Chinese and two clinically diagnosed FH patients were screened by multiplex polymerase chain reaction (PCR), long PCR, and single-strand conformation polymorphism (SSCP) analyses to detect mutations in the LDL receptor gene. Two gross deletions, six point mutations, and two polymorphisms were found. Additionally, the haplotype at nine polymorphic sites and the effect of mutations on LDL receptor function were examined.

MATERIALS AND METHODS

Subjects and lipids

Blood samples were collected after an overnight fast from 170 unrelated hyperlipidemic patients, ages 31–74 years (mean, 58 years), attending the lipid clinic of the Veterans General Hospital, Taipei and Wei Gong Memorial Hospital. Of these subjects, 112 (70 men and 42 women) had primary type IIa hyperlipidemia, and 58 (40 men and 18 women) had type IIb hyperlipidemia. These patients had cholesterol concentrations >6.20 mmol/l (range, 6.21–11.32 mmol/l) without tendon xanthomas. Type IIa patients had triglyceride values <2.27 mmol/l, and type IIb patients had concentrations above this value. Patients with familial defective apolipoprotein B (apoB) were excluded by testing for the apoB-3500 mutation (20, 21). In addition, a 12-year-old girl and a 40-year-old woman, who were clinically diagnosed with homozygous FH based on markedly increased concentrations of plasma LDL cholesterol (16.61 and 17.16 mmol/l, respectively) and the presence of skin and tendon xanthomas, were included in this study. Family members of patients with identified LDL receptor mutations and 100 unrelated Chinese subjects with normal fasting plasma lipid levels (cholesterol concentrations <5.17 mmol/l) were also recruited for the study with their consent. The procedures followed were in accordance with the current revision of the Helsinki Declaration of 1975. Blood samples from fasting subjects were drawn for DNA extraction and lipid measurements as described (20, 21).

Mutation analysis

Genomic DNA samples were amplified by PCR using primers (12, 22) specific for the promoter region and 18 exons of the LDL receptor gene (**Table 1**, PCR A–G). A 25 μl reaction contained 200 ng DNA, 0.4 μmol/l of each primer, 0.2 mmol/l deoxynucleotide triphosphates, 1.5 mmol/l $MgCl_2$, and 0.5 U *Taq* DNA polymerase (Promega). Amplification was set as denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min (Table 1, PCR A–F). Amplification for exon 4 (Table 1, PCR G) was set as denaturation at 94°C for 1 min and primer annealing and extension at 68°C for 5 min.

PCR products (3 μl) were mixed with an equal volume of 95% formamide buffer for SSCP analysis (21). Fragments showing mobility shifts of single strands were sequenced directly using the ABI Prism Dye Terminator Kit and an ABI Prism™ 377 DNA Sequencer (Applied Biosystems). All point mutations were verified by restriction analysis (**Table 2**) of PCR amplified products.

If the presence of a large deletion was indicated by electrophoresis of multiplex PCR products, a long PCR amplification was then performed (Table 1, PCR H and L). A 50 μl reaction contained 500 ng DNA, 0.4 μmol/l of each primer, 0.2 mmol/l deoxynucleotide triphosphates, 1.0 mmol/l $MgCl_2$, and 1 unit of ELONGASE enzyme mix (Gibco Brl). Amplification was set as denaturation at 94°C for 1 min, and primer annealing and extension at 68°C for 10 min. Shorter (deleted) fragments were subcloned into pGEM-T Easy (Promega) and sequenced as described.

cDNA constructs

The 2.8 kb *Bam*HI-*Sma*I fragment containing the LDL receptor coding region (cDNA ∼−19–2804, where +1 represents the first nucleotide to be translated) was excised from pLDLR3 (ATCC No. 57004) and subcloned into *Bam*HI and *Eco*RV sites of pcDNA3 (Invitrogen Corporation) to produce plasmid pcDNA3-

TABLE 1. Primers for PCR amplification of LDL receptor exons

PCR Mix	Amplified Region	Forward Primer	Reverse Primer	Frag. size (bp)
A	Promoter	CAGCTCTTCACCGGAGACC	ACCTGCTGTGTCCTAGCTGG	287
	Exon 1	CACATTGAAATGCTGTAATGACG	CTATTCTGGCGCCTGGAGCAAGCC	215
	Exon 5	AGAAAATCAACACACTCTGTCCTG	GGAAAACCAGATGGCCAGCG	180
B	Exon 9	CCTGACCTCGCTCCCCGGACCCCCA	GGCTGCAGGCAGGGGCGACGCTCAC	223
	Exon 2	TTGAGAGACCCTTTCTCCTTTTCC	GCATAATCATGCCCAAAGGGG	183
	Exon 16	CCTTCCTTTAGACCTGGGCC	CATAGCGGGAGGCTGTGACC	173
C	Exon 7	GGCGAAGGGATGGGTAGGGG	GTTGCCATGTCAGGAAGCGC	234
	Exon 3	TTCCTTTGAGTGACAGTTCAATCC	GATAGGCTCAATAGCAAAGGCAGG	196
	Exon 8	CCAAGCCTCTTTCTCTCTCTTCCAG	CCACCCGCCGCCTTCCCGTGCTCAC	176
D	Exon 15	AGAAGACGTTTATTTATTCTTTC	GTGTGGTGGCGGGCCCAGTCTTT	221
	Exon 6	TCCTCCTTCCTCTCTCTGGC	TCTGCAAGCCGCCTGCACCG	179
	Exon 18	GCCTGTTTCCTGAGTGCTGG	TCTCAGGAAGGGTTCTGGGC	135
E	Exon 10	ATGCCCTTCTCTCCTCCTGCCTCAG	AGCCCTCAGCGTCGTGGATACGCAC	278
	Exon 17	TGACAGAGCGTGCCTCTCCCTACAG	TGGCTTTCTAGAGAGGGTCACACTC	210
	Exon 12	TCTCCTTATCCACTTGTGTGTCTAG	CTTCGATCTCGTACGTAAGCCACAC	190
F	Exon 13	GTCATCTTCCTTGCTGCCTGTTTAG	GTTTCCACAAGGAGGTTTCAAGGTT	219
	Exon 14	CCTGACTCCGCTTCTTCTGCCCCAG	ACGCAGAAACAAGGCGTGTGCCACA	204
	Exon 11	CAGCTATTCTCTGTCCTCCCACCAG	TGGCTGGGACGGCTGTCCTGCGAAC	173
G	Exon 4	TGGTCTCGGCCATCCATCCCTGCAG	ACGCCCCGCCCCCACCCTGCCCCGC	437[a]
H	Exons 5–9	AGAAAATCAACACACTCTGTCCTG	GGCTGCAGGCAGGGGCGACGCTCAC	6,941
L	Exons 2–6	CCTTTCTCCTTTTCCTCTCTCTCAG	GCAAGCCGCCTGCACCGAGACTCAC	7,348

[a] PCR-amplified product was digested with *Ava*II to generate exon-containing 157 bp and 280 bp subfragments for single-strand conformation polymorphism analysis.

TABLE 2. LDL receptor mutations in hyperlipidemic Chinese

Mutation[a]	Nucleotide Alteration	Protein Alteration	Patient	Restriction Enzyme Test	Remarks
		residue number		*fragment sizes (bp)*	
W-18X	TGG→TGA (exon 1)	Trp_{-18}→Stop	D102	+*Dde*I (129, 86)	Reported (11, 15)
D69N	CGC→CAC (exon 3)	Asp_{69}→Asn	A133, L41	+*Mfe*I[b] (115, 23)	Reported (16, 18, 25)
R94H	CGC→CAC (exon 4)	Arg_{94}→His	A144	−*Fnu*4HI (230)	Reported (18, 30)
E207K	GAG→AAG (exon 4)	Glu_{207}→Lys	95-123	−*Mnl*I (294)	Reported (11, 12, 16, 17, 31)
C308Y	TGC→TAC (exon 7)	Cys_{308}→Tyr	95-035, D63[c]	+*Acc*I (133, 101)	Reported (16, 18)
I402T	ATC→ACC (exon 9)	Ile_{402}→Thr	D101	+*Hph*I (57, 113)	Reported (18, 35)
A410T	GCT→ACT (exon 9)	Ala_{410}→Thr	D142	+*Tsp*45I (127, 96)	Reported (34)
I602V	ATC→GTC (exon 13)	Ile_{602}→Val	A107, D96, D194	−*Eco*RV (219)	Novel[d]
A696G	GCT→GGT (exon 15)	Ala_{696}→Gly	D162	−*Pst*I (221)	Novel
Del e3-5	Exons 3–5 deleted	Deletion of 209 aa (44–252)	D72		Novel
Del e6-8	Exons 6–8 deleted	Deletion of 123 aa (252–374)	D254[c]		Novel

[a] Number of amino acids according to ref. (3).
[b] The *Mfe*I site generated by amplification created restriction site PCR using exon 3 forward primer and mismatch reverse primer (TTGTCGCAGTCCACTTGGCAAT).
[c] Homozygosity for the mutation.
[d] Normal variant allele, which is presumed to be a polymorphism.

LDLR. The point change in the LDL receptor cDNA was made by using QuickChange XL system (Stratagene) and confirmed by DNA sequencing. pcDNA3-LDLR/Del e3-5 was derived from pcDNA3-LDLR by replacing a 759 bp *Hae*II-*Nci*I fragment (cDNA ~150–908) with a 552 bp fragment excised from the deleted exons 2–6 long PCR clone of patient D72 (substitution of exons 3–5 with 420 bp intron). pcDNA3-LDLR/Del e6-8 was derived from pcDNA3-LDLR by replacing a 559 bp *Eco*RI-*Bsu*36I fragment (cDNA ~719–1277) with a 1,503 bp fragment from the deleted exons 5–9 long PCR clone of patient D254 (substitution of exons 6–8 with 1,313 bp intron). The replaced sequences were confirmed by restriction enzyme digestion and DNA sequencing.

Expression studies

COS-7 cells cultivated in DMEM containing 10% FCS were transfected with the various LDL receptor cDNA constructs by lipofection procedure. To inhibit the endogenous LDL receptor expression, cells were incubated in medium containing 0.2 μg/ml 25-hydroxycholesterol for 24 h prior to transfection. Forty-eight hours later, total RNA and proteins from transfected cells were prepared. The level of LDL receptor mRNA in transfected cells was quantitated by reverse transcription (RT)-PCR using the ThermoScript™ RT-PCR system (Gibco). The expressed LDL receptor proteins were examined by Western blot analysis using rabbit anti-LDL receptor polyclonal antibody (1:400 dilution, Progen) and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Boehringer Mannheim).

For flow cytometric analysis, cells were harvested by trypsin treatment 48 h after transfection, and cells in suspension were incubated with 0.4% heparin for 30 min to remove any surface-bound LDL. To quantitate the amount of receptor on the cell surface, the cells were incubated for 30 min at 4°C in primary antibody diluted 1:60 (v/v) in PBS, washed in PBS, and incubated further for 30 min at 4°C in FITC-conjugated goat anti-rabbit antibody (Zymed) also diluted 1:60 (v/v) in PBS. To measure the LDL receptor function, the cells were incubated for 30 min at 4°C in DiI-LDL (Molecular Probes) diluted in DMEM to a concentration of 4 μg/ml, washed with PBS, and incubated further for 30 min at 37°C. All cells were subsequently washed and resuspended in PBS for fluorescence activated cell sorter analysis. Cells were analyzed in a FACStar flow cytometer (Becton-Dickinson) equipped with an argon laser operating at 488 nm (antibody) or 514 nm (DiI). A forward scatter gate was established to exclude dead cells and cell debris from the analysis. 10^4 cells were analyzed in each sample.

The intracellular receptor protein was visualized by staining cells fixed in 4% paraformaldehyde and permeabilized in 70% ethanol. Cells were incubated for 60 min at room temperature with primary antibody diluted 1:50 in 1% BSA in TBS, washed in PBS, and incubated for 60 min at room temperature in FITC-conjugated secondary antibody diluted 1:50 in 1% BSA in TBS, washed in PBS. Lysosomes were detected using lysotracker (Molecular Probes) at 0.1 μmol/l for 60 min. The stained cells were analyzed using a Leica TCS confocal laser scanning microscope.

Haplotype analysis

Nine markers were analyzed to establish haplotypes at the LDL receptor locus. These markers were as follows: polymorphic *Sfa*NI site in exon 2; *Sma*I site in intron 7; *Aci*I site in exon 11; *Hin*cII site in exon 12; *Ava*II site in exon 13; *Msp*I site in exon 15; *Nco*I site in exon 18; hypervariable region-TA dinucleotide repeat marker at the 3′ end of the LDL receptor gene; and GAAG tetranucleotide repeat marker D19S394, 250 kb 5′ to the LDL receptor gene (5). For the dinucleotide and tetranucleotide repeat markers, the sense primers were fluorescently labeled. For the seven restriction fragment length polymorphism (RFLP) markers in the LDL receptor gene, PCR-amplified products were digested with the appropriate restriction enzyme and separated on 1.4% agarose or 8% polyacrylamide gel. Length of dinucleotide and tetranucleotide repeat markers was determined by electrophoresis of the PCR-produced fragments in a linear polyacrylamide gel on an automated MegaBACE Analyzer (Molecular Dynamics, Division of Amersham Pharmacia Biotech).

RESULTS

Identification of point mutations

The promoter region and 18 exons of the LDL receptor gene were amplified and screened for mutations by SSCP and DNA sequencing. Six reported (5) polymorphic changes, intron 7 *Sma*I, exon 8 *Stu*I, exon 11 *Aci*I, exon 12 *Hin*cII, exon 13 *Ava*II, and exon 15 *Msp*I, were detected (data not shown). Additionally, seven reported (5) point mutations (W-18X, D69N, R94H, E207K, C308Y, I402T, and A410T) and two novel variations (I602V and A696G) were identified (Table 2). The I602V variant was seen in two normolipidemic controls in addition to three hyperlipidemic patients. This then can be presumed to be a

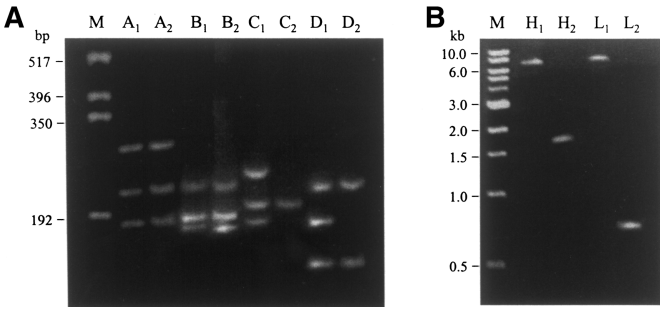

Fig. 1. A: Agarose gel (2.0%) electrophoresis results of multiplex polymerase chain reaction (PCR) products of D254 (lanes A_2, B_2, C_2, and D_2) and a normolipidemic control (lanes A_1, B_1, C_1, and D_1). The lanes marked A–D correspond to the multiplex PCR A–D in Table 1. Lane M (*Hin*fI digest of pGEM4 DNA) contains size markers. B: Agarose gel (0.7%) electrophoresis results of long PCR products of D254 (lane H_2), D72 (lane L_2), and a normolipidemic control (lanes H_1 and L_1). The lanes designated H and L correspond to the long PCR H and L in Table 1. The marker used was 1 kb ladder from New England BioLabs.

polymorphism (1.0% allele frequency). Other point changes, however, were not detected in our normolipidemic controls.

Identification of deletion mutations

Two novel deletions (Del e3-5 and Del e6-8) were identified (Table 2) by multiplex PCR and long PCR. Del e6-8 in patient D254 is a 5,277 bp deletion spanning from intron 5 to intron 8 and eliminating exons 6, 7, and 8 (**Fig. 1A**, lanes C_2 and D_2). Long PCR using primers to amplify a 6,941 bp fragment containing exons 5–9 detected a shorter (deleted) 1.7 kb fragment (Fig. 1B, lane H_2). Sequencing of the shorter fragment (**Fig. 2A**) revealed an intrastrand recombination event between *Alu* sequences in intron 5 and intron 8 (Fig. 2C). In patient D72, Del e3-5 eliminates a 6,632 bp fragment containing exons 3, 4, and 5 (Fig. 1B, lane L_2). Sequencing of the shorter fragment (Fig. 2B) demonstrated another intrastrand recombination between *Alu* sequences in intron 2 and intron 5 (Fig. 2C).

Expression of LDL receptor cDNA mutants

The effects of mutations on LDL receptor function were investigated by transient expression in COS-7 cells. Approximately equal levels of LDL receptor mRNA derived from each variant was expressed as quantitated by RT-PCR (data not shown). The LDL receptor protein that derived from each variant was examined by immunostaining of Western blot with LDL receptor polyclonal antibody (**Fig. 3**). No nonspecific polypeptide was detected in pcDNA3 vector-transfected cells (Fig. 3, lane 1). One hundred sixty kilodalton mature and 120 kDa precursor proteins, as well as an intermediate form, were detected in wild-type receptor cDNA-transfected cells (Fig. 3, lane 2). A novel intermediate protein that is probably a degradation product and an

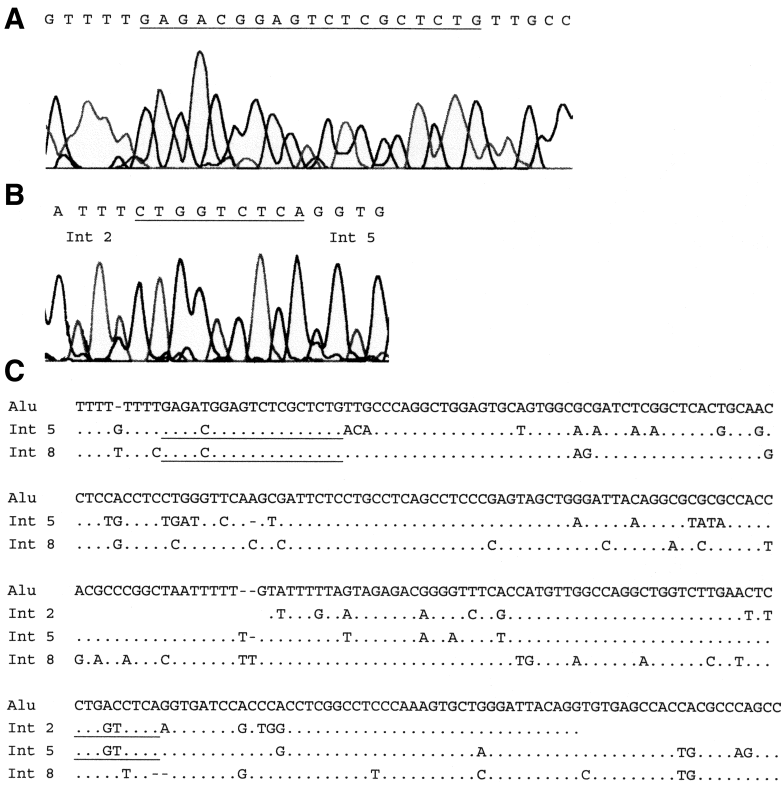

Fig. 2. Nucleotide sequences across the deletion joints in D254 and D72. The breakpoint regions are underlined. A: Sequences across the deletion joint in the Del e6-8 allele. B: Sequences across the deletion joint in the Del e3-5 allele. C: Alignment of *Alu* sequences of normal intron 2 (IVS2+32∼+137), normal intron 5 (IVS5+152∼+442), and normal intron 8 (IVS8+479∼+769) with the consensus *Alu* sequence (36). Dots indicate positions at which the sequences are identical.

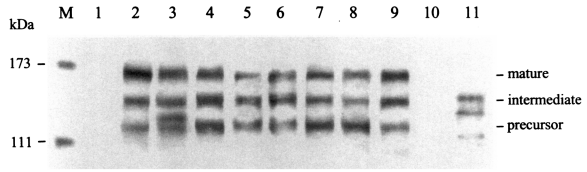

Fig. 3. Western analysis of LDL receptor cDNA variants. Total protein (10 μg) was separated on 6% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with LDL receptor polyclonal antibody. Molecular mass of LDL receptor protein is indicated as determined from a plot of migration distances of standard proteins against the logarithms of molecular masses. Lane 1, pcDNA3; lane 2, wild-type; lane 3, D69N; lane 4, R94H; lane 5, E207K; lane 6, C308Y; lane 7, I402T; lane 8, A410T; lane 9, A696G; lane 10, Del e3-5; lane 11, Del e6-8. Lane M contains molecular weight markers.

apparent reduction in mature receptor protein were seen in D69N transfected cells (Fig. 3, lane 3). Although the same molecular weight receptor proteins were seen with R94H, E207K, C308Y, I402T, and A410T mutations, the amounts of mature protein detected were reduced (Fig. 3, lanes 4–8). For A696G, both the processing and the level of the mature protein were close to those of wild type (Fig. 3, lane 9). Conversely, no receptor protein for Del e3-5 and a defect receptor protein for Del e6-8 were detected (Fig. 3, lanes 10 and 11).

Analyses of antibody-labeled cells and activity measurements revealed that A696G encodes receptors in amounts and activity comparable with the wild-type receptor (98% and 93%, respectively). Del e3-5 encodes receptors that cannot be detected and have no detectable residual activity. All other mutations impair the function, encoding receptors in reduced amounts (~13%–64%) and displaying ~12%–64% residual activity (**Table 3**).

Fluorescence microscopy of cells expressing the wild-type or the mutant LDL receptor was performed. As shown in **Fig. 4A**, the wild-type and A696G receptor proteins were located in a ring-shaped structure representing the cell surface, whereas most of the mutant receptors accumulated intracellularly. Colocalization studies using a lysosome staining dye (Fig. 4B) further established that A410T and Del e6-8 mutant proteins are retained in the endosomal/lysosomal regions, whereas D69N, R94H, E207K, C308Y, and I402T produce proteins that seem to be localized in the endoplasmic reticulum (ER) (Fig. 4C).

Lipoprotein concentrations and family studies

The clinical and biochemical features of the nine FH heterozygotes and two FH homozygotes are shown in **Table 4**. The plasma total cholesterol levels of these heterozygotes ranged from 6.21 to 10.03 mmol/l. Four of them (D102, A133, L41, and A144) had evidence of atherosclerotic disease. It was observed that both FH homozygotes had tendon xanthomas and atherosclerosis, in addition to high values of cholesterol (16.61 and 17.16 mmol/l). Currently, D254 is being treated by periodical plasmapheresis.

A total of 33 family members of patients A133, D63, D102, and D254 gave consent to be screened for the identified D69N, C308Y, W-18X, or Del e6-8 mutation. The D69N mutation was found in A133's son and grandson, although their cholesterol values were lower than 6.20 mmol/l (**Fig. 5A**). When D63's family was recruited and screened, results showed that both of her parents and three of her siblings also carry the mutation, which was cosegregated with hypercholesterolemia in this family (Fig. 5B). None of the daughters inherited the W-18X mutation from patient D102 (Fig. 5C). Patient D254 inherited Del e6-8 from her parents. Among her siblings, four heterozygous and one homozygous deletion were found. Eight of the ten individuals in the third generation also carried the deletion. With the exception of one individual (cholesterol value 4.94 mmol/l), the deletion was cosegregated with hypercholesterolemia in this family (Fig. 5D).

All of the above mutation carriers are informed and followed up at our out-patient clinics. Those carriers with normal phenotype will have periodic biochemistry examination. Lipid-lowering drugs, including fibric acid derivatives and statins, are prescribed for those carriers with elevated values of cholesterol.

Haplotype analysis on the mutant allele

The genotypes at nine polymorphic loci were examined in patients with W-18X, D69N, C308Y, or Del e6-8 mutations and recruited family members. As shown in **Table 5**, the

TABLE 3. Flow cytometric measurement of polyclonal antibody binding and DiI-LDL binding and internalization

Transfected Vector	Antibody 4°C	DiI-LDL 37°C
pcDNA3	1,404 ± 29 (0%)	5,002 ± 116 (0%)
pcDNA3-LDLR	78,377 ± 1,095 (100%)	141,737 ± 3,043 (100%)
pcDNA3-LDLR/D69N	43,112 ± 2,734 (54%)	80,246 ± 1,571 (55%)
pcDNA3-LDLR/R94H	50,932 ± 1,443 (64%)	92,067 ± 2,114 (64%)
pcDNA3-LDLR/E207K	20,107 ± 455 (24%)	34,017 ± 730 (21%)
pcDNA3-LDLR/C308Y	25,600 ± 323 (31%)	46,753 ± 643 (31%)
pcDNA3-LDLR/I402T	47,018 ± 255 (59%)	79,373 ± 1,704 (54%)
pcDNA3-LDLR/A410T	18,301 ± 1,655 (22%)	32,119 ± 656 (20%)
pcDNA3-LDLR/A696G	76,796 ± 793 (98%)	132,670 ± 2,393 (93%)
pcDNA3-LDLR/Del e3-5	1,422 ± 35 (0%)	5,148 ± 102 (0%)
pcDNA3-LDLR/Del e6-8	11,757 ± 164 (13%)	21,719 ± 194 (12%)

The cumulated fluorescence signals are the mean of three independent measurements and standard deviation. Values in parentheses represent the percentage of wild type.

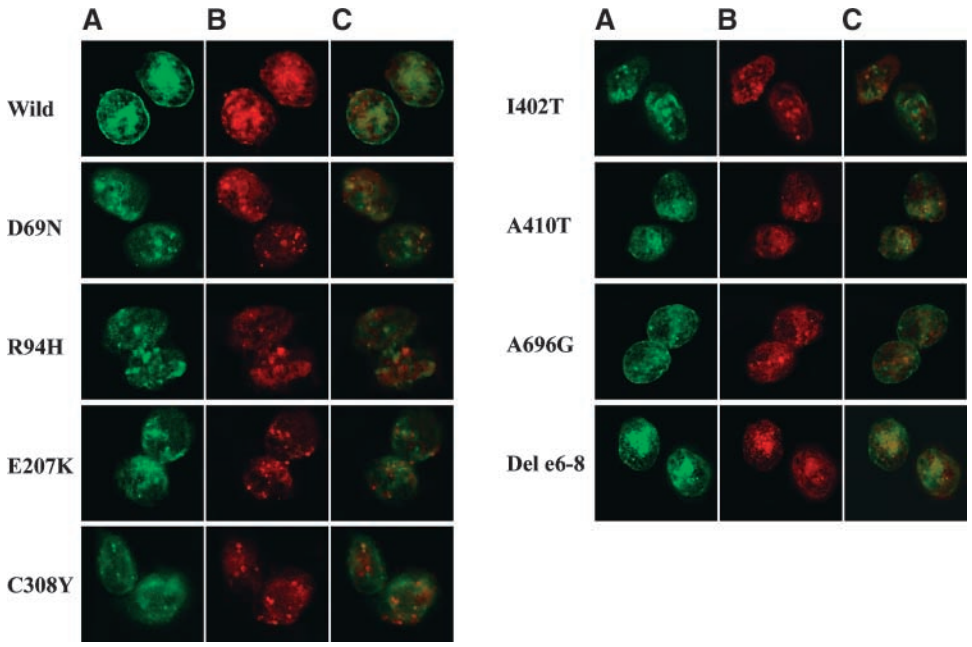

Fig. 4. Colocalization analysis of COS-7 cells transfected with pcDNA3-LDLR plasmids. Transfected cells were analyzed by fluorescence microscopy simultaneously for LDL receptor immunofluorescence (green, A) and lysosome fluorescence (red, B). The sum of these two images is also shown C. Original magnification 600×.

W-18X allele was associated with *Sfa*NI+/*Sma*I+/*Aci*I−/*Hin*cII+/*Ava*II−/*Msp*I+/*Nco*I+/(dTA)n 112nt/D19S394 239nt. The D69N allele in A133 was associated with *Sfa*NI+/*Sma*I+/*Aci*I+/*Hin*cII+/*Ava*II−/*Msp*I+/*Nco*I−/(dTA)n 112nt/D19S394 243nt. The lack of DNA from L41 family members precluded unambiguous resolution of this haplotype. When an unequivocal haplotype was deduced, one of the two alleles could be the same haplotype. The same haplotype of nine polymorphic sites, *Sfa*NI+/*Sma*I+/*Aci*I+/*Hin*cII+/*Ava*II−/*Msp*I+/*Nco*I−/(dTA)n 112nt/D19S394 255nt was noted with the two C308Y alleles in D63. When deducing an unequivocal haplotype, the C308Y allele in 95-035 could not be this haplotype. The two Del e6-8 alleles in D254 also shared the same haplotype: *Sfa*NI+/*Aci*I−/*Hin*cII+/*Ava*II−/*Msp*I+/*Nco*I+/(dTA)n 106nt/D19S394 263nt.

TABLE 4. Lipid characteristics of patients with LDL receptor mutation

Patient	Mutation	Sex	Age	Cholesterol	Triglycerides	Clinical Findings[a]
				mmol/l		
D102	W-18X	M	62	8.17	1.15	CAD
A133	D69N	M	65	8.04	1.47	CAD
L41	D69N	M	70	8.22	1.80	CAD
A144	R94H	M	76	6.21	0.91	CAD, DM
95-123	E207K	M	38	8.32	0.75	TIA
95-035	C308Y	M	37	7.62	5.73	DM
D63	C308Y/C308Y	F	12	16.61	0.80	CAD, TX
D101	I402T	F	40	6.61	0.46	
D142	A410T	M	69	10.03	1.59	
D72	Del e3-5	F	51	7.34	1.13	
D254	Del e6-8/Del e6-8	F	48	17.16	1.50	CAD, TX

[a] CAD, coronary artery disease; DM, diabetes mellitus; TIA, transient ischemic attack; TX, tendon xanthomata.

DISCUSSION

In the 170 unrelated hyperlipidemic patients and two clinically diagnosed FH homozygotes, eight point mutations and two deletions were found in a total of 12 patients. Compared with previous reports (15–18), the frequency of mutations identified in hyperlipidemic Chinese is less than expected. The discrepancy of the results may be due to the screening method, population stratification, and polygenic nature of the disease. The SSCP screening method does not detect every possible mutation. The subjects in the studies of Sun et al. (15), Mak et al. (16), Pimstone et al. (17), and Khoo et al. (18) were clinically diagnosed FH patients, whereas the subjects in our study only show elevated cholesterol values. Mutations in other genes, such as LDL receptor adaptor protein and adenosine triphosphate binding cassette transporters, may also contribute to the elevated cholesterol (23).

Although the two FH homozygotes were affected similarly to those elsewhere, the heterozygotes were much less severely affected than their counterparts in Western populations, and rarely exhibited tendon xanthomas or premature CAD. The lack of clinical presence has also been observed in Chinese FH heterozygotes in China (15, 19, 24). However, in the Chinese patients in Hong Kong, Canada, and Southeast Asia, heterozygous FH appears to manifest in a way similar to that seen in Western countries (16–18). This may be due to traditional low dietary fat consumption in Chinese in Taiwan and China. This indicates that conventional methods for diagnosis of FH, based mainly on lipid determinations and a family history of CAD, may not always allow an accurate diagnosis of the disease in all Chinese. A low-fat diet modulating the phenotype of Chi-

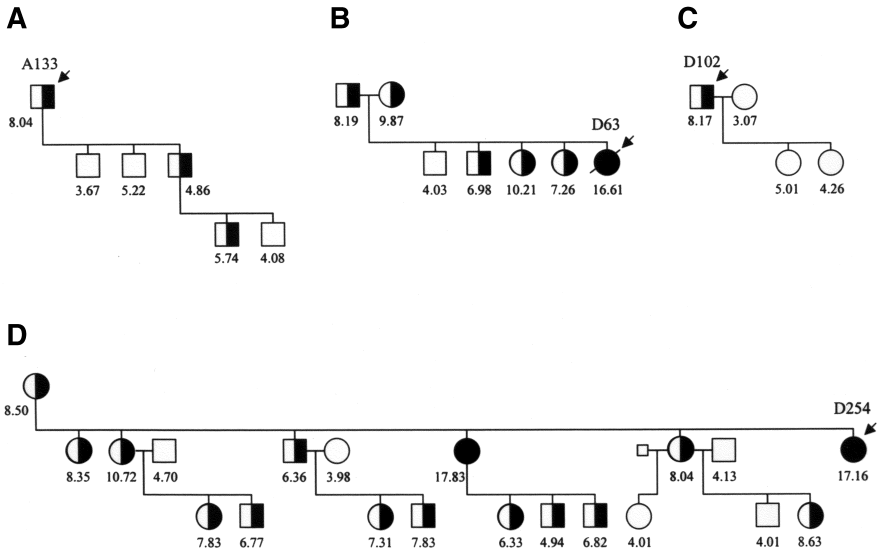

Fig. 5. Family pedigrees of familial hypercholesterolemia (FH) patients A133 (A), D63 (B), D102 (C), and D254 (D) with LDL receptor mutations. The index patient in each family is indicated with an arrow. Unaffected individuals are indicated with open symbols; those affected are shown with half-filled symbols (FH heterozygotes) or filled symbols (FH homozygotes). Symbols with a slash denote deceased individuals. The total plasma cholesterol concentration (in mmol/l) in the individual is shown below the symbol.

nese patients in Taiwan with heterozygous familial defective apoB-100 has also been reported (20, 21).

The W-18X mutation in the signal peptide predicts no LDL receptor protein synthesis (class 1). This mutation has also been seen in compound heterozygous FH patients from China (11, 15). The lack of haplotype reported precludes the prediction of the mutation origin.

The D69N mutation affects the highly conserved aspartate residue 69 in the second cysteine-rich repeats in the binding domain (3, 4). The novel intermediate and reduced mature receptor protein (Fig. 3) indicate abnormal processing and delayed transport of newly synthesized receptors to the cell surface (class 2B) similar to D69G and D69Y mutations reported earlier (11, 26). The LDL receptor was retained in the ER with 55% activity (Table 3). The mutation was found in two Chinese patients in Hong Kong and Malaysia, and previously in a British patient (16, 18, 25). Two patients (A133 and L41) originating from Fukien province of China were noted to have this mutation. Because Chinese in Hong Kong and Malaysia originated primarily from the southern Chinese population, the D69N mutation found in Chinese patients in Hong Kong, Malaysia, and Taiwan may have been inherited from a common ancestor. The results of the haplotype analysis also suggest that the D69N alleles of our two patients are likely identical by descent (Table 5). When the family members of A133 were screened for the D69N mutation, two additional carriers were found. However, their cholesterol values were relatively low (4.86 and 5.74 mmol/l, respectively; Fig. 5A) as compared with those Chinese patients in the studies of Mak et al. (16) and Khoo et al. (18) (11.0 and 8.7 mmol/l, respectively) and our index cases A133 (8.04 mmol/l) and L41 (8.22 mmol/l) (Table 4), suggesting an environmental or genetic effect on the phenotypic expression. Both the patient and carrier relatives are kept fully informed and adhere to a regular follow-up scheme.

The R94H mutation is located in the third cysteine-rich repeat in the binding domain. Although evolutionarily conserved among human, rat, rabbit, and cow (4, 27–29), arginine_{94} is not conserved among the seven cysteine-rich repeats (3). The mutation results in slower processing and delayed transport (class 2B). The mutation was also found in two Chinese patients and a Japanese patient (18, 30).

TABLE 5. Haplotype associated with W-18X, D69N, C308Y, and Del e6-8 mutations

		Haplotype/Genotype[a]								
Patient	Mutation	*Sfa*NI	*Sma*I	*Aci*I	*Hinc*II	*Ava*II	*Msp*I	*Nco*I	(dTA)n (nt)	D19S394(nt)
D102	W-18X	+	+	−	+	−	+	+	112	239
A133	D69N	+	+	+	+	−	+	−	112	243
L41	D69N	+/+	+/+	+/+	+/+	+/−	+/+	+/−	108/112	239/243
D63	C308Y	+	+	+	+	−	+	−	112	255
95-035	C308Y	+/+	+/+	−/−	+/−	+/−	+/+	+/+	106/106	243/247
D254	Del e6-8	+	del	−	+	−	+	+	106	263

[a] A plus sign denotes presence, and a minus sign denotes absence of restriction site.

No prediction of the mutation origin can be made due to the lack of haplotype reported.

The E207K mutation is located in the fifth cysteine-rich repeats in the binding domain. The mutation affects the highly conserved negatively charged Ser-Asp-Glu triplet at the COOH-terminus of all ligand binding repeats (3) and results in severely slow processing and delayed transport (class 2B). This mutation occurs at a CpG hot spot in which the G is mutable to A, C, or T (11). Such mutation has been detected in different ethnic groups (11, 12, 16, 17, 31) and might have occurred independently during evolution.

For C308Y, the cysteine residue 308 in the cysteine-rich repeat A of the epidermal growth factor (EGF) precursor homology domain is highly conserved and is important for the correct folding of the LDL receptor protein (3). The disruption of the disulfide linkage formation may lead to the reduced mature protein (class 2B) (Fig. 3), similar to other transport defect mutations nearby, C297Y (11) and C317S (13). The LDL receptor activity was reduced to 31% (Table 3). Previously, the C308Y mutation was found in two Chinese patients in Hong Kong (16) and two Chinese patients in Malaysia (18). In patient D63, the same haplotype of two C308Y alleles resulted from consanguineous marriage. This haplotype was the same as that reported [*Sfa*NI+/*Ava*II−/*Nco*I−/(dTA)n 10 repeats] in two Chinese patients in Hong Kong (32), but could not be deduced from 95-035 (Table 5). The data suggest limited multiple recurrent origins for C308Y in Chinese populations.

Mutations I402T and A410T were between cysteine-rich repeats B and C of the EGF precursor homology domain. The domain serves to position the ligand-binding domain to bind LDL on the cell surface. It is also required for the acid-dependent dissociation of lipoproteins from the receptor in the endosome during receptor recycling. The amount of receptor protein produced by both mutations was reduced (Fig. 3). I402T was reported as pathogenic in mediating uptake and degradation of LDL (33). The LDL receptor was retained in the ER with 54% activity (class 2B) (Table 3). A410T may be associated with a recycling-deficient phenotype (34). The mutant receptor retained 20% activity (Table 3) and gave rise to an endosomal/lysosomal staining pattern (class 5) (Fig. 4). Both I402T and A410T mutations have been reported previously in different ethnic groups (18, 34, 35). Due to the geographical distance and historic ethnic differences, it is probable that the two mutations have arisen independently in Chinese.

The A696G in the *O*-linked sugars domain was novel. The point change was not detected in our normolipidemic controls. A696G is a conservative amino acid substitution, and alanine at 696 is not conserved across species among human, rat, rabbit, and cow (4, 27–29). Although patient D162 had an elevated cholesterol value (6.77 mmol/l) in addition to hypertension, A696G is a rare sequence variation that does not affect LDL receptor function (Fig. 3, Table 3).

Two amino acid sequence variants in the EGF precursor homology domain were noted. The A370T variant (*Stu*I RFLP) was first reported in the South African Afrikaner population, where the frequency was found to be 8% (36). Among several Chinese groups studied, the A370T was only found once in our patients (0.3% allele frequency). The novel I602V variant (*Eco*RV RFLP) is a conservative amino acid substitution. Isoleucine$_{602}$ is a valine at this position of rat, rabbit, and cow LDL receptor proteins (27–29). The I602V variant was found with a frequency of 1% in both normal and hyperlipidemic Chinese.

Two novel deletions involving *Alu* sequences (37) were identified and characterized. Del e3-5 is caused by misalignment of *Alu* sequences in intron 2 and intron 5 and predicted to cause deletion of 209 aa (44–252), including cysteine-rich repeats II to VI in the binding domain (3). The resultant defect receptor protein cannot to be detected and is unable to bind with the LDL (class 2A) (Fig. 3, Table 3). A similar misalignment of *Alu* sequences in introns 5 and 8 is responsible for Del e6-8. The mutation is predicted to cause deletion of 123 aa (252–374), including the seventh disulfide-rich repeat in the binding domain and cysteine-rich repeats A and B in the EGF precursor homology domain (3). The resultant defect receptor retained 12% of its activity and was localized in the endosomal/lysosomal regions (class 5) (Table 3, Fig. 4). The same haplotype of two Del e6-8 alleles in D254 resulted from a known consanguineous marriage. The LDL receptor gene contains many highly redundant *Alu* sequences in its introns and flanking sequences, as well as in the 3′ noncoding region (4). A number of characterized deletions or insertions at the LDL receptor gene involve *Alu* sequences (5). Our results further support the hypothesis that *Alu* sequences are hot spots for recombinational events at the LDL receptor locus.

In summary, among more than 30 mutations noted in the Chinese, recurrent point mutations in unrelated patients were identified (11, 15–18, 32, and the present study). Although the incidence rate is too low to implicate common mutations due to a founder effect, the rapid RFLP detection methods may provide a practical direction and approach for further studies of hyperlipidemic Chinese patients. In FH, a complex genetic disease, to clearly define individual gene defects in patients is of prognostic value, making early detection, optimal treatment approaches, and a reduction in the risk factors associated with CAD highly possible.

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