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Expression of Mutations in the Low-density Lipoprotein Receptor Gene Associated with Familial Hypercholesterolemia in Taiwan

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism, primarily caused by mutation in the low-density lipoprotein (LDL) receptor gene. Previously five point mutations (D69N, C308Y, I402T, A410T, and A696G) in the LDL receptor gene were identified in Taiwanese patients with FH. The preliminary characterization of these mutations is reported here. The wild type LDL receptor cDNA was subcloned into eukaryotic expression vector and cDNAs containing the five mutations were constructed. In transfected COS-7 cells, the wild type cDNA expressed 160 kDa mature and 120 kDa precursor proteins. An apparent reduction in LDL receptor mRNA level and a novel intermediate protein were seen in D69N transfected cells. Although normal amount of LDL receptor mRNA was seen with the C308Y, I402T and A410T mutations, the amount of mature protein versus precursor protein was less than in the wild type. The LDL receptor mRNA and protein levels were close to those of wild type in A696G transfected cells. Molecular analysis of the LDL receptor gene can define the cause of patient's hyperlipidemia clearly and allow appropriate early treatment as well as antenatal and family studies.

Key words: LDL receptor mutations, cDNA expression

Introduction

The low-density lipoprotein (LDL) receptor is a cell surface glycoprotein that regulates plasma cholesterol by mediating endocytosis of LDL, the major cholesterol transport protein in human plasma (Brown and Goldstein, 1986). Mutations in the LDL receptor gene cause familial hypercholesterolemia (FH), a common autosomal dominant disease that affects about 1 in 500 in most populations (Goldstein *et al.*, 1995). 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 heart disease (CHD). The rare FH homozygotes occur in about 1 in 1 million and have a more severe disease than heterozygotes.

The human LDL receptor gene consists of 18 exons spanning 45 kb on chromosome 19p13 (Südhof *et al.*, 1985). The 5.3 kb mRNA, half of which constituting a long 3' untranslated region, encodes a protein of 860 amino acids. The N terminal 21 hydrophobic amino acids encoded by exon 1 comprise the signal sequence, which is cleaved from the protein during translocation into the endoplasmic reticulum (ER). The resultant 839-amino acid protein contains ligand binding domain (exons 2~6), epidermal growth factor (EGF) precursor homology domain (exons 7~14), O-linked sugars domain (exon 15), membrane spanning domain (exons 16 and 17), and cytoplasmic domain (exons 17 and 18) (Figure 1; Yamamoto *et al.*, 1984). The 120 kDa precursor receptor contains 1~2 N-linked and 9~18 O-linked carbohydrate chains, which undergoes several carbohydrate processing reactions in the Golgi apparatus en route to the cell surface, resulting in a 160 kDa mature receptor (Cummings *et al.*, 1983). Overall, more than 700 mutations including gross deletions, minor deletions, insertions, point mutations, and splice-site mutations scattered over the LDL receptor gene have been reported (Heath *et al.*, 2001). These mutations affect the synthesis, post-translational processing, ligand binding activity, internalization, or recycling of the LDL receptor.

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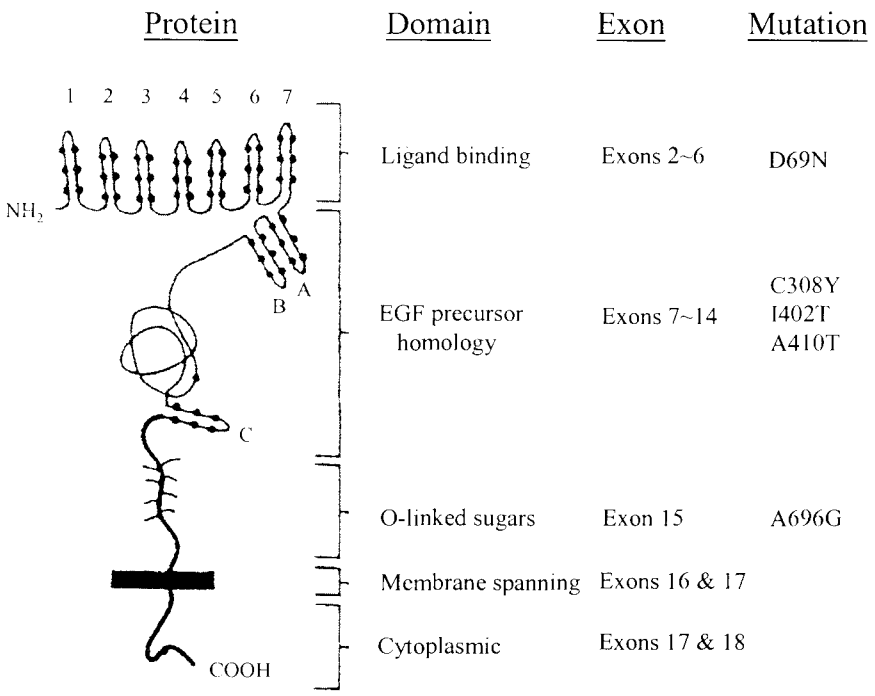

Figure 1. Domain structure of the human LDL receptor protein (left) as well as its relation to the exon organization and mutation of the LDL receptor gene (right). Exons 2~6 encode the ligand-binding domain, which is made up of seven ~40-amino acid repeats, each containing six cysteine residues (black circles) that form three intrarepeat disulfide bonds. Exons 7~14 encode the EGF precursor homology domain, which includes cysteine-rich growth factor repeats A, B and C. Exon 15 encodes O-linked sugar domain that is enriched in serine and threonine for O-linked carbohydrate chains. Exon 16 and 5'-end of exon 17 encode hydrophobic amino acids comprising the membrane-spanning domain. The remainder of exon 17 and exon 18 encode amino acids that make up the cytoplasmic domain.

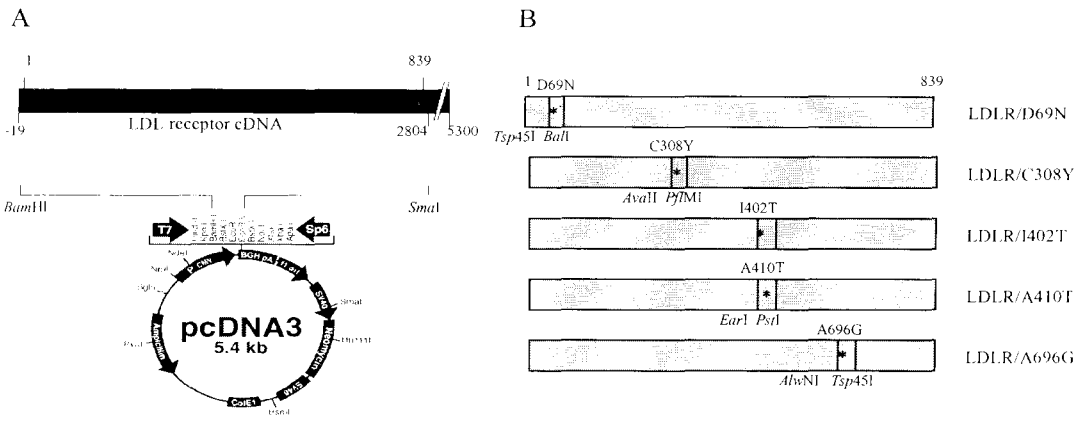

Figure 2. (A) Structure of pcDNA3-LDLR. The 2.8 kb LDL receptor cDNA (nucleotides -19 to 2804, where +1 represent the first nucleotide to be translated) was joined to 5.4 kb pcDNA3 as described in Materials and Methods. Amino acid 1 and 839 above the LDL receptor cDNA represent the amino and carboxyl termini of the mature receptor, respectively. The solid areas in pcDNA3 denote regions that contain cytomegalovirus enhancer-promoter (P CMV), bovine growth hormone polyadenylation signal (BGH pA), SV40 origin for episomal replication (SV40 ori), neomycin and ampicillin resistant genes for selection in *E. coli* or COS-7 cells. (B) Construction of mutated LDL receptor cDNA plasmids. cDNA is represented by a gray box. Location of the mutation is indicated by an asterisk (*). The restriction enzymes used to generate mutated fragment for replacement are indicated below the cDNA.

To date, over 30 mutations in the LDL receptor gene are known in the Chinese FH patients in China, Hong Kong, Canada, and Southeast Asia (Sun *et al.*, 1994; Mak *et al.*, 1998; Pimstone *et al.*, 1998; Khoo *et al.*, 2000). Previously, five missense mutations (D69N, C308Y, I402T, A410T, and A696G) were found during screening of the LDL receptor gene mutations in hyperlipidemic Taiwanese (Huang, 2001). In this study, the effect of these mutations on LDL receptor processing and stability was examined by transient expression in COS-7 cells and western blot analysis.

Materials and Methods

cDNA constructs

Plasmid pLDLR3 (ATCC No. 57004) contains the full length 5.3 kb LDL receptor cDNA. The 2.8 kb *Bam*HI-*Sma*I fragment containing the LDL receptor coding region (cDNA -19~2804) was excised from pLDLR3 and subcloned into *Bam*HI and *Eco*RV sites of pcDNA3 (Invitrogen corporation) to produce plasmid pcDNA3-LDLR (Figure 2A). Construction of mutated cDNA plasmids is shown in Figure 2B. Plasmid pcDNA3-LDLR/D69N was derived from pcDNA3-LDLR by replacing a 78-bp *Tsp*45I-*Bal*I fragment (cDNA 196~273) with a corresponding fragment containing D69N mutation. The fragments used to construct pcDNA3-LDLR/C308Y, pcDNA3-LDLR/I402T, pcDNA3-LDLR/A410T, and pcDNA3-LDLR/A696G are 98-bp *Ava*II-*Pfl*MI fragment (cDNA 942~1039) containing C308Y mutation, 158-bp *Ear*I-*Pst*I fragment (cDNA 1201~1358) containing I402T or A410T mutation, and 150-bp *Alw*NI-*Tsp*45I fragment (cDNA 2144~2293) containing A696G mutation, respectively. The replaced sequences were confirmed by restriction enzyme digestion and DNA sequencing.

Transfection and western blot analysis

Plasmid pcDNA3, pcDNA3-LDLR or mutated cDNA plasmids were transfected into COS-7 cells by lipofection procedure (Hawley-Nelson *et al.*, 1993). Forty-eight hr after transfection, cells were harvested and extracts prepared by freeze-thawing. Concentrations of proteins were determined using Protein Assay Dye Reagent (Bio-Rad). Proteins (10 μg) from transfected COS-7 cells were electrophoresed in 6% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane by X Cell II™ electroblotting apparatus (Novex) at 40 V for 40 min. After overnight blocking at 4℃, the membrane was stained with anti-LDL receptor polyclonal antibody (1:400 dilution, Progen) for 1.5 h at 25℃. After washing, the membrane was incubated for 1.5 h at 25℃ with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. The membrane was washed three times and subjected to colorimetric detection with NBT and X-phosphate.

RNA isolation and northern blot analysis

Total RNA was isolated from transfecied cells using RNAzol™ B (Tel-Test). The RNA (10 μg) was separated on a 1.0% agarose gel containing 2 M formaldehyde (80 V, 1.5 h) and transferred onto a Biotrans membrane (ICN). The 2.8-kb *Hin*dIII-*Xba*I fragment containing LDL receptor cDNA and the 0.9 kb *Bst*BI-*Sma*I fragment containing neomycin gene were excised from pcDNA3-LDLR, labeled with DIG-dUTP by random priming (BM biochemical) and used as probes. The membrane was prehybridized and hybridized at 42℃, washed twice at 62℃ for 15 min, and developed as described above. The membrane was quantitated by phosphorimaging (ImageMaster VDS, Pharmacia).

Results

Construction and expression of cDNA plasmids

Figure 2 outlines the strategy used to obtain wild type and mutated LDL receptor cDNA plasmids. In pcDNA3-LDLR, the entire coding region of LDL receptor is under the control of CMV promoter. Upon transfection into COS-7 cells, the recombinant plasmid reproduces via SV40 origin and transiently expresses a high level of LDL receptor protein. The mutated receptor in pcDNA3 was constructed by fragment replacement. The mutations examined were five missense mutations (D69N, C308Y, I402T, A410T, and A696G) found in Taiwanese FH patients (Huang, 2001).

Western analysis of LDL receptor variants

The LDL receptor protein derived from each variant was examined by immunostaining of

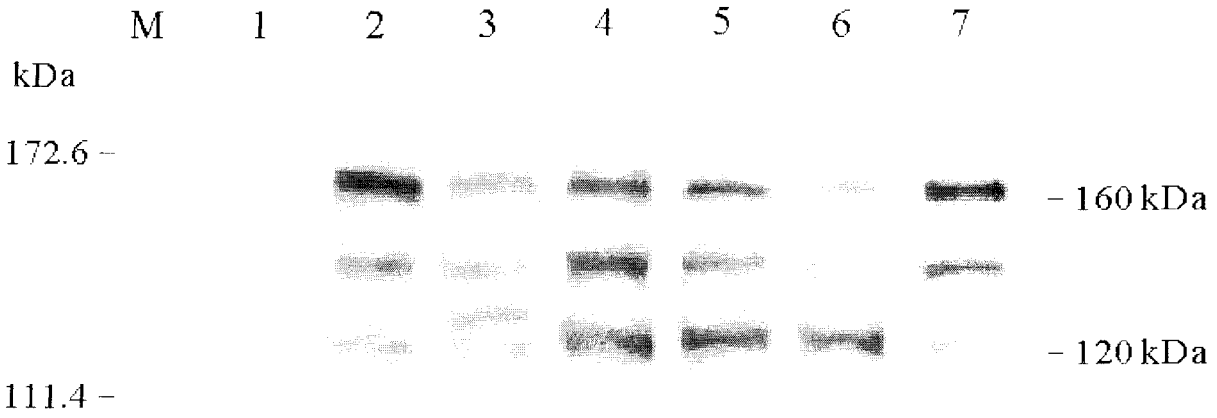

Figure 3. Western analysis of LDL receptor cDNA variants. Total protein (10 μg) was separated on 6% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with LDL receptor polyclonal antibody. Molecular mass of LDL receptor protein is indicated as determined from a plot of migration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

western blot with LDL receptor polyclonal antibody. No nonspecific polypeptide was detected in pcDNA3-transfected cells (Figure 3, lane 1). In addition, 160 kDa mature and 120 kDa precursor proteins as well as an intermediate form were detected in wild type pcDNA3-LDLR-transfected COS-7 cells (Figure 3, lane 2). An apparent reduction in LDL receptor protein level and a novel intermediate protein were seen in D69N transfected cells (Figure 3, lane 3). For C308Y, I402T and A410T mutations, the amount of mature protein versus precursor protein detected was less than that of wild type (Figure 3, lanes 4-6). The mature LDL receptor protein level was close to that of wild type in A696G transfected cells (Figure 3, lane 7).

Northern analysis of LDL receptor variants

The level of LDL receptor mRNA derived from each variant was quantitated by northern blot hybridization. The expressed neomycin mRNA level in transfected cells was used as an internal control. While C308Y, I402T, A410T, and A696G mutations did not cause a significant change in steady-state LDL receptor mRNA level (Figure 4, lanes 4-7), D69N mutation reduced the level of LDL receptor mRNA to 40% of wild type (Figure 4, lane 3).

Discussion

The previously identified LDL receptor mutations in hyperlipidemic Taiwanese were expressed in this study. The D69N mutation was a G→A substitution at nucleotide 268, which affects the highly conserved aspartate residue 69 in the second cysteine-rich repeat in the binding domain (Figure 1; Yamamoto *et al.*, 1984; Südhof *et al.*, 1985). The mutation was found in Chinese patients in Hong Kong (Mak *et al.*, 1998), Southeast Asia (Khoo *et al.*, 2000), and Taiwan (Huang, 2001). Upon transfecting D69N mutated cDNA into COS-7 cells, a decrease in LDL receptor mRNA level (Figure 4, lane 3) was observed. This could be attributable to an increased rate of mRNA decay. Whether or how this single nucleotide change may control the stability of LDL receptor mRNA remains unclear. In addition, the observed novel intermediate and reduced 160 kDa mature receptor protein (Figure 3, lane 3) indicate abnormal processing and delayed transport of newly synthesized LDL receptors from the ER to the plasma membrane. In addition to the D69N mutation, D69G and D69Y mutations also produced the phenotype of slow transport to the cell surface (Hobbs *et al.*, 1992; Rubinsztein *et al.*, 1993).

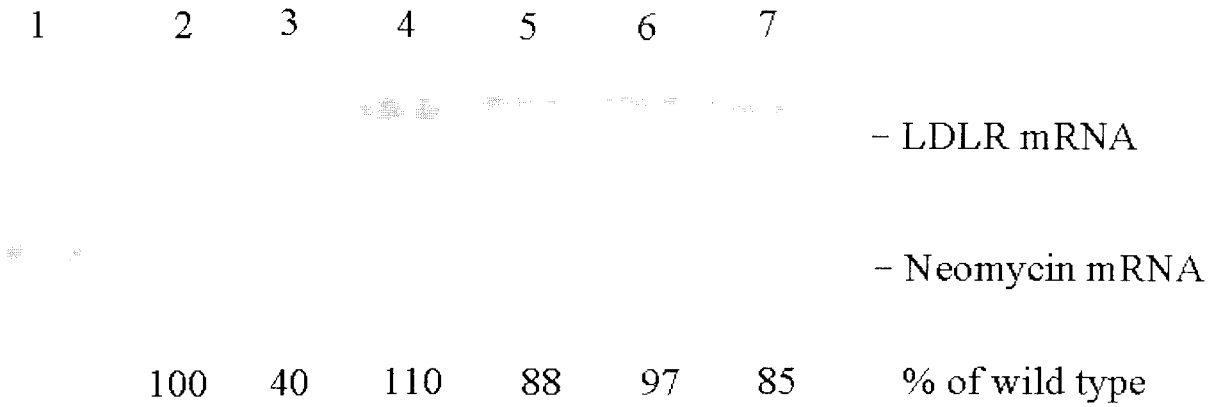

Figure 4. Northern analysis of LDL receptor cDNA variants. Total RNA (10 μg) was separated on 1% formaldehyde-agarose gel, transferred onto nylon membrane, and hybridized to LDL receptor probe (upper panel) or neomycin probe (lower panel). Lane 1, pcDNA3; lane 2, pcDNA3-LDLR; lane 3, pcDNA3-LDLR/D69N; lane 4, pcDNA3-LDLR/C308Y; lane 5, pcDNA3-LDLR/I402T; lane 6, pcDNA3-LDLR/A410T; lane 7, pcDNA3-LDLR/A696G.

For C308Y, the cysteine residue 308 in the cysteine-rich repeat A of the EGF precursor homology domain (Figure 1) is highly conserved and is important for the correct folding of the LDL receptor protein (Südhof *et al.*, 1985). In transfected cells, the amount of mature protein versus precursor protein produced by C308Y mutation was less than that of wild type (Figure 3, lane 4). The reduced mature receptor may result from disruption of the disulfide linkage formation, leading to production of an unstable protein, similar to other transport defect mutations nearby, C297Y (Hobbs *et al.*, 1992) and C317S (Maruyama *et al.*, 1995).

Mutations I402T and A410T were between cysteine-rich repeats B and C of the EGF precursor homology domain (Figure 1; Südhof *et al.*, 1985). 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 mature receptor protein versus precursor protein produced by both mutations was less than that of wild type (Figure 3, lanes 5 and 6). The I402T is reported pathogenic; cells expressing I402T mutation were seriously affected in mediating uptake and degradation of LDL (Ekström *et al.*, 2000). The A410T may be associated with recycling-deficient phenotype; the 160 kDa A410T receptor reached cell surface (though more slowly than normal), but was rapidly degraded (Hobbs *et al.*, 1990).

The A696G mutation in the O-linked sugars domain (Figure 1) was novel. The LDL receptor protein level was close to that of wild type in A696G transfected cells. The mutation is a conservative amino acid substitution, but alanine at 696 is not conserved across species among human, rat, rabbit, and cow (Russell *et al.*, 1984; Yamamoto *et al.*, 1984, 1986; Lee *et al.*, 1989). Therefore, A696G may be a rare sequence variation which does not affect LDL receptor protein function.

In summary, the effect of mutation on LDL receptor activity was examined by transient expression and western analysis. In FH, a complex genetic disease, to clearly define individual gene defects in patients is of prognostic value, making it possible to optimize treatment and prevent the risk of CHD.

Acknowledgments

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臺灣家族性高膽固醇血症患者低密度脂蛋白突變受體的表現

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摘　要

家族性高膽固醇血症(FH)爲低密度脂蛋白(LDL)受體突變導致的體染色體顯性遺傳疾病。先前於臺灣 FH 患者中發現五種 LDL 受體基因的點突變(D69N、C308Y、I402T、A410T、A696G)，本研究即對這些突變做初步記述。野生型的 LDL 受體 cDNA 被選殖入眞核表現載體，含上述突變的 cDNA 重組質體亦被構築。在轉移的 COS-7 細胞中，野生型的 cDNA 表現 160 kDa 成熟蛋白及少量的 120 kDa 前驅蛋白。含 D69N 突變的重組質體表現的 LDL 受體 mRNA 量明顯的降低，且出現異常的中間型前驅蛋白。含 C308Y、I402T 及 A410T 突變的重組質體所表現的 mRNA 量與野生型者相近，但所表現的成熟蛋白與前驅蛋白的比值均較野生型者少。含 A696G 的 mRNA 及成熟蛋白則均與野生型者相近。此 LDL 受體基因的分生研究可明確的定義患者的高血脂原因及進行出生前診斷、家族分析等。

關鍵詞：低密度脂蛋白受體突變，cDNA 表現

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