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SHORT COMMUNICATION

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Eight novel mutations and functional impairments of the LDL receptor in familial hypercholesterolemia in the north of Japan

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Abstract In the course of investigations of familial coronary artery disease in Hokkaido, the northland of Japan, we identified 13 families affected by familial hypercholesterolemia. Among them, we identified eight novel mutations of the low-density lipoprotein (*LDL*) receptor gene, four of which caused frameshifts: (1) a 7-bp deletion at nucleotide (nt) 578–584 (codon 172–174, exon 4); (2) a 14-bp insertion at 682 nt (codon 207–208, exon 4); (3) a 49-bp deletion at nt 943–991 (codon 294–310, exon 7); and (4) a one-base insertion of C to a stretch of C_3 at nucleotides 1687–1689 or codon 542. The others included (5) a T-to-C transition at nt 1072 causing substitution of Cys for Arg at codon 337 (C337R, exon 8); (6) a splice-site G-to-T substitution in intron 11; (7) a splice-site G-to-C substitution in intron 11; and (8) a G-to-T transition at nt 1731 causing substitution of Trp for Cys at codon 556 (W556C, exon 12). To disclose the functional consequences of novel mutations, we characterized each of these mutations by two assays in peripheral lymphocytes, i.e., uptake of fluorescently labeled LDL by LDL receptors, and measurement of cell surface-bound LDL receptor protein using specific monoclonal antibody against LDL receptor.

Key words LDL receptor · Familial hypercholesterolemia · Flow cytometry · Receptor uptake · Lipoproteins.

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Introduction

Heterozygous familial hypercholesterolemia (FH) results from inheritance of a single defective copy of a low-density lipoprotein receptor (*LDLR*) gene (Goldstein and Brown 1989). The disease is recognized clinically by striking elevations of LDL cholesterol in serum. Affected families display bimodally distributed LDL cholesterol levels consistent with an autosomal dominant trait. Typically, a family history of early coronary disease is evident. The clinical triad of FH is type IIa hypercholesterolemia, i.e., elevated LDL cholesterol in serum, xanthomas, and premature atherosclerosis. The *LDLR* gene is large and complex in structure; it spans 45 kb and consists of 18 exons and 17 introns. In addition, no mutational "hot-spot" has been identified, and mutations rarely recur among Caucasian families or in Japanese families residing in mainland Japan.

Functional activity of the LDL receptor traditionally has been measured using fibroblasts obtained from patients by invasive skin biopsy (Brown and Goldstein 1989). We and others recently developed a rapid-flow cytometric assay for LDLR activity in peripheral blood lymphocytes (Schmitz et al. 1993; Ranganathan et al. 1995). One procedure measures LDL uptake by the LDL receptor, and involves upregulation of LDL receptor in cultured cells, uptake of a fluorescently labeled LDL (DiI-LDL), and determination of the fluorescent signal by flow cytometry. The other approach measures cell surface-bound LDL receptor using a specific monoclonal antibody against LDL receptor, IgG-C7, which recognizes the N terminus of the LDL receptor protein. The LDL receptors are upregulated when lymphocytes are incubated in lipoprotein-free medium, and the amount of LDL receptor protein on cell surfaces is determined by its binding to IgG-C7.

In the present study, we identified eight novel mutations of the *LDLR* gene among families with FH, described clinical features of each pedigree, and studied the functional consequence of each mutation on the respective LDL receptors by analyzing peripheral lymphocytes using labeled LDL and flow cytometry.

Patients and methods

Patients and measurement of lipoproteins

All 13 families ascertained to have member(s) with hyperlipidemia were referred to one of us (M.T.) and followed clinically at the outpatient clinic of Hokkaido Hospital for Social Health Insurance. Blood samples collected after 12–16 h of fasting were prepared from essentially every family member. Lipid and lipoprotein concentrations were measured by procedures described previously (Williams et al. 1986); i.e., plasma cholesterol and triglyceride concentrations were assayed enzymatically, and concentrations of high-density lipoprotein (HDL) cholesterol were determined by the $MgCl_2$-dextran precipitation method. LDL cholesterol (LDL-c) was calculated by subtracting the HDL cholesterol (HDL-c) level from the LDL-c + HDL-c fraction separated by ultrasentrifugation in a Beckmann TL-100 ultracentrifuge as described elsewhere (Wu et al. 2000). Screening for the defective apoB3500 mutation was carried out according to the procedures previously described (Myant 1993). No apoB3500 mutation was observed here.

LDL receptor uptake assay by fluorescent-activated cell sorter (FACS) flow cytometry

Binding and uptake of a fluorescently labeled LDL (DiI-LDL; Molecular Probes, Eugene, OR, USA) by LDL receptors was measured in peripheral lymphocytes whose LDL receptors had been upregulated in primary culture. DiI-LDL taken up by cells was determined with a FACS flow cytometer. In brief, lymphocytes from patients and healthy controls were separated by centrifugation over Separate L (Muto Chemical, Osaka, Japan) and then suspended in RPMI1640 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 2 mM glutamate, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% fatty acid-poor bovine serum albumin (Sigma, St. Louis, MO, USA) at densities of approximately 1×10^6 cells/ml (Ranganathan et al. 1995). Lymphocytes were incubated for 72 h in a humidified chamber with 5% CO_2 to upregulate LDL receptors. Cultured cells were washed, then incubated with 1 μg (10 μg/ml final concentration) of DiI-LDL at 37°C for 2 h. Appropriate controls with cells only or with cells incubated with DiI-LDL and 2 mM ethyleneglycoltetraacetic acid (EGTA), were included to distinguish receptor-mediated uptake from nonspecific binding and uptake. After incubation, cells were washed and their fluorescent intensities were measured in a FACScan flow cytometer (Becton Dickinson, Mansfield, MA, USA). All results were expressed as mean intensity of fluorescence (MIF) after subtracting the background values (MIF typically less than 50) obtained in the presence of 2 mM EGTA. LDL receptor activities were represented as a percentage of the average MIF in healthy controls.

Measurement of cell surface-bound LDL receptor protein by FACS flow cytometry

We measured the amount of LDL receptor protein on the surfaces of peripheral lymphocytes whose LDL receptors had been upregulated in primary culture, using a specific monoclonal antibody against LDLR, IgG-C7. Cell surface-bound IgG-C7 was measured by flow cytometry according to procedures described elsewhere (Schmitz et al. 1993). In brief, lymphocytes with upregulated LDL receptors were incubated with 1 μg/ml IgG-C7 (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 4°C for 30 min. After washing, cells were incubated with biotinylated goat antimouse IgG (TAGO, Burlingame, CA, USA) at 4°C for 30 min and then incubated with PE-labeled streptoavidin (Molecular Probes) at 4°C for 30 min. The fluorescent intensity of bound IgGs in cells was determined by a FACSscan flow cytometer as described earlier. Appropriate controls, with cells only or with cells incubated with murine IgGs, were included to distinguish receptor-mediated uptake from nonspecific binding of immunoglobulins. Again, all results were expressed as MIF after subtracting the background values obtained with murine IgGs. The quantity of LDL receptor protein was represented as a percentage of the average MIF in healthy controls.

Polymerase chain reaction (PCR)

Genomic DNA was extracted from lymphocytes of FH patients (Ikegawa et al. 1999). The promoter region and each exon of the LDL receptor gene were individually amplified by PCR using specific oligonucleotides that encode intronic sequences flanking each of the 18 exons. The sequences of the primers were based on those described by Hobbs et al. (1992), except that we used 3′ antisense primers with 40-bp GC clamps for denaturing gradient gel electrophoresis (DGGE) analysis. Each segment was amplified according to procedures described previously (Tsukamoto et al. 1998).

Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out as described previously (Hattori et al. 1999). GC-clamped amplified DNA fragments were electrophoresed on 9% polyacrylamide gels containing a linear gradient of denaturant. Denaturing gradients were prepared from two stock solutions of 9% acrylamide: one without denaturant (0%) and one with 80% denaturant (100% denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide). The gradient for each exon was examined by perpendicular DGGE. Electrophoresis was performed at 150 V for 16 h at constant 60°C in TAE buffer (40 mM Tris at pH 7.4, 20 mM sodium acetate, 1 mM ethylene diamine tetraacetic acid). After electrophoresis, the gels were stained with ethydium bromide and examined under UV illumination.

Single-strand conformation polymorphism (SSCP) analysis

PCR-SSCP analyses were carried out according to procedures described elsewhere (Hirayama et al. 1998). Briefly, each PCR product was mixed with loading buffer, heated rapidly, cooled on ice, and applied to a 5% polyacrylamide gel containing 5% glycerol in 0.5X TBE buffer. Electrophoresis was performed under two different conditions: 150V for 16h at room temperature, and 240V for 16h at 4°C.

DNA sequencing

Each variant PCR product was sequenced according to methods previously described to identify the nature of the mutation, and confirmed by direct sequencing on both strands of the given fragment (Hopkins et al. 1999).

Results

Assay of LDL receptor uptake

Uptake of DiI-LDL by LDL receptors in cultured lymphocytes from probands was determined by FACS flow cytometry, as described by Ranganathan et al. (1995). Fresh lymphocytes do not bind or take up DiI-LDL because they do not express LDL receptor, whereas after culture in lipoprotein-deficient medium for 72h, LDLR is upregulated; in our experiments, uptake of DiI-LDL reached a plateau after 2h of incubation.

Figure 1a illustrates specific uptake of Dil-LDL into lymphocytes. Excess native LDL, 2mM EGTA, or 0.2mg heparin in the medium blocked the binding of Dil-LDL, but an excess of acetylated LDL or murine IgG did not. DiI-LDL uptake by lymphocytes was positively related to the results of LDL-dependent lymphocyte differentiation after stimulation with phytohemagglutinin, as described by Cuthbert et al. (1986; data not shown). A typical pattern of fluorescent intensity in cells that took up DiI-LDL is depicted in Fig. 2b.

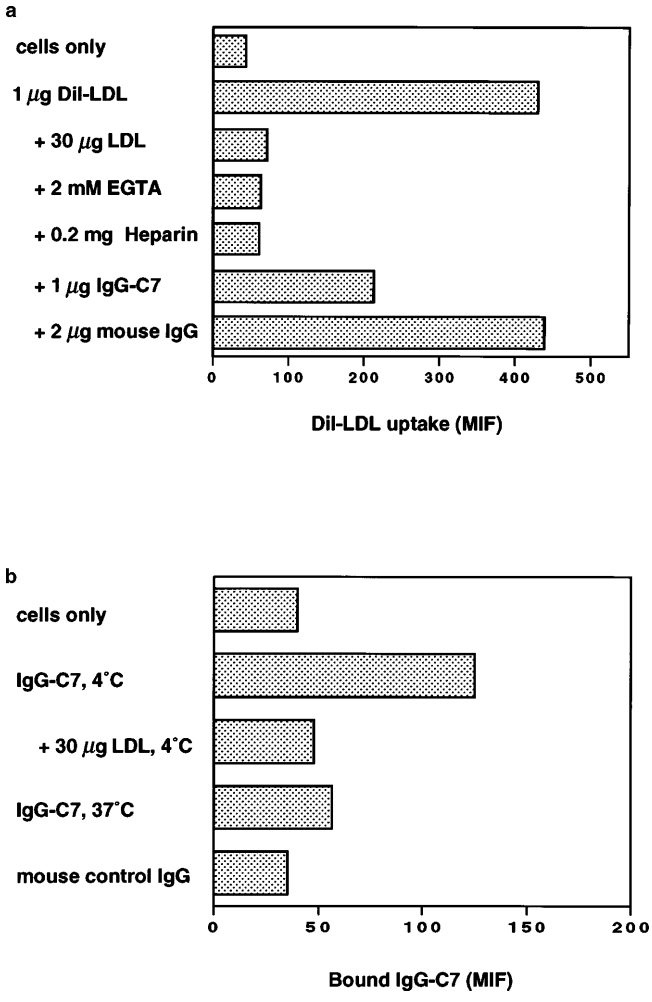

Fig. 1.a, b Specificity of the technique for measuring low-density lipoprotein (*LDL*) receptor protein and uptake (or binding) by fluorescent-activated cell sorter (FACS) flow cytometry. Peripheral lymphocytes were cultured in lipoprotein-deficient medium for 72h. **a** Cultured lymphocytes were incubated with 1μg of fluorescently labeled LDL (*DiI-LDL*) in the absence or presence of excess LDL, acetylated LDL, 2mM ethylene glycoltetraacetic acid (*EGTA*), 0.2mg heparin, or IgG-C7. **b** Lymphocytes were incubated at 4°C or 37°C with 1μg/ml IgG-C7 in the absence or presence of excess LDL

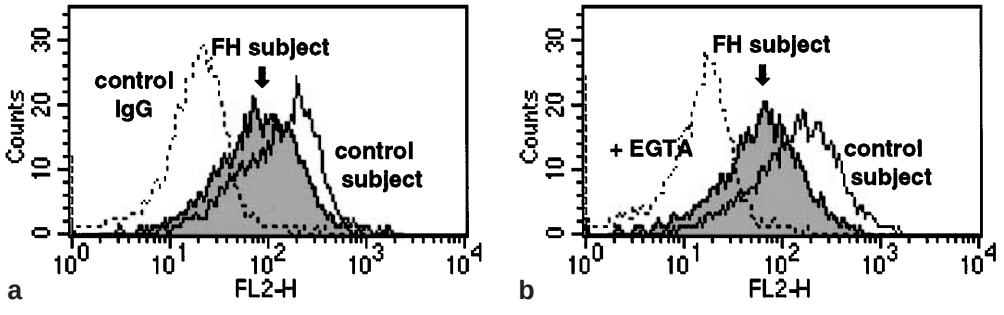

Fig. 2.a, b Actual LDL receptor protein and uptake (or binding) determined by FACS flow cytometry. LDL receptor protein in lymphocytes was detected with a specific monoclonal antibody against LDL receptor, IgG-C7 (**a**) and LDL receptor uptake (or binding) was measured by uptake of DiI-LDL (**b**). Cell-specific mean fluorescent intensities (*MIF*) in controls (*solid lines*) were calculated by subtraction of MIF of nonspecific binding of control IgG (*dotted line*) for the LDL receptor protein assay, or MIF in the presence of 2mM EGTA (*dotted line*) for LDL receptor uptake of DiI-LDL. Peaks representing data for an FH subject (Family D: III-1) are shown in *gray*

Table 1. Lipid profiles of familial hypercholesterolemia subjects in this study

Subjects	Age	TC (mmol/L)	TG (mmol/L)	LDL-c (mmol/L)	HDL-c (mmol/L)	LDLR Protein (%)	LDLR Uptake (%)
Family A							
II-3	63	8.69	0.91	7.37	0.80	91	100
Family B							
II-2	53	7.45	1.23	5.97	0.91	65	69
Family C							
I-1	65	6.34	0.72	4.63	1.30	75	86
II-3	31	5.46	0.42	4.11	1.16	77	77
II-5	29	6.15	0.67	3.93	1.94	44	91
Family D							
I-2	76	7.84	1.23	6.03	1.27	58	75
II-2	38	5.69	1.73	4.11	0.80	86	85
II-1	5	6.52	0.67	4.76	1.45	72	40
Family E							
II-1	64	7.34	1.46	5.69	0.98	40	49
Family F							
II-4	50	6.23	1.69	3.90	1.55	43	64
Family G							
II-4	45	8.28	0.78	6.70	1.22	58	66
III-1	23	8.82	1.28	7.27	0.96	—	—
Family H							
I-1	68	12.9	2.03	—	—	—	—
II-2	39	7.89	1.20	6.34	1.01	64	64

The average level of LDL uptake in the FH heterozygotes we examined was much lower than that in normal controls (101.0 ± 10.8%, mean ± SD) (Table 1). FH patients with null or nonsense mutations had lower levels of DiI-LDL uptake in general, although patients carrying the K790X mutation observed by Maruyama et al. (1995) displayed nearly normal uptake.

Surface-bound LDL receptor protein

LDL receptor protein on the surfaces of peripheral lymphocytes from FH subjects was determined using a specific monoclonal antibody (IgG-C7), after the LDL receptors were upregulated by incubation in lipoprotein-free medium. Fig. 1a shows that binding of IgG-C7 was blocked in the presence of excess native LDL. Incubation with IgG-C7 at 37°C also decreased the intensity of fluorescence markedly, because cell-surface LDLR bound with IgG-C7 was actively internalized into cytoplasm. Figure 2b displays a typical pattern of fluorescence in cells that bound IgG-C7. LDL receptor protein was lower than normal in FH subjects with frameshift or missense mutations (Table 1). FH subjects with the K790X mutation showed normal levels of LDL receptor protein.

Novel mutations, clinical features, and receptor dysfunction in eight FH families

Family A: 578–584del7bp in exon 4. The pedigrees of all eight families with novel mutations are shown in Fig. 3. Screening of exon 4 of the *LDLR* gene by DGGE and SSCP revealed that the proband of family A was heterozygous for a seven-base deletion of nts 578–584 in codons 172–174. This deletion would cause a frameshift, resulting in premature termination of 11 amino acids downstream. The mutation was found in one other member of the family (III-1), but individual I-1 was a suspected obligate carrier because she had died of ischemic heart disease at age 23 years. The proband (II-3) manifested corneal arcus, tuberous and Achilles tendon xanthomas on both legs (20.0/20.0 mm, rt/lt), and ischemic heart disease; he had been treated with plasma LDL apheresis. Levels of total cholesterol, total triglyceride, LDL cholesterol, and HDL cholesterol of the affected individuals belonging to each FH family we examined are listed in Table 1. This proband's total cholesterol (8.69 mmol/l) and LDL cholesterol (7.37 mmol/l) levels were markedly elevated, whereas his total triglyceride (0.91 mmol/l) and HDL cholesterol (0.80 mmol/l) levels were within normal ranges. We suspect that this pedigree carries some mechanism of LDL receptor dysfunction that could not be detected by our two functional assays, because the proband's membrane-bound LDL receptor protein and LDLR levels were normal (91% and 100% of controls, respectively).

Family B: 682Ins14bp, Exon 4. Screening of exon 4 of the *LDLR* gene revealed that the proband (II-2) was heterozygous for a 14-bp insertion (AGGACAAATCTGAC) at nt 682 between codons 207 and 208, which was a duplication of nts 668–681.This insertion would cause a frameshift, resulting in premature termination 42 amino acids downstream. The mutation was found in a brother of the proband (II-1), and individual I-2 is a suspected obligate carrier because he exhibits hypercholesterolemia. The proband manifested with corneal arcus, Achilles tendon xanthomas on both legs (20.0/21.0 mm, rt/lt), and ischemic heart disease; he had been treated with plasma LDL apheresis. In this patient, membrane-bound LDL receptor protein and

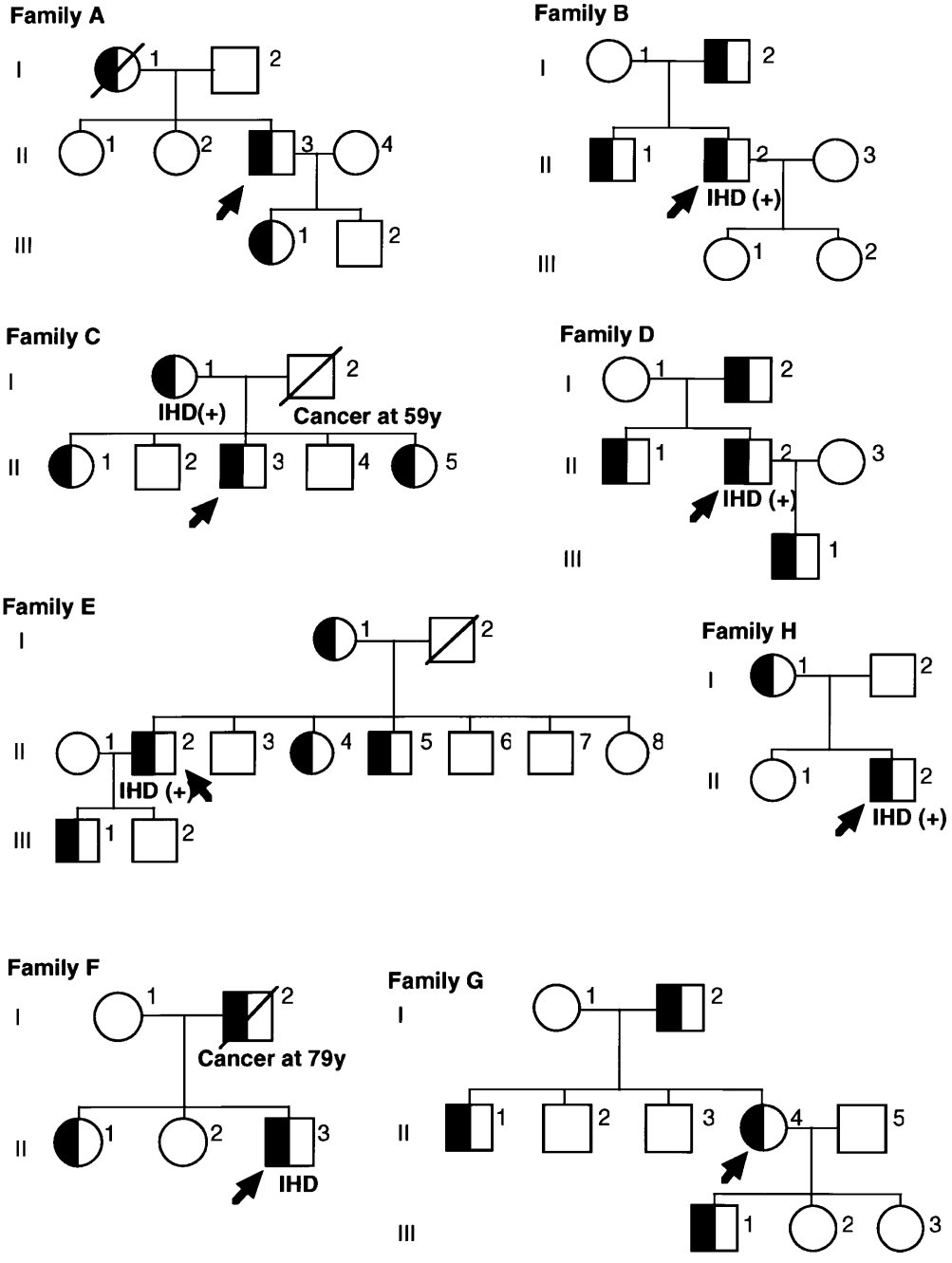

Fig. 3. Pedigrees of eight FH families. Each proband is indicated by an *arrow*. *Squares*, Males; *circles*, females; *half-solid squares and circles*, familial hypercholesterolemia; *IHD*, ischemic heart disease; AMI, acute myocardial infarction

LDL uptake were decreased to 65% and 69% of controls, respectively.

Family C: 946del49bp, exon 7. Screening of exon 7 of the *LDLR* gene in the proband revealed heterozygosity for a 49-bp deletion from nt 946 to nt 994 (codons 295–311). This deletion would cause a frameshift, resulting in premature termination 39 amino acids downstream. The proband manifested corneal arcus and Achilles tendon xanthomas (17.0/17.0 mm) on both legs. The mutation was found in two other members of the family (I-1 and II-5), whose lipoprotein profiles are shown in Table 1. The mean levels of total cholesterol, total triglyceride, LDL cholesterol, and HDL cholesterol among the affected individuals were 5.98 ± 0.46 mmol/l, 0.60 ± 0.16 mmol/l, 4.22 ± 0.36 mmol/l, and 1.46 ± 0.42 mmol/l, respectively. The mean levels of LDL receptor protein and LDL uptake in this pedigree were decreased to 65.3 ± 18.5% and 84.7 ± 7.1% of controls, respectively.

Family D: C337R, exon 8. The proband was heterozygous for a T-to-C transition at nt 1072. This transition would substitute arginine for cysteine at codon 337, abolishing a critical cysteine residue in the second Y-repeat of the EGF-precursor homology domain of the LDL receptor protein. The mutation was found in two other members of the family (Table 1). The proband manifested with Achilles tendon xanthomas (9.0/9.5 mm) on both legs and ischemic heart disease; he had been treated with plasma LDL apheresis.

The mean levels of total cholesterol, total triglyceride, LDL cholesterol, and HDL cholesterol among the carriers were 6.08 ± 1.08 mmol/l, 1.21 ± 0.53 mmol/l, 4.97 ± 0.98 mmol/l, and 1.17 ± 0.34 mmol/l, respectively. Mean levels of the LDL receptor protein and LDL uptake in this pedigree were decreased to 72% and 67% of controls, respectively. A 5-year-old boy (III-1) was diagnosed as affected on the basis of mutation status and defective receptor activities.

Family E: 1687insG, exon 11. The proband was heterozygous for a one-base (C) insertion in a stretch of three cytosines at nts 1687–1689 (codon 542). This insertion would cause a frameshift, resulting in premature termination 16 amino acids downstream. The mutation was found in four members of the family including the proband, and individual I-1 was a suspected obligate carrier as she had died of ischemic heart disease. The proband manifested with corneal arcus, Achilles tendon xanthomas (19.0/17.0 mm) on both legs, and ischemic heart disease, and had been treated with plasma LDL apheresis. His lipoprotein profile is shown in Table 1. His LDL receptor protein and LDL uptake were decreased to 40% and 49% of controls, respectively.

Family F: 1705+1G/T splicing consensus mutation, exon 11/intron 11 junction. The proband was heterozygous for a G-to-T transition at the first nucleotide of the splicing-donor site of intron 11. This insertion would cause a splicing defect, resulting in degradation of the mutant mRNA. The mutation was found in two members of the family including the proband, but individual I-2 was a suspected obligate carrier because he had exhibited hypercholesterolemia during his lifetime. The proband manifested with Achilles tendon xanthomas (16.0/16.0 mm) on both legs, and ischemic heart disease. The proband's lipoprotein profile is shown in Table 1. His LDL receptor protein and LDL uptake were decreased to 43% and 64% of controls, respectively.

Family G: 1705+1G/C splicing consensus mutation, exon 11/intron 11 junction. The proband was heterozygous for a G-to-C transition at the first nucleotide of the splicing-donor site of intron 11. As in Family F, this substitution would cause a splicing defect resulting in degradation of the mutant mRNA. The mutation was found in three members of the family including the proband; individual I-2 is a suspected obligate carrier because he has hypercholesterolemia. The proband manifested with extensor tendons of the hands and Achilles tendon xanthomas (19.0/23.0 mm) on both legs. The proband's LDL receptor protein and LDL uptake were decreased to 58% and 66% of controls, respectively.

Family H: W556C in exon 12. The proband was heterozygous for a G-to-T transition at nt 1731 (Fig. 3, Family H), which would substitute Cys for Trp at codon 556 in the third Y-repeat of the EGF precursor homology domain of the LDL receptor. The mutation was found in two members of the family. The proband manifested with corneal arcus, Achilles tendon xanthomas (15.0/16.0 mm) on both legs, and ischemic heart disease; he had been treated with plasma LDL apheresis. His LDL receptor protein and LDL uptake were each decreased to 64% of controls.

K790X mutation (exon 17) in five additional families. The K790X mutation (exon 17) was previously described in several mainland Japanese families (Maruyama et al. 1995). We identified it in five other unrelated pedigrees (data not shown).

Discussion

We have described here eight FH pedigrees carrying novel mutations in the *LDLR* gene, and characterized the dysfunction of each mutated LDL receptor by analyzing membrane-bound LDLR protein and Dil-LDL uptake by LDL receptors in peripheral lymphocytes, using FACS flow cytometry. Maruyama et al. (1995) had already reported five different point mutations of the LDL receptor among FH families living in mainland Japan: a splice-site mutation in intron 12, a C317S mutation, a P664L mutation, an E119K mutation, and a recurrent K790X mutation. In the present study, we detected eight novel mutations of LDLR among FH families living in Hokkaido, the northland of Japan. In addition, five unrelated FH families were found to carry the K790X mutation, although we did not observe any of the other four mutations that Maruyama's group had described in mainland Japanese families. Therefore, in general, specific mutations of the *LDLR* gene appear to recur only infrequently in Caucasian or Japanese populations.

The *LDLR* gene contains five domains that include a ligand-binding domain, an EGF-precursor homology domain, an O-linked sugar domain, a membrane-spanning domain, and a cytoplasmic tail domain. *LDLR* mutations have been classified into five functional classes on the basis of studies of fibroblast cells derived from skin biopsies (Russell et al. 1989, Hobbs et al. 1990, 1992).

Class 1 alleles fail to produce immunoprecipitable LDL receptor protein and are called null alleles. In the present study, we detected six nonsense, frameshift, or splicing mutants: 578–584del7bp in family A, 682ins14bp in family B, 946–994 del49bp in family C, 1687insG in family E, a 1705+1G/T splicing sequence mutation at the exon 11/intron 11 junction in family F, and a 1705+1G/C splicing sequence mutation at the exon 11/intron 11 junction in family G. Because all six of these mutations would fail to produce an intact receptor, they belong to class 1 (null alleles). Nonsense and frameshift mutations are the most frequent types of class 1 alterations reported for the *LDLR* gene, and they tend to be randomly distributed among its exons. Membrane-bound LDL receptor protein and LDL uptake activity in lymphocytes from our FH subjects with class 1 mutations showed marked decreases compared with controls.

Class 2 alleles encode proteins that are blocked either completely or partially in transport between the endoplasmic reticulum and the Golgi apparatus. We observed in Family H a substitution of cysteine for proline at codon 556

in the third Y-repeat of the EGF precursor homology domain, encoded by exon 12. Four other missense mutations in the Y-repeat of this domain have been functionally characterized (Hobbs et al. 1992). Because most of them belonged to class 2 (transport-defective alleles), we postulate that the P556C mutation reported here would cause the same class of deficiency because of its location. Membrane-bound LDL receptor protein and LDL uptake activity in lymphocytes from the proband of family H were decreased compared with controls (64% in each case).

Class 5 alleles encode receptors that do not recycle to the cell surface, because they fail to release their ligands in the endosome. In family D, a substitution of arginine for cysteine at codon 337 abolished a critical cysteine residue in the second Y-repeat of the EGF-precursor homology domain. The 5′ end of this domain, especially in the first Y1-repeat region, mediates acid-dependent dissociation of receptor and ligand in the endosome, an event essential for receptor recycling (Davis et al. 1987). Membrane-bound LDL receptor protein and LDL uptake activity in lymphocytes from the affected members of family D were decreased compared with controls (72% and 67%, respectively). The cysteine 337 residue appears to be critical for receptor recycling, in view of a report by Hobbs et al. (1992) that LDL receptors on fibroblasts from FH patient Naples-1, who carried a Cys-to-Arg substitution in this region, failed to release their ligands in the endosome and thus did not recycle to the cell surface. In fact, class 5 mutations (recycling-deficient alleles) cluster characteristically in the Y1-repeat region of the EGF-precursor homology domain (Hobbs et al. 1992). We observed no class 3 or class 4 alleles in the present study.

Patient II-5 in Family C showed normal LDLR uptake and a patient II-2 in Family D showed almost normal surface-bound LDLR protein despite reduced surface-bound LDLR protein in II-5 in Family C and reduced LDLR uptake in II-2 in Family D, respectively, in our functional measurements. One possible explanation for these discrepancies may relate to the inevitable necessity of forcing upregulation of LDL receptor in peripheral blood lymphocytes by means of depletion of lipoproteins from culture medium. Variation of this upregulation procedure among individual patients may cause such variability in outcome. In addition, it has been pointed out that the variability in phenotypes might be caused by other segregating genetic factors and environmental factors, such as the presence of unknown modifier genes interacting with *LDLR* gene. The phenotypic variabilities among the family members in the present study may be influenced by such factors. Also, members of FH families who carry mutant *LDLR* alleles sometimes do not fully express hypercholesterolemia as a result of nongenetic influences, including age. It has recently been reported that an autosomal recessive hypercholesterolemia (ARH), whose phenotype is similar to FH and has no apparent function defect in *LDLR*, was caused by the mutation of a putative adaptor protein (Garcia et al. 2001). Unequivocal diagnosis of borderline FH patients and exclusion of other causes of hypercholesterolemia such as ARH by means of a combination of approaches, including clinical, molecular genetic, and functional may help clinicians to invoke appropriate management protocols for members of FH families.

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