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Genetics

Identification of a point mutation in growth factor repeat C of the low density lipoprotein-receptor gene in a patient with homozygous familial hypercholesterolemia that affects ligand binding and intracellular movement of receptors

(gene amplification/epidermal growth factor precursor domain)

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ABSTRACT **The coding region of the low density lipoprotein (LDL)-receptor gene from a patient (MM) with homozygous familial hypercholesterolemia (FH) has been sequenced from six overlapping 500-base-pair amplified fragments of the cDNA from cultured skin fibroblasts. Two separate single nucleotide base changes from the normal sequence were detected. The first involved substitution of guanine for adenine in the third position of the codon for amino acid residue Cys-27 and did not affect the protein sequence. The second mutation was substitution of thymine for cytosine in the DNA for the codon for amino acid residue 664, changing the codon from CCG (proline) to CTG (leucine) and introducing a new site for the restriction enzyme *Pst* I. MM is a true homozygote with two identical genes, and the mutation cosegregated with clinically diagnosed FH in his family in which first cousin marriages occurred frequently. The amino acid change occurs in the center of growth factor repeat C in the epidermal growth factor precursor-homology domain of the protein, a region of highly conserved sequence between bovine and human LDL receptors, and results in slowed, but complete, maturation of the precursor to the mature form of the receptor and, despite its remoteness from the ligand-binding domain, in impaired binding of LDL. LDL receptors in MM's skin fibroblasts bind less LDL than normal and with reduced affinity. Thus this naturally occurring single point mutation affects both intracellular transport of the protein and ligand binding and occurs in growth factor-like repeat C, a region that has not previously been found to influence LDL binding.**

The low density lipoprotein (LDL) receptor is a cell-surface protein that mediates the specific recognition, uptake, and degradation of plasma LDL. During receptor-mediated endocytosis, LDL receptors on the cell surface cluster into coated pits that internalize to form endocytic vesicles from which the receptor recycles to the cell surface, while the ligand is degraded in lysosomes (1). The LDL receptor is synthesized as a precursor protein, of apparent molecular weight 120,000 by gel electrophoresis, and undergoes considerable posttranslational modification by the addition and remodeling of both O-linked and N-linked carbohydrate chains before it reaches the cell surface as a mature protein of apparent molecular weight 160,000 (2).

From cDNA cloning of LDL-receptor mRNA (3) and from the intron–exon structure of the gene (4) it has been predicted that the LDL receptor is an integral membrane protein of 839 amino acid residues that comprises a mosaic of several different domains. Analysis of naturally occurring mutations in the LDL receptor gene in patients with familial hypercholesterolemia (FH) and of constructed mutations has shed some light on the function of these different domains in the process of receptor-mediated endocytosis. The first NH_2-terminal 292 residues of the LDL receptor comprise the ligand-binding domain, containing seven cysteine-rich 40-amino acid repeats. Mutations in this domain can affect intracellular transport (5, 6) as well as ligand binding (7–9). The next adjacent domain comprises 400 amino acid residues with 35% homology with the epidermal growth factor (EGF) precursor (10). Within this domain are three cysteine-rich repetitive elements (A, B, and C) termed "growth factor-like repeats" (11), which are found in many cell-surface and secreted proteins. Mutational analysis has shown that repeat A, but not B, is required for binding of LDL (9), whereas other mutations in repeats A and B affect recycling of receptors (12). Deletion of the entire domain affects the ability of the receptor protein on the cell surface to bind LDL but has no effect on binding of ligands by the purified receptor on a nitrocellulose blot (12). Mutations in the EGF-precursor homology domain have also been found to prevent the maturation of the precursor protein (9), which is rapidly degraded and fails to reach the cell surface.

We have recently described the properties of the LDL-receptor protein in cultured skin fibroblasts from a FH subject (MM) in whom there is both impaired transport of the newly synthesized protein to the cell surface and impaired binding of LDL (13). In this paper, we have determined the sequence of the coding region of the LDL-receptor gene in MM, who is a true homozygote with identical defective genes, and show that the malfunctions are due to a single point mutation that changes proline 664 in growth factor-like repeat C to leucine.

SUBJECTS

Subject MM is a male Zambian of Asian-Indian origin whose parents are first cousins. His eldest brother died of coronary disease (age, 23) and, as described elsewhere, MM exhibits the clinical features of homozygous FH type IIB (14). Homozygous and heterozygous FH were diagnosed in MM's family on the basis of their plasma cholesterol concentration.

METHODS

Cultured Cells. Skin fibroblasts were maintained in culture as described (15). Total cytoplasmic RNA was isolated from cultured skin fibroblasts that had been preincubated for 48 hr

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Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; EGF, epidermal growth factor.

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in medium containing 10% (vol/vol) lipoprotein-deficient serum as described (16).

Gene Amplification. Double-stranded cDNA for the LDL-receptor mRNA was synthesized with a "cDNA synthesis system plus" kit (Amersham), using the protocol described in the kit for 1 μg of poly(A)-rich RNA, with the exceptions that 5–10 μg of total cytoplasmic RNA was substituted for poly(A)-rich RNA, and a 21-nucleotide oligomer complementary to bases 2609–2577 of the LDL-receptor mRNA was substituted for the primer(s) in the kit, to give a concentration of 50 μg/ml in the first-strand reaction. Based on the incorporation of $dCT^{32}P$, the yield of cDNA was ≈20 ng. For amplification (17), a Gene Amp DNA amplification kit was used (Perkin–Elmer/Cetus). The reaction mix (final volume, 100 μl) contained ≈2 ng of cDNA and two 21-nucleotide oligomers that delineated 0.5-kilobase (kb) fragments of the coding region (3, 4), to give a final concentration of 5 μg/ml each. The 5′ ends of the oligomers were phosphorylated prior to the amplification reaction using a standard protocol (18). The amplification comprised incubation of the reaction mix at 94°C for 2 min before addition of enzyme; this was followed by 30 cycles of 2 min each at 92°C, 48°C, and 72°C in turn, using an automated device (19). At the end of the 30th cycle, the samples were incubated for 15 min at 72°C to complete any partial strands. Amplified fragments were analyzed by agarose gel electrophoresis, with or without restriction enzyme digestion, using standard methods (18) and blotted with specific probes for the LDL-receptor cDNA as described (16). Probes specific for each segment were prepared by random primed synthesis labeling with $dCT^{32}P$ (20) of the equivalent amplified fragments of plasmid pLDLR3 (a full-length cDNA clone for the human LDL receptor, kindly provided by D. Russell, Dallas). Amplified fragments were excised from low-melting-point agarose gels (Sigma) and cloned into the *Sma* I site of M13mp18 (21), and their nucleotide sequence was determined using a Sequenase kit (Cambridge Bioscience, Cambridge, U.K.). For each fragment, at least one clone with an insert in each orientation was sequenced; where differences were found at least two of each orientation were sequenced or the amplified fragment was analyzed by restriction enzyme digestion.

Genomic DNA was isolated from whole blood or from the nuclear pellets of cultured skin fibroblasts and cultured lymphoblasts (16) essentially as described by Kunkel *et al.* (22). Genomic DNA was amplified with two 21-nucleotide oligomers that encompassed exons 13 and 14 of the LDL-receptor gene as described above. The reaction mix (100 μl) contained 1 μg of genomic DNA and 0.5 μg each of oligomers M and N (see Fig. 3), and the amplification protocol included incubation for 20 min at 94°C and 5 min at 65°C before the addition of enzyme; this was followed by 30 cycles of alternately 1 min at 91°C and 5 min at 65°C, using a Techne programable DW-Block (GRI, Essex, U.K.).

RESULTS

To determine the structural basis for defects in LDL-receptor function in MM's fibroblasts (13), the sequence of the coding region of the mRNA for the LDL receptor was determined from six overlapping amplified fragments of ≈0.5 kb each. With each pair of oligomers, when the amplified material was fractionated on an agarose gel and blotted with a series of ^{32}P-labeled probes corresponding to the coding region of the receptor, a single band of expected molecular weight was detected. In each case the MM fragment was indistinguishable in size from a normal fragment.

When each amplified fragment from MM was sequenced, two consistent differences were found between the wild-type sequence (3) and the sequence of the cDNA in MM. In fragment AB comprising nucleotides −25 to 470 of the coding region, there was a single base change such that the codon for amino acid residue 27 was changed from UGU in the wild type to UGC in MM; this base change does not affect the cysteine residue at this position. This single base change was detected in five of five M13 clones of fragment AB and was found in inserts in both orientations. Other changes were also detected in fragment AB, but each was present in only one of five clones and probably represents errors in the amplifications.

The second difference, shown in Fig. 1, occurred in fragment IJ of MM's cDNA where there was a single base change such that the codon for amino acid residue 664 was changed from CCG in the wild type to CUG in MM, substituting leucine for proline at this position. This mutation introduces a new site for the restriction enzyme *Pst* I in MM's cDNA, as shown in Fig. 2. To confirm that this base change was not an error introduced into the single M13 clone as an artefact of the amplification reaction, and to determine whether MM was homozygous or heterozygous for this mutation, the whole amplified fragment IJ was digested with *Pst* I. When the digested fragments were analyzed by blotting with a labeled probe specific for fragment IJ, a band of 0.43 kb was detected in normal cDNA, whereas a band of 0.34 kb was detected in MM's cDNA. No fragment of 0.43 kb could be detected in the *Pst* I-digested amplified fragment IJ from two separate preparations of cytoplasmic RNA from MM's fibroblasts (Fig. 2).

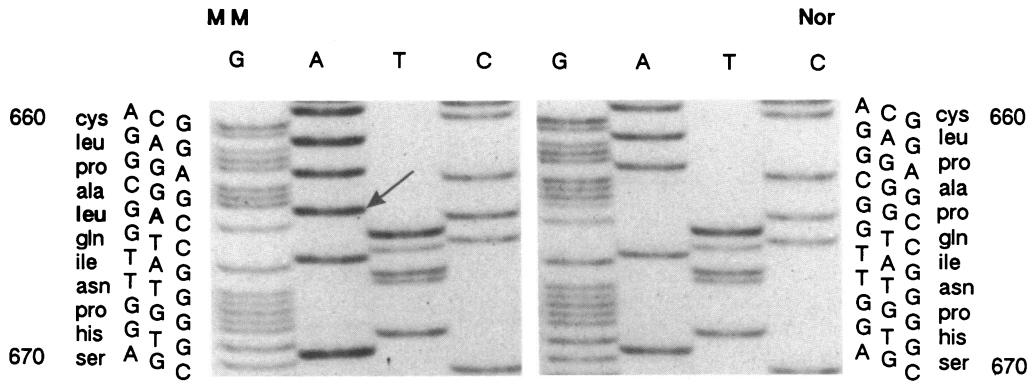

FIG. 1. Partial sequence of fragment IJ of the LDL-receptor cDNA. Part of a sequencing gel of single M13 clones of amplified fragment IJ of normal (Nor) cDNA and MM's cDNA is shown; the sequence shown is the noncoding strand and therefore the codons appear as anticodons for the amino acid residues shown. Nucleotide number 2054 (where 1 is the adenine of the AUG initiator codon), indicated by an arrow, is changed from guanine in the normal fragment to adenine in MM, so that amino acid residue 664 is changed from proline in the normal protein to leucine in MM.

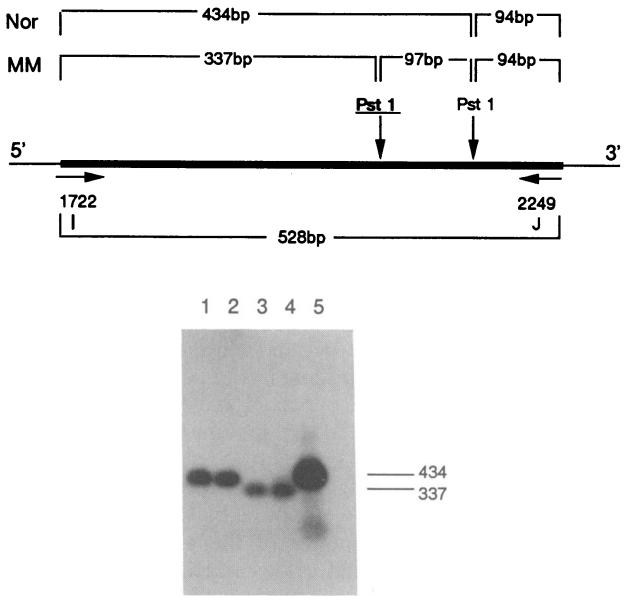

FIG. 2. Sites for the restriction enzyme *Pst* I in normal (Nor) fragment IJ and MM's fragment IJ of the LDL-receptor cDNA. (*Upper*) Diagram of the 528-base-pair (bp) region of the LDL-receptor cDNA delineated by oligonucleotides I and J (represented by horizontal arrows). The positions of the *Pst* I restriction enzyme site at position 2155 in the normal cDNA and of the new *Pst* I site at position 2058 in MM's cDNA are indicated by vertical arrows; also shown is the expected size of fragments obtained after *Pst* I digestion of amplified fragments IJ. (*Lower*) Blot of an agarose gel of *Pst* I-digested fragment IJ from two preparations of normal fibroblast cDNA (lanes 1 and 2) and two separate preparations of MM's fibroblast cDNA (lanes 3 and 4), each prepared from different preparations of cytoplasmic RNA. Also shown (lane 5) is the *Pst* I digest of amplified fragment IJ from plasmid pLDLR3. The blot was hybridized with a ^{32}P-labeled probe specific for the IJ region of the LDL-receptor cDNA.

The presence of the new *Pst* I site made it possible to examine the inheritance of the mutation in the proband's family. However, since the LDL-receptor gene contains numerous *Pst* I sites, it was necessary to amplify a short region of the gene, encompassing exons 13 and 14, to detect the mutation reliably in genomic DNA. Fig. 3 shows the results obtained when the amplified fragment MN of the LDL-receptor gene from several members of MM's family, in which there are several first cousin marriages (Fig. 4), was cut with *Pst* I and the fragments were analyzed by blotting with a probe specific for fragment IJ of the LDL-receptor cDNA. Members of the family who were diagnosed as homozygous had two copies of the mutant gene, whereas heterozygotes had one normal gene and one mutant gene.

DISCUSSION

We have described the defective function of LDL receptors in cultured skin fibroblasts from homozygous FH subject MM in detail elsewhere (13, 16). The LDL-receptor mRNA in MM is 5.3 kb and is synthesized at an apparently normal rate; it is also subject to regulation by sterols in the same way as in normal cells (16). A precursor protein of normal mobility on SDS gels is synthesized at approximately the same rate as in normal cells, but its rate of maturation in MM's cells is delayed so that it has an abnormally long half-life (3 hr rather than 0.5 hr). However, all of the precursor protein eventually reaches the cell surface where it recycles normally, although its half-life is 6 hr rather than 10 hr as in normal cells (13). In fibroblasts cultured in the absence of sterols there is approximately the same amount of LDL-receptor protein as in normal cells, but, whereas almost all of the receptors in normal cells are in the mature recycling form, in MM's cells nearly half of the receptor protein is in the precursor form unable to participate in uptake of LDL (13).

As well as this defect in intracellular transport of the receptor protein, LDL receptors on the surface of MM fibroblasts show a reduced affinity for both ^{125}I-labeled-LDL and very low density lipoprotein with β-mobility. Furthermore, taking into account the reduced number of recycling receptors in MM's cells compared to normal, LDL receptors in MM's cells bind less ligand per mole of protein than in normal cells. Once bound, the ligand is internalized and degraded normally. These functional differences, together with the observation that MM's cells also bind less of a monoclonal antibody to the bovine LDL receptor (23) per mole of LDL-receptor protein on the cell surface than do normal cells (13), show that there is a major structural defect in the LDL-receptor protein in MM's cells that is different from those described previously (2).

Of the two single base changes found consistently in the amplified fragments of the LDL-receptor cDNA from MM's cells only one involved a change in the amino acid sequence of the protein, that of proline 664 to leucine. This mutation introduced a new restriction enzyme site that was present in both copies of MM's LDL-receptor gene, confirming that he was a true homozygote with two functionally identical genes. The mutation also cosegregated with clinically diagnosed FH in MM's large family, which contained two more homozygotes and numerous heterozygotes. Thus it is likely that all of the defective functions of the LDL receptor in MM skin fibroblasts can be ascribed to this single amino acid change.

Proline 664 occurs in growth factor-like repeat C, a disulfide- and proline-rich region of the EGF-precursor homology domain of the LDL-receptor protein. This sequence is highly conserved between bovine and human LDL-receptor genes (2), suggesting that it has an important functional role. Because of the large number of proline residues it is likely that this region is of irregular structure, and the difference in amino acid sequence between it and the other two growth factor-like repeats A and B, which resemble more closely the sequence found in the EGF precursor itself, suggests that it is folded differently from them (4). The mutation of proline to leucine might be expected to have a marked effect on the folding of the protein, as it would release the constraint on the flexibility of the chain caused by the rigidity of peptide bonds adjacent to proline (24). Mutations of proline to leucine in other proteins have been found both to markedly stabilize the protein, as in the case of the tryptophan synthase α-subunit (25) and the λ repressor protein (26), and to slightly destabilize it, as in the case of T4 lysozyme (27). Experiments to compare the stability of the LDL-receptor protein in MM cells and normal cells are necessary.

Previously described mutations in the LDL-receptor gene that affect ligand binding have all been localized to the ligand-binding domain (7–9) or to growth factor repeat A, which is adjacent to the ligand-binding domain (9). Thus it is of considerable interest that LDL binding is also affected by a mutation in growth factor-like repeat C, which is separated from this previously identified binding region by at least 300 amino acid residues in the primary structure of the protein (4). These observations suggest that the receptor protein could be folded so that growth factor-like repeat C is normally in contact with the ligand-binding region or that the change in folding of this region is sufficient to disrupt the normal folding of the binding domain. A third possibility is that the change in structure may disrupt some interaction between receptor protein molecules on the cell surface that is essential for optimum ligand binding. Our observations on the effect of monoclonal antibody 10A2 on the binding of LDL by MM's receptors and normal LDL receptors in skin fibroblasts

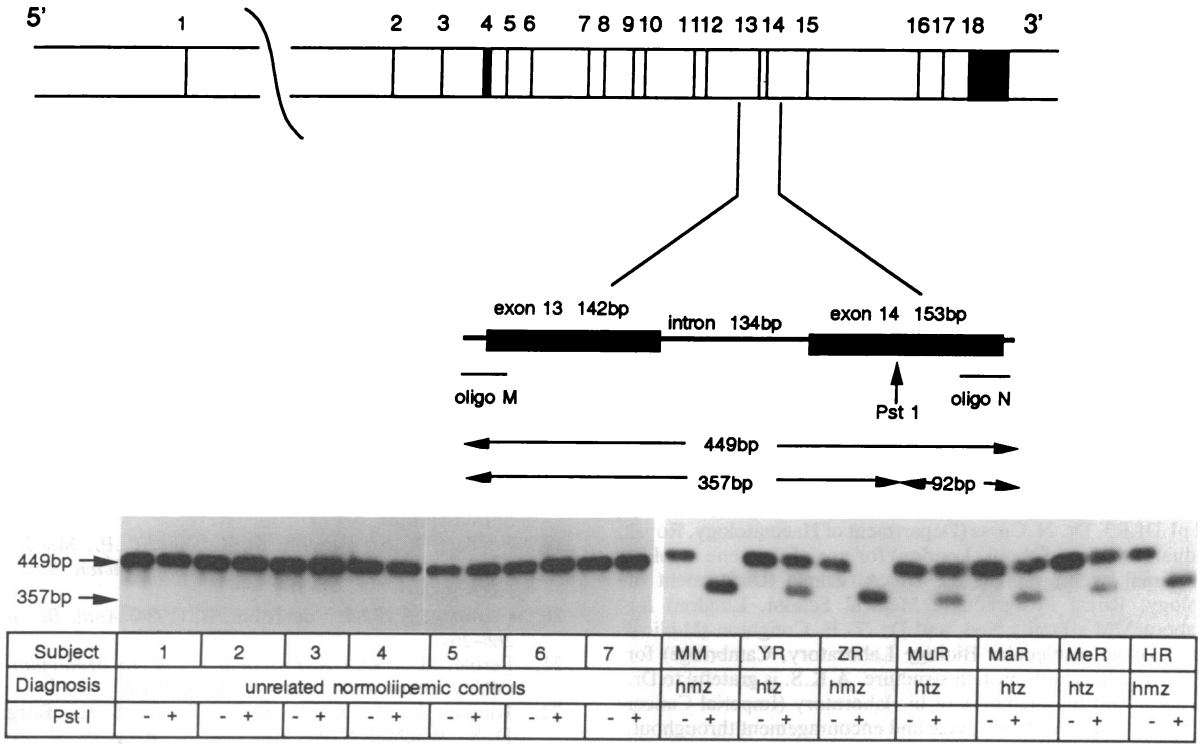

FIG. 3. (*Upper*) Diagram of the LDL-receptor gene with the exons (1–18) marked in black. The expanded region shows exons 13 and 14, and the two 21-nucleotide oligomers M and N that were used to prime the amplification of this region of the genomic DNA. Oligomer M was complementary to the noncoding strand and overlapped the intron–exon junction at the start of exon 13; oligomer N was complementary to the coding strand and overlapped the intron–exon junction at the end of exon 14 (4). The position of the new *Pst* I site in MM's genomic DNA is indicated by an arrow; the expected size of the fragments obtained after *Pst* I digestion of fragment MN is also shown. (*Lower*) Results obtained when genomic DNA from members of MM's family (Fig. 4) and from control subjects was amplified with oligomers M and N as primers, and the amplified DNA then was digested with *Pst* I, analyzed by agarose gel electrophoresis, and blotted with a ^{32}P-labeled probe specific for fragment IJ of the LDL-receptor cDNA (see Fig. 2). Fragment MN from normal subjects was unaffected by *Pst* I; that from homozygotes (hmz) was completely digested, whereas that from heterozygotes (htz) was partially digested.

would lend some support to this (13). In this context it is also of interest that a constructed mutant LDL-receptor protein in which the entire EGF-precursor homology domain was deleted was unable to bind the ligand when on the cell surface in intact cells but was able to bind apparently normally when isolated on a nitrocellulose blot (12).

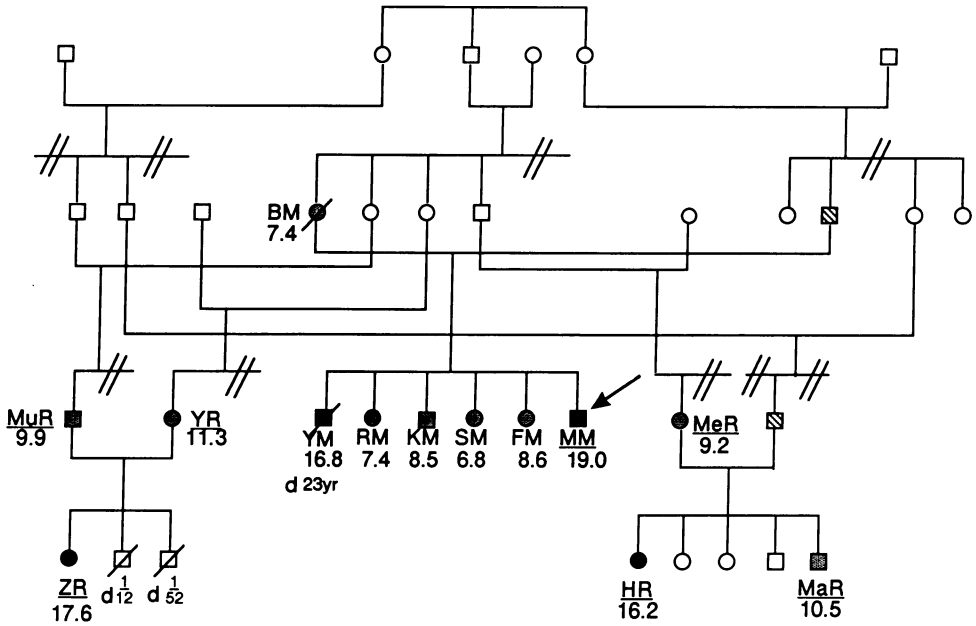

FIG. 4. Inheritance of the mutation at proline 664 in MM's family. The index patient MM is indicated by an arrow. Those members of the family with clinically diagnosed FH are indicated by filled symbols (homozygotes) and stippled symbols (heterozygotes), with their plasma cholesterol values (mmol/liter) shown below. Obligate heterozygotes not examined are indicated by hatched symbols. MM's brother (YM) and mother (BM) both died of coronary heart disease. Those members of the family whose genomic DNA was analyzed are underlined.

Several different mutations in the LDL-receptor gene that affect transport of the newly synthesized protein to the cell surface have been described (28). One of these, the Lebanese mutation, is also located in growth factor-like repeat C, where the codon for Cys-660 is changed to a stop codon. The truncated protein produced is not processed and becomes rapidly degraded (29). Of more interest in relation to the mutation in MM's gene is that of FH 429 (28) in which the glycine at 544 is changed to valine. In FH 429 cells, the receptor is not processed to the mature form and is again more rapidly degraded than normal; thus the effect of this mutation on processing and transport is different from that of the proline → leucine at 664 in the LDL receptor in MM's cells.

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