Characterization of Three Mutations of the Low Density Lipoprotein Receptor Gene in Italian Patients With Familial Hypercholesterolemia


Three gross rearrangements of the low density lipoprotein receptor (LDL-R) gene were recognized during a survey of 23 unrelated Italian subjects with familial hypercholesterolemia (FH). Restriction endonuclease data were obtained by Southern blotting and hybridization with exon-specific probes. Proband FH-29 is heterozygous for a 4-kb deletion, which eliminates exons 13 and 14. This mutation is similar to that previously reported by other investigators in one Italian homozygous and two British and Canadian heterozygous patients. Proband FH-30 is homozygous for a 5.5-kb insertion caused by a duplication of exons 16 and 17 of the LDL-R gene. LDL-R mRNA isolated from skin fibroblasts of FH-30 was found to be larger than normal mRNA (5.6 versus 5.3 kb), in concordance with the insertion of the 236 nucleotides corresponding to exons 16 and 17. Proband FH-44 was found to have >25-kb deletion, which eliminates the first six exons and the promoter region of the gene. This is the first example of a deletion that eliminates the promoter as well as the ligand-binding domain of the LDL-R gene. In the skin fibroblasts of this patient, the level of LDL-R mRNA was approximately half that found in control fibroblasts. We designate the new mutations found in FH-30 and FH-44 as FHVerona and FHBologna, respectively, after the names of the Italian cities where the two patients were born.

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Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the gene encoding the plasma low density lipoprotein (LDL) receptor. FH is a heterogeneous condition, since at least four classes of functional defects have been observed that disrupt the synthesis, intracellular transport, LDL binding capacity, and internalization of the LDL-receptor complex. During the past few years, the availability of cDNA and genomic clones has made it possible to characterize the mutations of the LDL receptor (LDL-R) gene at the DNA level. A large array of mutations, including deletions, insertions, nonsense, and missense types, have been found so far in various countries and ethnic groups. These mutations are scattered throughout the 45 kilobases (kb) of the LDL-R gene on chromosome 19.

During the past 2 years, we have undertaken a survey of Italian FH patients through a collaborative study involving several lipid clinics and aimed at investigating the frequency and features of the major structural rearrangements of the LDL-R gene. So far, we have screened 23 unrelated FH families living in various districts of Italy. DNA screening conducted by the use of 10 restriction enzymes allowed us to identify three patients with gross mutations of the LDL-R gene. Clinical and biochemical details of the 23 FH patients as well as the results of DNA screening and restriction fragment length polymorphism haplotype analysis will be reported elsewhere. In the present report, we illustrate the results of the analysis of the three gross mutations. Two of them (one insertion and one deletion) are new mutations.

Methods

The patients included in this study and designated FH-29, FH-30, and FH-44 were selected from a
survey of 23 unrelated Italian FH families. The diagnosis of FH was based on plasma cholesterol and LDL values in the proband and in the first-degree relatives, and, in all probands, on the assay of LDL-R activity on cultured skin fibroblasts. Skin fibroblasts that were at the third to sixth passage were used. Briefly, $1.5 \times 10^9$ cells were plated in 60-mm plastic petri dishes and cultured for 5 days in Dulbecco’s modified Eagle’s medium (DMEM; ICN-Flow, Milano, Italy) and 50% fetal calf serum (GIBCO, Milano, Italy). The medium was replaced daily. After 5 days, the medium was removed, and the cells were washed in phosphate-buffered saline and then incubated for 2 days in DMEM containing 10% lipoprotein-deficient serum to induce the expression of LDL-R. The cells were then incubated in the same medium containing [125I]-LDL at the concentrations of 25, 5, 15, 25, 50, and 100 μg/ml. Freshly isolated human plasma LDLs ($d=1.019-1.063$ g/ml) were iodinated with $[^{125}I]$NaI. [125I]-LDL specific activity was between 80 and 200 cpm/μg protein. Incubations were performed either in the presence or absence of a 10-fold excess of unlabeled LDL. Specific binding, internalization, and degradation of [125I]-LDL was calculated by subtracting the values obtained in the presence of unlabeled LDL from those obtained in its absence. Skin fibroblasts of a normolipemic subject, representative of our series of four control individuals, were used as control cells and incubated in parallel with the probands’ fibroblasts. The value of [125I]-LDL degradation found in the probands’ fibroblasts (at the medium concentration of [125I]-LDL of 25 μg/ml) is given as a percentage of the corresponding value found in the control cells.

Genomic DNA was isolated from 20 ml whole blood and digested overnight with 5–15 units restriction endonucleases/μg DNA at the conditions suggested by the manufacturer (Amersham, Buckinghamshire, England; Boehringer-Mannheim, Mannheim, F.R.G.). In the first screening for gross mutations, each DNA sample was digested with these restriction enzymes: BamHI, Bgl II, EcoRI, EcoRV, HindIII, Kpn I, Pst I, Pvu II, Stu I, and Xba I. In some experiments, DNA was also digested with Nco I. Digested DNA was separated by electrophoresis on 0.8% agarose gel and transferred to Hybond-N filters (Amersham) by the Southern blotting method. Total cellular RNA was isolated by extraction in guanidine thiocyanate from cultured skin fibroblasts, which had been maintained for various periods of time (0–48 hours) in a lipoprotein-deficient medium. Total RNA (15 μg) was denatured in 50 μl 50% formamide, 2.2 M formaldehyde, and 3-N-morpholino)propanesulfonic acid (MOPS) buffer containing 20 mM MOPS, 5 mM sodium acetate, and 1 mM Na₂EDTA and was separated on a 1.2% agarose gel in MOPS buffer. After separation, RNA blots were prehybridized and hybridized in the following solutions: 50% formamide, 3x standard saline citrate (SSC: 0.45 M NaCl, 0.045 M sodium citrate), 3x SSC and 0.1% SDS; 0.5x SSC and 0.1% SDS at room temperature; and 0.1x SSC and 0.1% SDS at 42°C. Washed membranes were then exposed to Hyperfilm-MP (Amersham) x-ray films at −80°C. Densitometric analysis of x-ray films was accomplished using an LKB-XL laser densitometer (LKB, Bromma, Sweden).

**Results**

During the DNA screening of the 23 unrelated FH patients, we found three gross mutations of the LDL-R gene. The individuals affected by these mutations were designated FH-29, FH-30, and FH-44.

**Proband FH-29**

Proband FH-29 is a 29-year-old man with the following plasma lipid values: total cholesterol = 488 mg/dl; triglycerides = 84 mg/dl; high density lipoprotein (HDL) cholesterol = 59 mg/dl; and apolipoprotein (apo) B = 259 mg/dl. [125I]-LDL degradation by skin fibroblasts was 48% of that found in control fibroblasts. Four first-degree relatives of proband FH-29 were affected by primary hypercholesterolemia and tendon xanthomas.

The proband’s DNA digested with several enzymes and hybridized with the cDNA probe pTZ1 was found to contain, in addition to the normal fragments, other fragments shorter (e.g., after DNA digestion with Bgl II, Pvu II, and Stu I) or longer (e.g., after DNA digestion with Kpn I and Nco I) than their.
normal counterparts. This preliminary screening suggested that FH-29 was heterozygous for a deletion located 3' to exon 12 because, by using exon-specific probes, we found that all restriction sites from exons 1–12 were maintained (data not shown). A cDNA probe specific for exons 15–17 was used to define the 3' boundary of the deletion, taking advantage of the fact that a Stu I restriction site is present in intron 15. In control DNA, Stu I digestion resulted in two fragments that were 14 and 8 kb long (Figure 1). In FH-29 DNA, the 8-kb fragment was maintained, and the 14-kb fragment was present in a reduced amount, in addition to a shorter fragment of 10 kb (Figure 1). The presence of a normal 8-kb fragment indicated that in FH-29, the Stu I site in intron 15 was maintained, thus suggesting that exon 15 was not involved in the deletion. Had exon 15 been deleted, we would not have been able to see the 10-kb fragments that were 14 and 8 kb long (Figure 1).
abnormal fragment (Figure 1). When FH-29 DNA digests were hybridized with a cDNA probe complementary to exons 13 and 14, the abnormal bands previously seen with other probes (e.g., probe A in Figure 1) were no longer detectable. However, in FH-29 the intensity of the hybridization signal of the normal fragments was half of that found in control. This observation is consistent with the idea that proband FH-29 is heterozygous for a deletion of approximately 4 kb that eliminates exons 13 and 14 (see the summary diagram in Figure 1). To ascertain whether the deleted allele of FH-29 was transcribed into a shorter LDL-R mRNA, Northern blot analysis of RNA isolated from skin fibroblasts was performed. Figure 2A shows that the mRNA species isolated from the proband's fibroblasts was indistinguishable from that found in the control cell line.

**Proband FH-30**

Proband FH-30 is a 32-year-old man with the following plasma lipid values: total cholesterol=573 mg/dl, triglycerides=62 mg/dl, and HDL cholesterol=43 mg/dl. 125I-LDL degradation by cultured skin fibroblasts was 16% of that found in the control cell line.

The hybridization of the proband's DNA with pTZ1 cDNA demonstrated an abnormal restriction pattern with several enzymes such as EcoRI, BamHI, Bgl II, and EcoRV. For example, the digestion of control DNA with EcoRV produced two fragments of 6.5 and approximately 23 kb; in FH-30 DNA, the ~23-kb fragment was missing, having been replaced by a larger (~25-kb) fragment. These abnormal restriction patterns cosegregated with the disease in the family. DNAs of the proband's parents and son, all affected by primary moderate hypercholesterolemia, were found to contain both the normal and the abnormal fragments (see example in Figure 3, panels B and D). Taken together, these observations indicated that proband FH-30 was homozygous for a gross mutation of the LDL-R gene. However, some restriction data obtained with other enzymes (Kpn I, Stu I, Pvu II, and Nco I) appeared to conflict with this hypothesis. For example, in control DNA Kpn I digests followed by hybridization with the pTZ1 probe, three fragments of 9, 13, and 20 kb were produced; the same fragments with the same relative intensity were present in proband FH-30, together with another 5.5-kb fragment of low intensity (Figure 3A). The same results were found after DNA digestion with Stu I, Pvu II, and Nco I (data not shown).

To clarify this point, the proband's DNA was screened with exon-specific probes, starting from the 3' end of the coding region of the gene, where the rearrangement was presumed to be located. When the proband's DNA was digested with BamHI and hybridized with an exon 18--specific probe, we confirmed that FH-30 was homozygous for an abnormal fragment of high molecular weight (Figure 3B). The same was observed after Bgl II, EcoRI, and EcoRV digestion (data not shown). Surprisingly, however, when the same probe was used to hybridize DNA digested with Kpn I and Stu I, the fragments obtained in FH-30 were identical to those found in control subjects (Figure 3C), thus indicating that the 5.5-kb fragment previously detected did not contain sequences complementary to the exon 18 cDNA probe. The same results were obtained with Pvu II and Nco I (data not shown).

When this study was repeated using a probe complementary to exons 15–17, the following pattern emerged in FH-30. The digestions with BamHI (Figure 2). Photographs of Northern blot analyses of mRNA isolated from skin fibroblasts and hybridized with cDNA probe pTZ1 and a full-size cDNA probe of human β-actin. A: Low density lipoprotein receptor (LDL-R) mRNA in proband FH-29 (FH29) and in one of his first-degree relatives (FH28), a carrier of the same mutation of the LDL-R gene. B: LDL-R mRNA, isolated from skin fibroblasts from proband FH-30 (FH 30), that had been incubated in lipoprotein-deficient serum for 3 (3h) and 6 (6h) hours, respectively. C, LDL-R mRNA isolated from a control cell line; 28S and 18S, migration of 28S and 18S human ribosomal RNA.
Kpn Bam HI Kpn I Stu I

kb

20_9_5.5

>_20 kb

FIGURE 3. Photographs of Southern blot analyses of proband FH-30 (FH^) DNA. A: DNA digested with Kpn I and hybridized with cDNA probe pTZ1; B and C: DNA digested with BamHI, Kpn I, and Stu I and hybridized with a cDNA probe specific for exon 18; D and E: DNA digested with Bam HI, Kpn I, and Stu I and hybridized with a cDNA probe specific for exons 15-17. FH30 het., DNA isolated from the heterozygous father of proband FH-30. Lane C, DNA from control cell line.

ure 3D), Bgl II, EcoRI, and EcoRV (data not shown) confirmed the presence of a larger fragment; the digestions with Kpn I, Stu I (Figure 3E), Nco I, and Pvu II (data not shown) revealed the presence of the additional 5.5-kb fragment. These data indicated that the 5.5-kb abnormal fragments contained sequences that were specifically recognized by the exon 15-17 probe. The observation that with several enzymes (Kpn I, Nco I, Stu I, and Pvu II) the size of the abnormal fragment was of the same order of magnitude (5.5 kb) suggested that in the mutated allele, the restriction sites for these enzymes were repeated 5.5 kb downstream from their expected positions in the sequence of the normal allele. Our restriction enzyme data are consistent with the idea that in FH-30, there is duplication of a 5.5-kb DNA fragment that contains exons 16 and 17. DNA digestion with enzymes that do not have restriction sites in the DNA segment encompassing exons 16 and 17 produces fragments of higher molecular weight. By contrast, DNA digestion with enzymes that have restriction sites in duplicated region generates, in addition to the normal fragments, an abnormal fragment of 5.5 kb (Figure 4).

As a corollary to this model, one would expect the LDL-R mRNA originating from the mutated allele to be larger than its normal counterpart. Figure 2B shows that LDL-R mRNA isolated from proband FH-30 consisted of a single species of mRNA migrating more slowly than its normal counterpart. The difference between the two mRNAs was approximately 0.3 kb.

Proband FH-44

Proband FH-44 is a 53-year-old woman with the following lipid values: total cholesterol=393 mg/dl, triglycerides=150 mg/dl, and HDL cholesterol=30 mg/dl. 125I-LDL degradation by skin fibroblasts was 45% of that found in control cells. Also, the
proband's mother and sister were affected by primary moderate hypercholesterolemia.

DNA screening with the cDNA probe pTZ1 revealed the presence of abnormal restriction fragments with several enzymes. From these data, we inferred that proband FH-44 was heterozygous for a gross mutation (presumably a deletion) located in the 5' half of the gene, since the abnormal fragments were detectable with a probe complementary to the first 11 exons of the gene. When this probe was used to hybridize DNA digested with Kpn I and BamHI, we found the following in FH-44 (Figure 5): 1) a normal

**Figure 4.** Diagram of the 3' end of the low density lipoprotein receptor (LDL-R) gene in control (C) and proband FH-30 (FH 30) DNA. According to this model, in proband FH-30, restriction sites of enzymes Stu I, Kpn I, Pvu II, and Nco I, located between introns 15 and 17, are duplicated 5.5 kb downstream from their normal positions. See text for additional details concerning experiments with other restriction endonucleases. * Restriction sites in exons.

**Figure 5.** Photographs of Southern blot analyses of proband FH-44 (FH44). FH-44 DNA was digested with BamHI, Kpn I, and BamHI-Kpn I, hybridized with a cDNA probe specific for exons 1-11 (probe A), and rehybridized with a cDNA probe specific for exons 1-4 (probe B). FH44s, sister of proband FH-44. Lane C, DNA from control cell line.
(15.5-kb) and an abnormal (13-kb) fragment after BamHI digestion; 2) a normal (20-kb) and an abnormal (>30-kb) fragment after Kpn I digestion; and 3) a normal fragment of 11 kb and an additional fragment of 13 kb after Kpn I-BamHI double digestion. These findings indicated that in FH-44, the Kpn I site located in intron 2 was deleted (see diagram shown in Figure 6A) and suggested that the large Kpn I extra fragment originated from the loss of this site and that the deletion most likely eliminated the adjacent exons (2 and 3). To corroborate this finding, the filter shown in Figure 5 was rehybridized with a probe specific for exons 1-4. We found that in FH-44, the abnormal BamHI and Kpn I fragments were no longer detectable, and the normal fragments showed half the intensity found in control DNA. The failure to detect the abnormal fragments by the exon 1-4 probe suggested that the first four exons were involved in the deletion. To define the 3' boundary of the deletion, DNA was digested with EcoRI and BamHI and hybridized with a cDNA probe specific for exons 5-7. In control DNA, EcoRI digestion produced two fragments of 1.5 and 8.5 kb, whereas BamHI produced a single fragment of 15.5 kb. Two fragments of 1.5 and 3.3 kb were generated by EcoRI and BamHI double digestion (Figure 7). In FH-44, there were additional 7.5-kb EcoRI and 13-kb BamHI fragments; the double digestion generated an abnormal fragment of 2.8 kb (Figure 7). This finding can be explained if one assumes that the EcoRI restriction sites in exon 5 and intron 6 are deleted (Figure 7). Taken together, these findings provide indirect evidence that in FH-44, the deletion spans from intron 6 to an "ill-defined" region several kilobases 5' to exon 1, as shown in Figure 6B.

Densitometric analysis of a Northern blot (data not shown) of LDL-R mRNA in proband FH-44 fibroblasts revealed that the ratio between β-actin and LDL-R mRNAs was 11.9±3.2 in control fibroblasts and 5.0±1.6 in the proband's fibroblasts.

**Discussion**

In the present study, we report the characterization of three gross mutations of the LDL-R gene in three unrelated Italian FH patients. These mutations were detected during a screening of 23 Italian FH families. Proband FH-29 was found to be heterozygous for a 4-kb deletion, which eliminates exons 13
and 14. This mutation bears strong similarities to that previously reported in British and Canadian FH heterozygous patients, and an Italian homozygous patient. By analogy to previous findings, this deletion is presumably due to a recombination event involving Alu repetitive sequences that are in the same orientation in introns 12 and 14. The elimination of exons 13 and 14 is expected to produce an mRNA shorter than the normal mRNA (provided that the splicing process is not affected by the deletion). In addition, it may be predicted that the deletion of exons 13 and 14 and the fusion of exon 12 to exon 15 during splicing leads to a frame shift in mRNA and the occurrence of a premature stop codon in exon 15. A shorter mRNA was documented in the homozygous FH patient reported by Hobbs et al. In proband FH-29, we were unable to detect the abnormal mRNA species, presumably because the latter was present in a very low concentration. This is not surprising, as several recent reports have demonstrated that mutations affecting mRNA translation (e.g., nonsense mutations) are often associated with a decreased intracellular level of the abnormal mRNA. In proband FH-29, ^125^I-LDL binding was 46% of that found in a control cell line. This is consistent with the idea that only the normal allele is functional in this patient.

Proband FH-30 was found to be homozygous for a 5.5-kb insertion at the 3' end of the gene. The results of the restriction mapping strongly suggest that the insertion derives from a duplication of a segment of DNA containing exons 16 and 17. The insertions of large stretches of DNA are much rarer than are deletions in the LDL-R gene, and we are not aware that a mutation similar to that in proband FH-30 has been reported previously. As elegantly demonstrated in a previous study concerning the duplication of exons 2-8 of the LDL-R gene, the most likely mechanism for explaining the FH-30 mutation is an unequal crossing over involving introns 15 and 17. This would generate two mutated alleles, one with a duplication and the other with a deletion of exons 16 and 17. In proband FH-30, LDL-R mRNA was found to be larger than the normal mRNA (5.6 versus 5.3 kb), in good agreement with the insertion of 236 nucleotides corresponding to the transcripts of exons 16 and 17. We predict that in this abnormal mRNA, the translation reading frame of the duplicated exon 16 would continue for 48 nucleotides before the appearance of a stop codon. As a consequence, the mutated LDL-R protein would contain the normal membrane-spanning domain, the 39 amino acids of the cytoplasmic domain encoded by the first exon 17, and an abnormal sequence of 16 novel amino acids (encoded by the duplicated exon 16), replacing the last 11 amino acids of the cytoplasmic domain normally encoded by exon 18. Therefore, the receptor protein of FH-30 would be slightly longer than the normal receptor and would have an abnormal sequence in its carboxyl terminal end. In this patient, we found a reduction of both ^125^I-LDL binding and internalization at 37°C (25% of control values) in cultured skin fibroblasts. Studies are now in progress.
to further clarify the binding/internalization defect and the molecular pathology of the LDL-R protein in this patient.

Proband FH-44 was found to be heterozygous for a large deletion. Although we were unable to define the 5' boundary of this mutation accurately, our restriction data indicate that the deletion is larger than 25 kb and eliminates the first six exons as well as the promoter region of the gene. So far, only a few deletions have been described that involve the 5' end of the LDL-R gene. Hobbs et al. found that 63% of French-Canadian FH heterozygotes have a large deletion of the LDL-R gene that removes the promoter and the first exon of the gene. A 6-kb deletion also removing the promoter and the first exon was reported in an American FH homozygote. On the other hand, deletion of the ligand-binding domain (exons 2-6) was reported in two FH heterozygotes from Canada.

So far as we know, the mutation found in FH-44 is the first deletion reported that simultaneously eliminates the promoter region and the ligand-binding domain of the LDL-R gene. This mutation is associated with a 50% reduction of LDL-R mRNA in skin fibroblasts. In keeping with these findings, the 125I-LDL binding and internalization in skin fibroblasts was 47% and 58% of control values, respectively. Interestingly, we recently identified a new unrelated FH patient (FH-50) who has the same mutation as proband FH-44. Both probands come from the same geographical area in northern Italy.

We propose to designate the two novel mutations reported in this study as FH\textsubscript{Venezia} (FH-30) and FH\textsubscript{Bologna\textsc{i}} (FH-44), from the cities in the central and northern districts of Italy where the two patients were born.

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