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A Large Deletion in the LDL Receptor Gene—The Cause of Familial Hypercholesterolemia in Three Italian Families: A Study That Dates Back to the 17th Century (FH-Pavia)

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Summary

In the LDL-receptor gene, a large rearrangement causing hypercholesterolemia was detected in three apparently unrelated families living in northern Italy. In all probands, binding, internalization, and degradation of ^{125}I-LDL measured in skin fibroblasts were found to be 40%–50% of control values, indicative of heterozygous familial hypercholesterolemia (FH). Southern blot analysis revealed that the probands were heterozygous for a large (25-kb) deletion of the LDL-receptor gene eliminating exons 2–12. The affected subjects possessed two LDL-receptor mRNA species: one of normal size (5.3 kb) and one of smaller size (3.5 kb). In the latter mRNA, the coding sequence of exon 1 is joined to the coding sequence of exon 13, causing a change in the reading frame and thereby giving rise to a premature stop codon. The receptor protein deduced from the sequence of the defective mRNA is a short polypeptide of 29 amino acids, devoid of any function. Tracing these three families back to the 17th century, we found both their common ancestor and the possible origin of the mutation, in a region which is called "Lomellina" and which is located in southwest Lombardy, near the old city of Pavia. Therefore we named the mutation "FH-Pavia."

Introduction

Familial hypercholesterolemia (FH) is a common inherited disorder with autosomal dominant inheritance, characterized by a selective increase of LDL in plasma, giving rise to tendon and skin xanthomatosis and to premature atherosclerosis (Goldstein and Brown 1989). The genetic defect in FH resides in the gene coding for the cell-surface receptor for LDL, located on the short arm of chromosome 19 (Yamamoto et al. 1984; Südhof et al. 1985). So far, about 60 different mutations (deletions, insertions, exon duplications, nonsense mutations, and missense mutations) have been characterized at the DNA level (for review, see Hobbs et al. 1990; also see Lelli et al. 1991*a*, 1991*b*; Rüdiger et al. 1991). The vast majority of the large rearrangements of the LDL-receptor gene are deletions, of different sizes, along the entire length of the gene. The marked heterogeneity of the molecular defects underlying FH suggests that most families have their own particular defect. However, in certain populations, isolated because of geographical, social, or religious reasons, a high percentage of FH patients carry the same mutation apparently stemming from a common ancestor through a "founder effect" (Lehrman et al. 1987*c*; Aälto-Setala et al. 1989; Leitersdorf et al. 1989*b*; 1990).

In the present paper we describe a novel LDL-receptor gene mutation resulting in a 25-kb deletion of exons 2–12. This mutation was detected in three probands with heterozygous FH and in some of their family members. The three families live in different parts of northern Italy and are apparently unrelated.

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Subjects and Methods

Subjects

Table 1 summarizes the clinical characteristics of the probands and FH cases of the three families—M., S., and P. The diagnosis of FH was made according to the guidelines of the European Atherosclerosis Society (Study Group, European Atherosclerosis Society 1988). The clinical features of all the affected members of the families were consistent with heterozygous FH. The subjects XI.9, XI.16, and XI.18 were deceased, but XI.9 and XI.16 were presumably affected, since they had a history of hypercholesterolemia and premature coronary heart disease. All the examined subjects gave informed consent.

Biochemical Analysis

Plasma lipoproteins were separated by preparative ultracentrifugation in a Beckman 50.4 Ti rotor (Warnick and Albers 1982), and cholesterol and triglycerides were assayed by enzymatic methods (Bucolo and David 1973; Allain et al. 1974).

Southern Blot Analysis

High-molecular-weight genomic DNA was prepared from peripheral blood by standard methods (Maniatis et al. 1982, pp. 458–459). Genomic DNA (5–10 μg) was digested to completion according to the manufacturer's conditions (Amersham International, UK), by using 5–10 U/μg for each of the following restriction enzymes: *Pvu*II, *Stu*I, *Nco*I, *Ava*II, *Apa*LI, *Xba*I, *Bam*HI, *Eco*RI, *Bgl*II, *Hin*dIII, *Kpn*I, *Sac*I, and *Eco*RV. Subsequently, DNA fragments were separated by size on 0.7%–0.8% (w/v) agarose and were transferred to nylon membranes (Hybond-N; Amersham International, UK) by Southern blotting (Maniatis et al. 1982, pp. 382–386). Probes were labeled with α^{32}P-dCTP by using a random oligonucleotide priming kit (Amersham International, UK). Two cDNA clones of the human LDL-receptor gene were a gift from Dr. D. W. Russell (Dallas): pLDLR-2HH1 is a plasmid which contains a *Bam*HI insert of 1.9 kb corresponding to the last eight exons of the gene, and pTZ1 is a plasmid which contains the whole coding sequence of the gene, in a *Hin*dIII insert of 2.6 kb.

Table 1

Summary of Data Concerning FH-Pavia Pedigree

Pedigree Position (sex)	Age (years)	LDL Cholesterol (mg/dl)	Tendon Xanthomas[a]	Arcus Corneae	Myocardial Infarction[a]	Carrier of Mutation	
						Documented	Probable[b]
XIII.2 (M)	17	244				Yes	
XIII.4 (F)	17	260				Yes	
XII.3 (M)	48	346	+	+		Yes	
XII.6 (F)	24	236				Yes	
XII.7 (M)	42	402	+	+		Yes	
XII.9 (F)	44	298	+			Yes	
XII.16 (M)	26	392				Yes	
XI.2 (F)	74[c]	270	+	+	+	Yes	
XI.6 (M)	63	316	+	+		Yes	
XI.7[d] (M)	69	288	+	+	+	Yes	
XI.9 (F)	50[c]				+		Yes
XI.10 (M)	76	254	+			Yes	
XI.15 (F)	54	270				Yes	
XI.16 (M)	47[c]				+		Yes
XI.22[e] (F)	51	325				Yes	
XI.23[f] (M)	55	380	+	+	+	Yes	

Note.—FH individuals of generation XIII, XII, and XI, families belonging to M., S., and P., are boxed in fig. 1.

[a] A plus sign (+) denotes that condition is present.

[b] Individuals with clinical history of hypercholesterolemia and coronary heart disease.

[c] Death from myocardial infarction.

[d] Proband of family M.

[e] Proband of family S.

[f] Proband of family P.

Prehybridization and hybridization of the filters were carried out according to standard procedure (Maniatis et al. 1982, pp. 387–389). Filters were subjected to autoradiography on Kodak XAR-5 film for 24–48 h at −80°C. Densitometric scanning of X-ray films was performed by using a laser densitometer (UltroScan XL; Pharmacia LKB Biotechnology, Sweden).

Fibroblast Cultures

Skin biopsies were taken from the three probands and from four control subjects. Explants were cultured in 25-cm^2 flasks in Dulbecco's modification of Eagle's medium (DMEM), 100 IU of penicillin/ml, and 50 μg of streptomycin/ml, 2 mM glutamine, and 15% FCS (Gibco, UK), under 95% air and 5% CO_2.

Northern Blot Analysis

Total cellular RNA was extracted, in guanidine-thiocianate (Chomczynsky and Sacchi 1987), from cultured skin fibroblasts and was maintained in lipoprotein-deficient serum (LPDS) for 15 h. Total RNA (15 μg) was denatured in 50 μl of 50% formamide, 2.2 M formaldehyde, and 1 × MOPS buffer (20 mM 3-(N-morpholino)-propane-sulfonic acid, 5 mM sodium acetate, and 1 mM Na_2 EDTA) and was separated on 1.2% agarose in 1 × MOPS buffer. After separation, RNA was transferred to Hybond-N membranes and hybridized with either a probe specific for exons 15–17 or a probe specific for exon 1. The same membranes were stripped and rehybridized with the cDNA clone pHFβA-1 of human β-actin. β-Actin mRNA was used as an internal standard to ascertain the amount of RNA. Prehybridization and hybridization were performed according to a procedure described elsewhere (Lelli et al. 1991*b*).

cDNA Synthesis

Total RNA from cultured fibroblasts of the probands was used to synthesize single-stranded cDNA. The reaction was performed by using a cDNA synthesis kit (GIBCO-BRL, UK), 10 μg of RNA, and an oligonucleotide exon 13–specific primer (13 reverse [13rv]: 5′ GTTGTGGAAGAGGACCATATC 3′). Single-stranded cDNA was also synthesized from a control subject. In this case we used an oligonucleotide exon 4–specific primer (4 reverse [4rv]: 5′ ACGAACTGCCGAGAGATGCAC 3′). The single-stranded cDNA was ethanol precipitated, and PCR was performed in the same test tube.

PCR

The RNA-DNA hybrids were used to amplify the junction between exon 1 and exon 13 (in FH probands) or between exon 1 and exon 2 (in a control subject). To amplify the junction exon 1–exon 13 in FH subjects, we used an exon 1–specific primer (1 forward [1fr]: 5′ CGAGTGCAATCGCGGGAAGC 3′) and the antisense exon 13–specific primer used for the synthesis of the first-strand cDNA (i.e., 13 rv; see above). To amplify the junction exon 1–exon 2 in the control subject, we used both the same exon 1–specific primer (i.e., 1fr) and the same antisense exon 4–specific primer (i.e., 4rv) that were used for the synthesis of single-stranded cDNA, as specified above. The first cycle was used to synthesize the second strand of the cDNA and was carried out at 93°C for 7 min, at 60°C for 1 min, and at 72°C for 3 min; the following 25 cycles were carried out at 93°C for 1 min, at 60°C for 30 s, and at 72°C for 3 min; in the last cycle the time for the extension was 7 min. An asymmetric PCR was performed to obtain single-stranded DNA (Innis et al. 1990); the molar ratio of the two primers was 100:1. One-tenth of the amplified material was fractionated by gel electrophoresis on 1.5% agarose, to check both the amount and size of the single-stranded DNA, and the remaining amplified material was purified by using a Centricon-100 microconcentrator (Amicon Division, W. R. Grace, USA), and was sequenced directly.

Sequencing

Sequencing of single-stranded DNA was performed by the dideoxynucleotide chain-termination method (Sanger et al. 1977), using the enzyme Sequenase (United States Biochemical, USA) and γ-^{32}P ATP as labeled nucleotide (Amersham International, UK). The primers used to sequence both strands were the same as those used in the PCR reaction (see above).

Assay of LDL Receptor Activity

LDL (1.019–1.063 g/ml) was separated, by sequential ultracentrifugation, from a control pool of fresh plasma. LPDS was prepared according to standard procedure (Goldstein et al. 1983). LDL and LPDS were used after an extensive dialysis against 0.154 M NaCl and 0.001M Na_2EDTA; protein concentration was determined by the Lowry method (Lowry et al. 1951). LDL was iodinated with [$Na^{125}I$], according to a modification of McFarlane's technique (Goldstein et al. 1983). The final specific activity of [^{125}I]-LDL was 150–300 cpm/ng of protein.

Freshly prepared [^{125}I]-LDL was used in each experiment. Cultured fibroblasts from the three probands and from control subjects were assayed for [^{125}I-]-LDL binding, internalization, and degradation, according to a standard procedure (Goldstein et al. 1983). Specific binding, internalization, and degradation of [^{125}I]-LDL were calculated by subtracting values obtained in the presence of unlabeled LDL from those obtained in its absence (Goldstein et al. 1983). In each experiment, fibroblasts from a control subject were incubated at the same time and with the same batch of labeled LDL as were the cells of the probands.

Genealogical Studies

In our attempt to discover a common ancestor of the three families, we traced them back 13 generations, to the year 1615. The information we obtained came from three sources: interviews with the family members, local council records, and parish archives. The latter provided essential data with regard to birth, marriage, and death.

For generations X and XI (fig. 1), it was possible to consult clinical records of deceased subjects. For preceding generations, there was a step-by-step analysis of each married couple. It was assumed that the carrier of the disease was the partner who either died prematurely or had siblings who died prematurely. It should be noted that in three cases (VII.1, VII.6, and VII.9) the local priest wrote next to the name the Latin words *repentina mors* or *repentinus morbus* (literally "sudden death" and "acute and severe illness"), suggesting that the person in question experienced sudden death caused by a heart attack.

Results

Genomic DNA Analysis

The results of Southern blot analysis of genomic DNA from all probands are summarized in figure 2. The first evidence of a large rearrangement of the LDL receptor gene came from hybridization performed with probes complementary to the 3′ half of the gene. Both DNA digestion with several enzymes and autoradiography showed, in all probands, that, besides normal fragments, additional fragments either smaller or larger were present (fig. 3). When probes complementary to the 5′ half of the gene were used, autoradiograms did not show abnormal bands, but only bands whose intensity was half that of control DNA (fig. 3). These findings suggested that the probands were heterozygous for a deletion involving the first 12 exons (fig. 2).

In order to define the 3′ boundary of the deletion, DNA was digested with *Xba*I, *Ava*II, and *Ava*II/*Xba*I and was hybridized with a probe specific for exons 11–15. Figure 4 shows that the *Xba*I site located in intron 12 was maintained, whereas the *Ava*II site in intron 11 was deleted. These results indicated that the 3′ boundary of the deletion was located in the 5′ half of intron 12 (figs. 2 and 4). To define the 5′ boundary of the deletion, DNA was digested with several enzymes and was hybridized with a probe specific for exons 1–4. Autoradiograms showed that the *Eco*RI, *Bgl*II, and *Xba*I restriction sites located in intron 1 and more closely adjacent to exon 1 were maintained (fig. 2), suggesting that exon 1 was not involved in the deletion. To confirm this, FH DNA was subjected to *Kpn*I/*Bam*HI double digestion and was hybridized with probes complementary to exon 1 and to exons 13–14. In all probands, both probes detected (*a*) normal size bands (5- and 5.5-kb band, respectively) possessing half the intensity of the corresponding bands in control DNA; (*b*) an abnormal size band of about 7 kb, produced by the *Kpn*I and *Bam*HI sites in intron 14 and 5′ to exon 1, respectively. This fragment, recognized by both probes, results from the bridging of exon 1 to exon 13.

Northern Blot Analysis

Northern blot analysis revealed two LDL-receptor mRNA species in skin fibroblasts taken from the probands; one species had a normal size of 5.3 kb, and the other had a smaller size of about 3.5 kb (fig. 5). This is consistent with the foreseen deletion of 1,778 bp in the transcript. Both mRNA species were detectable either by using a probe specific for exons 15–17 or a probe specific for exon 1.

Molecular Characterization of the Mutant mRNA

To ascertain whether, in FH probands, exon 1 sequence was followed by exon 13 in FH mRNA, the cDNA spanning the junction of these exons was amplified by asymmetric PCR and then was sequenced. By using 1fr and 13rv primers, we obtained an amplified fragment of 282 bp. Both strands of the amplified fragments were sequenced. The sequence of the amplified single-stranded DNA showed that exon 1 was normally transcribed, and its last nucleotide G was joined to the first two nucleotides GA of exon 13 (fig.

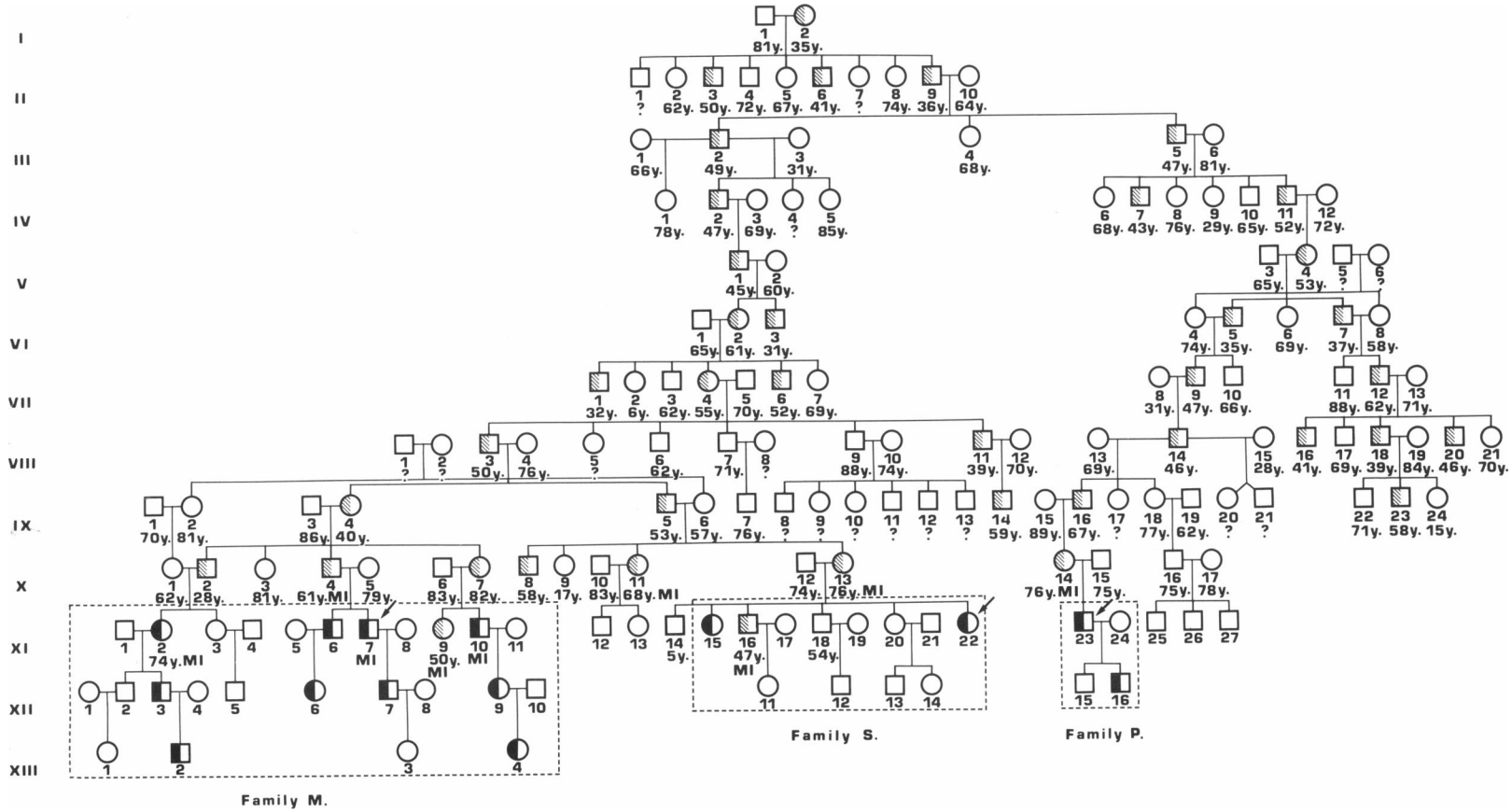

Figure 1 Pedigree of FH-Pavia family, 1615–1991. Numbers indicate age at death. The squares denote males, and the circles denote females; ◪ and ◔ = presumed heterozygote carrier of the mutation; ◧ and ◐ = documented heterozygote carrier of the mutation. MI = myocardial infarction. The arrows denote probands.

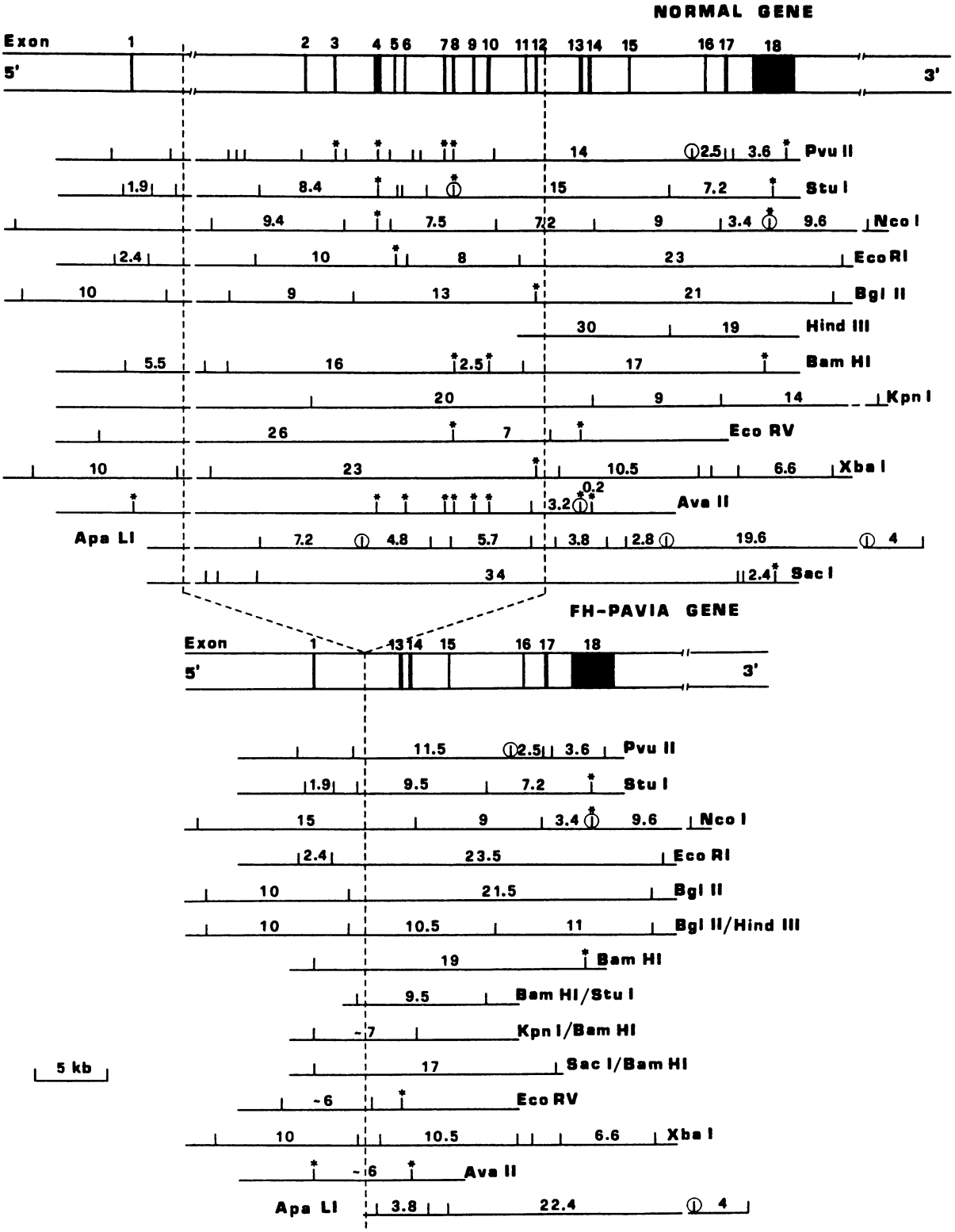

Figure 2 Restriction maps of the normal LDL-receptor gene and of the FH-Pavia gene. The *Ava*II and *Apa*LI polymorphic sites in exon 13 and intron 15 were not present in the mutated gene. * = Site in cDNA; and ⊙ = polymorphic site.

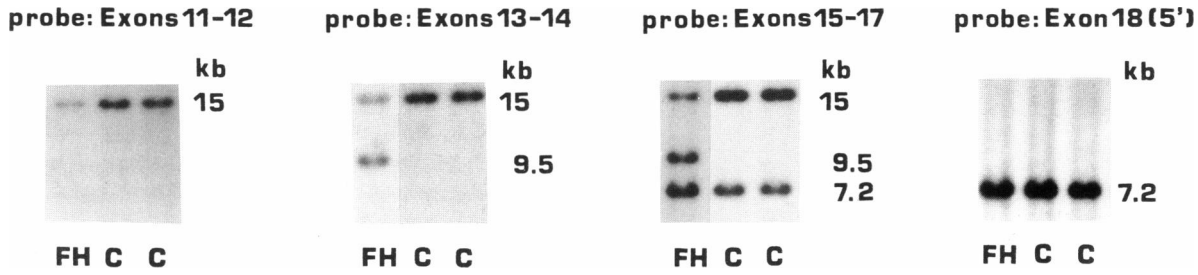

Figure 3 Southern blot analysis of *StuI* digest in a control (lanes C) and in a proband (lanes FH), after hybridization with exon-specific probes.

6, *left panel*). This abnormal junction changed the reading frame, giving rise to a sequence of 28 novel codons followed by the premature appearance of a TGA stop codon. In control mRNA the sequence of exon 1 was followed by that of exon 2, as expected (fig. 6, *right panel*).

Assay of LDL Receptor Activity

Specific binding, internalization, and degradation of [^{125}I]-LDL were measured in fibroblasts taken from the proband of each of the three families and from four control subjects and were markedly reduced (by

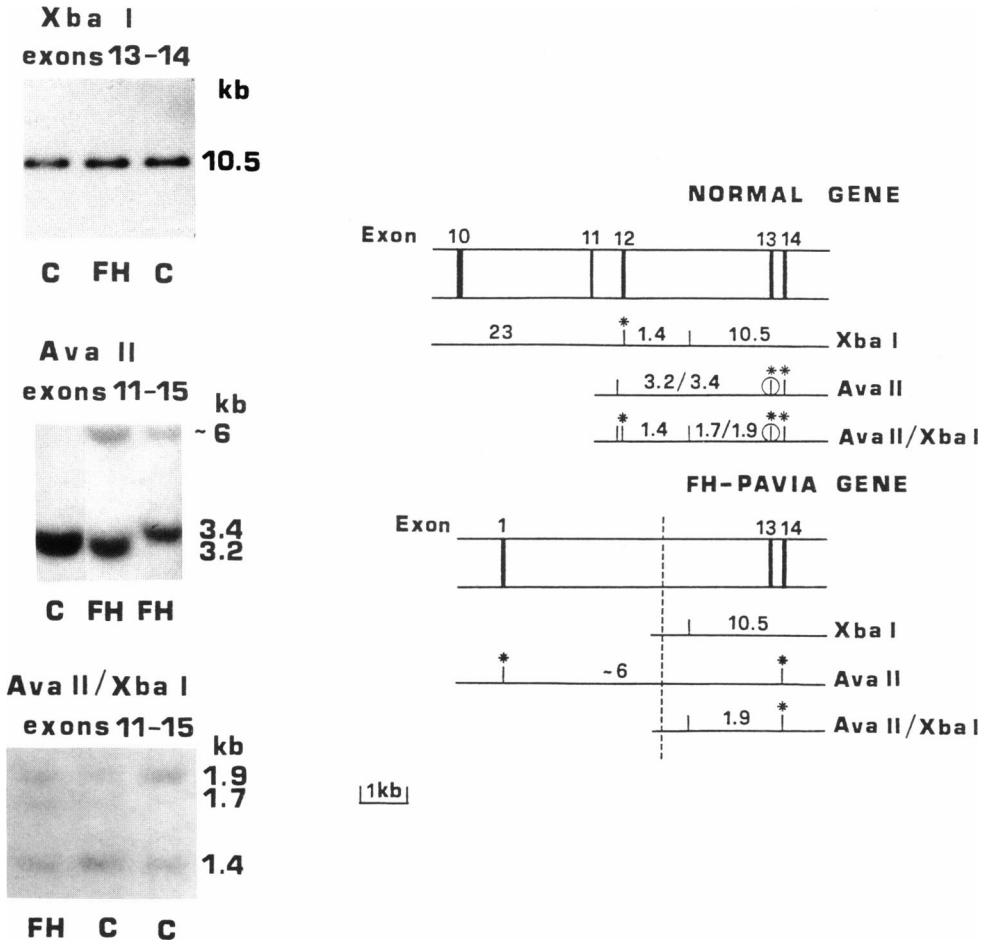

Figure 4 Southern blot analysis of *Xba*I, *Ava*II, and *Ava*II/*Xba*I digests in a control (lanes C) and in a proband (lanes FH). The exon-specific probes used for hybridization are indicated. Details of restriction map of mutated and normal genes are shown. Symbols are as in fig. 2.

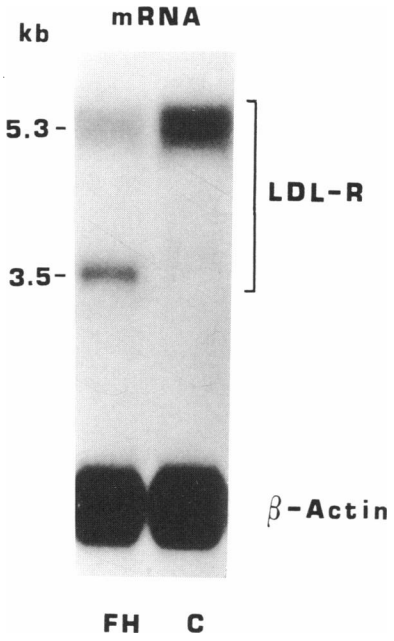

Figure 5 Northern blot analysis of LDL-receptor mRNA isolated from skin fibroblasts of proband of FH-Pavia (lane FH) and of a control (lane C) and hybridized with a probe specific for exons 15–17. The same result was obtained after hybridization with a probe specific for exon 1.

40%–55%, vis-à-vis control values) in proband fibroblasts.

Haplotype Analysis

To identify the haplotype which cosegregated with the disease in all affected FH heterozygotes, haplotypes were constructed by using the following restriction endonucleases: *Stu*I, *Ava*II/*Xba*I, *Apa*LI/*Bam*HI, *Pvu*II, and *Nco*I (Hobbs et al. 1990). As shown in figure 7, all affected individuals had the following haplotype: V1 (absence of *Ava*II site), A1 (absence of *Apa*LI site), and N2 (presence of *Nco*I site). Since the *Stu*I and *Pvu*II digestions gave abnormal fragments (S* and P*, respectively), we attempted to derive the haplotype of these enzymes. This was only possible for *Pvu*II, since the polymorphic site lies 3′ to the deletion. All affected members were found to be P2 (presence of *Pvu*II site) and were denoted P*2 (fig. 7). The haplotype S*V1A1P*2N2 was present in all the affected members of the three families, strongly suggesting a common LDL-receptor gene defect.

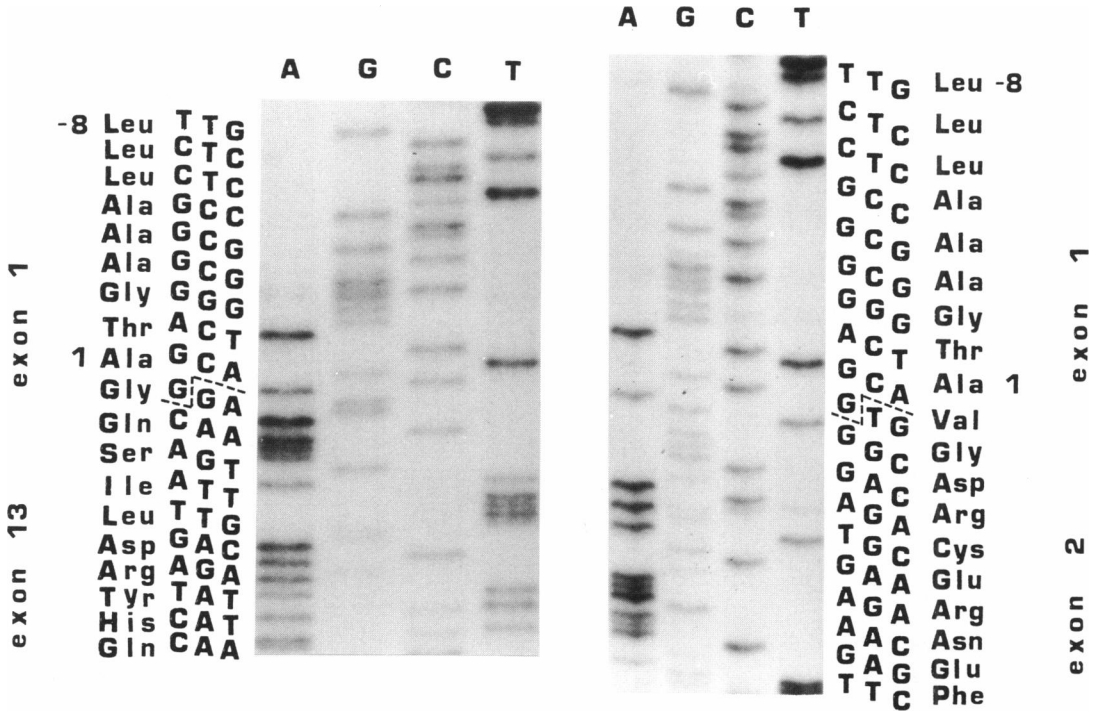

Figure 6 *Left panel,* cDNA nucleotide sequence corresponding to the junction between exon 1 and exon 13 in an FH-Pavia proband. *Right panel,* cDNA nucleotide sequence corresponding to the junction between exon 1 and exon 2 in a control subject. The fourth codon (TGC) of exon 2 of cDNA of the control subject differs from the sequence TGT of the corresponding codon published by Yamamoto et al. (1984). Both codons encode cysteine. The T-C transition represents a common polymorphism (Soutar 1991).

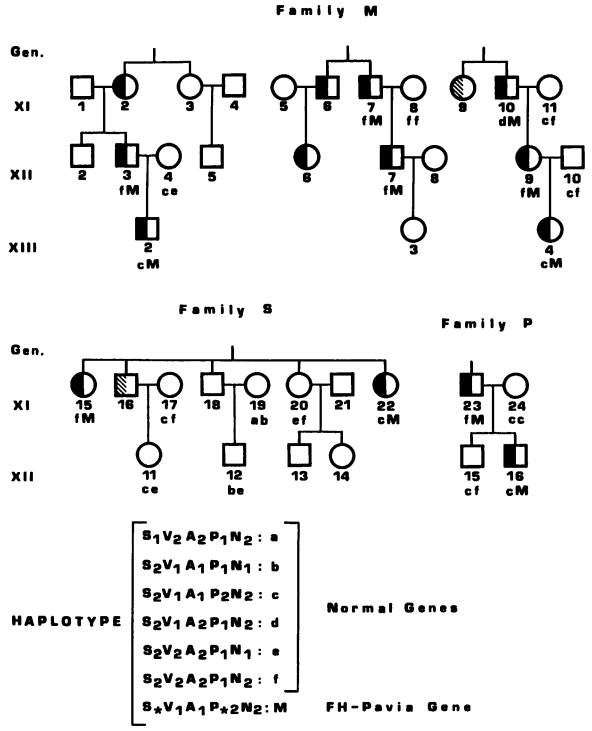

Figure 7 Haplotype analysis in some members of the three families. Restriction enzymes with polymorphic sites in LDL-receptor gene are *Stu*I (S), *Ava*II/*Xba*I (V), *Apa*LI/*Bam*HI (A), *Pvu*II (P), and *Nco*I (N). Genotype 1 indicates the absence of the restriction site, and genotype 2 indicates the presence of the restriction site. * = presence of an abnormal fragment. Hybridizations were carried out by using an exon-specific probe. Symbols are as in fig. 1.

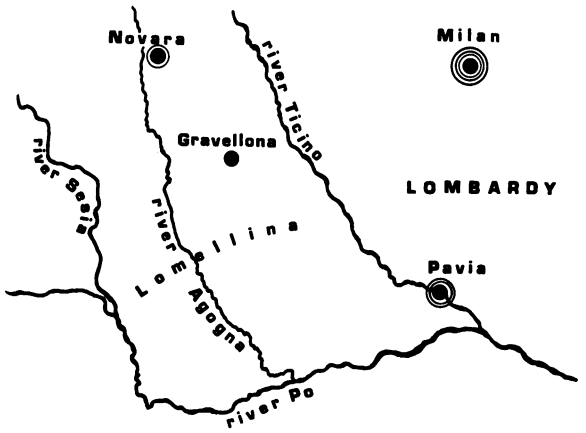

Figure 8 Geographical map of the area of northern Italy (Lomellina) identified as the starting point of the mutation designated FH-Pavia.

Historical Survey

The three families were apparently unrelated. The disease was present in all three branches of family M. and had its origin in two brothers and a sister in the 10th generation. Brother (X.2) died at 28 years of age during the First World War. It is likely that this subject was a FH heterozygote, since hypercholesterolemia and ischemic heart disease were present among his descendants.

A relationship between families M. and S. was discovered at generation IX, where we found the respective ancestors (IX.4 and IX.5), who died prematurely. It is most likely that their father (VIII.3) was the parent affected, since he died at 50 years of age, whereas his wife died at 76 years of age. This subject was born in 1835 in Gravellona (a small town near the city of Pavia, in the farming district of Lomellina) (fig. 8).

The relationship between family P. and families M. and S. was found only after tracing them back 12 generations, to the middle of the 17th century. The common ancestor (II.9) was a man born in Gravellona in 1641, who died in 1677. One of his sons (III.2) initiated the line which in time led to families M. and S., and the other son (III.5) initiated the line leading to family P. The common ancestor (II.9) probably inherited the disease from his mother (I.2), who was born in 1615 and who died at 35 years of age; his father (I.1) was probably unaffected, since he died at 81 years of age.

Starting from generations VI and V and tracing back to generation II, we found a surname that the members of the two principal branches of the family tree had in common; "Piccolini" was the surname of subjects VI.2, VI.3, V.1, V.4, IV.2, IV.11, III.2, III.5, and II.9. This surname is exactly the same as the name of the village in which subject I.1 was born and which is only a few kilometers from Gravellona. Therefore, Gravellona has been identified as the starting point of the mutation in question, as of the second half of the 17th century.

Discussion

Here we describe a novel mutation of the LDL-receptor gene in three families in northern Italy. The molecular analysis of the defective gene showed that about 25 kb, including exons 2–12, were deleted. All the affected members of the three families were heterozygous for the mutation. This deletion and the one we have described elsewhere—i.e., FH-Bologna-1 (Lelli

et al. 1991*b*)—are the largest deletions (Kajinami et al. 1990) reported in this gene. The mutated allele was transcribed into a smaller-size (3.5-kb) mRNA. We demonstrated that in this mRNA the sequence corresponding to exon 1 was correctly spliced to exon 13. The receptor protein deduced from the sequence of defective mRNA is a short polypeptide of 29 amino acids, devoid of any function. Therefore we can infer that this mutation belongs to the first class of the functional LDL-receptor protein defects (i.e., no detectable receptor protein [Goldstein and Brown 1989]).

Both the molecular characterization of the defective gene and the haplotype analysis showed that in all the affected subjects the mutation cosegregated with the same haplotype. The haplotype V1A1P2N2 present in the affected members of the three families is not rare, either in our study population or in other populations. Its frequency ranges from .21 (Leitersdorf et al. 1989*a*) to .27 (authors' unpublished observation).

The mechanism responsible for the deletion of exons 2–12 could involve the Alu-repetitive sequences present in intron 1 (Lehrman et al. 1987*a*) and in intron 12 (Horsthemke et al. 1987), which are oriented in the same direction. A recombination between Alu sequences has been documented as the cause of other deletions, both in this gene and in other genes (Hobbs et al. 1986; Lehrman et al. 1986, 1987*b*; Horsthemke et al. 1987). One way of testing this hypothesis would be to apply PCR to genomic DNA in order to amplify the junction between introns 1 and 12. Unfortunately, both the lack of information on the sequence of intron 1 (>10 kb in size) and intron 12 (3 kb in size) (Südhof et al. 1985) and the fact that all probands are heterozygotes makes this a difficult undertaking. The alternative would be to prepare a genomic library, subclone the fragment of the mutated LDL-receptor gene, and sequence it. This work is now in progress in our laboratory.

In our reconstruction of the family tree, we traced back from one generation to the preceding one, assuming that the parent affected by the disease was the one who either had died prematurely or had close relatives who had died prematurely. Clearly, there must be a certain degree of error in the method followed, since the causes of premature death might have been other than coronary heart disease. Such a consideration is of some relevance to a historical period covering some three centuries in which the various aspects of life in that area of northern Italy were particularly harsh (Arecchi 1983). However, the step-by-step historical investigation led us back to a common ancestor of the three families M., S. and P., thus supporting our assumption with regard to the family members affected by FH in each generation. Since Pavia is the capital of the province where Gravellona is located (fig. 8), we named this mutation "FH-Pavia."

Although there are no complete surveys of LDL-receptor gene mutations in a large number of FH families in ethnically heterogeneous populations, the available data seem to suggest that, in most populations, FH is caused by a large variety of LDL-receptor gene defects. Only in certain populations which, because of geographical, social or religious reasons, have remained isolated for centuries, do a high percentage of FH cases carry the same mutation, through a "founder gene" effect. Clusters of families with the same mutation were found in the French-Canadian population (Leitersdorf et al. 1990), in the Finnish population (Aälto-Setala et al. 1989), in the Christian Lebanese (Lehrman et al. 1987*c*), and in the Afrikaners in South Africa (Leitersdorf et al. 1989*b*).

Italy is a country which over the centuries has had not only numerous but also lengthy contacts with foreign populations (Calvi 1874). Consequently, there are good grounds for claiming that our population is of heterogeneous origin. It was therefore surprising to find the same mutation causing FH in three families living in different parts of northern Italy. A historical investigation into these families supplied us with the common ancestor. This report is the second example of a historical investigation carried out in order to discover the common ancestor in different families with the same mutation causing FH; the first report dealt with Afrikaans-speaking South Africans whose common ancestor arrived in South Africa in 1692 (Torrington and Brink 1990).

Our examination was limited to three families, and we have not yet extended our study to looking into all the descendants of the common ancestor. Such an extended investigation could lead to the discovery of other families carrying this mutation and, consequently, to timely therapeutic intervention in order to delay and perhaps even prevent the clinical onset of coronary heart disease.

Acknowledgments

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