A mutation (−49C>T) in the promoter of the low density lipoprotein receptor gene associated with familial hypercholesterolemia

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Abstract **We have identified a mutation (−49C>T) in the low-density lipoprotein receptor (LDLR) gene in a Spanish familial hypercholesterolemia (FH) patient. The mutation maps within repeat 3 of the LDLR gene promoter. This region binds Sp1 and collaborates with repeat 2 in the regulation of LDLR gene by sterols. To evaluate whether the mutation influenced the activity of the promoter, luciferase reporter plasmids containing 296 bp of the proximal promoter region were constructed. In transient transfection assays in HepG2 cells, the mutation resulted in an 80% reduction of promoter activity. Also, gel-shift assays demonstrated that the mutation severely affects Sp1 binding. However, the mutated promoter still retains the ability to respond to low sterol concentrations. As the analysis of the LDLR gene did not reveal any other changes, we conclude that the −49C>T mutation is the cause of FH in the patient. The analysis of the proband's pedigree indicated that not all the members of the family having the mutation disclose a FH phenotype. These results support the view that factors other than the presence of the mutation are important in the determination of the clinical phenotype in FH.**—Mozas P., R. Galetto, M. Albajar, E. Ros, M. Pocoví, and J. C. Rodríguez-Rey. **A mutation (−49C>T) in the promoter of the low density lipoprotein receptor gene associated with familial hypercholesterolemia.** ***J. Lipid Res.*** **2002.** 43: **13–18.**

Supplementary key words gene expression • familial hypercholesterolemia • deoxyribonucleic acid • fetal bovine serum • low-density lipoprotein receptor gene

Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by elevated levels of plasma LDL, tendon xanthomas, and an increased frequency of coronary heart disease (CHD). FH is caused by mutations in the gene coding for the low-density lipoprotein receptor gene (LDLR), which result in an impaired clearance of LDL from plasma (1). Since the first description of the molecular basis of this disorder, a great number of mutations have been described (2, 3). Although most of the mutations map in the coding region of the gene, an increasing number of mutations in the regulatory regions are currently being described (4–9).

The basic regulatory region of the LDLR gene is located within 177 bp of the proximal promoter and consists of a TATA-like sequence and three imperfect direct repeats of 16 bp. Repeats 1 and 3 contain binding sites for the transcription factor Sp1 and contribute to the basal expression of the gene (10). Sterol-dependent repression of transcription is mediated by a 42 bp region encompassing repeats 2 and 3 (11). As mentioned, repeat 3 is a positive element that binds Sp1. On the other hand, in the presence of sterols, repeat 2 confers strong repression on repeat 3 (12). Sp1 and the sterol regulatory element-binding protein (SREBP), which bind to a 10 bp sequence within repeat 2, cooperate in the repression of repeat 3 (13).

Due to the importance of repeat 3 in LDLR gene regulation, it is not unexpected that mutations in this repeat affecting the binding of Sp1 result in FH phenotypes. Accordingly, four different mutations in repeat 3 giving rise to FH phenotypes have been described. They map at positions −42, −44 (6), −43 (5) and −45 (9), all within the 10 bp se-

Abbreviations: apoE, apolipoprotein E; CHD, coronary heart disease; EMSA, electrophoretic mobility shift assay; FH, familial hypercholesterolemia; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor gene; SREBP, sterol regulatory element binding protein; SSCP, single-strand conformation polymorphism.

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quence ACTCCTCCCC (coordinates −50 to −41), which resembles the consensus GC box sequence identified as the Sp1 binding site (14).

Here we describe a new mutation in repeat 3 of the LDLR gene at position −49 in several members of a Spanish FH family. This mutation decreases LDLR promoter activity in reporter gene assays and also impairs the ability of repeat 3 to bind Sp1 in gel retardation assays. Interestingly, some carriers of this mutation do not disclose hypercholesterolemia, suggesting the existence of other factors in the development of the FH phenotype.

METHODS

FH proband and family

The proband was a male born in 1961, referred for evaluation to the Lipid Clinic of Hospital Clínic, Barcelona, in 1995 because of hypercholesterolemia. At age 33 he developed effort angina and was admitted for coronary angiography, which revealed 80% occlusion of one coronary vessel. An angioplasty was performed, but angina recurred after eight months. A restenosis was found at repeat angiography and was treated by placement of an intraluminal stent. He practiced non-competitive sports regularly, had never smoked, had a blood pressure of 115/74 mm Hg, and his body mass index was 24.6 Kg/m^2. No corneal arcus, xanthelasma, or tendon xantomas were observed on clinical examination. Serum cholesterol was 9.95 mmol/l, HDL cholesterol (HDL-C) 0.98 mmol/l, LDL cholesterol (LDL-C) 8.15 mmol/L, triglycerides 1.80 mmol/l, and lipoprotein(a) 10 mg/dl. After 2 months on a Mediterranean-type hypolipidemic diet, the serum LDL-C was reduced by 25% to 6.07 mmol/l. Simvastatin treatment (20 mg daily) was started, resulting in an additional 55% reduction of LDL-C to 3.67 mmol/l. Addition of colestipol (10 g daily) further lowered LDL-C to 2.38 mmol/l, and combined simvastatin-colestipol therapy has been continued for the last 3 years.

There was a strong family history of hyperlipidemia and coronary heart disease. The proband's mother had severe hypercholesterolemia, but was free of coronary artery or other vascular diseases. One maternal uncle was known to have a lipid phenotype typical of heterozygous FH and had died of myocardial infarction at age 58. The maternal grandparents lived to old age, but the grandmother suffered from cardiac angina since age 60, and she died of myocardial infarction at age 90. Both were of Spanish origin. The proband's mother and his youngest brother also were referred to the Lipid Clinic in 1995 for evaluation of hypercholesterolemia. The mother had bilateral corneal arcus and xanthoma of the Achilles' tendon. She had no non-lipid cardiovascular risk factors, and she already followed a hypolipidemic diet. Her on-diet LDL-C was 7.20 mmol/l and was lowered to 3.28 mmol/l after treatment with simvastatin (20 mg daily), a 54% reduction. The brother had no classical cardiovascular risk factors, but had a LDL-C of 6.40 mmol/l. He was advised to follow a hypolipidemic diet and showed an excellent response, with a LDL-C reduction of 35% to 4.16 mmol/l. Their plasma lipid lipoproteins levels are shown in **Table 1**. This study was approved by the Ethics Committee of the Hospital Clínic i Provincial, Barcelona. All the participants gave their informed consent.

Mutation identification and apolipoprotein E (apoE) genotyping

All the exons, the exon/intron junctions as well as 660 bp of the proximal promoter of the LDLR gene were analyzed for the presence of mutations by single strand conformation polymorphism (SSCP) methods. All the procedures and the primers used have already been described (15). The presence of the mutation in the promoter region was subsequently determined by EcoRI digestion of a PCR fragment amplified with the primers 5′-GAAAATCACCCCACTGCGAA-3′ and 5′-ACCTGCTGTG TCCTAGCTGG-3′. The underlined base shows the mismatch that produces an EcoRI site when the mutation is present. ApoE genotyping was carried out as previously described (16).

TABLE 1. Characteristics of the proband and his relatives

Subject	Age	Gender	CHD[a]	TC	LDL-C	HDL-C	TG	Mutation
I-1	−	M	−	NA	NA	NA	NA	NA
I-2	−	F	+(61)	NA	NA	NA	NA	NA
II-1	−	M	NA	NA	NA	NA	NA	NA
II-2	−	M	+(58)	NA	NA	NA	NA	NA
II-3	70	F	−	6.83	4.25	2.04	0.95	−
II-4	66	F	−	9.30	7.20	1.50	1.28	+
II-5	67	M	−	5.02	3.36	1.19	1.03	−
III-1	39	M	+(33)	9.95	8.15	0.98	1.80	+
III-2	39	M	−	4.97	3.08	1.63	0.54	−
III-3	37	M	−	4.61	3.08	0.85	1.44	−
III-4	36	M	−	7.98	6.40	1.28	0.63	+
III-5	−	F	NA	NA	NA	NA	NA	NA
III-6	30	F	−	5.83	4.18	1.04	1.35	+
III-7	27	F	−	6.27	4.07	1.86	0.73	+
IV-1	6	M	−	4.17	2.33	2.17	0.34	−
IV-2	6	M	−	4.22	2.56	1.52	0.29	+
IV-3	6	F	−	4.27	2.38	1.68	0.44	−

Subjects' numbering according to the pedigree in Fig. 4. Data expressed in mmol/l. NA, data not available.

[a] CHD Coronary Heart Disease (age of onset).

Reporter plasmids

A 276 bp fragment of the LDL-R gene promoter extending from position −225 to position +51 was amplified with primers modified to contain *Hin*dIII and *Bgl*II sites and cloned in the PCR vector pGEMT(Promega, Madison, WI). After excision with these enzymes, the fragments were cloned in the promoterless luciferase vector pXP2 (17) and used in transient expression assays.

Cell culture, transfections, and luciferase assays

HepG2 cells were cultured in DMEM supplemented with 10% FBS and antibiotic/antimicotic mix (Life Sciences). The day prior to transfection the cells were seeded in T6 plates at a cell density of 150,000 cells per well. The following day the medium was replaced by 2 ml of Optimem (Life Sciences). For transfection we used 1.5 μg of plasmid and DOTAP(Roche, Postfach, Switzerland) according to manufacturer's instructions. Fifty ng of the plasmid pRL-SV40 were included in each transfection as a control of transfection efficiency. The DNA-cells mixtures were incubated for 6 h and then the cells were incubated for another 48 h in the medium indicated before analyzing luciferase gene expression with the Dual Luciferase Report Assay System (Promega). Duplicate cells were assayed for each transfection condition and at least three independent transfection assays were performed.

SL-2, a *Drosophila* cell line devoid of Sp-1, was cultured in Schneider medium supplemented with 10% FBS. To obtain nuclear extracts containing Sp-1, SL-2 cells were transfected with the expression plasmid pPac-SP1 (18).

Electrophoresis mobility shift assay (EMSA)

Binding reactions were carried out in 20 μl of annealing buffer (20 mM Hepes pH 7.5, 50 mM KCl, 0.175 mM EDTA, and 5% glycerol) containing 1.5 μg of polydIdC and 0.4 μg of ssDNA in the presence or absence of 5 μg of nuclear extracts. The reac-

tions were incubated at 4°C for 10 min prior to the addition of 0.25 pmol of the ^{32}P-labeled DNA probe. The reactions were then incubated for 15 min at 4°C. The DNA-protein complexes were resolved from the free probe by electrophoresis at room temperature on a 5% polyacrylamide gel in 0.25 × TBE buffer (9 mM Tris pH 8, 90 mM boric acid, 20 mM EDTA). The sequences of the double stranded oligonucleotides used were LDL-Rwt: 5′-TGCAAACTCCTCCCCCTGCTAGAAA-3′ and LDL-Rmut: 5′-TGCAAATTCCTCCCCCTGCTAGAAA-3′. The mutated base is underlined in the sequence. Nuclear extracts were prepared either from SL-2 cells (used as negative control) or from Sp1-transfected cells, following the procedure described (19). In the supershift experiments, 2 μg of the Sp1-specific antibody SC-59X (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction prior to the addition of the labeled oligonucleotide.

RESULTS

Genomic DNA from 36 individuals with a clinical diagnosis of FH was analyzed for the presence of mutations in the LDLR gene. For that purpose, all the exons, the exon/intron junctions and the 660 bp of the proximal promoter region were studied by SSCP. As expected, most individuals showed mutations in the coding sequence of the LDLR gene, which could explain the FH phenotype (15). An abnormal SSCP pattern of the proximal LDLR 5′ fragment was observed in one of the patients (**Fig. 1A**). Sequencing analysis of this fragment revealed a C>T transition at the position −49 (Fig. 1B and C). This mutation was confirmed by restriction analysis of PCR fragments amplified by using mismatched primers that create an artificial EcoRI site when the mutation is present (Fig. 1D). The existence of major deletions or insertions was also tested by Southern blot with negative results. The mutation was found mapped within repeat 3 of the LDLR gene promoter, a region known by its importance in LDLR gene regulation (10, 11), and thus could constitute the basis of the FH phenotype present in this individual.

To test the possible influence of this mutation on the properties of the LDLR gene promoter, the strength of the promoter harboring the mutation was compared to that of the normal allele. For that purpose, 276 bp fragments extending from position −225 to position +51 from either mutant or normal alleles were cloned at the 5′ end of the luciferase gene of the promoterless plasmid

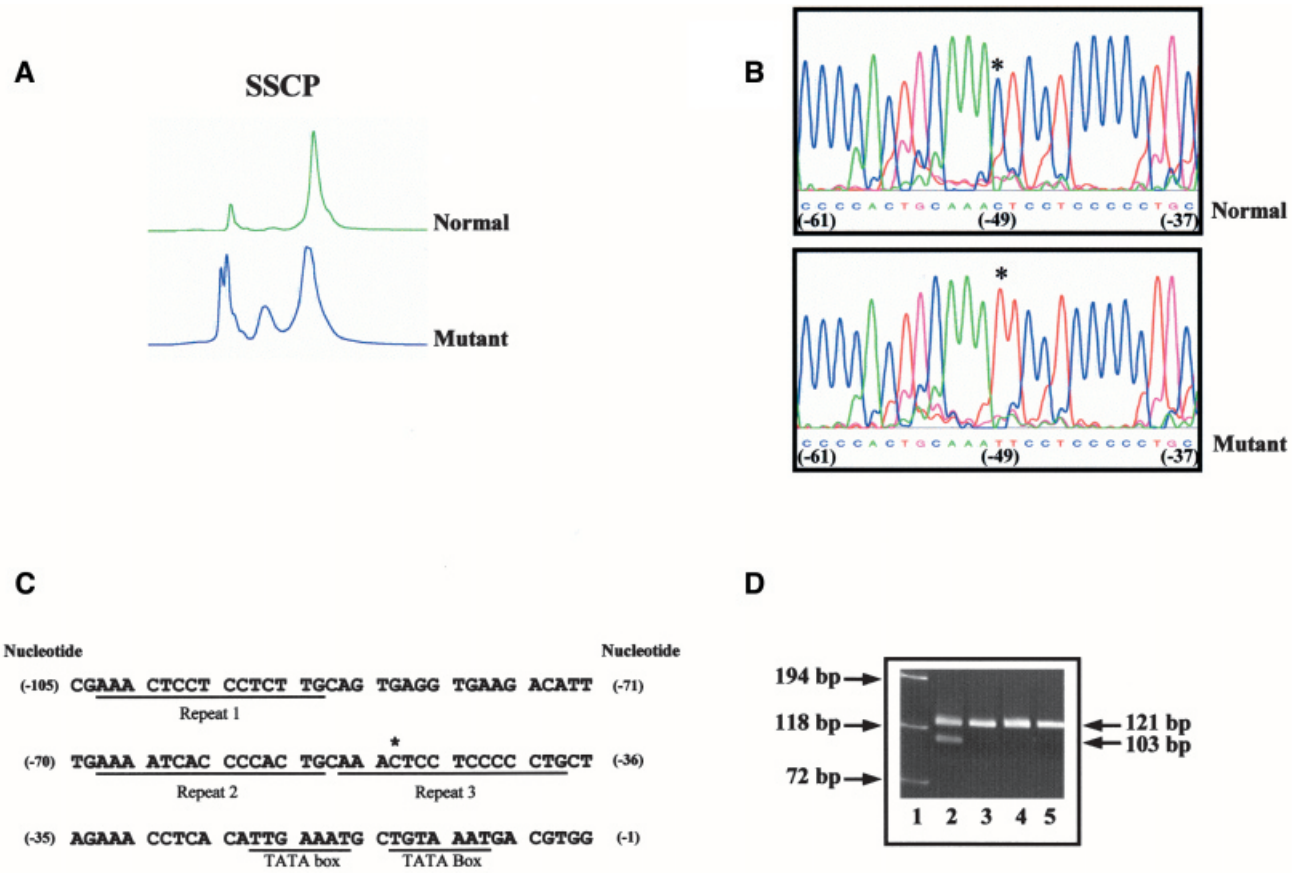

Fig. 1. Identification of the −49C>T mutation in the LDL-R gene promoter. A: SSCP profile of the index case showing the presence of an abnormal SSCP pattern in the fragment corresponding to the proximal 5′ region of the LDL-R gene. B: Comparison of the nucleotide sequences of the normal and mutant alleles showing a C>T mutation at the −49 position. C: Nucleotide sequence of the proximal promoter of the LDL-R gene. The major regulatory regions are underlined. The position of the mutation identified in this work is shown as an asterisk above the sequence. D: Detection of the mutation by restriction analysis of fragments originated by PCR with the primers 5′GAAAATCACCCCACTGCGAA3′ and 5′ACCTGCTGTGTCCTAGCTGG3′ which create an artificial *Eco*RI site when the mutation is present. Lane 1: MW marker. Lane 2: Heterozygote Lanes 3–5: Homozygotes for the wild type allele.

pXP2. The transcriptional activity of the promoter was tested in transient transfection assays in HepG2 cells in culture. In the presence of serum, the promoter carrying the mutation disclosed approximately 20% of the luciferase activity of the normal promoter. The experiments were also carried out in the absence of serum. In this situation the luciferase activity of both the mutant and the normal allele increased by a factor of 3–4.5, the mutant allele showing 15% of the activity of the normal allele (**Fig. 2**).

The only transcription factor known to bind to repeat 3 is Sp1, which binds to the 10 bp sequence ACTCCTCCCC (10, 11). To test the influence of the mutation on the binding properties of the sequence, EMSA were performed with labeled oligonucleotides corresponding to either the normal or the mutant alleles. The experiments were carried out in the presence of extracts of the cell line SL-2 transfected with a Sp1 expression vector. SL-2 is a cell line of *Drosophila* origin which does not produce Sp1 (18) and, therefore, extracts of SL-2 were used as Sp1-negative controls. The extracts of pPac-SP1-transfected SL-2 cells retarded the labeled oligonucleotide corresponding to the normal allele (**Fig. 3**, lane 3). The retarded band must contain Sp1 because *i*) it was not retarded by non-transfected SL-2 cells (Fig. 3, lane 2), and *ii*) it is further retarded with an antibody specific for Sp1 (Fig. 3, lane 4). The same experiments were performed with a labeled mutant oligonucleotide using the same amounts of protein extracts (Fig. 3, lanes 5–7). The results indicated that the mutation alters the ability to bind Sp1. However, the mutant oligonucleotide still retains a certain ability to bind Sp1, but to a much lesser extent than the wild type oligonucleotide. Together with the transient transfection assays reported above, these results supported the hypothesis that the −49C>T mutation is the basis of the FH phenotype.

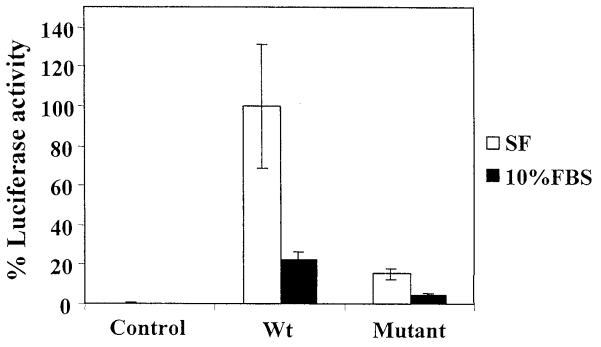

Fig. 2. Influence of the −49C>T mutation on promoter strength in a transient transfection assay. Fragments containing either the normal of the mutant promoter were cloned in the 5′ of the luciferase gene of pXP2. The constructs were transfected to cultures of HepG2 cells. After transfection the cells were cultured either in aserum- free medium (empty bars) or in a medium containing 10% fetal calf serum (black bars). The luciferase activity was assayed in cell extracts 48 h after transfection. The promoterless pXP2 plasmid was used as a negative control. The results were expressed as a percentage of the activity of the construction of the wild type allele in serum free conditions.

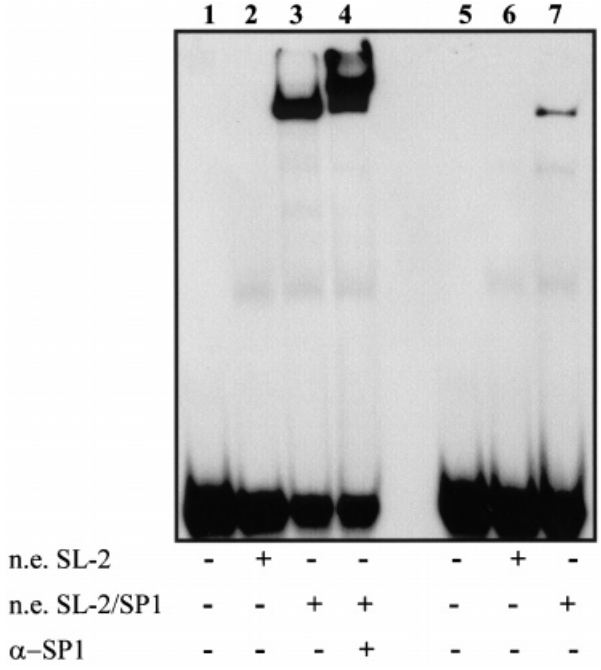

Fig. 3. Gel shift assays to determine the effects of the mutation on Sp1 binding. Labeled double-stranded oligonucleotides corresponding either to the normal or to the mutant allele were retarded in the presence of extracts of either SL-2 cells or Sp1-transfected SL-2 cells. Lane 1, normal oligonucleotide and no protein extract; Lane 2, normal oligonucleotide and SL-2 extracts; Lane 3, normal oligonucleotide and extracts of Sp1- transfected SL-2 cells; Lane 4, the same as lane 3, but with the prior incubation in the presence of a specific anti-Sp1 antibody; Lane 5, mutant oligonucleotide and no protein extract; Lane 6, mutant oligonucleotide and SL-2 extracts; Lane 7, mutant oligonucleotide and extracts of Sp-1 transfected SL-2 cells. NE, nuclear extract.

The study of the proband's pedigree showed a high prevalence of hyperlipidemia and CHD. Twelve members of the family were analyzed for the presence of the mutant allele. The results indicated that five additional family members were carriers of the mutation. However, only two of them (II-4 and III-4) disclosed the FH phenotype (**Fig. 4** and Table 1). The other three, two young women and a child (III-6, III-7, and IV-2 respectively), had blood cholesterol levels below the 90th percentile of the Spanish population (20, 21). ApoE genotyping indicated that all the members of the family were ε3/ε3 carriers.

DISCUSSION

In this study we have identified a point mutation in the promoter region of the LDLR gene in a FH family from Spain. This was the only base change identified in the LDLR gene of the proband after an extensive analysis including all the coding sequences, the intron/exon junctions, and 660 bp of the proximal promoter. Also, there were no major rearrangements of the sequence of the gene. As shown by our transient transfection assays, the mutation impairs the function of the promoter and its ability to bind Sp1 in vitro. These data lead us to conclude that

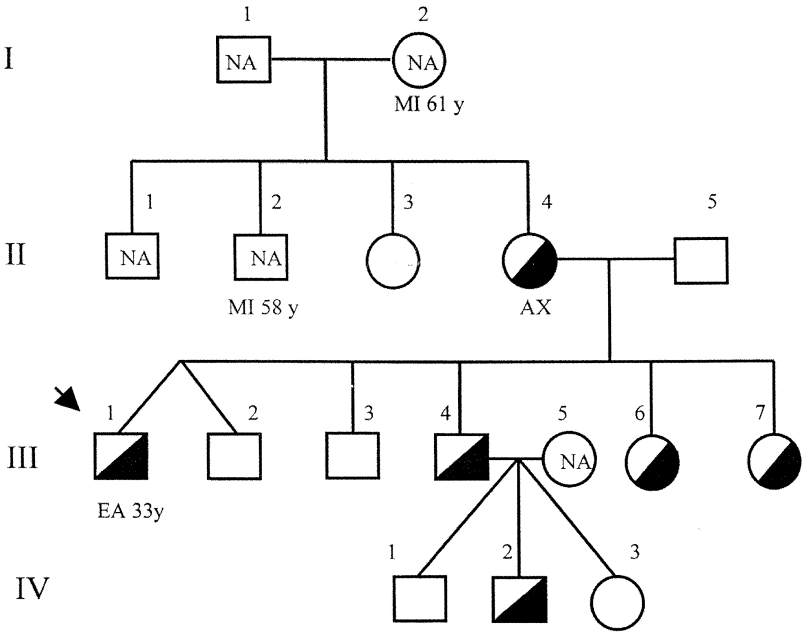

Fig. 4. Structure of the proband's family. Family members with the −49C>T mutation are indicated by half- filled symbols. Those members with cardiovascular disorders are shown together with the age of onset when known. MI, myocardial infarction and the age of presentation; AX, Achilles' tendon xanthomata; EA, effort angina; NA, data not available. The individual characteristics of the members of the family are shown in Table 1.

the C>T change at −49 is likely to be the cause of the FH present in the proband.

The −49C>T mutation maps at repeat 3 of LDLR gene promoter. Repeat 3 is a positive element which binds Sp1 and in this way contributes to the basal level of expression of LDLR gene. In addition, repeat 3 collaborates with repeat 2 in the regulation of LDLR expression by sterols. As Sp1 is the only transcription factor that binds to repeat 3, it is reasonable to think that the severity of the phenotype will depend on the way the mutation affects Sp1 binding. Of four mutations in repeat 3 producing a FH phenotype reported to date, only two, located at −43 and −45, have been characterized in terms of Sp1 binding and promoter strength. The −43 mutation completely abolished the ability of repeat 3 to bind Sp1 and, accordingly, the strength of the mutant promoter was approximately 5% of normal. FH patients heterozygous for the −43 mutation disclosed plasma cholesterol levels of 11.5 mmol/L(5). On the other hand, the only patient heterozygous for the −45 mutation described to date had lower blood cholesterol levels (8.5 mmol/l), consistent with the fact that repeat 3 still retains some ability to bind Sp1, and that the mutant promoter activity is about half the activity of the normal allele(9). The −49C>T mutation described here does not completely abolish the ability of repeat 3 to bind Sp1. Indeed, the mutant promoter had 20% of the activity of the normal promoter. In agreement with these data, the blood cholesterol levels of the proband are similar to those reported for the carrier of the −45 mutation (9).

It has been reported that repeat 3 collaborates with repeat 2 in the regulation of LDLR gene expression by SREBP (13). However, our reporter gene experiments indicated that both the normal and mutated allele expression responded to serum deprivation with similar increases in reporter gene activity. Therefore, the mutant allele still retains the ability to respond to low cholesterol levels. In fact, this could help explain the good response of the hypercholesterolemic members of this family to both dietary and pharmacological cholesterol-lowering treatments.

The study of the kindred showed that not all the family members that carry the mutation show typical FH phenotypes. This result is consistent with the mild phenotype and suggests that other factors might participate in the development of the heterozygous FH phenotype. ApoE is a major determinant of the FH phenotype (22). As all the members of the family had the ε3/ε3 genotype, a role for apoE cannot be invoked. It should be mentioned that the individuals carrying the mutation but not showing the FH phenotype in this family are either children or young women. It is well known that age and gender are common factors affecting LDL metabolism (23, 24). Perhaps because the mutant allele still shows residual activity, a plausible explanation is that hypercholesterolemia only arises in those conditions in which there is a low expression of the normal LDLR gene.

In summary, we report here a mutation in repeat 3 of the LDLR gene that impairs promoter activity by altering its ability to bind Sp1. This is a LDLR mutation with incomplete dominance of hypercholesterolemia, an observation that supports the influence of other genetic or environmental factors in determining the clinical phenotype of heterozygous FH.

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