Original Paper

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A Xanthomatosis-Susceptibility Gene May Exist in a Syrian Family with Familial Hypercholesterolemia

Abstract

Familial hypercholesterolemia (FH) is an autosomal-dominant inherited disorder characterized by high serum low-density lipoprotein (LDL)-cholesterol concentrations, xanthoma formation, and premature atherosclerosis. Homozygous individuals die of vascular disease as children or young adults; heterozygous persons are at high risk for premature cardiovascular death. Mutations in the LDL-receptor gene are responsible for FH. We studied 49 members of a consanguineous Syrian kindred containing 6 homozygous individuals from the same pedigree. Half of the homozygotes had giant xanthomas, while half did not, even though their LDL-cholesterol concentrations were elevated to similar degrees (>14 mmol/l). Heterozygous FH individuals from this family were also clearly distinguishable with respect to xanthoma size. We performed DNA analysis and were successful in identifying a hitherto not described mutation in this family's LDL receptor. DNA sequence analysis of the LDL-receptor gene revealed a T to C substitution at nucleotide 1,999 in codon 646 of exon 14. We next conducted a segregation analysis, which suggests that a susceptibility gene may explain the formation of giant xanthomas in this family. We raise the hypothesis that the appearance of giant xanthomas in this FH family is controlled by a second gene acting in an autosomal-dominant or recessive fashion. Elucidation of this 'xanthoma' gene may shed additional light on LDL-cholesterol deposition.

Introduction

In 1964, Khachadurian [1] described 12 patients with marked hypercholesterolemia and extensive xanthomatosis. The patients represented 10 sibships; the high incidence of consanguinity in the sibships led Khachadurian to conclude that the patients were homozygotes and that the inheritance pattern in these sibships conformed to that of an incompletely dominant gene. Khachadurian observed that homozygous persons had high cholesterol levels and extensive xanthomas, as well as severe vascular disease already in childhood. Heterozygous persons on the other hand, had more moderate cholesterol levels and developed xanthomas only later in life. Kwiterovich et al. [2] confirmed these observations in their examination of 236 children from 90 pairs of parents, one of whom had

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elevated LDL-cholesterol and one of whom did not. Brown and Goldstein [3] were instrumental in clarifying the nature of this disease. The primary defect in familial hypercholesterolemia (FH) is a mutation in the gene specifying the receptor for plasma LDL. The gene is located on the short arm of chromosome 19 [4]. FH is characterized by a selective elevation of a single fraction of cholesterol-carrying lipoproteins, namely, low-density lipoprotein (LDL)-cholesterol, a selective deposition of LDL-derived cholesterol in macrophage-like scavenger cells, and inheritance as an autosomal-dominant trait with a gene dosage effect.

Xanthomatosis, an abnormal deposition of lipids in various parts of the skin and tendons, is a hallmark phenotype in FH. Khachadurian [1] observed giant xanthomas in his homozygous patients and commented that the severity of atherosclerosis in his families paralleled the xanthomatous lesions. Hata et al. [5] studied the relationship between xanthomatosis and atherosclerosis and concluded that if the causation of their common tissue alterations could be defined, mechanisms common to the two conditions could be elucidated. We examined a large consanguineous FH kindred from Syria. We were able to identify two groups of homozygotes with similarly high LDL-cholesterol concentrations. One group had giant xanthomas and one did not. We identified a hitherto not described mutation in the LDL-receptor in this family. Analysis of the family tree has caused us to raise the hypothesis that a second autosomal gene is responsible for giant xanthomas in this family. Identification of this gene could be important in elucidating mechanisms of LDL-cholesterol incorporation.

Methods

Genetic Field Work

The subjects belong to a large kindred with FH and marked xanthomatosis living in and around Damascus, Syria. The study was approved by the Humboldt University's committee on the protection of human subjects and written informed consent in the Arabic language was obtained from participating adults and from the parents of family members < 18 years of age. A total of 49 family members were recruited and an extended pedigree, as well as a core pedigree, were drawn. Past medical and family histories were taken from each subject. Body weight and height were measured. Blood pressure and heart rate were measured by an oscillometric automated method (Dinamap, Johnson & Johnson, Evansville, Ind., USA) every 2 min in the supine position. An electrocardiogram (ECG) was taken in the resting state. The subjects underwent a physical examination concentrating on the cardiovascular system as well as the documentation of xanthomatous lesions and arcus corneae. The Achilles tendons were measured in selected individuals with slide calipers at the level of the internal malleolus. Skin lesions and arcus corneae were photographed in color. Venous blood samples from all subjects were collected into two 10-ml K-EDTA tubes. The plasma was separated from red cells after centrifugation at 4 °C. Whole blood and plasma samples were kept at 4 °C until analyzed.

Total cholesterol, HDL-cholesterol and triglycerides were determined in plasma immediately after collection in Damascus, Syria, and later in Berlin, Germany. Lipoprotein(a) [Lp(a)] was also determined in Berlin, Germany, in addition to total cholesterol, HDL-cholesterol and triglycerides. Total cholesterol was determined on the Cobas-Mira Hoffmann-La Roche automated analyzer (Basel, Switzerland) with the CHOD-PAP method. Triglycerides were measured using the GPO-PAP, also an enzymatic colorimetric test. HDL-cholesterol was determined enzymatically using PEG-modified enzymes and sulfated a-cyclodextrin and dextran sulfate. Lp(a) was analyzed by an immunoturbidimetric method making use of a specific unimate 3 Lp(a) antiserum. LDL-cholesterol was calculated according to the Friedewald formula.

Genomic DNA was prepared from 10 ml of whole blood by a standard method. Microsatellite analysis was performed using a marker, D19S394, closely linked to the LDL-receptor gene, in order to match our phenotype data and check the pedigree for paternity and any inconsistencies. The marker analysis was performed using the ABI Prism Genotyping System, including PCR 9600 thermocyclers, an ABI 373 DNA Sequencer, Genescan 1.2.2-1 and Genotyper software from Applied Biosystems Division of Perkin Elmer Corporation (Foster City, Calif., USA). Polymerase chain reactions (PCR) were performed according to Day et al. [6]. The forward primer was labeled with TET fluorescent dye. Electrophoresis and detection were carried out according to the manufacturer's protocol.

All 18 exons and the promotor of the LDL-receptor gene of an individual homozygous for FH were amplified using primers flanking the intron-exon junctions. Using the Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Division of Perkin Elmer) we obtained sequencing products for each PCR amplicon, which were subsequently electrophoresed for 14 h at 2,500 V ± and 37 W on the ABI 373 fluorescence scanning DNA sequencer. The denaturing 5% polyacrylamide gel (acrylamide/bisacrylamide 29:1) contained 7 M urea, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA. The gel thickness was 0.4 mm and the well-to-read length was 48 cm. The raw and digital gel data were analyzed with the Sequencing Analysis Software and the Navigator Software (Applied Biosystems Division of Perkin Elmer).

We genotyped the entire family for the found point mutation by a method based on the introduction of an artificial restriction site [7]. For this application, we used a modified primer, which creates a new restriction site for the restriction endonuclease AcyI, to amplify a fragment with the mutation near its 3′ end. 1.5 pmol of forward (5′-CCT TGT GGA AAC TCT GGA ATG TTC T-3′) and reverse (5′-CAT TGC TCA GGG TGG TCC TCT GAC-3′) oligonucleotides were used as a primer set. PCR reaction was performed in a final volume of 15 μl containing 3 mM MgCl$_2$, 200 μM of each dNTP and 0.5 U TaqGold (Perkin Elmer). After an initial denaturation at 95 °C for 10 min, 40 cycles were carried out consisting of a heat-denaturation step of 30 s at 95 °C, an annealing step of 30 s at 68 °C, and an extension step of 1 min at 72 °C. This step was followed by a final enzymatic extension at 72 °C for 10 min. 6 μl of PCR product were subsequently digested with 2 U AcyI at 50 °C over 1 h. High-resolution acrylamide gel electrophoresis was required to separate the short fragment deriving from the 3′ end of the amplification product. A

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Table 1. Cholesterol, triglyceride, and Lp(a) values for homozygous, heterozygous, and normal (FH) family members

	Homozygous	Heterozygous	Normal
Total cholesterol, mmol/l[1]	17.7±2.5	7.8±1.2	4.5±0.9
LDL-cholesterol, mmol/l[1]	16.5±2.3	6.2±1.0	2.6±0.6
HDL-cholesterol, mmol/l	0.6±0.1	0.9±0.2	1.3±0.4
Triglycerides, mmol/l	1.3±0.7	2.3±1.7	1.4±1.1
Lp(a), mg/dl[2]	61.6±37.8	35.9±33.6	18.7±10.5

Data are given as mean ± SD.
[1] Groups differ significantly (p < 0.05).
[2] Cannot be expressed in SI units.

12% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide 19:1) was used for this application. The gel was stained with ethidium bromide.

Statistical Analysis

We performed complex segregation analysis using the program POINTER in this family [8]. The ascertainment probability was assumed to be 75% (0.75). We reached this conclusion because individuals with FH and large xanthomas are very likely to be under medical care and consequently to have been brought to our attention and included in our study. The incidence of the phenotype considered (giant xanthomas in carriers of at least one copy of the FH mutation) was set at 5% (0.05). In addition, we performed a sensitivity analysis by varying the ascertainment probability from 0.001 to 1 (by using the values of 0.001, 0.050, 0.100, 0.200, 0.500, 0.750, 0.800 and 1.000) and the frequency of the phenotype from 0.001 to 0.1 (by using the values of 0.001, 0.010, 0.050, and 0.100).

Results

After extended family analysis, it was possible to integrate the 49 subjects we examined into all 203 family members in one pedigree, where all genetic links and loops were shown. Two pairs of common ancestors could be traced back for all affected family members in all sub-pedigrees extending over six generations. These common ancestors had the same family name, leading us to hypothesize that they stem from a common ancestor. Among the 49 investigated persons, we identified 6 homozygous, 33 heterozygous, and 10 normal individuals. We based the phenotypic characterization on LDL-cholesterol measurements. Data covering the lipid and lipoprotein profiles of our subjects from this family are given in table 1. All had normal blood pressures, none were obese, and physical findings were confined to the cardiovascular system, the eyes, and the skin. Cardiac murmurs and bruits were audible in all clinically homozygous persons and all homozygotes had arcus lipoides. On the basis of pheno-

typic appearance, it was possible to classify FH-affected individuals into two groups, one with moderate, and another with extensive xanthoma development. The xanthomas were remarkably larger by direct measurement in half of the homozygous subjects. In half of the heterozygous subjects, small xanthomas were found while in the other half, no xanthomas were apparent. A core pedigree was drawn in order to clearly depict this difference in the phenotype between subfamilies and the interrelationships of the individuals. This pedigree comprises 16 investigated individuals and is shown in figure 1. Figure 2 illustrates the degree of xanthomatosis within the two groups. Each group consists of three FH homozygotes differing in the size of the xanthomatous lesions. The age, gender and BMI did not differ significantly, while the values for all biochemical parameters were similar. Table 2 outlines the characteristics of these homozygous individuals. All subjects also share the same allele for the LDL receptor marker in the homozygous state, as demonstrated by the segregation analysis of alleles in the family.

As shown in figure 1 (core pedigree), only one defective allele (4) segregates within the family. To identify the underlying genetic defect, DNA sequence analysis of the entire LDL-receptor gene of a homozygous individual (personal ID 118) was performed. Our analysis revealed the presence of a single T to C transition, in nucleotide 1,999, the first of the three of codon 646 in exon 14, resulting in an arginine for cysteine substitution. Figure 3 shows part of the sequence data clearly demonstrating the mutation in its homozygous state. To genotype the entire family, we used a method based on the introduction of an artificial restriction site using a modified primer during the PCR, which creates a new *Acy*I restriction site. Direct mutation detection analysis was in complete accordance with data retrieved from phenotypic characterization, biochemical investigations and segregation analysis with

Fig. 1. Core pedigree demonstrating the consanguineous marriages and phenotypic differences. Large xanthomas in heterozygous and homozygous persons are marked with 'XL', while common xanthomas are labeled with 'X'. Unmarked symbols from living individuals did not have xanthomas. Numbers below the symbols represent alleles for the LDL-receptor marker and allele 4 is the allele which cosegregates with the LDL-receptor mutation.
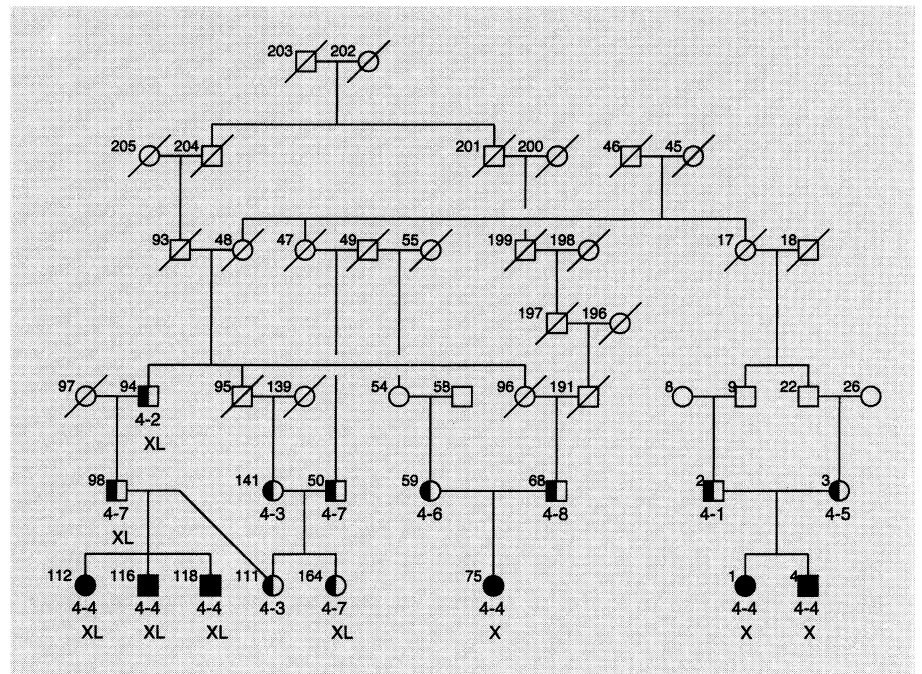
Table 2. Demographic and laboratory data from 6 homozygous FH individuals

|	Giant xanthoma			Small xanthoma		
Subject ID number (see family tree)	116	118	112	1	4	75
Gender, M/F	M	M	F	F	M	F
Age, years	15	12	27	17	15	14
BMI	19.7	18.1	21.5	17.6	25.5	16.8
Total cholesterol, mmol/l	15.6	18.1	14.2	16.9	17.6	19.2
LDL-cholesterol, mmol/l	14.7	17.1	13.2	16.0	16.4	17.5
HDL-cholesterol, mmol/l	0.5	0.5	0.6	0.7	0.8	0.6
Triglycerides, mmol/l	0.9	1.2	0.9	0.6	0.9	2.5
Lp(a), mg/dl[a]	11	27	68	83	43	123

Three individuals had giant xanthomas, while 3 did not. With the exception of the xanthoma characteristic and variability in their Lp(a) values, the subjects were similar. BMI = Body mass index (weight, kg/height, m²).
[a] Cannot be expressed in SI units.

Fig. 2. A Giant xanthomas of individual (116) on the left compared to smaller xanthomas on the knees of individual (75) on the right. **B** Giant xanthomas on the hands of individual (112) on the left, compared to smaller xanthomas on the hands of individual (1) on the right. **C** Giant xanthoma on elbow of individual (118) on the left compared to small xanthoma on elbow of individual (4) on the right. All subjects were homozygous for the LDL-receptor mutation; numbers represent photo documentation and are not pedigree designations.

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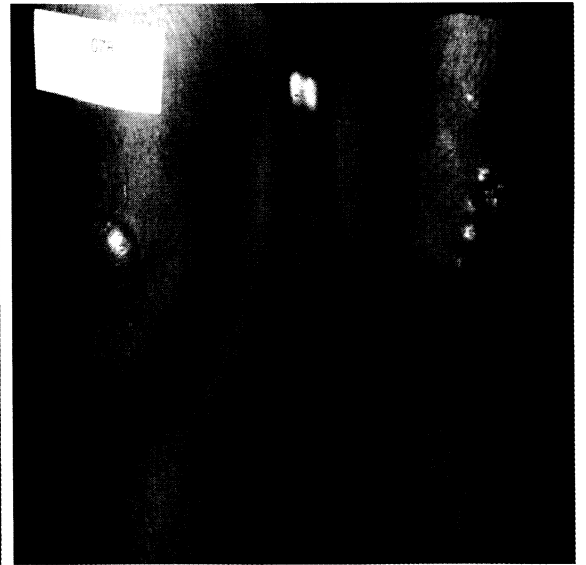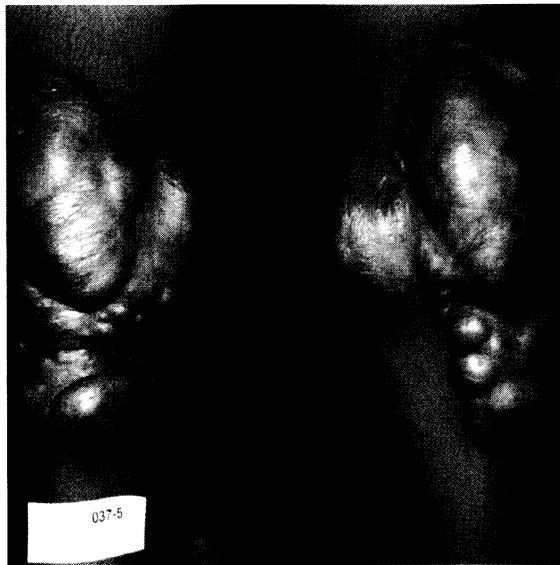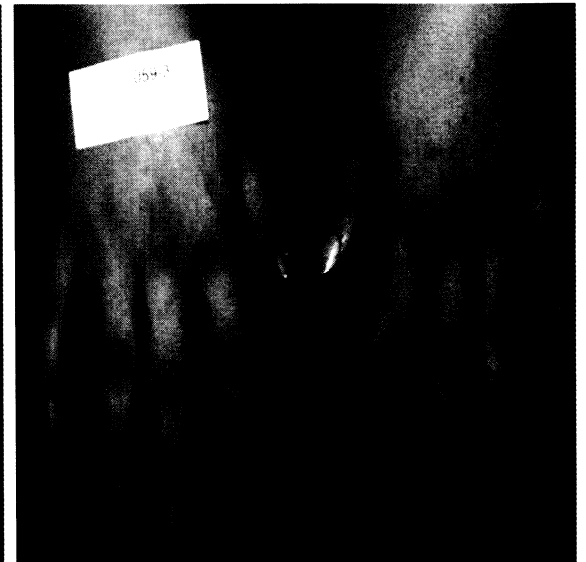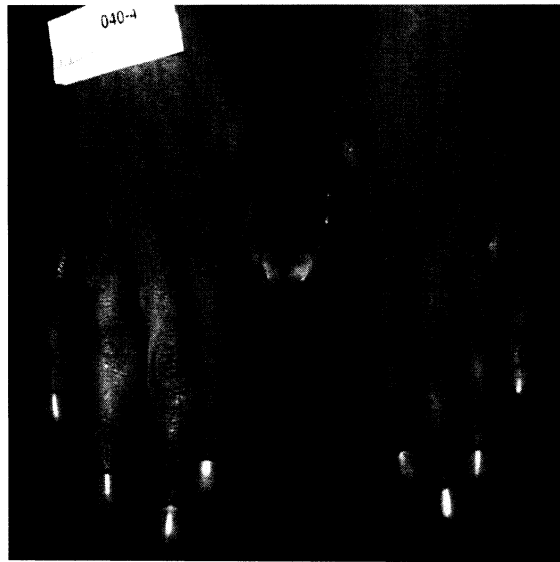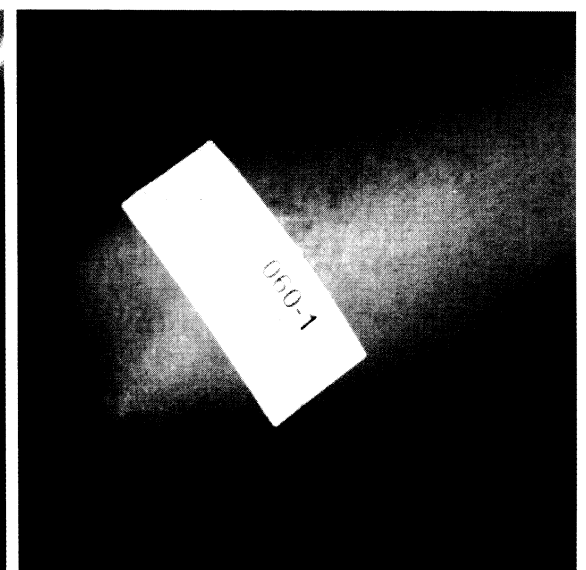
Fig. 3. Shown is part of the coding DNA sequence of exon 14 of the LDL-receptor gene showing the T to C substitution of nucleotide 1,999 in codon 646 in a homozygous individual. Wild-type sequence is shown in the upper portion, while the mutation is in the lower portion.
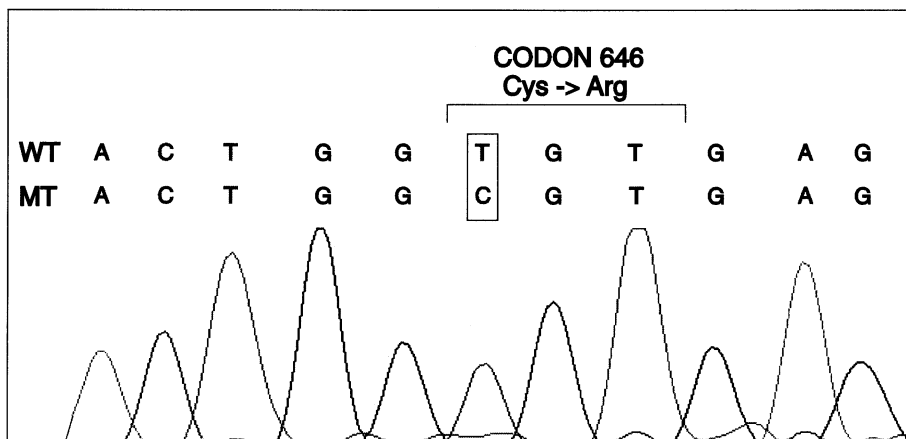

Fig. 4. Shown is the DNA test we developed for direct mutation detection. Direct DNA analysis in normal (upper band), heterozygous (double bands), and homozygous (lower band) individuals from a subpedigree. The method is based on the introduction of an artificial restriction site during the PCR, followed by enzymatic digestion and high-resolution acrylamide-gel electrophoresis (see Methods).
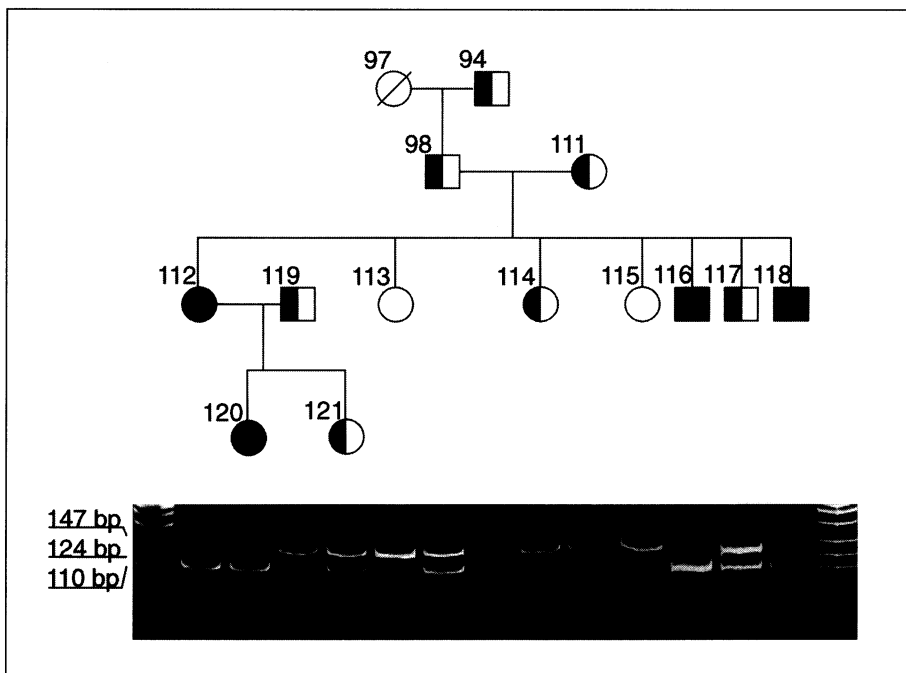
the flanking microsatellite marker. Figure 4 shows direct DNA test results for a representative subsample of the family. The discrepancy in the phenotype and especially the xanthoma formation between FH-affected individuals is best explained by the presence of a genetic factor.

We compared the hypothesis of no genetic influence (all cases occurring by chance with the population frequency of the disease), an autosomal dominant model (frequency of the disease allele q = 0.0135, penetrance in hetero- and homozygotes for the disease allele = 0.9999 and in homozygotes for the wild-type allele = 0.0235) and an autosomal-recessive model (q = 0.2235, penetrance in homozygotes for the disease allele = 1.0000 and in homo-

zygotes and heterozygotes for the wild-type allele = 0.0001). We found that both the autosomal-dominant and the autosomal-recessive models were significantly better (χ^2 12.365 with 2 degrees of freedom for the autosomal-dominant hypothesis, p < 0.003 and of 11.800 for the autosomal-recessive hypothesis, p < 0.003) than the nongenetic hypothesis. The likelihood ratio for the autosomal-dominant hypothesis versus the autosomal-recessive hypothesis was only 1.325:1, a value which is clearly not statistically significant. The addition of a multifactorial component did not significantly improve the likelihood of either the autosomal-dominant or the autosomal-recessive hypothesis.

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Multifactorial inheritance alone did not explain the pedigree significantly better than the hypothesis of no genetic influence. All results given above were obtained assuming an ascertainment probability of 0.75 and a phenotype frequency of 0.05. When we independently varied the ascertainment probability between the extremes 0.001 and 1.000 and the frequency of the phenotype between 0.001 and 0.100 as described above, we found that in all cases both the autosomal-dominant and the autosomal-recessive hypothesis explained the data significantly better than the hypothesis of no genetic influence ($p < 0.030$ in all cases, data not shown). In addition, in all cases, the autosomal-dominant hypothesis had a higher likelihood than the autosomal-recessive hypothesis, although this difference was in no case statistically significant. Parameter estimates were very stable towards variation in the ascertainment probability, while being dependent on the frequency of the phenotype assumed in the population (data not shown). The results led us to conclude that the findings suggest the existence of a 'xanthoma' gene acting in an autosomal-dominant or possibly autosomal-recessive fashion.

Discussion

There are two important findings in this study. First, we describe a new mutation in the LDL-receptor causing FH in a large, Syrian kindred. Second, we present evidence suggesting the existence of a second gene leading to the formation of giant xanthomas. This latter observation provides evidence for the hypothesis that xanthoma formation itself is subject to genetic variance independent of serum LDL-cholesterol concentrations. Elucidation of such a gene and clarification of its actions could be important to our understanding of atherogenesis.

Hobbs et al. [9] recently reviewed the molecular genetics of the LDL-receptor gene in FH. They commented that 71 mutations in the LDL-receptor gene had been characterized at the time of their report and they contributed 79 additional mutations. Clarification of these 150 mutations provides great insight into the structure/function relationships of the receptor protein and are relevant to the clinical manifestations of FH. The LDL-receptor gene is located on the distal short arm of chromosome 19 (p13.1–p13.3), spans 45 kb, and is comprised of 18 exons [10]. Exon 1 encodes the signal sequence, exons 2–6 encode the ligand binding domain, while exons 7–14 encode a region that shares sequence identity to the human epidermal growth factor precursor gene. Non-

sense, frameshift, and missense mutations have all been described in exon 14. The new mutation we describe is similar to, but distinct from a mutation in the same exon described by Lehrman et al. [11] in a Christian-Lebanese, Syrian family. Their mutation involved stop codon 660 and resulted in a nonsense TGC to TGA substitution. As a result, the receptor was truncated and retained in the endoplasmic reticulum. Our mutation deletes a cysteine at codon 646 in the EGF precursor homology domain. A previously described mutation in a French Canadian population involving a tyrosine for cysteine substitution within the same codon was shown to result in a functional class 2A allele [12]. Class 2A alleles encode proteins that completely block the transport between the endoplasmic reticulum and the Golgi apparatus [9]. Therefore, we expect this mutation to act in a similar fashion.

Xanthomas are a well-recognized clinical finding which directs the physician's attention to cholesterol metabolism disturbances and increased cardiovascular risk [13]. Hata et al. [5] studied 86 patients with xanthomas and concluded that the combined occurrence of xanthomas and atherosclerotic disease was striking. They noted that individuals with tuberous and tendon xanthomas invariably had marked elevations in LDL-cholesterol, while triglycerides and high-density lipoprotein (HDL)-cholesterol values were normal. Our subjects had tuberous and tendon xanthomas, and indeed, they all had markedly elevated LDL-cholesterol concentrations. Half the homozygous individuals featured giant xanthomas, namely huge, disfiguring lesions often requiring multiple operations for their removal. The giant xanthomas of our homozygous individuals closely resemble those described by Khachadurian [1]. However, his homozygous patients all had such lesions.

The 49-member subpedigree we elicited allowed us to perform a genetic analysis on the basis of xanthoma size in homozygous and heterozygous individuals. We found evidence for the existence of a separate 'xanthoma' gene in this family, responsible for giant xanthomas in homozygous persons and early or more prominent xanthomas in heterozygous persons. We have not been able to conduct long-term observations of this kindred and were also unable to accrue sufficient information to state for certain whether or not the presence of the xanthomatosis phenotype represents an added risk for premature atherosclerosis. However, we suggest on the basis of previous observations [5] that this may indeed be the case. In addition, we are actively searching for other families with this condition to enable a linkage analysis.

The pathology of FH is characterized by accelerated atherosclerosis and prominent lipid accumulation in macrophages and other stromal cells of the aortic and mitral valves, skin, tendons, and variably other extravascular sites [14]. Xanthomas from FH individuals feature marked lipid accumulation in histiocytic foam cells, but no lipid deposits in the endothelium of blood vessels in the lesions themselves. Xanthoma cholesterol turnover in FH patients has been studied. Bhattacharyya et al. [15] used isotopic cholesterol and biopsied xanthomas sequentially. They were able to show a dynamic exchange between cholesterol in xanthomas and that in the plasma. Sugiyama et al. [16] have studied the immunohistochemical distribution of lipoprotein epitopes in xanthomas from patients with FH. They found that oxidatively modified LDL-cholesterol was associated with macrophages and occurred intracellularly, while LDL-cholesterol epitopes were detected extracellularly. Sugiyama et al. [16] suggested that oxidized LDL-cholesterol is likely to play a pathogenetic role in the lipid accumulation by macrophages in xanthomas and also suggested a role for Lp(a). Interestingly, probucol, which has considerable antioxidant properties and inhibits the uptake of oxidized LDL by macrophages [17], typically leads to regression of xanthomas but not necessarily to regression of atherosclerosis. Kajinami et al. [18] recently reported a heterozygous FH patient, whom they treated with pravastatin 20 mg/day and probucol 1,000 mg/day. His xanthomas were greatly reduced in size and his LDL-cholesterol decreased 75 mg/dl; however, his coronary artery disease unfortunately progressed.

The role of LDL-cholesterol and its uptake by macrophages in the pathogenesis of atherosclerosis and production of xanthomatosis is established [19, 20]. Excellent animal models of FH have been developed. Massive xanthomatosis and atherosclerosis in cholesterol-fed LDL-receptor-negative mice have been described [21]. Similarly, cholesterol feeding results in xanthomatosis in mice that have been made deficient in apolipoprotein E [22]. Other in vitro and in vivo studies have shown that LDL-cholesterol undergoes an oxidative modification that targets it for uptake by the macrophage through specific receptors, namely the acetyl-LDL or scavenger receptors [23]. These receptors have been implicated both in the deposition of LDL-cholesterol in the vascular wall and elsewhere, as well as in host defense mechanisms against infections. Such receptors and mechanisms related to their action may provide us with interesting candidate genes to explain the phenomenon of giant xanthomatosis.

In summary, we have described a novel Syrian FH family with a mutation in exon 14 of their LDL-receptor gene. A subpedigree and phenotypic characterization of their xanthomas strongly suggest the existence of a second mutation in this family, responsible for giant xanthoma formation. We suggest that elucidation of a xanthoma gene might be of mechanistic importance in LDL-cholesterol uptake by macrophages.

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