Am. J. Hum. Genet. 68:653–660, 2001

Use of Homozygosity Mapping to Identify a Region on Chromosome 1 Bearing a Defective Gene That Causes Autosomal Recessive Homozygous Hypercholesterolemia in Two Unrelated Families

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Familial hypercholesterolemia (FH) is a common inherited disorder of metabolism characterized clinically by high levels of low-density lipoprotein (LDL) in plasma owing to reduced catabolism. This leads to accelerated atherosclerosis and thus to an increased risk of coronary heart disease. FH is usually caused by defects in the gene for either the LDL receptor or apolipoprotein B (apoB), the ligand for the LDL receptor. Elsewhere, we have described two unrelated patients with phenotypic homozygous FH. Both patients were offspring of consanguineous unions, and linkage to either the gene for the LDL receptor or the gene for apoB was excluded in both. Their cells in culture do not degrade LDL, despite the presence of normal surface binding of LDL to the LDL receptor. This observation suggests that the patients may be homozygous for a defective gene that encodes a component of the internalization pathway. We first excluded linkage of the defect to known genes for proteins reported to be involved in internalization of receptors in clathrin-coated pits. We then performed genomewide homozygosity mapping. Genotyping of 500 polymorphic markers in three affected and seven unaffected members of the first pedigree showed that recessive hypercholesterolemia in this family is localized to a single chromosomal region on 1p36-p35. Genotyping of two affected and five unaffected members of the second pedigree provided further evidence of linkage to this locus, thereby mapping the disease-causing gene to a 12-cM region on chromosome 1p36-p35, with a combined LOD score of 5.3 in these unrelated families. Identification of the gene in this region may lead to new insights into the mechanisms of LDL receptor–mediated uptake of LDL by cells and may help to identify further genetic risk factors for premature atherosclerosis.

Introduction

Familial hypercholesterolemia (FH [MIM 143890]) is an inherited disorder of metabolism that is characterized clinically by an increased concentration of low-density lipoprotein (LDL) cholesterol in the serum, which results in accelerated atherosclerosis and the deposition of cholesterol in the peripheral tissues. The disease is usually caused by defects in the gene for the LDL receptor, a cell-surface protein that mediates the specific and regulated uptake and degradation of LDL and some other cholesterol-rich lipoproteins. FH is inherited as an autosomal dominant trait and has a gene dosage effect, in that heterozygous FH patients with one defective copy of the LDL-receptor gene usually have an LDL cholesterol concentration in plasma that is twofold higher than normal, whereas homozygous FH patients frequently have an LDL level that is fivefold higher than normal. If left untreated, many heterozygous FH patients develop xanthomas and show signs of coronary heart disease by early middle age, whereas all homozygous FH patients have these manifestations of the disease, as well as aortic root disease, beginning in early childhood (Goldstein et al. 1995). A defect in the gene for apolipoprotein B (apoB), which is the major protein component of LDL and is also the ligand for the LDL receptor, results in a similar, but generally less severe, phenotype (Myant 1993). However, in studies conducted in various geographical regions, careful genetic analysis of FH patients has failed to reveal a defect in the genes for the LDL receptor or apoB, suggesting that defects in other genes can result in severe inherited hypercholesterolemia (Sun et al. 1994; Miserez and Keller 1995; Day et al. 1997).

We have described elsewhere two unrelated patients, one of Turkish and the other of Asian Indian origin, with a clinical diagnosis of homozygous FH that is clearly not due to defects in the genes for the LDL re-

Received November 15, 2000; accepted for publication January 2, 2001; electronically published February 9, 2001.

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0002-9297/2001/6803-0011$02.00

ceptor or apoB (Norman et al. 1999). In both patients, the disorder appears to be inherited as an autosomal recessive trait and does not cosegregate with alleles of the LDL-receptor or apoB genes. In cultured Epstein-Barr virus–transformed lymphoblasts from both patients, an apparently normal LDL-receptor protein is synthesized and transported to the cell surface, where it binds LDL with normal affinity. However, the ligand is not degraded, apparently because the receptor-ligand complex is not internalized by the cells, although the transferrin receptor is internalized normally, a result suggesting that the internalization defect is specific for the LDL receptor (Norman et al. 1999). Both probands are the offspring of consanguineous parents, suggesting that the affected individuals in each pedigree are likely to be homozygous for a region of the genome harboring the same defective gene allele inherited from a common ancestor.

In this report we describe the results of a genomewide scan with polymorphic genetic markers. In both pedigrees, the same single region of ~12 cM on chromosome 1 fulfilled the criteria for homozygous inheritance of alleles in the affected offspring but not their unaffected siblings, a result suggesting that the disease gene lies in this region. A preliminary report of some of these data has been presented elsewhere (Eden et al. 2000).

Subjects and Methods

Patients

The clinical characteristics of the two index patients in this study have been described in detail elsewhere (Norman et al. 1999). For the present study, fasting blood samples were collected from additional members of both pedigrees for analysis of plasma lipid and lipoprotein concentrations and for isolation, by standard procedures, of genomic DNA from white blood cells (Maniatis 1989). Informed consent was obtained from all participants. For estimation of ethnicity-specific allele frequencies, anonymous DNA samples from unrelated Turkish individuals ($n = 25$) were kindly provided by D. Booth; DNA samples from unrelated Asian Indian individuals ($n = 25$) were provided by B. Knight.

Genotyping

The genomewide screen was performed with a combination of both the Human Genome Mapping Project (HGMP)/Todd marker set (Reed et al. 1994) and the ABI PRISM Linkage Mapping Set, version 2 (PE Biosystems), which are selected from the Généthon human linkage map (Dib et al. 1996). Amplification conditions were optimized for each primer pair in a 5-μl reaction with a Perkin-Elmer 9600 thermal cycler. Pooled PCR products were separated by electrophoresis on 6% polyacrylamide gels on an ABI 377 DNA sequencer (Applied Biosystems). Alleles were sized by GENESCAN, version 2.1.1, and were scored with GENOTYPER software, version 2.0 (PE Biosystems), with respect to 6-carboxy x-rhodamine (for Linkage Mapping Set 2) and 6-carboxy tetramethylrhodamine (for the HGMP/Todd set) 500 size standards. After linkage analysis of the genomewide data, saturation mapping of candidate regions included additional markers selected from the Généthon human linkage map (Dib et al. 1996) or from the Genetic Location Database (LDB); primers for the latter were kindly provided by R. Plomin and colleagues. Interloci mapping distances were taken from the Généthon human linkage map (Dib et al. 1996) or from the LDB, relative to other Généthon markers on this map.

Data Analysis

We initially performed nonparametric and parametric, single- and multipoint genomewide linkage analysis, using GENEHUNTER software (Kruglyak and Lander 1995). GENEHUNTER provides multipoint parametric LOD-score calculations, nonparametric pedigree analysis, and maximum-likelihood reconstruction of haplotypes. In the absence of allele frequencies for specific ethnic groups, frequencies for marker loci were assumed to be equal for the initial genomewide scan. However, for the fine mapping of candidate regions, ethnicity-specific allele frequencies were derived from 25 DNA samples from the Turkish population (for pedigree 1) and 25 DNA samples from the Indian population (for pedigree 2). Parametric analyses were performed assuming an autosomal recessive model of inheritance with 98% penetrance and a disease-allele frequency of .002. The FASTLINK program LINKMAP (Cottingham et al. 1993) was also used for multipoint linkage analysis of markers on selected chromosomes, under the same model of inheritance. All LOD scores shown in the

Table 1

Chromosomal Localization of Genes for Protein Components of Clathrin-Coated Pits and Clathrin Adapter Complex 2

Protein	Gene	Chromosomal Localization[a]
Clathrin heavy chain-1	*CLTC*	17q11-qter
Clathrin heavy chain-2	*CLTC2*	22q11.2
Clathrin light chain-A	*CLTA*	12q23-24
Clathrin light chain-B	*CLTB*	4q2-q3
AP2 large subunit (β1)	*AP2B1*	17q11.2-17q12
AP2 large subunit (α1)	*AP2A1*	19q13.3
AP2 medium subunit (μ2)	*AP2M1*	3q28
AP2 small subunit (σ2)	*AP2S1*	19q13.2-13.3
Disabled-1 (Dab-1)	*DAB1*	1p31-32

[a] From the Human Genome Database.

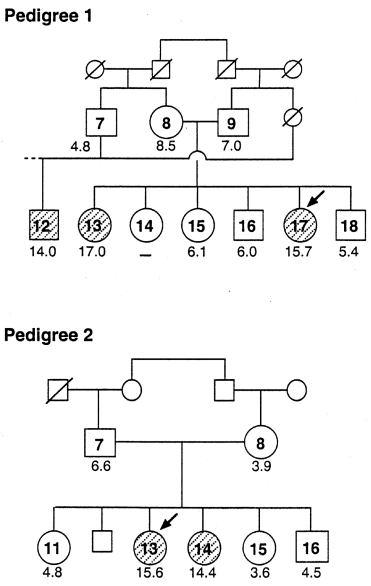

Figure 1 Pedigrees of families with autosomal recessive hypercholesterolemia not caused by mutations in the LDL-receptor gene. The pretreatment plasma cholesterol concentration (mM/l) of each individual is indicated below each symbol; the plasma cholesterol level for individual 14 in pedigree 1 was reported to be in the normal range. Symbols enclosing dotted lines indicate affected individuals, and symbols containing numbers indicate individuals who were included in the genetic analysis. Arrows indicate probands.

Results section for both GENEHUNTER and LINKMAP are from parametric analyses. The classical LOD-score threshold of 3 was taken as the criterion for linkage, on the basis that the segregation pattern in pedigree 1 indicated a single-locus etiology. Such a "dense-map threshold" has been shown to be appropriate for multistage linkage analyses (Kruglyak and Daly 1998).

Results and Discussion

The defect in internalization of the LDL-receptor protein in cultured lymphoblastoid cells from the two patients first prompted us to investigate candidate genes for proteins involved in trafficking of other receptors (for review, see Heilker et al. 1999). Receptor-mediated endocytosis of receptors that are located in clathrin-coated pits is thought to involve binding of the cytoplasmic domain to the $\mu 2$ component of adapter protein complex–2 (AP-2), another component of which binds to the clathrin. However, this has never been demonstrated directly for the LDL receptor, and we have already shown that the sequence and expression of $\mu 2$ in the patients described here are not different from those in control subjects (Norman et al. 1999). It has been suggested that the cytoplasmic tail of the LDL-receptor protein binds directly to the heavy chain of clathrin (Kibbey et al. 1998). We therefore analyzed the sequence and relative level of expression of the mRNA for the ubiquitously expressed heavy chain of clathrin. The cDNA was amplified by reverse transcriptase–PCR from total cellular mRNA, but nucleotide sequencing did not reveal any sequence variants in the index patients (data not shown). All other candidate genes for components of AP-2 and for other clathrin chains (listed in table 1) were excluded by the pattern of inheritance of polymorphic markers flanking each gene of interest. It has also been shown that the cytoplasmic tail of the LDL receptor can interact with Dab-1 in the yeast two-hybrid system (Tromsdorff et al. 1998), but linkage of the defect to this gene was excluded in the families described here.

The search for mutations in or linkage to candidate genes was not fruitful, and we therefore decided to screen for regions of the genome where only the affected individuals shared homozygosity. A preliminary screen with 500 highly polymorphic microsatellite markers, spaced at average intervals of 8 cM, was performed with DNA from the 10 individuals indicated in the family tree of pedigree 1, shown in figure 1. This revealed no markers with shared homozygosity restricted to the three affected individuals, and some markers were uninformative in this pedigree, because the parents were homozygous at the locus, although typing of a small percentage of markers was not of sufficient quality. To achieve the desired ~8-cM map density, additional markers from the Généthon human linkage map were typed. At this stage, a single marker (D1S2734 at 1p35) fulfilled the criteria for homozygosity mapping in the

Table 2

Two-Marker Parametric LOD Scores in Regions Identified as Potential Candidates on the Basis of a GENEHUNTER Analysis of the 8-cM Genomewide Map

Chromosomal Region[a]	Markers[b]	Map Distance[b] (cM)	LOD Score[c]
1p36.2	D1S214–D1S2667	10.5	−2.9
1p36-35	D1S199–D1S2734	6.6	1.64
2q21	D2S347–D2S114	10.1	−1.06
4q31-32	D4S415–D4S1535	12.4	−2.47
5q12-13	D5S424–D5S641	9.5	.19
5q15-21	D5S644–D5S409	6.4	−2.23
9q22	D9S387–D9S1690	6.5	.46
12p13	D12S356–D12S336	5.7	−.57
14q13	D14S587–D14S75	2.0	−1.09
15q26	D15S207–D15S120	6.1	−.02
17q12	D17S250–D17S934	4.0	−1.04

[a] Chromosomal regions giving a parametric LOD score ⩾.8 after analysis by GENEHUNTER.

[b] Markers and map distances were taken from the Généthon human linkage map (Dib et al. 1996).

[c] Two-marker multipoint parametric LOD scores were calculated by LINKMAP analysis, under an autosomal recessive mode of inheritance with 98% penetrance.

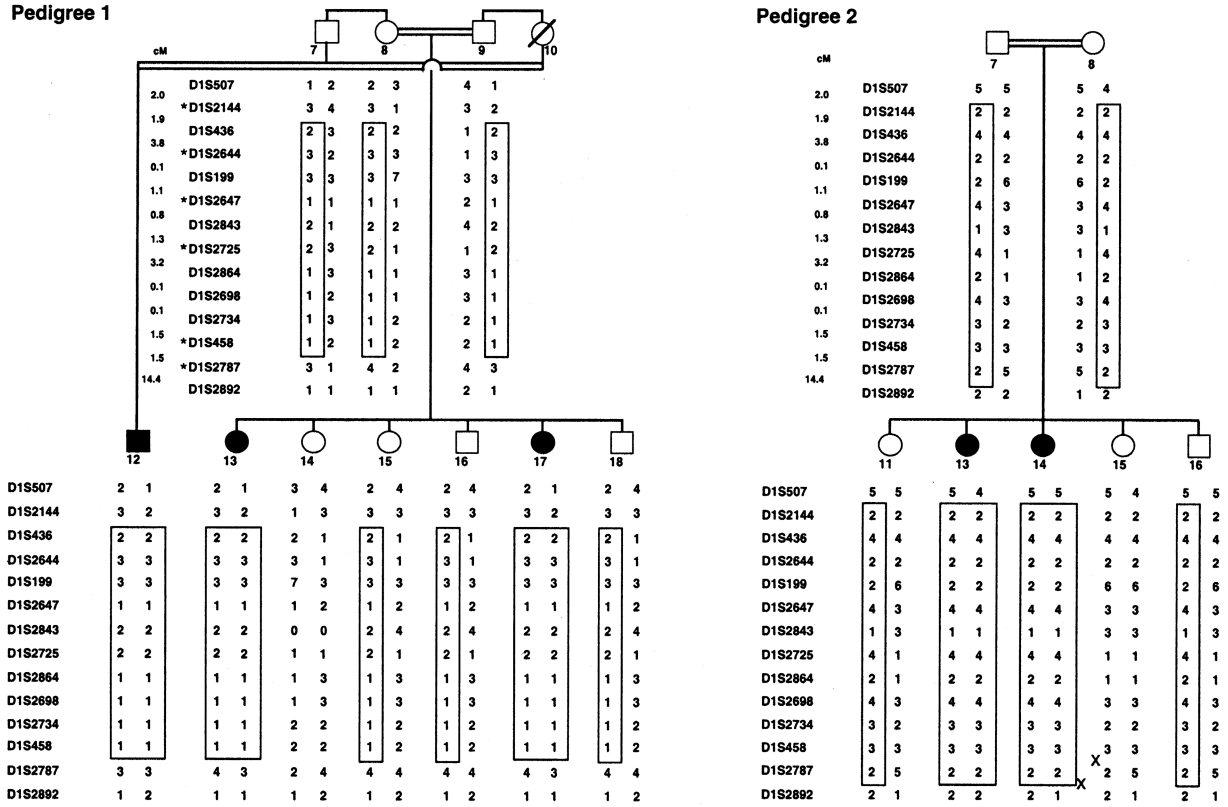

Figure 2 Haplotypes for the region of homozygosity on chromosome 1p36-35. Haplotypes for individuals in pedigrees 1 and 2, with the affected individuals shown as filled symbols, were as constructed by GENEHUNTER (Kruglyak and Lander 1995); the approximate genetic distances between the markers were taken from the Généthon human linkage map (Dib et al. 1996) or from the LDB. Markers accompanied by an asterisk (*) were taken from the LDB and are shown on the left of the markers. Boxes indicate the region of homozygosity in affected individuals. Also indicated is the region on chromosome 1p32 that has been mapped in pedigrees with autosomal dominant hypercholesterolemia (Hunt et al. 2000; Varret el al. 1999).

pedigree (Lander and Botstein 1987). Analysis of the genomewide scan data by GENEHUNTER, under an autosomal recessive model of inheritance with 98% penetrance, resulted in a parametric LOD score >2.0 for two regions, on chromosomes 1p35 (LOD = 2.4) and 12p13 (LOD = 2.5). Additional regions in which the parametric LOD score was ⩾0.8 were observed on chromosomes 1, 2, 4, 5, 9, 14, 15, and 17. However, the size of the pedigree that can be analyzed with GENEHUNTER software is restricted because of software limitations (Kruglyak and Lander 1995). Thus, information from only one of the unaffected siblings in the youngest generation of pedigree 1 could be included in these GENEHUNTER analyses, and data from individuals 15, 16, and 18 were disregarded. Inspection of the genotypes for the putative candidate regions in the pedigree revealed that, with the exception of the region on chromosome 1, the haplotypes shared by at least two of the three affected individuals were not restricted to affected individuals in the pedigree, because they were also shared by at least one unaffected sibling. On chromosome 1, only the three affected individuals shared the same haplotype in the region and were homozygous for one of the markers (D1S2734). We confirmed this by performing two-point analysis of the markers in these putative candidate regions, using the LINKMAP component of the FASTLINK program (Cottingham et al. 1993), which permits inclusion of all family members. This analysis showed that, when all the unaffected sibs were included, only chromosome 1 produced a LOD score >1.0 (table 2).

When the genomewide screen was performed on seven individuals in pedigree 2 (figure 1), many more markers were uninformative, a result that may reflect a greater degree of consanguinity in this pedigree. However, the two affected siblings were again homozygous at D1S2734 (although not for the same allele as in pedigree 1), and no unaffected members shared this genotype. In pedigree 2, one other region on chromosome 9q21-q22 also fulfilled the criteria for homozygosity mapping; however, this region was excluded in pedigree 1, because the two affected siblings had inherited dif-

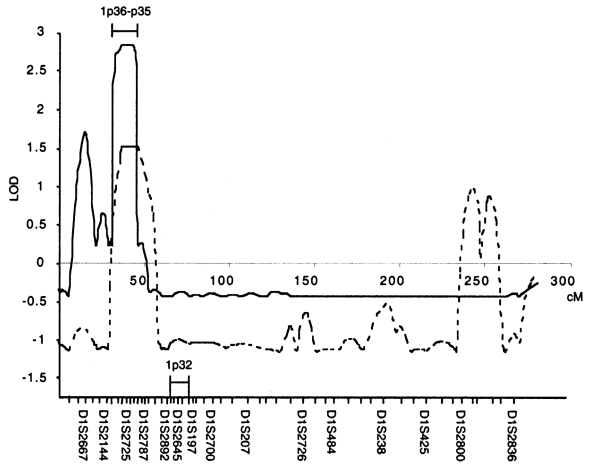

Figure 3 Linkage of the defect to chromosome 1p36-1p35. Parametric LOD scores for linkage on chromosome 1 were determined by GENEHUNTER (Kruglyak and Lander 1995), under an autosomal recessive model of inheritance in which it was assumed that the disease phenotype was 98% penetrant and that the disease-gene frequency was .002. Pedigree 1, solid line; pedigree 2, dashed line. Individuals 15, 16, and 18 in pedigree 1 could not be included because of the constraints of the software.

ferent paternal alleles and because the genotype shared by one of the affected siblings and the affected cousin was also shared by an unaffected sibling.

Fine mapping with markers spaced at ~1-cM intervals across the region of interest on chromosome 1 revealed a 12-cM region of homozygosity shared by all three affected individuals in pedigree 1 and also revealed a larger overlapping region of homozygosity in the two affected siblings in pedigree 2 (fig. 2). However, the haplotype of the putative mutant allele was different in the two pedigrees, which is not surprising because the families are of different racial origin and are therefore unlikely to share a common founder. When the data were analyzed by GENEHUNTER, peak parametric LOD scores of 2.83 in pedigree 1 and 1.51 in pedigree 2 were observed at D1S2725 (fig. 3), giving a combined LOD score of 4.34. No combined LOD score >1.5 was observed elsewhere in the genome (fig. 4). As described above, information from only one of the unaffected individuals in pedigree 1 could be included in the GENEHUNTER analysis of all the markers on chromosome 1, but the observed LOD scores for these individuals under this model of inheritance approached the maximum possible values of 3.09 for pedigree 1 and 2.13 for pedigree 2. Under the same model of inheritance, a three-locus (D1S2843, D1S2725, and D1S2864) multipoint analysis of the whole family, using FASTLINK, produced a LOD score at D1S2864 of 3.26 in pedigree 1 and 2.08 in pedigree 2. Again, these results approached the theoretical maximum values, assuming a fully informative marker of 3.47 for pedigree 1 and of 2.13 for pedigree 2.

Three recent studies have described genome mapping in families of index patients who had a clinical diagnosis of inherited hypercholesterolemia but in whom linkage to the genes for the LDL receptor and apoB had been excluded. In two of these studies, inheritance of autosomal dominant hypercholesterolemia, with a clinical diagnosis of typical heterozygous FH, was mapped to chromosome 1p32 in a single large pedigree from Utah (Haddad et al. 1999; Hunt et al. 2000) and to 1p34.1-p32 in a group of 13 families of western European origin (Varret et al. 1999). The region of overlap on chro-

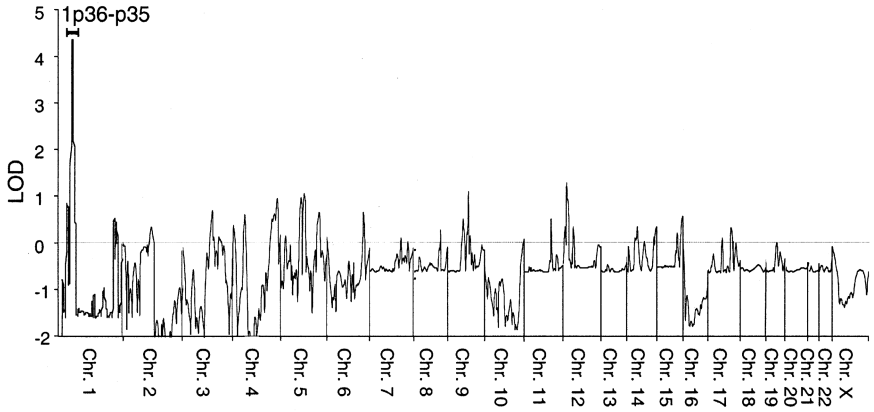

Figure 4 Combined LOD scores obtained in a genomewide screen with polymorphic markers in pedigrees 1 and 2. A genomewide screen, with markers located at ~8-cM intervals, was performed for the individuals as indicated in figure 1. Combined LOD scores were determined by GENEHUNTER analysis, but individuals 15, 16, and 18 in pedigree 1 could not be included because of the limitations of the software. The linkage analyses were performed under an autosomal recessive model of inheritance in which it was assumed that the disease phenotype was 98% penetrant and that the disease gene frequency was .002.

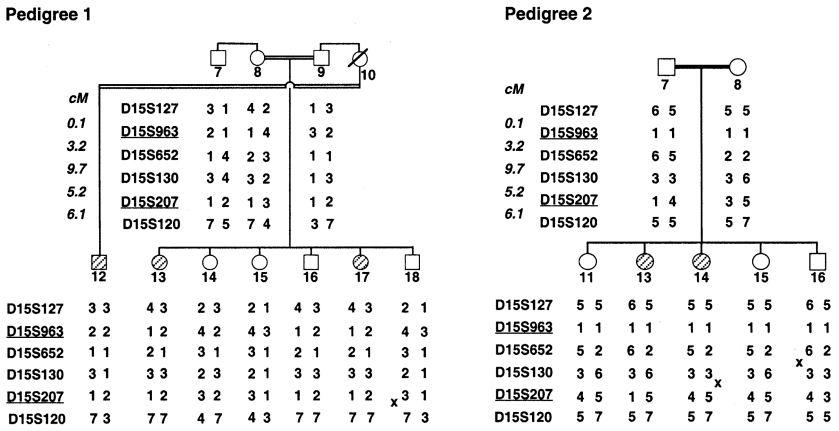

Figure 5 Exclusion of homozygosity in pedigrees 1 and 2 on chromosome 15q25-q26. Genotyping was performed with markers spanning a region on chromosome 15 that has recently been mapped in Sardinian families with a variant form of inherited autosomal recessive hypercholesterolemia (Ciccarese et al. 2000); the markers that delineate the 16-cM region of linkage on 15q25-q26 in that study are underlined. X indicates recombination events.

mosome 1 in these two studies is ~6 cM, lying between D1S2130 and D1S197, and is adjacent to, but clearly does not overlap, the region of chromosome 1 identified in the present study (fig. 3). The affected offspring in the present study were heterozygous for markers throughout the region identified in the families from Utah and France. Furthermore, the phenotype in the patients described here is apparently recessive, and it therefore differs from that in the patients in these two studies, although it is known that the penetrance of hypercholesterolemia in heterozygous FH can vary in different populations, even in individuals with the same gene mutation (Sun et al. 1994; Pimstone et al. 1998). Although we cannot exclude the possibility that in other environments some individuals heterozygous for the defective gene that we are seeking may be hypercholesterolemic, there is clearly a strong gene dosage effect in these two families, as is found in patients with FH due to mutations in the LDL-receptor gene (Goldstein et al. 1995). Interestingly, Varret and coworkers (1999) did not find linkage to their chromosome 1 locus in all the families tested, a result they interpreted as evidence for other genetic loci predisposing to hypercholesterolemia.

In the third study, mapping was performed in several Sardinian families of index patients who had a clinical phenotype of homozygous FH but did not have a defect in the LDL-receptor gene and whose cells in culture exhibited what appeared to be completely normal LDL-receptor activity. However, whole-body scans with labeled LDL showed that in vivo uptake of LDL by the liver was reduced, a result that led the authors to suggest that the genetic defect might be restricted to hepatic uptake of LDL (Zuliani et al. 1995). Genome mapping revealed linkage, with a LOD score of 4.0, to a 16-cM region on chromosome 15, although there did not appear to be a common haplotype carrying the putative disease gene in this relatively isolated population (Ciccarese et al. 2000). Again, the phenotype in the Sardinian families appears to differ from that in the pedigrees reported here, because we observed impaired internalization of an apparently normal LDL-receptor protein by cells in culture. We were unable to detect any linkage to chromosome 15 in the families reported here (as shown in fig. 5). Although the two siblings in pedigree 1 were homozygous for markers in this region, one of the unaffected siblings was also homozygous, and their affected cousin had inherited different alleles at this locus. In pedigree 2, the two affected siblings had inherited different paternal alleles in this region. Thus, our data exclude linkage of a recessive disorder to this locus in both families.

We conclude that the gene defect in the families described here has not been mapped previously and is likely to lie in the 1p36-35 region of chromosome 1. In pedigree 2, the computed linkage to this region was not as strong, because there were only two affected individuals and the family was much smaller. As stated above, the two families are unlikely to share a common ancestor; however, the clinical phenotype of the affected individuals is similar, as is the defect in their cells in culture, and these similarities suggest that they share the same gene defect. On the other hand, the affected individuals could equally well have defects in different genes whose protein products lie on the same cellular pathway, thereby resulting in the same phenotype. Nor can we formally exclude, at this stage, the possibility that the phenotype in the families described here has

arisen as the result of interaction between two or more gene variants. Resolution of these issues awaits the identification and characterization of the gene concerned. We have not yet identified any strong candidate genes in the region of 1p36-35, but the full genome sequence of this region is not available (Sanger Centre Chromosome 1 web site). Since cells from the affected individuals exhibit deficient internalization of LDL, analysis of differential expression of mRNA or protein in these cells compared with cells from normolipemic controls may assist in the identification of the defective gene.

Acknowledgments

We are grateful to the British Heart Foundation for grant BHF PG/98062. David Booth (Royal Free Hospital School of Medicine, London) and B. Knight (MRC Clinical Sciences Centre) kindly provided anonymous DNA samples for the determination of allele frequencies, and we thank A. Dearlove (UK HGMP Resource Centre, Cambridge), T. Aitman (MRC Clinical Sciences Centre), and R. Plomin and colleagues (SGDP Research Centre, Institute of Psychiatry, London) for providing primers. We are grateful to Simon Gregory (Sanger Centre, Cambridge) and Steven Wiltshire (Wellcome Trust Centre for Human Genetics, Oxford) for helpful discussions. We are indebted to K. Taylor (Birmingham City Hospital) for allowing access to the patients in pedigree 2, and we thank members of both families for their willing cooperation.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Généthon, http://www.genethon.fr
Genome database, http://gdbwww.gdb.org
Genetic Location Database, http://cedar.genetics.soton.ac.uk
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/, for familial hypercholesterolemia (MIM 143890)
Sanger Centre Chromosome 1 web site, http://www.sanger.ac.uk/HGP/Chr1/

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