Mapping a Gene Involved in Regulating Dietary Cholesterol Absorption

The Sitosterolemia Locus Is Found at Chromosome 2p21

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Abstract

The molecular mechanisms regulating the amount of dietary cholesterol retained in the body as well as the body's ability to selectively exclude other dietary sterols are poorly understood. Studies of the rare autosomal recessively inherited disease sitosterolemia (OMIM 210250) may shed some light on these processes. Patients suffering from this disease appear to hyperabsorb both cholesterol and plant sterols from the intestine. Additionally, there is failure of the liver's ability to preferentially and rapidly excrete these non-cholesterol sterols into bile. Consequently, people who suffer from this disease have very elevated plasma plant sterol levels and develop tendon and tuberous xanthomas, accelerated atherosclerosis, and premature coronary artery disease. Identification of this gene defect may therefore throw light on regulation of net dietary cholesterol absorption and lead to an advancement in the management of this important cardiovascular risk factor. By studying 10 well-characterized families with this disorder, we have localized the genetic defect to chromosome 2p21, between microsatellite markers *D2S1788* and *D2S1352* (maximum lodscore 4.49, $\theta = 0.0$). (*J. Clin. Invest.* 1998. 102:1041–1044.) Key words: genetics • sitosterolemia • linkage analyses • chromosomal localization

Introduction

Sitosterolemia is a lipid disorder first described by Bhattacharyya and Connor in 1974 (1). The disease is characterized by the presence of tendon and tuberous xanthomas, premature atherosclerotic disease, absence of a family history of premature coronary artery disease, and normal to occasionally elevated plasma cholesterol levels (2). Premature fatal myocardial infarction was the presenting feature in the next three families reported with this condition (2). Sitosterolemia shares several clinical characteristics with the well-characterized homozygous familial hypercholesterolemia (FH),[1] including expression of tendon xanthomas in the first ten years of life, and development of aortic stenosis and premature atherosclerosis in the first several decades. However, homozygotes for sitosterolemia usually have normal to moderately elevated total sterol levels, in contrast to the profound hypercholesterolemia in FH homozygotes. Sitosterolemia is further distinguished from FH by its autosomal recessive mode of inheritance and the diagnostic elevation of plasma phytosterols. This condition may be underreported, as the detection of plasma phytosterols requires the use of HPLC or capillary GLC (3). Sitosterol is clearly the major plant sterol species, hence the name sitosterolemia, although many other plant sterols are also significantly elevated in the plasma. The term phytosterolemia may therefore be preferable. Segregation analyses of a large Amish pedigree showed that the trait showed an autosomal recessive pattern of inheritance (4). The true prevalence of this disorder is not known, but > 40 individuals with this condition have been reported worldwide (3).

Under normal circumstances, our diets contain almost equal amounts of cholesterol and plant sterols. However, only 30–60% of total dietary cholesterol and $< 5\%$ of total plant sterols are retained by the body (5). The liver excretes most of the absorbed noncholesterol sterols rapidly into bile, almost unchanged, such that the net absorption of these sterols is almost negligible (5). In sitosterolemia, affected individuals show an increase in the absorption of total dietary sterols, with failure to discriminate between different sterol species and a failure to excrete absorbed noncholesterol species rapidly into bile (6–10). This leads to greatly expanded body pool size of both cholesterol and sitosterol. Clinical studies of affected individuals show that, in addition to the defects of absorption and excretion of sterols, whole body cholesterol biosynthesis is depressed (6, 11, 12). Treatments that normally result in an increase in cholesterol biosynthesis in normal individuals fail to do so in affected individuals (12). Additionally, analyses of liver and intestinal biopsies from affected individuals show that activities of many of the enzymes involved in cholesterol biosynthesis pathway are depressed (9, 11–13), suggesting that intracellular sterol pools are being sensed as "replete." However, the expression of the LDL receptor in liver and intestinal biopsies was found to be elevated, suggesting discordant regu-

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1. *Abbreviations used in this paper:* FH, familial hypercholesterolemia; SREBP, sterol regulatory element binding protein.

lation (11). The structural genes for LDL receptor and some of the enzymes along the cholesterol biosynthetic pathway have been excluded as sites of the defect (14). Similarly, transcriptional factors that are thought to play a major role in regulating many of these genes, sterol regulatory element binding proteins (SREBPs), have also been excluded (14). To identify the genetic defect in sitosterolemia, a genome-wide scan and linkage analyses of ten well-characterized pedigrees was undertaken.

Methods

Pedigrees. All probands were identified on the basis of clinical findings of tendon and tuberous xanthomas, the suspicion of sitosterolemia as a diagnosis, confirmed by the diagnostic elevation of the plasma sitosterol levels determined by either HPLC or capillary gas-liquid chromatography as previously described. Pedigrees 100, 200, 500, 700, 800, 2200, and 2300 have been previously described (4, 9, 15–19). Informed consent was obtained from all participants. Although individuals 100.1 and 2200.75 are dead, their DNA was available from fibroblast cultures established before death. Individuals 500.24, 500.27, 2200.76, 2200.79, 2200.81, 2200.82, 2200.83, 2200.84, and 2200.87 were not available for genotyping. All of these, except 2200.84, are unaffected siblings, as determined by plasma sitosterol levels.

Genotyping. Genomic DNA was extracted from whole-blood fibroblast cultures or transformed lymphoblastoid cell lines, as previously described (14). Genome-wide linkage analyses were performed using primer sets from the Weber version 8 screening set (Research Genetics, Birmingham, AL). Reverse primers from each set were end-labeled with γ-^{32}P-ATP, using standard techniques. PCR amplification of the microsatellite repeats was performed as previously described (14), and the products separated by denaturing urea-acrylamide gels, dried and analyzed by phosphorimager. Additional markers were analyzed using FAM-labeled primers (Research Genetics) and the PCR products analyzed by gel separation and fluorimetric detection using a fluorimager (Molecular Dynamics, Sunnyvale CA). Allele sizing was determined using CEPH family DNA, 1331-01 and 1331-02.

Linkage analyses. Linkage analyses were performed using the computer package LINKAGE (20, 21). The PREPLINK function was recompiled as a macro to run on a Macintosh using a Microsoft Excel worksheet. This checks the pedigrees for misinheritances of the alleles and generates an appropriately formatted output file. Two-point linkage was performed using MLINK, running on a DEC station 5000/200, Ultrix v4.1 operating system. Autosomal recessive inheritance and a disease prevalence of 1:5000 were specified. Allele frequencies were obtained from the CEPH family database. Multipoint analysis was performed using GENEHUNTER (22).

Results

We have assembled 10 families with the diagnosis of sitosterolemia, some of which have been reported previously (Fig. 1). There are two Japanese families (pedigrees 700 and 800), one Amish family (pedigree 2200), one Asian Indian family (pedigree 500), one Finnish family (pedigree 400), and one Dutch family (pedigree 2500); the remaining families are from the United States, and are of Caucasian origins (pedigrees 100, 200, and 300). DNA from 22 affected individuals, 11 unaffected siblings, and 17 parents was available for genotyping. The plasma sitosterol levels in affected individuals, their obligate heterozygous parents, unaffected siblings (from all except pedigree 700), and unrelated normal controls are shown in Fig. 2. Sitosterol levels in pedigree 700 have been reported previously (23). We have also included plasma sitosterol levels from other known sitosterolemia individuals on file in our databases. As can be seen, the diagnosis of affected status can be made definitively. Based solely on the plasma sitosterol levels, the heterozygous and normal control states are indistinguishable. Total plasma sterol levels in affected individuals may only be marginally elevated relative to control populations and do not correlate with the presence of accelerated atherosclerosis, suggesting that elevated cholesterol per se is not responsible for the increased cardiovascular risk.

A genome-wide scan was performed using a microsatellite screening set at an average interval of 25 cM, using families 100, 200, 500, 700, 800, and 2200. All of the autosomal chromosomes were examined. A significant two-point lodscore (3.49, $\theta = 0.1$) was obtained with marker *D2S1788.* For the remaining autosomes, the highest maximum lodscore obtained was 0.74, $\theta = 0.2$, for one telomeric marker on chromosome 19 (data not shown). Mapping markers at an average density of 10-cM intervals, using all of the families, gave a maximum lodscore of 4.49, $\theta = 0.0$, with the marker *D2S1346* (Table I). A multipoint analysis is shown in Fig. 3. By comparing the shared haplotypes within sibling pairs and examining for informative recombination events, we located the disease locus between markers *D2S1788* and *D2S1352* (Fig. 3). This region spans almost 15 cM. Family 800 showed no informative recombination events within the area of interest. Family 400 had one affected child, and his other two siblings did not share any haplotypes with the affected individual. Areas of haplotype sharing were made within pedigrees only and are shown by the closed areas. The unshared regions, by virtue of a recombination event on the maternal or the paternal chromosome, are shown in open

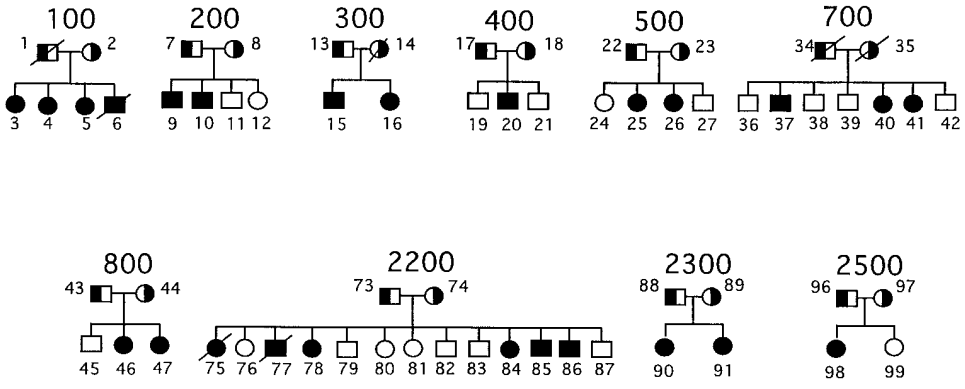

Figure 1. Sitosterolemia pedigree trees. In the pedigrees assembled for this study, affected individuals are shown by filled symbols. Only those individuals for whom both serum sitosterol values and DNA were available were included in the analyses. A total of 17 parents, 22 affected individuals, and 11 unaffected siblings were genotyped.

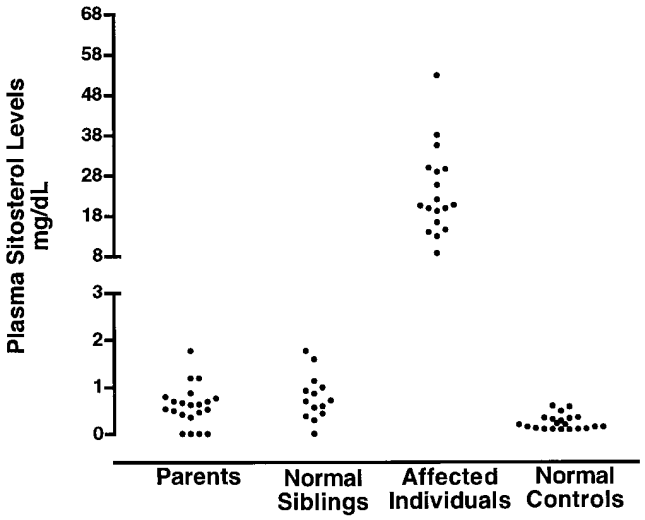

Figure 2. Plasma sitosterol levels in affected individuals, their obligate heterozygous parents, unaffected siblings, and normal controls. Plasma sitosterol levels were determined by capillary GLC or HPLC for all of our pedigree volunteers shown above as well as for 20 normal individuals chosen at random. Additionally, data on file on other families not included in this study are also shown. Note the break in the y-axis. In general, most unaffected individuals had plasma sitosterol levels < 1 mg/dL. Of the three parents and three siblings who had values higher than 1 mg/dL, all of these were measured by HPLC; these values may reflect the different technique used. None was higher than 2 mg/dL. All of the affected individuals had plasma sitosterol values > 8 mg/dL, and many of these values were obtained while the patients were in treatment.

areas. In families 300 and 500, two recombination events were detected. These were inferred for family 300 because the mother was not available for genotyping. For family 500, distinct haplotypes could be constructed for both parents. For all of the pedigrees analyzed, haplotype analyses showed that all of the affected individuals share the region between *D2S1788* and *D2S1352.* Markers *D2S1346* and *D2S1348* are very close to each other (< 0.5 cM), and their order has not been determined accurately. No evidence of genetic heterogeneity has been detected in our pedigrees. As sitosterolemia appears to

Table I. Two-point Lodscores between Microsatellite Markers and Sitosterolemia

	Recombination fraction, θ						
Locus	0.00	0.02	0.10	0.20	0.30	Z max	θ max
D2S1325	-∞	1.36	1.89	1.4	0.74	1.91	0.08
D2S1788	-∞	**3.25**	**3.49**	2.57	1.42	**3.59**	0.08
D2S1348	**4.41**	**4.19**	3.31	2.19	1.14	**4.41**	0.00
D2S1346	**4.49**	**4.27**	3.37	2.23	1.16	**4.49**	0.00
D2S1352	-∞	1.83	2.62	1.93	1.02	2.65	0.08
D2S1337	-∞	−0.46	0.89	0.84	0.48	0.95	0.14
D2S441	-∞	−6.27	−1.90	−0.54	−0.11	0.00	0.5
D2S1328	-∞	−5.36	−1.30	−0.23	0.03	0.03	0.3

Two-point lodscores were calculated with an autosomal recessive pattern of inheritance, a phenocopy rate of 0, and a disease prevalence of 1:5,000. Significant lodscores (> 3.0) are in boldface.

be very rare and the disease locus in pedigrees drawn from different racial backgrounds maps to the same region, the likelihood that mutations in different genes along a common metabolic pathway are responsible for causing the same or similar disease is reduced.

Discussion

The disease locus for sitosterolemia maps to 2p21, to an interval no larger than 15 cM, spanned by the microsatellite markers *D2S1788* and *D2S1352.* Because the families used for these analyses are of different racial origins and no evidence of genetic heterogeneity was found, these results would suggest that there is only one locus that is responsible for causing this rare disease. Although this region is too large for application of positional cloning, a number of cDNAs have been mapped to this region and could be considered as candidate genes. For example, the genes for 3-hydroxyanthranilate 3,4-dioxygenase, dioxin-inducible cytochrome P450 (*CYP1B1*), sodium/calcium exchanger 1 precursor, lutropin-choriogonadotropic hormone receptor and FSH receptor, amino acid transporter protein, phosphatidylinositol-glycan biosynthesis class F protein, a 130-KD leucine-rich protein, genes homologous to the *Drosophila*

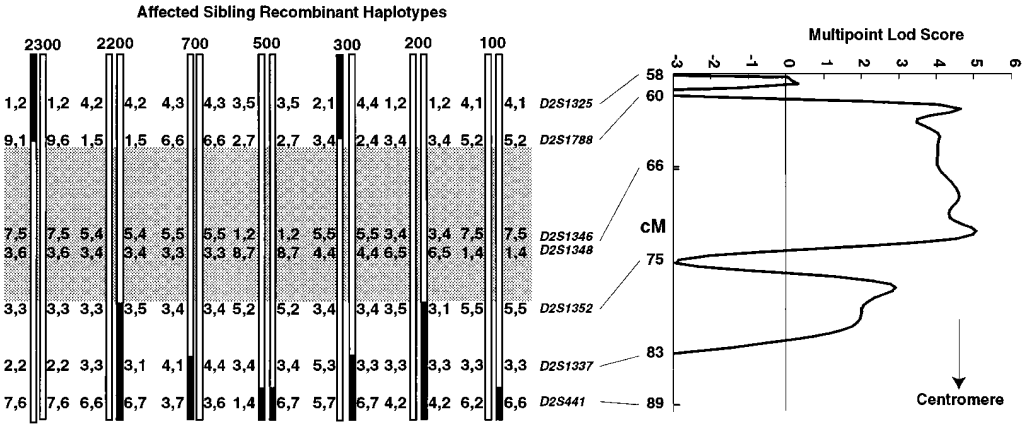

Figure 3. Multipoint and haplotype analyses of informative markers to localize the sitosterolemia gene. Multipoint analysis between markers *D2S1325* and *D2S441* was performed as described in Methods. The multipoint analysis is shown graphically on the right and the haplotype analysis on the left. Only those haplotypes that had an informative recombination are shown. The allele assignments are as indicated. The maximal area of localization is indicated by the shaded rectangle. The upper border is marked by the marker *D2S1788*, which is recombinant in pedigrees 300 and 2300. The lower border is defined by recombination events in pedigrees 200 and 2200 for marker *D2S1352.* For family 300, the mother was not available for genotyping and her haplotypes were therefore inferred.

melanogaster dosage compensation regulator, serum protein MSE55, TIS11B protein, and calmodulin all map to this region. More than 100 unidentified transcripts also have mapped to this region. Of the known genes that map to this region, none seem likely candidates genes based on their known and/or proposed functions, which are based on homology.

Normal individuals show almost no net dietary absorption of non-cholesterol sterols. This may result from a highly selective intestinal absorptive process, allowing cholesterol, but not other sterols, to enter the body, or by a very rapid and preferential clearance of these noncholesterol sterols by the liver into bile, giving the appearance of selective sterol absorption. Equally feasible is that both of these mechanisms may be operative. The identification of the gene in sitosterolemia is therefore likely to be very informative of these processes.

The other pathological features of sitosterolemia are increased foam cell formation, leading to widespread tendon and tuberous xanthoma formation with accelerated atherosclerosis. Although the mechanisms by which atherosclerosis is accelerated are not fully worked out, sitosterol and cholesterol per se are unlikely to be the key sterols that lead to the cascade of differential gene expression changes and foam cell formation. Sitosterol infusions in rats or man do not recapitulate the discordant gene regulation (24), and the plasma levels of cholesterol in many affected individuals with accelerated atherosclerosis are frequently not elevated (2, 3). However, as many other sterol species such as shell-fish sterols (8), are also absorbed, and many of these also are partially metabolized by the body to oxy-sterols (2, 3), any one of these compounds could be responsible for these changes (25). It is known that cholesterol needs to be metabolized before it can be "sensed" by the cell. Considerable advances have been made in identifying transcriptional factors that regulate intracellular cholesterol homeostasis, namely SREBPs, as well as the novel mechanisms by which SREBPs are activated. However, the intracellular sterol "sensor" has not been definitively identified. Although a number of sterol species have been shown to act as potent repressors of this pathway, both the exact sterol species that is sensed and the intracellular compartment that this occurs in have not been elucidated. While we believe that the gene product defective in sitosterolemia is primarily involved in selective transport of cholesterol across the plasma membrane, the pattern of discordant gene regulation observed suggests that these two pathways are linked. The elucidation of the gene defective in sitosterolemia may therefore shed some light on these processes.

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