Sitosterolemia: exclusion of genes involved in reduced cholesterol biosynthesis

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Abstract Sitosterolemia (phytosterolemia) is a rare autosomal recessively inherited disorder that is characterized by premature coronary artery disease, xanthomas, and increased plasma plant sterols and 5α-stanols. Affected individuals show an increased absorption of both cholesterol and sitosterol from the diet, decreased bile clearance of these sterols and their metabolites resulting in markedly expanded whole body cholesterol and sitosterol pools. Biochemical studies have shown that the regulation of the cholesterol biosynthetic pathway may be abnormal in this condition. In particular, the activities and mRNA for the biosynthetic enzymes, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and HMG-CoA synthase are low in liver biopsy specimens isolated from affected individuals, suggesting replete intracellular cholesterol pools. However, the membrane expression of hepatocyte low density lipoprotein receptors was increased, suggesting discordant regulation. Segregation analyses in three families for the genes for HMG-CoA reductase, HMG-CoA synthase, and LDL-receptor excluded these as sites of mutation. In view of the previously described discordant regulation of the above genes in sitosterolemia, the two major regulatory genes for this pathway, sterol regulatory element binding proteins (SREBP-1 and -2), were also examined. These genes did not segregate with the disease and were thus excluded. Two other genes involved in cholesterol absorption and chylomicron secretion, namely acyl coenzyme A:cholesterol acyltransferase (ACAT) and microsomal triglyceride transfer protein (MTP) were also examined for segregation and similarly excluded. Although the gene defect in sitosterolemia therefore remains to be elucidated, important candidate genes have been excluded.—Patel, S. B., A. Honda, and G. Salen. **Sitosterolemia: exclusion of genes involved in reduced cholesterol biosynthesis. *J. Lipid Res.* 1998.** 39: **1055–1061.**

Supplementary key words sitosterolemia • genetics • cholesterol biosynthesis • gene exclusion

Sitosterolemia (also known as phytosterolemia, OMIM number 210250) is a rare autosomal recessively inherited disorder, characterized by the presence of tendon and tuberous xanthomas, premature coronary artery disease causing lethal myocardial infarction in males, but with normal to only moderately elevated plasma cholesterol levels (1–3). Clinically, it is distinguishable from familial hypercholesterolemia by its pattern of inheritance and from cerebrotendinous xanthomatosis by absence of neurological involvement and by the diagnostic hallmark of elevated plasma sitosterol level (with other phytosterols). Sitosterol is the major plant sterol component in our diets. Under normal circumstances, plant sterols are poorly absorbed by the intestine and the total phytosterol levels in the plasma are less than 1 mg/L, compared with a range of 10–60 mg/dL reported for patients with sitosterolemia (2). Routine enzymatic cholesterol measurements do not discriminate between cholesterol and phytosterols, and the detection of elevated plant sterols and 5α-stanols is made by capillary-gas or high performance liquid chromatography analyses of plasma (2, 4). This, in part, may allow for under-reporting of this condition, hence its true prevalence is not known.

The molecular defect in sitosterolemia has not been identified. Segregation of plasma sitosterol levels in 250 individuals in a large Amish kindred with five affected siblings showed that the pattern of inheritance fit best a rare single gene recessive inheritance model (5). Reports of further pedigrees in the literature have supported this conclusion (see ref. 3). Although a variety of hypotheses have been advanced regarding the possible site of primary defect (1), it is pertinent to discuss the normal cholesterol and phytosterol physiology to place these in context.

The normal daily diet in the US contains about 250–350 mg of plant sterols and 400–600 mg of cholesterol. Absorption of dietary cholesterol may range from 30 to 60%, but only 5% or less of dietary phytosterols are absorbed (6). Of the absorbed phytosterols, more than 80% are excreted in the bile unchanged (6). The exact mecha-

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; MTP, microsomal triglyceride transfer protein; SREBP, sterol regulatory element binding protein; HMG-CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; PAC, plasmid artificial chromosome.

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nism of the absorption of sterols in the gut has not been fully elucidated, but the rate of sterol net esterification has been suggested to play a major role (7–9). Almost 70–80% of the absorbed cholesterol is esterified and transported as cholesteryl ester in the chylomicron particles, but of the small amounts of phytosterols absorbed, the majority of these are transported as free sterols, with only 10–20% being esterified (10). Conversely, the molecular mechanisms by which free sterols are excreted into the bile is also not well characterized (11, 12). It is not known how free cholesterol is transported into bile across the plasma membrane, but the relative proportions of phytosterols to cholesterol excreted suggest that phytosterols are preferentially excreted (6, 13, 14). The mechanism for this 'selectivity' has not been elucidated. Studies in sitosterolemic patients show that this sterol discrimination between cholesterol and other phytosterols and shell fish sterols, exhibited by both the intestine and the liver, is impaired; both phytosterols and cholesterol show increased absorption, and both are also very poorly excreted into bile (15).

Finally, studies of whole body cholesterol synthesis suggest that in sitosterolemic subjects, relative to controls, synthesis of cholesterol and turnover of both sitosterol and cholesterol are reduced significantly (6, 13, 14, 16). Examination of the rate-limiting enzyme for cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase showed that the activity of this enzyme, in both isolated monocytes, liver or ileal biopsy specimens was considerably reduced (17–22). Both the mRNA and mass of this enzyme were decreased, but the catalytic efficiency was normal, suggesting that the structural gene might not be defective. In contrast, the LDL-receptor activity, as measured by radiolabeled LDL binding and degradation, showed normal or increased activity, suggesting discordant regulation of the LDL receptor and HMG-CoA reductase genes (18). That this finding is not a direct inhibition of sitosterol is suggested by animal studies where either dietary feeding or infusion of sitosterol led to an increase in the hepatic activity of HMG-CoA reductase (23). However, incubation of polarized CaCo-2 cells with sitosterol (or stigmasterol) resulted in a significant reduction of both the mRNA and protein for HMG-CoA reductase (24). The observations cannot be easily reconciled. Sitosterol did, however, competitively inhibit the rate-limiting enzyme for bile acid synthesis, cholesterol 7α-hydroxylase, and the activity of this enzyme in sitosterolemic patients is also decreased, although the mRNA for cholesterol 7α-hydroxylase was not reduced (23, 25, 26).

Based on the above, a number of hypotheses have been put forward to explain the primary defect in sitosterolemia. *1*) The defect involves a gene product important in the esterification/de-esterification cycle for sterol absorption and metabolism; *2*) the defect involves structural or regulatory gene for cholesterol biosynthetic pathway; or *3*) the defect involves a putative sterol 'transporter' expressed in the intestine and the liver.

In this study, we have examined the activities of HMG-CoA reductase, synthase and the LDL receptor in the liver of four sitosterolemic homozygous individuals. Although the HMG-CoA reductase has been previously shown to be discordantly regulated relative to HMG-CoA synthase and LDL-receptor (27, 28), the reduced activity of HMG-CoA synthase, in the face of normal or increased LDL-receptor activity would be highly suggestive of discordant transcription regulation. The promoter elements for the LDL receptor and HMG-CoA synthase genes are highly homologous and functional studies have suggested that these are tightly and coordinately regulated (29). By using polymorphic markers close to or within the genes (30, 31), we also examined some of these candidate genes as causative for the disease. In three families with sitosterolemia, our data show that the genes for acyl coenzyme A:cholesterol acyltransferase (ACAT), microsomal triglyceride transfer protein (MTP), HMG-CoA reductase, HMG-CoA synthase, and LDL-receptor did not segregate with this disorder.

These studies exclude any defect of the structural genes for the rate-limiting enzymes controlling cholesterol biosynthesis. We also examined the regulatory sterol element binding protein (SREBP) transcriptional factor genes (32) as primary causes of the defect. These genes also did not segregate with the disease.

METHODS

Pedigrees

All studies have been approved by our respective Institutional Review Boards and written consent was obtained from all our participants. The diagnosis of sitosterolemia was based on the clinical findings, and the presence of elevated levels of plasma plant sterols and 5α-stanols. The pre-treatment plasma sitosterol levels in all affected individuals were greater than 8 mg/L, as determined by capillary-gas–liquid chromatography. **Figure 1** shows the sitosterol levels in affected individuals, obligate heterozygotes, unaffected siblings, and normal controls. These data include individuals from the current study as well as those on file in our laboratories. The measurements demonstrate that the diagnosis of sitosterolemia can be made with considerable certainty as the levels in affected individuals are very clearly elevated. Although one of the obligate heterozygotes (parents) and two of the siblings have slightly elevated plasma sitosterol levels (1.8 mg/dL, 1.8 mg/dL, and 2.84 mg/dL), these are the exception. In general, the obligate heterozygotes, siblings, and the normal control values are indistinguishable. All families (**Fig. 2**), except pedigrees 300 and 400, have been previously reported (33–36).

Liver biopsy specimens

Liver biopsy specimens (~500 mg) were obtained for diagnostic histology from four untreated sitosterolemic patients (ages 26–35 years, 3 sisters, pedigree 100, and 1 male, pedigree 200) during ileal by-pass surgery performed as therapeutic intervention for symptomatic coronary artery disease. The patients had not been treated with diets or drugs for 2 months leading up to the surgical intervention. Liver specimens from normal control subjects were obtained from 19 subjects (11 female, 9 males, ages 16–67 years) from the Liver and Tissue Procurement and Distribution System (National Institutes of Health, contract NO1-DK 62274, University of Minnesota Hospital, Minneapolis, MN). All individuals were healthy when they died accidentally and their

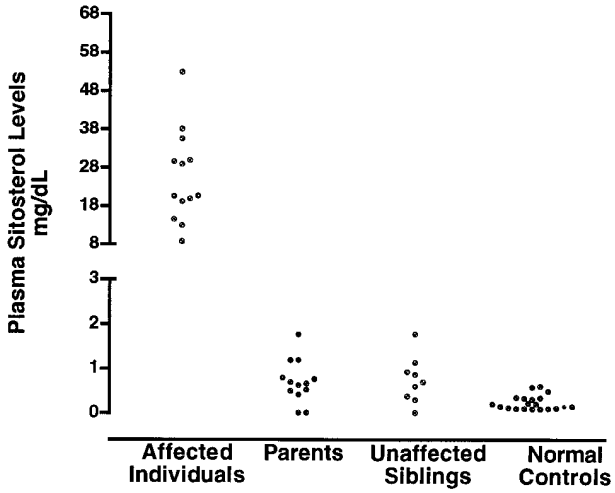

Fig. 1. Plasma sitosterol levels in affected, obligate heterozygotes, siblings, and normal controls. Plasma sitosterol levels were measured as described in Methods. In addition, we have included data from four further pedigrees for a more complete characterization. The median plasma sitosterol levels were: in affected individuals (n = 15) 20.6 mg/dL, in obligate heterozygotes (parents, n = 11) 0.67 mg/dL, in unaffected siblings (n = 8) 0.9 mg/dL, and in normal controls (n = 20) 0.18 mg/dL. In contrast to the affected status, a ready distinction cannot be made between obligate heterozygotes, siblings (who may or may not be heterozygous for the defective allele) and normal controls based upon their plasma sitosterol levels. There are three 'outliers,' with slightly higher values. There were no gender differences. There is a difference in the scale for the y-axis.

livers became available because no suitable recipient could be found. The specimens were received on dry ice and stored at −70°C for less than 3 months prior to use.

Assay of HMG synthase activity

Cytosolic fractions from liver were prepared by differential ultracentrifugation (22, 37) and were dialyzed for 24 h at 4°C against a 1000-fold excess of 20 mmol/L potassium phosphate buffer, pH 7.7, containing 0.1 mmol/L EDTA and 0.5 mmol/L dithiothreitol to inhibit contaminating HMG-CoA lyase activity (38). HMG-CoA synthase activity was assayed according to the method of Scharnagl et al. (39) with minor modifications.

Assay of HMG-CoA reductase

The activity of the microsomal total HMG-CoA reductase was measured as described previously (18, 22).

DNA isolation

Peripheral white blood cells (WBC) were used to extract DNA using DNAzol (Gibco BRL, Bethesda, MD) according to manufacturers' conditions.

Genotyping

Oligonucleotides for genotyping, Weber Screening Set 8A, were obtained from Research Genetics (Research Genetics, Huntsville, AL). Oligonucleotides for genotyping for HMG-CoA reductase, and MTP were as previously described (40, 41). For the ACAT locus, a GT dinucleotide repeat within intron 13 was used (T-Y. Chang, Dartmouth Medical School, NH). For the LDL receptor, the marker *D19S394* (GDB Accession ID 198395) was used. This marker is at the 3′ end of the LDL receptor gene (Dr. Jonathan Cohen, UT Southwestern Medical Center, TX). For SREBP-2, although a chromosomal map position is known, no fine mapping or close polymorphic markers were available. With the help of Dr. Ian Dunham (The Sanger Center, UK) a PAC contig around this region was screened by PCR for the presence of SREBP-2 sequences. A positive hit was found for PAC 41K5 from the RPCI1 human PAC library, which is also positive for the polymorphic marker *D22S307* (GDB Accession ID 190622). More information is obtainable from http://www.sanger.ac.uk/HGP/Chr22/. With the help of Dr. Michael Lovett (UT Southwestern Medical Center, TX), HMG-CoA synthase was mapped to YACs 801D5 and AA2A10. However, no polymorphic markers that mapped to these YACs were available in the databases, hence markers spanning this region were used for segregation analyses. Polymorphic marker alleles were amplified by PCR using 30–60 ng of genomic DNA. The reaction mix of 10 μl contained 1 μmol/L primers, spiked with [^{32}P] end-labeled reverse primer, 200 μmol/L of each deoxynucleotide triphosphate, 1 unit of Taq polymerase (Gibco BRL) and 1.5 mmol/L magnesium chloride. Amplification was performed in a Hybaid thermocycler (denaturation 94°C for 5 min, 30 cycles, 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s, followed by 10 min at 72°C). An equal volume of 95% formamide dye was added, and samples were denatured by heat and analyzed by 6% denaturing polyacrylamide gel electrophoresis. The gels were dried and subjected to autoradiography or phosphorimage analyses. Allele sizes were scored relative to each other.

Binding of low density lipoproteins to liver membranes

LDL binding to liver membranes was performed as previously described (18). High affinity (receptor-mediated) binding was determined as the difference between total binding (assayed in the absence of unlabeled LDL) and non-specific binding (assayed in the presence of 40-fold excess unlabeled LDL).

Linkage analyses

Linkage analyses were performed using the LINKAGE computer program package (30, 42). Autosomal recessive pattern of inheritance was assumed. Linkage analyses were performed at a recombination fraction (θ) of 0.01, 0.1, 0.2, 0.3, and 0.5. A lod score of 3 or more is taken to indicate evidence of significant linkage to the disease (30).

RESULTS

Hepatic activities of HMG-CoA synthase, reductase, and LDL receptor-mediated binding

Table 1 lists the enzymatic activities of hepatic HMG-CoA synthase, reductase, and the surface LDL receptor binding activities in 4 sitosterolemic (3 females and 1

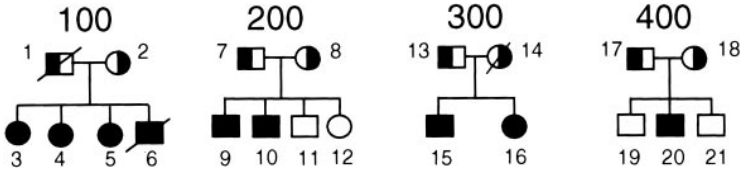

Fig. 2. Sitosterolemia pedigrees analyzed in this paper. Obligate heterozygotes are indicated by the partially filled symbols. Unaffected siblings are indicated by open symbols, as there are no diagnostic features to allow us to assign a heterozygous or a homozygous normal genotype, based on biochemical parameters.

TABLE 1. Activities of HMG-CoA synthase, reductase, and LDL receptor binding activities in subjects with sitoterolemia and control subjects

Subjects	Microsomal Activity		Hepatocyte Membrane
	HMG-CoA Synthase	HMG-CoA Reductase	LDL Receptor-Mediated Binding
	pmol/mg protein·min		*ng/mg protein*
Controls (n = 19)	360 ± 45	78 ± 23	95 ± 8
Sitosterolemia (n = 4)	162 ± 20[a]	19 ± 6[a]	252 ± 30[a]

Enzymatic and receptor activities were measured as described in the Methods. All data were normalized for protein content as indicated (±SEM). Statistical comparisons were made using non-paired Student's *t*-test. Activities of HMG-CoA synthase and reductase in the microsomes isolated from affected individuals showed a significantly reduced level of activity relative to the normal controls. Activity of the LDL receptor on the hepatocyte membrane preparations was increased by 2.7-fold relative to controls.

[a] $P < 0.05$.

male) and 19 control subjects (11 females and 9 males). Cytosolic HMG-CoA synthase activity was decreased by 55% and the HMG-CoA reductase by 76%. However, the LDL receptor activity, as judged by high affinity binding, was increased by 270% relative to the normal control subjects. Thus key enzymes in the cholesterol biosynthesis pathway were inhibited, in combination with an increase LDL receptor activity, suggesting discordant regulation of this pathway.

Exclusion of candidate genes

Figure 3 shows the pattern of inheritance in families 100 and 200 for polymorphic markers for the HMG-CoA reductase and the LDL receptor genes. The trinucleotide repeat polymorphic marker for HMG-CoA reductase is located 10 kb from the 3′ end of exon 2 of the gene (41). For pedigree 100, all the affected sisters have inherited different alleles, as have the two affected brothers in family 200 (Fig. 3, middle panel, HMG-CoA reductase). This, therefore, excludes this locus as a site of the mutation. Previous studies have showed that the HMG-CoA reductase activity and enzyme levels were reduced in liver biopsy specimens from affected individuals in the face of normal or increased expression of the LDL receptors (18–20, 35). It is possible, but highly unlikely, that the LDL receptor gene is mutated and the HMG-CoA reductase gene is normally regulated. Based on the extensive knowledge of LDL receptor function, and that any mutations affecting this locus show an autosomal dominant pattern of inheritance (43), we do not consider this locus as a strong candidate gene. However, to exclude this locus, we segregated the LDL receptor in families 100 and 200. The LDL receptor locus did not segregate with the disease (Fig. 3, bottom panel, LDL receptor). The father in family 200 was homozygous for this marker, therefore this pedigree was uninformative. HMG-CoA synthase is coordinately regulated with the LDL receptor. However, the enzyme activity of this enzyme was also found to be lower in liver biopsy specimens from affected individuals relative to controls (Table 1). Hence we examined this locus for segregation with the disease. Although our mapping studies showed that the locus for HMG-CoA synthase was fine-mapped to a YAC contig, no close (<5 cM) polymorphic markers in the databases were available. Therefore, we analyzed the segregation of a set of markers close to the HMG-CoA synthase locus. As both HMG-CoA synthase and reductase are on the same chromosome, chromosome 5, we also analyzed markers at an average distance of 25 cM apart for evidence of linkage to this entire chromosome (**Table 2**). Marker *D5S1457* (Table 2) maps to within 10 cM of HMG-

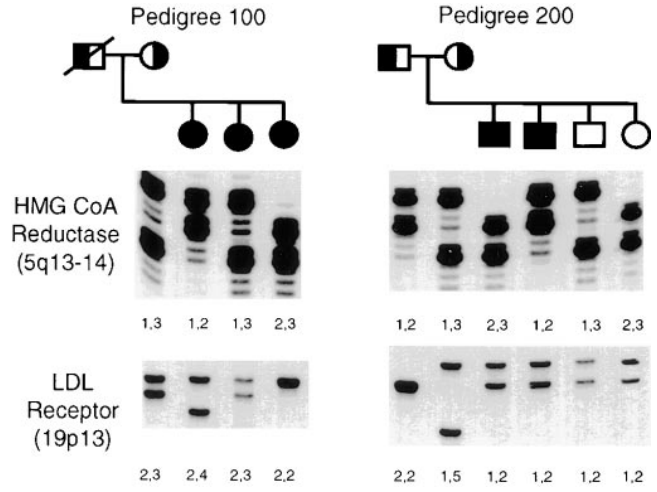

Fig. 3. Exclusion of HMG-CoA reductase and the LDL receptor loci. In two families (top panel), we examined for segregation for the HMG-CoA reductase and LDL receptor loci. For both pedigrees 100 and 200, affected individuals have inherited different HMG-CoA reductase alleles from their parents, thus excluding this locus (middle panel). For the LDL-receptor, pedigree 200 was not informative, as the father was homozygous for the marker, and all the children had inherited the same allele from their mother (bottom panel). However, for pedigree 100, none of the affected sisters inherited the same LDL receptor alleles, thus excluding this locus.

TABLE 2. Chromosome 5 summed lod scores

Marker	Distance from Telomere	Recombinant Fraction, θ		
		0.1	0.2	0.3
	cM			
CHLC.GATA145D10	6	−1.54	−0.64	−0.24
CHLC.GATA134BO3	38	0.41	0.39	0.23
D5S1457	63	−0.24	0.07	0.07
D5S1501	92	−0.13	0.05	0.04
D5S1462	113	−1.38	−0.44	−0.22
D5S2501	126	−1.97	−0.87	−0.34
D5S816	149	−1.20	−0.43	−0.14
D5S820	173	−1.39	−0.54	−0.19
D5S1471	188	−2.03	−0.81	−0.29
D5S1456	192	−1.89	−0.77	−0.28
D5S408	221	−1.12	−0.53	−0.23

The estimated distance of the markers from the telomere is indicated in column 2. Two-point linkage analyses were performed as described in Methods. For our pedigrees, at a recombination fraction θ = 0.2, our maximum estimated lod score for linkage, using SIMLINK analyses, was 2.89. The highest lod score measured was 0.42 at a θ of 0.1 (see also Fig. 4). The majority of the data show negative lod scores, suggesting that none of the markers on chromosome 5 segregate with the disease.

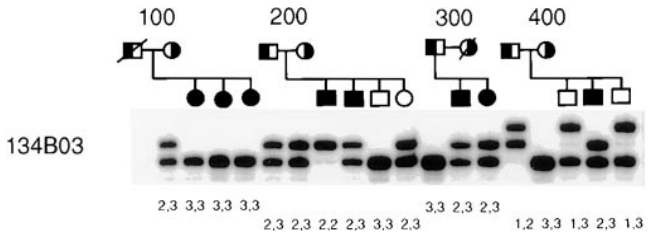

Fig. 4. Pattern of allele distribution for marker *CHLC.GATA 134BO3* on chromosome 5. As this marker had given a positive lod score (Table 2) and the candidate genes are located relatively close to this marker, we show the pattern of allele inheritance to demonstrate that this result is not likely to be of any significance. For pedigree 100, in the absence of the paternal allele pattern, we cannot rule out this locus. For pedigrees 300 and pedigrees 400, one of the parents is homozygous for this marker, hence the pattern of inheritance is non-informative. Hence the basis for exclusion is based upon the allele inheritance pattern for pedigree 200, where both affected brothers have inherited different alleles.

CoA synthase. No evidence of segregation was found. However, one marker, *CHLC.GATA134BO3*, gave a positive lod score. Examination of the allele inheritance pattern (**Fig. 4**) showed that three of the four families were non-informative for this marker. For pedigree 100, for example, the absence of the parental allele patterns makes it difficult to conclude evidence for linkage for this marker. One family (pedigree 300, Fig. 4) that was informative showed non-segregation. Additionally, the lod score was not high enough to suggest evidence for linkage of this chromosomal position with sitosterolemia.

The transcriptional factors SREBP-1 and -2 have been shown to regulate the transcription of genes involved in homeostasis of cholesterol within the cell. In view of the potential dysregulation of HMG-CoA reductase and LDL receptor, we examined these genes as candidate genes. For SREBP-2, we identified a marker, *D22S307*, that is no more than 100 kb apart from the SREBP-2 locus. This marker was informative in both families shown. No co-segregation for the SREBP-2 marker with sitosterolemia was found (**Fig. 5**). For SREBP-1, no polymorphic repeats

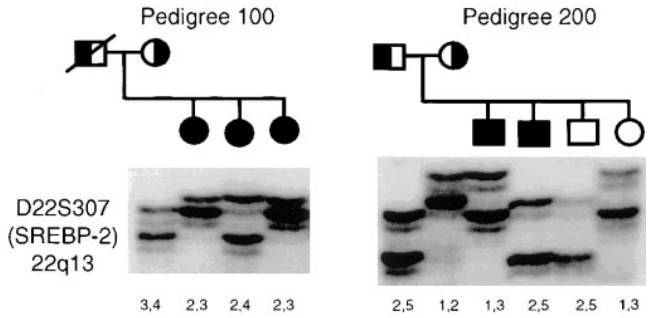

Fig. 5. Exclusion of SREBP-2 locus. Both the pedigrees analyzed were informative for the marker, *D22S307*, and show that the affected individuals have inherited different alleles. This marker is within 100 kb of the SREBP-2 locus, and it is highly unlikely that a recombination event could have taken place for all three sisters in pedigree 100 and for both affected brothers in pedigree 200 to account for these results.

TABLE 3. Chromosome 17 summed lod scores

Marker	Distance from Telomere	Recombination Fraction, θ		
		0.1	0.2	0.3
	cM			
D17S1308	0	−1.09	−0.35	−0.10
D17S1303	31	−0.72	−0.21	−0.05
D17S1293	61	−1.97	−0.81	−0.30
D17S1290	90	−2.25	−0.90	−0.33

Analyses were performed as described for Table 1. The four markers used span 90 cM from the telomere of chromosome 17. SREBP-1 locus is estimated to be about 65–70 cM away from the telomere. As can be seen, none of the markers spanning this region showed evidence of linkage, thus excluding this site.

have been reported. We therefore segregated markers at an approximate 25 cM spacing on chromosome 17, spanning its chromosomal map position (44). The results are shown in **Table 3**. No evidence of linkage was found.

Because the absorption of sterols could be affected by the rate of esterification, we also examined the ACAT gene for segregation (45). A trinucleotide repeat within the gene was used. Again, no co-segregation was found (**Fig. 6**). As the activity of microsomal triglyceride transfer protein is known to be an absolute requirement for absorption of dietary lipids, we also examined this gene. Although mutations in the MTP gene are responsible for causing abetalipoproteinemia (46), this protein is known to mediate transfer of neutral lipids, such as cholesteryl ester into nascent lipoprotein particles in the ER (47), hence subtle mutations of this gene could conceivably be responsible for non-selective absorption of sterols from the diet. However, this locus did not segregate with this disease (**Fig. 7**).

DISCUSSION

Sitosterolemia is an autosomal recessively inherited disorder characterized by the presence of tendon and tuberous xanthomas and premature coronary artery disease in the face of normal or mildly elevated plasma LDL levels (2, 3). The latter, together with the pattern of inheritance, distinguishes this relatively rare lipid disorder from familial hypercholesterolemia (43). The hallmark of sitosterolemia is elevated plasma phytosterol levels. This biochemical abnor-

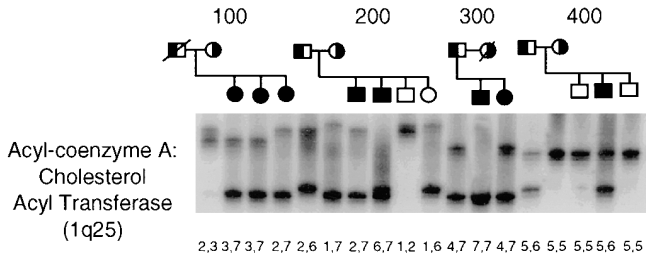

Fig. 6. Exclusion of ACAT locus. The ACAT gene was segregated by the use of a GT repeat sequence in intron 13 of this gene. In three pedigrees (100, 200, and 300), the ACAT alleles do not segregate with the disease state, thus excluding this locus.

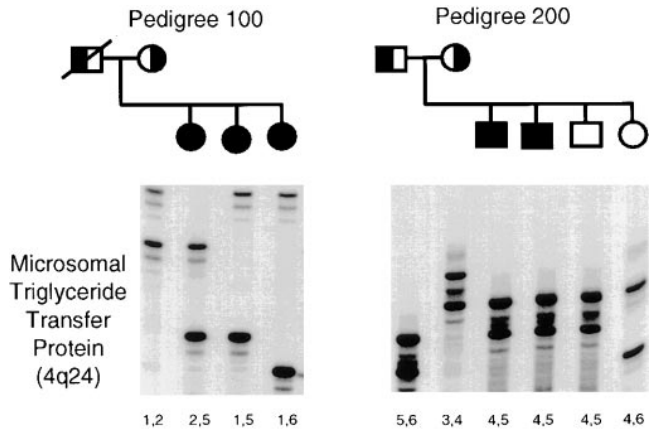

Fig. 7. Exclusion of MTP locus. Segregation of the MTP locus was examined in two families. The polymorphic marker is located in intron 10 of this gene. For pedigree 100, none of the affected sisters have inherited similar alleles, thus excluding this locus. For pedigree 200, an unaffected son has inherited the same alleles as the affected brothers, thus excluding this locus.

mality is both diagnostic as well as informative of the homozygous state. The heterozygous state is indistinguishable from the normal phenotype. Although there have been reports of minor differences between the heterozygous state and normals, these reports have not been confirmed on larger numbers of individuals to be robust (16, 48). To date, no heterozygote has been reported with an 'intermediate' level of plasma sitosterol. As sitosterol is the major component of phytosterols, this condition has been termed both sitosterolemia or phytosterolemia, respectively.

In sitosterolemia, absorption of all sterols is apparently greatly increased, including that for cholesterol. Hence hyperabsorption and loss of selectivity are present. This is not the only defect; the excretion of both cholesterol and phytosterols into bile by the liver is also greatly impaired (13, 14). As a consequence, both the cholesterol and sitosterol body pools are elevated (13, 14, 16). Additionally, the activities of a number of enzymes along the cholesterol biosynthetic pathway are reduced. HMG-CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis, and HMG-CoA synthase, which catalyzes the formation of HMG-CoA, were reduced in liver specimens isolated from affected individuals (Table 1). Our studies show that the structural genes for HMG-CoA reductase and synthase are not the sites of defect. The transcriptional regulatory genes SREBP-1 and -2 that are known to regulate these genes (32) also do not appear to be the defective. Whilst this excludes a genetic defect involving these gene products, abnormal regulation at the level of the protein has not been ruled out.

The other known defect in sitosterolemia involves sterol discrimination, both at the intestinal level and at the hepatic level for bile excretion of free sterols. Our knowledge of the molecular processes that control cholesterol transport into and out across cell membranes is poorly understood. We do not understand the molecular mechanism(s) by which dietary cholesterol enters the intestinal cells, whether sterol esterification is a necessary and absolute step required for this process, or whether this process is wholly dependent on carrier-mediated or facilitated transport. One difference between absorption of dietary cholesterol and sitosterol is that a large proportion of cholesterol is esterified (75% or more) compared to sitosterol (25% or less) appearing in the chyle. One candidate for this is the activity of ACAT. We have tested the ACAT gene cloned by Chang and colleagues (45) as a candidate gene. This gene was also conclusively excluded as defective in sitosterolemia. However, the possibility that the genetic defect involves another 'ACAT' cannot be ruled out, as there may be more than one ACAT-like activity that may be involved in sterol esterification. Another gene that is known to be involved in dietary lipid absorption is MTP. Genetic defects in MTP are now known to cause abetalipoproteinemia (46). MTP can transfer neutral lipid, including cholesteryl esters (47), into chylomicron particles and its activity is necessary for chylomicron synthesis and secretion. A subtle mutation in this protein could conceivably also cause loss of selectivity for sterol esters. However, our segregation analyses also excluded this gene as a cause of defect.

The elucidation of the gene defect in sitosterolemia may therefore shed some light on the pathways that are involved in dietary sterol transport across the intestinal cell and from the liver into bile.

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