

Phenotypic heterogeneity of sitosterolemia

Jian Wang,* Tisha Joy,* David Mymin,[†] Jiri Frohlich,[§] and Robert A. Hegele^{1,*}

Robarts Research Institute and University of Western Ontario,* London, Ontario, Canada; University of Manitoba,[†] Winnipeg, Manitoba, Canada; and St. Paul's Hospital and University of British Columbia,[§] Vancouver, British Columbia, Canada

Abstract Sitosterolemia is a rare autosomal recessive disorder of lipoprotein metabolism characterized by xanthomas and increased plasma concentrations of plant sterols, such as sitosterol. Causative mutations occur in either the *ABCG5* or *ABCG8* gene, each of which encodes a sterol half-transporter expressed in the intestine. We report five Canadian subjects with nonsense mutations in these half-transporters: four related Caucasian subjects were homozygous for the *ABCG8* S107X mutation, and one unrelated Japanese-Canadian subject was homozygous for a complex insertion/deletion (I/D) mutation in *ABCG5* exon 3. A female subject with each mutation was symptomatic with coronary atherosclerosis: a 5-year-old *ABCG8* S107X homozygote and a 75-year-old *ABCG5* exon 3 I/D homozygote; these represent the extreme ends of the spectrum of vascular involvement in sitosterolemia. The largest reductions in plasma concentrations of sitosterol and LDL-cholesterol were seen with ezetimibe or bile acid sequestrant treatment, and less dramatic reductions were seen with statin drug treatment. These findings extend the range of clinical phenotypes in sitosterolemia caused by nonsense mutations in either *ABCG5* or *ABCG8*.—Wang, J., T. Joy, D. Mymin, J. Frohlich, and R. A. Hegele. Phenotypic heterogeneity of sitosterolemia. *J. Lipid Res.* 2004. 45: 2361–2367.

Supplementary key words intestinal absorption • sterols • bile acids • transporters • genomic DNA • mutation • sequence analysis

Sitosterolemia [Mendelian Inheritance in Man (MIM) 210250] is a rare disorder of lipoprotein metabolism that was first described in two affected sisters in 1974 (1). Subsequent examination of a large Amish kindred revealed an autosomal recessive inheritance pattern (2). Beginning in childhood, some patients with sitosterolemia develop tendinous and/or tuberous xanthomas, whereas others have eruptive xanthomatosis, arthritis and arthralgias, and hemolysis, which is thought to arise from the increased osmotic fragility of erythrocytes (1–6). The presence of coronary heart disease (CHD) is variable but can be striking in some cases. Patients with sitosterolemia can be clinically

differentiated from patients with other childhood xanthomatoses, such as familial hypercholesterolemia, by the inheritance pattern, or cerebrotendinous xanthomatosis, by the absence of neurological involvement or cataracts (7, 8).

The biochemical hallmark of sitosterolemia is markedly (>30-fold) increased plasma concentrations of plant sterols, with sitosterol being the most abundant species (1, 9). In an average diet, 200 to 300 mg of plant sterols are consumed daily, with <5% being absorbed in healthy individuals. Most absorbed sitosterol is resecreted into the intestinal lumen, leaving minimal sitosterol and other plant sterols in the plasma of normal subjects (10). However, patients with sitosterolemia fail to resecret absorbed plant sterols, so that >60% of ingested sitosterol is absorbed (4, 11–15). Furthermore, patients with sitosterolemia have a generalized hyperabsorption of dietary sterols, including cholesterol and shellfish sterols, leading to increases in plasma concentrations of cholesterol and plant sterols, such as sitosterol (16). Homozygotes have not only increased intestinal absorption but also impaired biliary excretion of sterols. In contrast, heterozygotes are clinically normal, although some may have slightly increased plasma sitosterol (6, 17, 18).

Candidate gene analysis excluded metabolism-related genes in sitosterolemia (19). After mapping of the sitosterolemia locus to chromosome 2p21 (20), mutations in two ATP binding cassette (ABC) transporter genes, *ABCG5* (MIM 605459) and *ABCG8* (MIM 605460), were found to be causative for sitosterolemia (21–23). *ABCG5* and *ABCG8* each contains 13 exons. The proteins encoded by these genes, called sterolin-1, encoded by *ABCG5*, and sterolin-2, encoded by *ABCG8*, possess the characteristic ABC motif in the N terminus. However, the C terminus contains only 6 transmembrane domains compared with the usual 12-transmembrane domains of other ABC transporters (23). Sterolin-1 and -2 are thus termed “half-transporters” (23). They are expressed primarily in the liver and in-

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¹ To whom correspondence should be addressed.
e-mail: hegele@robarts.ca

testine (21–23). Since the discovery of these two genes, several mutations responsible for sitosterolemia have been described (21–27). We present two contrasting mutations in subjects with sitosterolemia.

METHODS

Study subjects

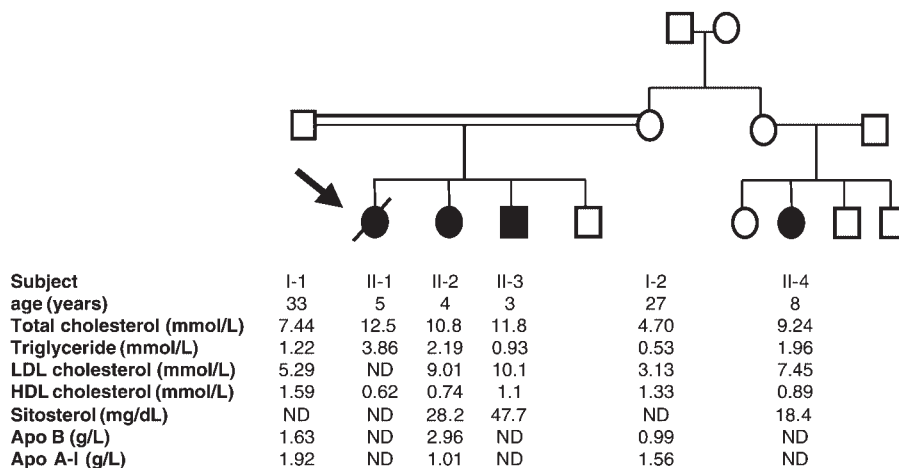
Family 1: ABCG8 S107X subjects II-1 to II-4. In 1997, a 5 year-old girl (subject II-1) presented to the local hospital with abdominal pain and acute respiratory distress. On arrival, she was asystolic and could not be revived. Her past medical history was significant for anemia as well as for recurrent episodes of abdominal pain. Autopsy revealed significant narrowing of not only the coronary ostia but also the right and left anterior descending arteries. There were widespread atheromas in the aorta, pulmonary arteries, and mitral valve leaflets. There was also a xanthoma measuring 1.2 cm × 0.4 cm over the sacrum. Postmortem serum cholesterol was 12.5 mmol/l (normal reference range is 3.0–4.4

mmol/l). The cardiac pathology of this child has been previously reported (27). The family structure is shown in Fig. 1.

The proband's family belonged to a religious isolate. Two of the proband's three siblings were subsequently referred for evaluation. The proband's 4 year old sister (subject II-2) was noted to be less than fifth percentile for both height and weight, with an otherwise normal physical examination. Her 3 year-old brother (subject II-3) had an entirely normal physical examination. The parents were well with no CHD history. Her 8 year old asymptomatic first cousin (subject II-4) was also evaluated for sitosterolemia. Serum lipoproteins were measured in parents (subjects I-1 and I-2), two siblings (subjects II-2 and II-3), and the first cousin (subject II-4). Markedly increased plasma sitosterol, using capillary gas chromatography, was detectable in the three affected children. Baseline biochemical values are shown in Fig. 1.

Family 2: ABCG5 E3 I/D subject II-1. A 75 year old Japanese-Canadian woman was referred for evaluation of hyperlipidemia, as reported (28). She had developed xanthelasmas and eruptive xanthomas at age 20. By age 44, she had typical angina pectoris and total cholesterol of 7.5 mmol/l. Coronary angiography at age 62 revealed single-vessel arterial disease with moderate aortic stenosis. Medical history was also significant for hypertension, hypothy-

Pedigree 1: ABCG8 S107X



Pedigree 2: ABCG5 exon 3 I/D

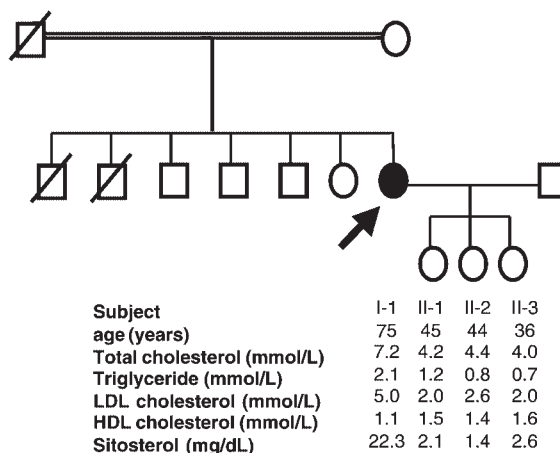


Fig. 1. Pedigree structure for the two sitosterolemia kindreds. Proband is indicated by arrows, and affected subjects are indicated by solid symbols. There is known consanguinity between subjects I-1 and I-2 in pedigree 1 and between the parents of subject I-1 in pedigree 2. ApoB, apolipoprotein B; ND, not determined.

roidism, and several transient cerebral ischemic attacks with no permanent neurological sequelae. On examination, she had bilateral xanthelasmas, xanthomas on the neck and chest, bilateral Achilles tendon xanthomas, a left patellar tendon xanthoma, and extensor tendon xanthomas of both hands. A loud cardiac murmur compatible with aortic stenosis was noted. She had dry atrophic skin in both lower limbs, with weak lower extremity pulses, and arterial bruits in the abdominal aortic region and bilaterally in the renal regions.

The family structure is shown in Fig. 1. The proband's parents were first cousins. Her 94 year old mother developed hypertension and angina after age 65, whereas her father died at age 94 with no history of CHD or hypercholesterolemia. One of the proband's brothers had a myocardial infarction at age 29 and died of a second myocardial infarction at age 30. An autopsy report revealed significant diffuse coronary atheroma formation with diffuse myocardial scarring. Another brother died accidentally, but the proband's four remaining siblings were healthy (Fig. 1). Her three daughters, subjects III-1, III-2, and III-3, aged 45, 44, and 36, respectively, were also all healthy. Before referral, the proband had been treated with several HMG-CoA reductase inhibitors (statins), with little effect on plasma lipoproteins. Untreated plasma total cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol were 7.50, 1.40, 3.20, and 1.20 mmol/l, respectively. Plasma plant sterol concentrations were measured using gas-liquid chromatography and showed total phytosterols of 52.2 mg/dl, of which 22.3 mg/dl was sitosterol and 15.2 mg/dl was campesterol. Upon diagnosis of sitosterolemia, colestipol and dietary management were instituted. Changes in plasma cholesterol and sitosterol after treatment using statins, colestipol 15 g daily, or ezetimibe 10 mg daily are shown in **Table 1**.

DNA isolation and sequence analysis

Informed consent was obtained from all subjects or their guardians, and the Institutional Review Board of the University of Western Ontario approved the study. Genomic DNA from the five study subjects was isolated from whole blood (Puregene; Gentra Systems, Minneapolis, MN). Exons 1–13 of *ABCG8* were amplified using reported primers (25). Exons 1–13 of *ABCG5* were amplified using the primers shown in **Table 2**. The final volume of 50 μ l contained 32 pmol of each primer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 2.5 units of *Taq* platinum DNA polymerase (Life Technologies, Mississauga, Ontario, Canada). DNA amplifications were performed with denaturing at 94°C for 5 min, followed by 30 cycles of a denaturing step at 94°C, an annealing step at either 59°C for *ABCG5* reactions or 57°C for *ABCG8* reactions, and an extension step at 72°C, each for 30 s. A final extension step at 72°C was performed for 10 min. Amplifi-

cation products were electrophoresed on 1.5% agarose gels and purified with the QIAEX II gel extraction kit (Qiagen, Inc., Mississauga, Ontario, Canada). Purified DNA fragments were sequenced by the chain termination method using the ABI 377 Prism Automated DNA Sequencer and analyzed using Sequence Navigator Software (both from PE-Applied Biosystems, Mississauga, Ontario, Canada).

RESULTS

Clinical and biochemical attributes

From the *ABCG8* S107X kindred, subjects II-1 to II-4 each had high LDL-cholesterol and low HDL-cholesterol, with corresponding changes in apolipoprotein A-I (apoA-I) and apoB concentrations. Three of the four affected children had depressed HDL-cholesterol (Fig. 1, Table 1). All three surviving affected children had significantly increased sitosterol concentrations. The proband's father had increased LDL-cholesterol and apoB, whereas the mother had more normal concentrations. The three surviving affected children were treated at different times with cholestyramine, atorvastatin, or ezetimibe for a minimum of 3 months, and the plasma lipoprotein and sitosterol responses are shown in Table 1. For these subjects, the greatest improvements in lipoproteins and sitosterol were seen on ezetimibe treatment, with somewhat less improvement on bile acid sequestrants; statin treatment had the smallest effect on plasma lipoproteins.

In subject *ABCG5* E3 I/D II-1, treatment with colestipol resulted in a 20% decrease in total cholesterol, a 45% decrease in LDL-cholesterol, and a 46% decrease in sitosterol. She had noted mild regression of her xanthomas with this treatment. Since coronary bypass grafting and aortic valve replacement at age 62, she has had no further symptoms (28). Plasma lipoproteins on various treatments, each for a minimum of 3 months, are shown in Table 1. Her adult children, all obligate heterozygotes, had normal plasma lipoproteins and sitosterol (Fig. 1).

DNA analysis

In kindred 1, sequencing of genomic DNA from affected subjects showed no mutations in the coding regions of *LDLR*, *ARH*, and *ABCG5* genes, nor was there any

TABLE 1. Lipoprotein variables in treated sitosterolemia subjects

Pedigree	Medication	TC	TG	LDL-C		HDL-C	Sitosterol
				mmol/l	mg/dl		
Pedigree 1	No treatment	10.6 ± 1.0	1.69 ± .06	8.94 ± 1.2	0.94 ± 0.18		30 ± 11
	Atorvastatin 10–20 mg/day	7.28 ± 0.22	0.82 ± 0.11	5.74 ± 0.25	1.21 ± 0.02		ND
	Cholestyramine 4–8 g/day	5.63 ± 1.03	0.83 ± 0.59	3.70 ± 1.19	1.60 ± 0.28		23 ± 4.7
	Ezetimibe 10 mg/day	3.90 ± 0.62	1.81 ± 0.05	2.12 ± 0.65	1.42 ± 0.03		12 ± 3.1
Pedigree 2	No treatment	7.5	1.4	3.2	1.2		22.3
	Colestipol 5 g/day	5.6	1.9	2.4	1.8		14.7
	Atorvastatin 20 mg/day	7.0	1.5	4.4	1.6		27.3
	Ezetimibe 10 mg/day	4.43	2.55	1.92	1.36		ND

HDL-C, high density lipoprotein cholesterol; LDL-C low density lipoprotein cholesterol; ND, not determined; TC, total cholesterol; TG, triglycerides. Means ± SEM are shown based on three subjects in pedigree 1, with single treated values for subject II-1 from pedigree 2.

TABLE 2. Oligonucleotides for genomic amplification and sequencing of *ABCG5*

Exon(s)	Forward Primer	Reverse Primer
1	5'-CCCCAACTGAAGCCACTCTGG-3'	5'-AGCCCGAAGTGCCAGACTGG-3'
2	5'-GCAGAGACTGAAACACGTTAGG-3'	5'-AGCAGTTCCATTCACAGTCAG-3'
3 and 4	5'-GTATCCTGCTGTGTCCTTAACACC-3'	5'-AAGCGTAGGCGAAGAGTGGAG-3'
5	5'-GCTGCCTCTTTCATGTCCTCC-3'	5'-CTGCACACACACAGAAGAAGC-3'
6	5'-TACATGTTTACTTCCCACCGC-3'	5'-AAGTGTGAGCCACTGTGCCTG-3'
7	5'-AGTGCATCGCTACCCTTGTGATC-3'	5'-TCCAGGCAGAAGTCTGAGATG-3'
8 and 9	5'-TATGGCCAGTACTCCTGTACC-3'	5'-AAAGAGGTGCACCTCCAGCAC-3'
10	5'-TTGTCCTGTGGATGGCAGGTC-3'	5'-TGTAGATCCTCCAGAGCAGA-3'
11	5'-AACTTCTCCCACCCACTCAC-3'	5'-CCATAACCATTATCAGTTCTC-3'
12	5'-GGACTATTGCCAGAGCCTACTG-3'	5'-CCTGTTGACCTTTGACTGGAG-3'
13	5'-CCAAGCGCTTGCTAAGTACCC-3'	5'-CAGTCATGCACAGTCGGCAGC-3'

mutation in the region of *APOB* encoding the receptor binding domain of apoB (data not shown). However, DNA sequencing revealed homozygosity for a nonsense mutation in *ABCG8*: this was a C→G transversion in the second position of codon 107 encoding a premature stop codon instead of the normal serine (Fig. 2). Sequencing showed that the two younger siblings and the first cousin were also homozygous for the S107X mutation, whereas both parents were heterozygous. Additional sequence analyses showed that the *ABCG8* S107X mutation was absent from the genomic DNA of 360 healthy Caucasians. In contrast, subject *ABCG5* E3 I/D II-7 had no mutations in *ABCG8* but instead had a complex mutation in *ABCG5* exon 3, described in the legend to Fig. 2. Because of the complexity of this mutation, we refer to it as *ABCG5* exon 3 del388-428/ins388-408 or *ABCG5* E3 I/D. This mutation was absent from 300 healthy subjects of various ethnicities.

DISCUSSION

We report nonsense mutations in *ABCG5* and *ABCG8* and associated clinical phenotypes in Canadian subjects. Each of four Caucasian subjects with the *ABCG8* S107X mutation, which encodes a protein that is truncated by ~80%, was ascertained under age 10 and had profound hyperlipidemia, with the index case showing marked skin manifestations and premature severe atherosclerosis with CHD. Also, in the Japanese-Canadian woman with sitosterolemia, we found a novel complex deletion/insertion mutation in *ABCG5* called E3 I/D, which encodes a protein that is truncated by >50% with a lengthy abnormal C-terminal sequence. One subject with each mutation was symptomatic with coronary atherosclerosis: a 5 year old girl with the *ABCG8* S107X mutation and a 75 year old woman with the *ABCG5* exon 3 I/D mutation, who represent the extreme ends of the spectrum of vascular involvement in sitosterolemia. In the surviving *ABCG8* S107X homozygotes, treatment with ezetimibe or bile acid sequestrants caused the largest reductions of plasma LDL cholesterol and sitosterol, whereas treatment with statin drugs was associated with less LDL-cholesterol reduction. Similarly, the homozygote for the *ABCG5* I/D exon 3 mutation has marked reductions in both plasma cholesterol and sitosterol with both bile acid sequestrants and ezetimibe, with less reduc-

tion on statin treatment. There are now >12 different *ABCG5* mutations and >24 different *ABCG8* mutations (21–28) in sitosterolemia; our findings extend the range of clinical phenotypes seen in subjects with sitosterolemia as a result of nonsense mutations in *ABCG5* or *ABCG8*.

Like our subject, most Asian probands with sitosterolemia, particularly those of Japanese origin, had mutations in *ABCG5* (21–27). In this ethnic group, *ABCG5* exon 9 is most commonly affected, and the most common mutation is R389H. In contrast, mutations in *ABCG8* occur mainly in Caucasians, and the most common mutation is Y361X (21–27). To date, no sitosterolemic individual has been reported to have mutations in both *ABCG5* and *ABCG8*. Careful genotype-phenotype surveys suggest that there are no clinical differences between patients with mutations in *ABCG5* or *ABCG8* (21). The two youngest sitosterolemia patients to die from cardiac complications (subject II-1 from family 1 and a 13 year old Amish boy) each had mutant *ABCG8* (22, 27). Shared haplotypes in unrelated kindreds have suggested the existence of founder effects for sitosterolemia attributable to *ABCG8* mutations (29). In contrast, most Japanese probands with *ABCG5* were homozygous without a high degree of haplotype sharing, implying that founder effects may be less important in sitosterolemia caused by *ABCG5* mutations (29). A screening analysis of *ABCG5* in 48 healthy Japanese subjects confirmed the absence of the exon 3 I/D mutation (30).

Subjects *ABCG8* S107X II-1 to II-4 demonstrated a certain degree of clinical and biochemical heterogeneity (Table 2), of which the most dramatic were the cardiac and skin manifestations in subject II-1. Subjects II-1 to II-4 each had increased LDL-cholesterol and apoB and depressed HDL-cholesterol and apoA-I, consistent with the findings reported previously in sitosterolemia (14, 31). The fatal coronary atherosclerosis in subject II-1 is not explained by differences in intermediate biochemical traits. It may instead be explained by unmeasured background genetic or environmental factors. Interestingly, the parents of the proband, both of whom were heterozygotes for *ABCG8* S107X, had some biochemical disparities. Specifically, the father had increased LDL-cholesterol and apoB, and the mother had concentrations that were within the normal range. Increases in apoB-containing lipoproteins have been inconsistently observed in obligate heterozygotes for sitosterolemia (29–32). The disparate biochemi-

olemic subjects (33). When the enterohepatic circulation of bile acids is interrupted with bile acid sequestrants, cholesterol 7 α -hydroxylase activity increases to maintain bile acid synthesis (33–35). This increases the requirement for cholesterol biosynthesis, but HMG-CoA reductase among patients with sitosterolemia cannot be further upregulated. The demand for bile acid synthesis is satisfied through increased catabolism of plasma LDL, with concomitant decreases in plasma cholesterol and plant sterols (11). Bile acid sequestrants thus provide an alternative pathway for cholesterol removal among patients with sitosterolemia. The average plasma sterol reduction is ~50% with cholestyramine (18, 36, 37), and the subjects we studied had reductions of plasma total cholesterol and LDL cholesterol that were in this range.

The favorable responses to ezetimibe, which is believed to function as an inhibitor of cholesterol absorption, are somewhat consistent with the findings in a recent double-blind, randomized, placebo-controlled, relatively short study in patients with sitosterolemia (38). Sitosterol concentrations decreased by 21%, and similar reductions in total sterols and apoB were also observed. The significant and progressive reductions in plasma plant sterol concentrations in patients with sitosterolemia were felt to be consistent with the hypothesis that ezetimibe inhibits the intestinal absorption of plant sterols and cholesterol, leading to reduced plasma concentrations (38).

The regulation of *ABCG5* and *ABCG8* expression is poorly understood. The two genes are arranged head-to-head and are separated by only 374 bp, which apparently harbors a bidirectional transcriptional promoter (23, 39). Increased gene expression results in decreased net sterol absorption (40). Coexpression of both transporters is critical for targeting to the apical cell surface (41). Overexpression of human *ABCG5* and *ABCG8* genes in mice decreases the absorption of dietary cholesterol and increases biliary sterol secretion (42). Conversely, *ABCG5/ABCG8*^{-/-} double-knockout mice showed increases in plasma cholesterol and decreases in biliary cholesterol after cholesterol feeding (43), suggesting that the two gene products can move sterols back into the intestinal lumen and also secrete sterols into the bile.

Thus, we report clinical phenotypes in five subjects with nonsense mutations in *ABCG5* and *ABCG8*. These extend the mutational spectrum in sitosterolemia, including a between-genotype difference in the severity of cardiovascular involvement and in the response to medications. It is possible that closer evaluation of these pathways will suggest new treatments both for patients with sitosterolemia and for individuals in the general population with milder disturbances in sterol metabolism. **FIG**

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