A missense mutation in the *Abcg5* gene causes phytosterolemia in SHR, stroke-prone SHR, and WKY rats

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Abstract **Sitosterolemia is an autosomal recessive disorder caused by mutations in the ABCG5 or ABCG8 half-transporter genes. These mutations disrupt the mechanism that distinguishes between absorbed sterols and is most prominently characterized by hyperabsorption and impaired biliary elimination of dietary plant sterols. Sitosterolemia patients retain 15–20% of dietary plant sterols, whereas normal individuals absorb less than 1–5%. Normotensive Wistar Kyoto inbred (WKY *inbred*), spontaneously hypertensive rat (SHR), and stroke-prone spontaneously hypertensive rat (SHRSP) strains also display increased absorption and decreased elimination of dietary plant sterols. To determine if the genes responsible for sitosterolemia in humans are also responsible for phytosterolemia in rats, we sequenced the *Abcg5* and *Abcg8* genes in WKY *inbred*, SHR, and SHRSP rat strains. All three strains possessed a homozygous guanine-to-thymine transversion in exon 12 of the *Abcg5* gene that results in the substitution of a conserved glycine residue for a cysteine amino acid in the extracellular loop between the fifth and sixth membrane-spanning domains of the ATP binding cassette half-transporter, sterolin-1. The identification of this naturally occurring mutation confirms that these rat strains are important animal models of sitosterolemia in which to study the mechanisms of sterol trafficking.**— Scoggan, K. A., H. Gruber, and K. Larivière. **A missense mutation in the *Abcg5* gene causes phytosterolemia in SHR, stroke-prone SHR, and WKY rats. *J. Lipid Res.* 2003.** 44: **911–916.**

Supplementary key words sitosterolemia • sterolin-1 • ATP binding cassette half-transporter • spontaneously hypertensive rats • plant sterols

Sitosterolemia (MIM 210250), also known as phytosterolemia, is a rare autosomal recessive disorder characterized by increased absorption and decreased elimination of dietary plant sterols, as well as abnormally low cholesterol biosynthesis (1–4). Affected individuals have high levels of plasma plant sterols, namely 18–72 mg/dl versus 0.3–1.0 mg/dl (W. M. N. Ratnayake, and E. Vavasour, unpublished observations) and normal to slightly elevated blood cholesterol levels. Patients exhibit tendon and tuberous xanthomas, accelerated atherosclerosis, and premature coronary artery disease. Recently, mutations in the ATP binding cassette (ABC) half-transporter genes ABCG5 and ABCG8 have been shown to cause sitosterolemia in humans (5, 6). These genes are oriented on chromosome 2p21 in a head-to-head arrangement, are separated by 375 bp, and each contains 13 exons (7). ABCG5 and ABCG8 genes are members of the ABC transporter family and encode for sterolin-1 and sterolin-2, respectively. These proteins are expressed in liver and intestine and consist of an N-terminal ATP binding site and six transmembrane domains at the C terminus. On the basis of their importance in sitosterolemia and recent expression in transgenic mice (8), these proteins are thought to pump plant sterols out of intestinal cells into the gut lumen, and out of liver cells into the bile duct. Functional ABC transporters comprise two ATP binding sites and 12 membrane-spanning domains (9, 10). Consistent with these half-transporters functioning as heterodimers, mutations in either ABCG5 or ABCG8, but not in both genes simultaneously, have been found in sitosterolemia patients (5–7, 11, 12). To date, no mutations have been identified in these genes in other species. Lu et al. have identified a number of polymorphisms in *Abcg5* and *Abcg8* in several mouse strains (13) and although some of these polymorphisms altered amino acids, none of them correlated with increased plasma plant sterol levels. Similar to phytosterolemic patients, specific rat strains have been shown to retain high levels of plasma plant sterols and to have blood and cell membrane cholesterol deficiencies (14–17). Normotensive Wistar Kyoto inbred (WKY *inbred*) rats, spontaneously hypertensive rats (SHRs), and stroke-prone spontaneously hypertensive rats (SHRSPs) contained 12% to 15% plant sterols in the sterol fraction of serum compared with 2% to 6% in nine different rat

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strains fed commercial rat chow (18). Ikeda et al. (18) further demonstrated that WKY *inbred* and SHRSP rats deposit three to four times higher levels of plant sterols in serum when fed a 0.5% plant sterol diet. The proportion of plant sterols in the sterol fraction was ~25% to 35% (33.7 mg/dl) in WKY *inbred* and SHRSP rats and 6% to 12% (8.39 mg/dl) in WKA and Wistar control rats (18). In all tissues, the deposition of campesterol was higher than that of sitosterol. These high serum plant sterol values are similar to those obtained from phytosterolemic patients (4) and by Ratnayake et al. in SHRSP rats (15). The increased accumulation of plant sterols in these rats may be due to enhanced intestinal absorption and decreased biliary excretion (18). The SHRSP strain was derived from the SHR strain (19–21) that had been developed previously from the WKY *inbred* strain (22). Their serum triacylglycerol levels do not differ significantly (23), and it is well established that dietary plant sterols have no effect on triglycerides in both rats (15) and humans (24). When fed a high cholesterol/cholate diet, the plasma cholesterol levels are significantly higher in normotensive WKY *inbred* than in SHR and SHRSP strains even though these three rat strains have increasing systolic blood pressures in that order (25). The SHR and SHRSP strains are widely used animal models for hypertension and hemorrhagic stroke and may also be suitable models for studying mechanisms of differential absorption of various sterols. To ascertain the mechanism of increased dietary plant sterol retention in these rats, we determined the genomic structures for the rat *Abcg5* and *Abcg8* genes and their mRNA tissue expression patterns, and subsequently identified the mutation responsible for phytosterolemia in rats.

MATERIALS AND METHODS

Database searches and PCR approach to determine exon/intron boundaries

Accession numbers AF312714.2 and AF351785.1, corresponding to rat *Abcg5* and *Abcg8* cDNA sequences, respectively, were used to search databases for any homologous genomic DNA sequences using the Basic Local Alignment Search Tool (BLAST). Rat clone CH230-359E1 (AC112747.1) and clone CH230-65H6 (AC120701) were identified from the Rat Genome Database. Using these genomic sequences, we were able to determine the intron sequences flanking all of the exons in the ABC half-transporter genes except for *Abcg5* exons 2, 8, and 9, and *Abcg8* exons 4, 5, 6, and 8. To determine the remaining exon/intron boundary sequences and estimate intron sizes, rat genomic fragments were amplified using primers selected from the cDNA sequences (**Table 1**). These fragments were subsequently cloned (TOPO TA Cloning Kit, Invitrogen Life Technologies, Canada) and sequenced.

Subject samples

Liver tissue samples were obtained from the following rats: SHRSP (SHRSP from Seac Yoshitomi, Fukuoka, Japan, *inbred*,

TABLE 1. Oligonucleotide primers used for amplification of *Abcg5* and *Abcg8* introns

Forward Primers			Reverse Primers		
Primer Name	Position in cDNA[a]	Sequences 5′ to 3′	Primer Name	Position in cDNA[a]	Sequences 5′ to 3′
Abcg5					
Abcg5-i1-F	139–159	GGAGGAAGGCTCAGTTACAGG	Abcg5-i1-R	269–249	TTTCCTGTCCCACTTCTGCT
Abcg5-i2-F	256–268	GTGGGACAGGAAAATCCTCA	Abcg5-i2-R	407–388	ACACTTCCCCTTCCAAGGTC
Abcg5-i3-F	398–416	GGGGAAGTGTTTGTGAACG	Abcg5-i3-R	521–502	CCGTGTATCTCAGCGTCTCC
Abcg5-i4-F	500–519	CGGGAGACGCTGAGATACAC	Abcg5-i4-R	629–610	AGTTGCCGATCATTTGGTCT
Abcg5-i5-F	618–638	TGATCGGCAACTATAATTTTG	Abcg5-i5-R	773–754	CCAAGAGGAGGACGATATGA
Abcg5-i6-F	787–806	CAGGAACCGCATTGTAATTG	Abcg5-i6-R	893–874	TGCCACAGAACACCAACTCT
Abcg5-i7-F	902–921	GAGATGCTCGGCTTCTTCAA	Abcg5-i7-R	1,037–1,018	TCTGGACTCGCTTGTACGTC
Abcg5-i8-F	1,121–1,140	CCCATGGTTCCTTTCAAAAC	Abcg5-i8-R	1,238–1,218	CAAGACGCATAATCACAACCT
Abcg5-i9-F	1,330–1,349	GCTGTTGTACCAGCTTGTGG	Abcg5-i9-R	1,462–1,443	GAGCAGCATCTGCCACTTCT
Abcg5-i10-F	1,465–1,484	CTATGTGCTGCATGCTCTCC	Abcg5-i10-R	1,582–1,563	AGCGGCAGAGAAGTATCCAA
Abcg5-i11-F	1,653–1,674	TTGTCAACAGCATAGTGGCTCT	Abcg5-i11-R	1,770–1,749	TGGAAGGTAAAGTAACCCAGGA
Abcg5-i12-F	1,778–1,799	TGTTGTGAGATTCTTGTGGTCA	Abcg5-i12-R	1,895–1,876	CAATGAATTGGATCCCTTGG
Primer Name	Position in cDNA[b]	Sequences 5′ to 3′	Primer Name	Position in cDNA[b]	Sequences 5′ to 3′
Abcg8					
Abcg8-i1-F	114–133	GCTCAGACGACCAAAGAGGA	Abcg8-i1-R	227–206	GGTGAAGTAGAGGCTGTTGTCA
Abcg8-i2-F	224–244	CACCTACAGTGGTCAGTCCAA	Abcg8-i2-R	343–324	CGAGACCTCCACGGTAACTT
Abcg8-i3-F	370–389	GCATCCGAAATCTGAGCTTC	Abcg8-i3-R	498–479	CTGATTTCATCTTGCCACCA
Abcg8-i4-F	609–628	CTGACTTTCATCGCCCAGAT	Abcg8-i4-R	746–728	CCCGCGTACGTATGTGTTG
Abcg8-i5-F	732–750	ACATACGTACGCGGGGTGT	Abcg8-i5-R	880–861	CGGGACAAAGTTCTCACCAG
Abcg8-i6-F	1,002–1,022	CAGCACATGGTGCAGTACTTT	Abcg8-i6-R	1,148–1,128	TGCAAGTAATCGAGCCTTCTC
Abcg8-i7-F	1,181–1,201	CGACTTTCTGTGGAAAGCTGA	Abcg8-i7-R	1,296–1,278	GTATCATCCCGGGCAGCTC
Abcg8-i8-F	1,260–1,279	AACTGTGGAACTGCTGCTGA	Abcg8-i8-R	1,373–1,355	TGCTCCATGGATGAACAGG
Abcg8-i9-F	1,465–1,486	TCATGATAGGAGCACTCATTCC	Abcg8-i9-R	1,569–1,549	TGTACAGTCCGTCCTCCAGTT
Abcg8-i10-F	1,546–1,565	ATGAACTGGAGGACGGACTG	Abcg8-i10-R	1,649–1,630	GGGCATCCCATAGATGATGA
Abcg8-i11-F	1,791–1,810	TGCTGCAACGCTCTCTACAA	Abcg8-i11-R	1,925–1,906	AATCTGCATCAGCCCTGAGA
Abcg8-i12-F	1,940–1,959	CATTTACACCACGCAGATCG	Abcg8-i12-R	2,055–2,036	TGCCAATGACGATGAGGTAG

[a] GenBank accession number AF312714.3.
[b] GenBank accession number AF351785.2.

TABLE 2. Oligonucleotide primers[a] used for amplification of *Abcg5* and *Abcg8* exons

	Abcg5		*Abcg8*	
Exon	Forward Primer	Reverse Primer	Forward Primer	Reverse Primer
1	AGCCAGACAGGACACCAGAG	TAGGGTGGGAAGCCTAGCTC	AGAATCCTGGCCTAGCCAAC	TCAGTTTCATCTTGCCTCCA
2	GGGTCCTACTCTGCCTTTTGT	CCTCCCAGAGTCTGCCTTAC	CCCTCCTGTCTGCTTCTCTG	CCCACCCCTGAACATTCTATT
3	AAAGTGCCCCCATTCTCAC	CAGGAAAGGGGACATCAGG	CTCTGAATGGCTCAGCTTCC	ATCGTACGGGTGAAAAACCA
4	CCAAGACTGCGTCTCCTACC	TGCTGAGGCACCTGATCTC	CAGGTAAGCCCTGCAGAAAC	TCCAGCTGAACTGGGTCTTC
5	AGTCATGGAGACAGCAGCAG	CGGGAACACATGGAGGATA	GAAGAAGTTGCCCCTGGAC	GGACAGGTTGTAGGCTCAGG
6	ACGATGCTAGGCAATGGTTC	TGGGATGAGATGTTGAGTCG	CCTGAGCCTACAACCTGTCC	GACAGCAAATGACTGTGTCCA
7	GGCTGGGAAGCACACACTA	AAGATTTCCAAAAAGCCCTGA	CAGGTCTCTGCCTTTCTGCT	ACCACCAGATCTTCCCATCA
8	TGTCCATTCTGTGTGTGTGC	ATGAGCATGAAGAGCCAAGC	GATGGGAAGATCTGGTGGTG	GGCAGAAGACAGAGACAGAGAGA
9	AGCTGGCTTGGCTCTTCAT	GATAGATGTGGGGGAGAGAGC	TCGGGTGATAAGGTCACAGA	TCCCACTGTCCCGAAGTCT
10	CCTCAGCAGTGTGGTGACTG	TGACCCAGGGGAACTGAA	CCCACGGCATTACAAGAGAT	CATGGCTGAGTGTTTCCGTA
11	TGATAGTGTGCGGAGAGAGAA	TCAGTTGACCCTTGACCACA	TGGTGTCGGCTCCATGTC	CCTACAGAGGCCTGGCTAA
12	GCATAAAGACGTACCCTTTCCA	CCCTGGGAAATCGCTTACTT	CCATGCGACTAACACTTGGA	CAGCAGCACTTGGATTGAGA
13	GAAGTGCCTGAGGGCTGA	GATGCCAGGGTCACAGATG	TCAATCCAAGTGCTGCTGAG	CGATGCTGCTTGAGATCTGT

[a] Primer sequences are given in the 5′ to 3′ direction.

SPF, maintained in the Animal Resources Division of Health Canada for 2 years); SHR [Tac:N(SHR) (Okamoto-Aoki Strain), *outbred*, bred in a closed colony, MPF, Taconic Farms, Inc., Germantown, NY]; WKY (WKY/NMol@Tac, *inbred*, MPF, Taconic Farms, Inc.); WKY [Tac:N(WKY), *outbred*, MPF, Taconic Farms, Inc.]; Sprague-Dawley [Crl:CD(SD)IGSBR, *outbred*, Charles River Canada, Saint-Constant, Quebec]; diabetes-prone and control BB (BBdp and BBc, respectively, Animal Resources Division of Health Canada). Genomic DNA was extracted from the tissue samples using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Chemical Co., St. Louis, MO).

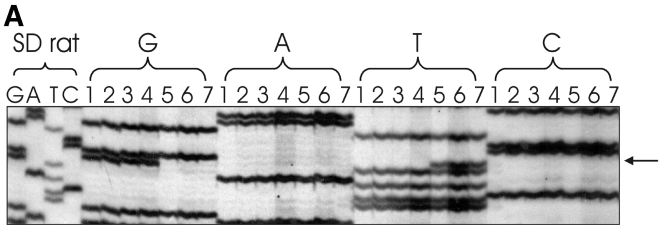
DNA sequencing analysis

Rat genomic DNA was subjected to PCR and direct DNA sequencing in order to screen the *Abcg5* and *Abcg8* genes for sequence variations. Primers were designed based on our deduced genomic structures of both genes and were used to amplify all 26 exons from the intronic sequences flanking each exon (**Table 2**). PCR amplification conditions were optimized for each primer pair, and the products were subsequently subjected to exonuclease I and shrimp alkaline phosphatase treatment (Exo-Sap-It, Amersham, Piscataway, NJ; USB Corp., Cleveland, OH) and then

TABLE 3. Exon-intron boundaries and organization of rat *Abcg5* and *Abcg8* genes

	Exon		Intron				Exon	
#	Size	3′ End Sequence	Splice Donor	#	Size	Splice Acceptor	5′ End Sequence	#
	bp				*bp*			
Abcg5								
1[a]	210	GTCCTTCAGCGTCAG	gtaaggggacccc	1	612	atttctttaaag	CAACCGTGTCGGGCC	2
2[b,c]	122	TCTTAGGTAGCTCAG	gtaagcgcctcga	2	14,228	ttgtcgcccctag	GCTCAGGGAAAACC	3
3[a]	137	TCCTACCTCCTGCAG	gtgggcgtgtccc	3	85	ccctttcctgcag	AGCGATGTCTTTCTG	4
4[a]	99	TTCTACGACAAGAAG	gtacttttagtta	4	2,340	gtgtctcttacag	GTAGAGGCAGTCCTG	5
5[a]	133	TCCTTCAGGACCCCA	gtaagtgggacac	5	1,316	tctttgctggcag	AGGTCATGATGCTTG	6
6[a]	140	TCTGAGCTCTTCCAC	gtaagggaacacc	6	901	gtggtccaatcag	CACTTCGACAAAATT	7
7[a]	130	CCTTTGATTTCTACA	gtaagtgcatttt	7	664	gggaaacttttag	TGGACTTGACATCGG	8
8[c]	214	CGGCGTTCTCCTGAG	gtaagagcctt	8	103	gtttggttttcag	GAGAGTAACGAGAAA	9
9[c]	206	ACGCTGTGAACCTCT	gtaagtgcctgtg	9	910	ccttccatgccag	TTCCCATGCTGAGAG	10
10[a]	139	CAGCGTGTGTTACTG	gtaaggtggtgtc	10	2,813	tcgtgtttttctag	GACTCTGGGCTTGT	11
11[a]	186	ATCTGGATTTATCAG	gtaagaagaaat	11	4,940	tctctttcttaag	AAACATAGAAGAAAT	12
12[a]	113	TGAACTTCACTTGTG	gtaagtatcctatt	12	1,857	ttctccttggcag	GTGGCTCCAACACTT	13
13[a]	641	GTGGAGTACAGAGAA						
Abcg8								
1[a]	173	CTCCAGGATGCTTCA	gtgagtgacctag	1	3,347	tgtctcccagcag	AGCCTCCAGGACAGC	2
2[a]	102	GATCTCACCTACCAG	gtaggggcacatg	2	1,788	cctctccccacag	GTGGACATGGCCTCTC	3
3[a]	157	TCATAGGGAGCGCAG	gtaccacagagac	3	3,254	ctgggtttgtcag	GCTGCGGGAGAGCCA	4
4[c]	237	CAGCGAGAAAACGG	gtaaccagtgggc	4	389	agcctgccctcag	GTGGAAGACGTGATT	5
5[c]	133	TCCTGTGGAACCCAG	gtgaggcctggga	5	86	gataccccccag	GAATCCTCATCCTGGA	6
6[c]	270	CTGCTGACTTCTACG	gtgagtgagtaaa	6	2,912	tcttctgcttgcag	TGGACTTGACGAGCAT	7
7[a]	163	CACCTATGCAGTCAG	gtactgagagaag	7	80	ctgttcccaacag	CCAGACCCTCACACAG	8
8[c]	81	TACCACCCTGATCCG	gtaaatcaacctc	8	1,235	tcctttctttcag	TCGTCAGATTTCCAAT	9
9[a]	200	ATGTCGTCTCCAAAT	gtgagtgtcacccg	9	164	cccccatctccag	GTCACTCGGAGCGGTC	10
10[a]	77	ATTTCTTTGCCAAG	gtcagggccagga	10	552	ctgtgctttgcag	GTCCTCGGTGAGCTG	11
11[a]	268	CAACCTGTGGATAG	gtgaggcctgcc	11	1,204	ttgctgtcttcag	TACCTGCATGGATTT	12
12[a]	128	CCCCGGAGACGCG	gtacgtagcgagg	12	87	tgtctgtgtccgcag	ATGGTCACTGCCATG	13
13[a,b]	2,838							

[a] GenBank accession number AC112747.
[b] GenBank accession number AC120701.
[c] Genomic fragments that were PCR amplified using primers indicated in Table 1, then cloned and sequenced.



B

	Glu	Phe	Tyr	Gly	Leu	Asn
Wild type	GAG	TTC	TAT	GGC	CTG	AAC
Mutant	GAG	TTC	TAT	TGC	CTG	AAC
	Glu	Phe	Tyr	**Cys**	Leu	Asn

Fig. 1. A guanine-to-thymine transversion in the *Abcg5* gene is present in rats with phytosterolemia. A: DNA sequence analysis of the coding strand of exon 12 of the rat *Abcg5* gene displays a guanine-to-thymine transversion at nucleotide 1,811 in both alleles (arrow) of WKY *inbred* (lane 5), SHR (lane 6), and SHRSP (lane 7) rat strains that is not present in SD (lane 1), BBc (lane 2), BBdp (lane 3), and WKY *outbred* (lane 4) rats. DNA sequence from the autoradiograph is given in the forward orientation starting at nucleotide 1,802. B: Nucleotide and amino acid comparison of wild-type and mutant sequences as a result of the guanine-to-thymine transversion at position 1,811. The guanine-to-thymine transversion is boxed, with the subsequent amino acid change in boldface.

manually sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham; USB Corp.) according to the manufacturer's recommended conditions. Denatured sequencing reactions were immediately loaded and electrophoresed through a 6% acrylamide gel (SequaGel-6, National Diagnostics, Atlanta, GA). Gels were transferred onto filter paper, dried for 2 h at 80°C, and exposed to X-ray film (KODAK BIOMAX MX, Mandel Scientific, Guelph, ON) for 18 h at −80°C. Autoradiographs were examined for sequence changes. All nucleotide, codon, and exon numbering corresponds to GenBank accession numbers AF312714.3 and AF351785.2 (http://www.ncbi.nlm.nih.gov/).

Restriction enzyme analysis

Several control rat strains were screened for the presence of the G1811T transversion by PCR amplification of exon 12 of the *Abcg5* gene followed by *Hae*III restriction analysis (New England Biolabs, Beverly, MA). The single nucleotide transversion deletes a unique *Hae*III restriction site.

Northern blot analysis

Rat multiple-tissue poly(A)$^+$ RNA Northern blots (Ori-gene, Bethesda, MD) were hybridized with radiolabeled rat *Abcg5* cDNA, rat *Abcg8* cDNA, or β-actin cDNA according to the manufacturer's recommended conditions. Primers designed to *Abcg5* (forward 5′-GCTCTGAAGCCAGACAGGAC-3′; reverse 5′-GTTCAGGACAGGGGTAACCA-3′) or *Abcg8* (forward 5′-GAGGACTCAAGTGCCCTAGC-3′; reverse 5′-GTAGATAGGGGTGCCAGACG-3′) transcripts were used to PCR amplify probes from cDNA prepared using rat liver total RNA. Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies) as per the manufacturer's recommended conditions. Rat *Abcg5* or *Abcg8* cDNA was radiolabeled by incorporation of [α-^{32}P]dCTP into the PCR product, and unincorporated nucleotides were removed using G-50 Micro columns (Amersham) as per the manufacturer's instructions. The activity of the probes was determined by scintillation counting, and 1–3 × 10^6 cpm of denatured probe was added per ml of hybridization solution.

RESULTS

Genomic structure of *Abcg5* and *Abcg8* rat genes

Genomic information for *Abcg5* and *Abcg8* genes was obtained by comparing the full-length cDNA transcripts present in GenBank to sequences deposited in the Rat Genome Database using BLAST. Two clones, CH230-359E1 (AC112747.1) and CH230-65H6 (AC120701), were identified that contained partial genomic fragments for both *Abcg5* and *Abcg8*. These sequences enabled us to quickly determine many of the exon/intron boundaries of the ABC half-transporter genes and importantly, the intron sequences flanking many of the exons of these genes. The remaining exon/intron boundaries and intron sizes were determined by sequencing cloned PCR products produced using exon-specific primers and rat genomic DNA (Table 1). Our results (**Table 3**) have now been confirmed by updated versions of clones CH230-359E1 (AC112747.3) and CH230-65H6 (AC120701.4) from the Rat Genome Database. Similar to the human and mouse genes, the rat *Abcg5* and *Abcg8* genes are arranged in a head-to-head orientation, and each gene is composed of 13 exons and 12 introns. The *Abcg5* gene spans ~33 kb of genomic DNA and the *Abcg8* gene spans about 20 kb, with 379 bp separating their respective initiation codons.

Mutation detection

SHRSP, SHR, and WKY *inbred* DNA was examined for a mutation in the *Abcg5* and *Abcg8* genes. PCR and direct sequencing analysis identified a guanine-to-thymine transversion at nucleotide position 1,811 (codon 583) in exon 12 of both alleles of the *Abcg5* gene (**Fig. 1A**). This transversion results in the substitution of a highly conserved glycine residue for a cysteine amino acid in the large extracellular loop between transmembrane domains 5 and 6 of sterolin-1 (Fig. 1B and **Fig. 2**), whereas *Abcg5* exon 12 from the WKY *outbred* rat, along with three other rat

Sterolin-1	568						601
WKY-N1/SHR/SHRSP	FQKYCCEI	LVVNEFY	**C**	LN	FTCG-GSNTS	VPNNPMC	
Rat	FQKYCCEI	LVVNEFY	G	LN	FTCG-GSNTS	VPNNPMC	
Mouse	FQKYCCEI	LVVNEFY	G	LN	FTCG-GSNTS	MLNHPMC	
Human	FQKYCSEI	LVVNEFY	G	LN	FTCG-SSNVS	VTTNPMC	
A.thaliana	LIRWAFQG	LCINEFS	G	LK	FDHQNTFDVQ	TGEQALE	

Fig. 2. Glycine residue at codon 583 is conserved through evolution. ABCG5 half-transporter amino acids 568 to 601 from several different species are shown. The highly conserved glycine residue is boxed and the cysteine mutation of the WKY *inbred*, SHR, and SHRSP rat is in boldface.

TABLE 4. Single nucleotide polymorphisms detected in the rat *Abcg5* and *Abcg8* genes

Exon/Intron	Variation	Position (Nucleotide)	SD	BBc	BBdp	WKY *outbred*	WKY *inbred*	SHR	SHRSP	Amino Acid Change
Abcg5-I3	C/T	14 of intron 3	C	T	C	C	C	C	C	No
Abcg5-E7	C/T	72 of exon 7	C	C	C	C	T	T	T	No
Abcg5-E8	C/T	147 of exon 8	T	C	T	T	T	T	T	No
Abcg5-E9	C/T	139 of exon 9	T	C	T	T	T	T	T	No
Abcg5-E9	T/C	160 of exon 9	C	T	C	C	C	C	C	No
Abcg8-E5	C/A	93 of exon 5	C	A	C	C	C	C	C	No
Abcg8-I6	G/A	2879 of intron 6	A	G	G	A/G	A	A	A	No

strains (SD, BBc, and BBdp), displayed only wild-type sequence (Fig. 1A). *Hae*III restriction enzyme digests of *Abcg5* exon 12 were also negative for the presence of the G1811T mutation in four additional rat strains, Wistar, Long-Evans, Wistar-Furth, and full diabetic (data not shown).

A polymorphism was also present in *Abcg5* exon 7 on both alleles from SHRSP, SHR, and WKY *inbred* rats that was not found in WKY *outbred*, SD, BBc, and BBdp rat strains (**Table 4**). This cytosine-to-thymine transition does not result in an amino acid substitution.

Several novel polymorphisms were also present in the seven rat strains sequenced, and these are listed in Table 4.

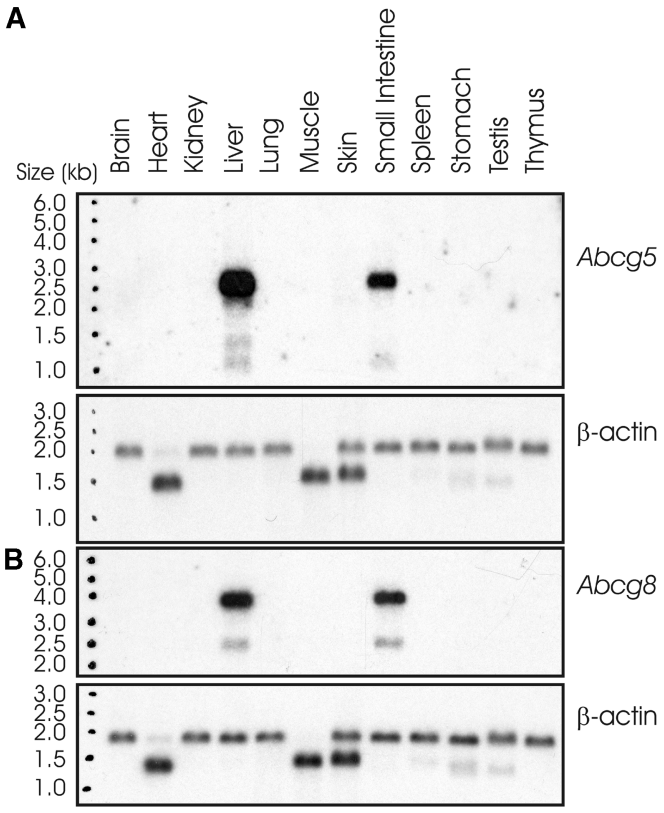


Fig. 3 The rat *Abcg5* and *Abcg8* genes are predominantly expressed in liver and small intestine. Results of Northern blot analyses of poly(A)$^+$ RNA from a variety of rat tissues (see Materials and Methods) are shown, demonstrating in A the expression of *Abcg5* (upper box) and β-actin (lower box) transcripts, and in B, the expression of *Abcg8* (upper box) and β-actin (lower box) transcripts.

Tissue distribution of rat *Abcg5* and *Abcg8* mRNA

Northern blot analyses demonstrated that the rat *Abcg5* gene is predominantly expressed in liver and small intestine, with a major transcript size of 2.6 kb and fainter transcript sizes of 1.1, 1.3, and 2.2 kb (**Fig. 3A**). Overexposure of the rat multiple tissue Northern blot resulted in the detection of faint *Abcg5* transcripts in brain, kidney, and skin (data not shown). Rat *Abcg8* mRNA expression is also predominant in liver and small intestine (Fig. 3B). Both of these tissues displayed an intense 3.9 kb transcript and a faint 2.6 kb transcript.

DISCUSSION

In the present study, we report the first identification of a mutation in the rat *Abcg5* gene that is responsible for phytosterolemia. This guanine-to-thymine transversion results in the substitution of a highly conserved glycine residue for a cysteine amino acid in the large extracellular loop between transmembrane domains 5 and 6 of the ABC half-transporter protein, sterolin-1. This mutation was present in both alleles of exon 12 of the *Abcg5* gene in WKY *inbred*, SHR, and SHRSP rats. Our results correlate with previous reports demonstrating increased absorption and retention of plant sterols in the serum and tissues of WKY *inbred*, SHR, and SHRSP rats (14, 15, 18), and are consistent with the identification of other homozygous missense mutations in the human ABCG5 or ABCG8 genes in sitosterolemia patients (5, 6, 11, 12). The homozygous mutation coincides with the recessive nature of the disease and with the development and inbreeding of these specific rat strains. The SHRSP *inbred* rat strain was developed from the SHR strain that is maintained in a closed colony (19–21). The SHR strain was derived previously from the normotensive WKY *inbred* rat strain (22). Based on the above information and on the absence of the glycine-to-cysteine amino acid substitution in the eight different rat strains tested, the data strongly suggest that the alteration at codon 583 represents a mutation. Formal proof of the mutation, however, will require functional analyses of the mutant protein.

The ABCG5 half-transporter was initially speculated to act as a heterodimer with the ABCG8 half-transporter, because mutations in sitosterolemia patients have been found exclusively in ABCG5 or ABCG8, but never together (5–7, 11, 12). Graf et al. (26) have now demonstrated that ABCG5 and ABCG8 are N-linked glycosylated,

physically interact, and require one another for transport from the endoplasmic reticulum to apical membranes. Our missense mutation in the extracellular loop between transmembrane domains 5 and 6 occurs near the canonical N-glycosylation sites of sterolin-1. We speculate that the amino acid substitution, which results in the addition of a sulfhydryl group, alters the tertiary structure of the protein, thereby preventing its interaction with sterolin-2. Consequently, assembly of the heterodimer and subsequent translocation from the endoplasmic reticulum into plasma/apical membranes will not occur, resulting in complete loss of ABC transporter function; however, one cannot rule out the possibility that the mutant ABC transporter may be properly expressed in plasma/apical membranes and still not function. Because expression of human ABCG5 and ABCG8 in mice caused a marked reduction in plasma levels of plant sterols (8), a loss of transporter function may lead to increased retention of plant sterols, presenting as phytosterolemia. Therefore, it appears that the SHR and SHRSP rat strains are excellent animal models for hypertension, hemorrhagic stroke, and phytosterolemia. Our results demonstrate that WKY *inbred*, SHR, and SHRSP rat strains represent the first naturally occurring animal models for the human disorder sitosterolemia, and are important models for studying the mechanisms of sterol trafficking.

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