*MUTATION IN BRIEF*

# Mutations in the Human ATP-Binding Cassette Transporters *ABCG5* and *ABCG8* in Sitosterolemia

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Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: LA1203/2-1

*Communicated by Johannes Zschocke*

**Phytosterolemia or Sitosterolemia is a rare autosomal recessive disorder characterized by highly elevated plasma levels of plant sterols and cholesterol as a consequence of hyperabsorption and impaired biliary secretion of sterols. The disease is caused by mutations in two half size ATP-binding cassette transporters, *ABCG5* and *ABCG8*. We have analyzed the genomic sequence of *ABCG5* and *ABCG8* in five well-characterized patients with Sitosterolemia. In the first patient we found a heterozygous mutation in exon 8 of the *ABCG5* gene leading to a premature termination of the protein (Arg408Ter). This German patient is the first European showing a mutation of the *ABCG5* gene. In a second patient we found a novel heterozygous mutation in exon 5 of *ABCG8* (c.584T>A; Leu195Gln). Both patients were heterozygous for the identified mutation, but no mutation could be identified on the other chromosome. In three further analyzed patients we found mutations in exons 7, 9 and 11 of the *ABCG8* gene, respectively, of which two result in a premature termination signal for translation products. One of these patients was compound heterozygous (Trp361Ter and Arg412Ter), the other was homozygous for Trp361Ter. The third patient was homozygous for an amino acid exchange (Gly574Arg). In conclusion this report describes one novel mutation affecting a highly conserved amino acid and two previously identified mutations in the *ABCG8* gene. In addition, we identified for the first time a mutation in the *ABCG5* gene of a European Sitosterolemia patient.** 

KEY WORDS: ATP-binding cassette transporters; *ABCG5*; *ABCG8*; beta-sitosterol; Sitosterolemia; cholesterol

## INTRODUCTION

Sitosterolemia (MIM# 210250) is a rare autosomal recessive disorder, first described by Bhattacharyya and Connor in 1974, characterized by hyperabsorption and retention of cholesterol and other sterols like plant (e.g.

Received 28 January 2002; revised manuscript accepted 26 April 2002.


**DOI: 10.1002/humu.9047**

beta-sitosterol) and shellfish sterols from the intestine and the inability to excrete these sterols into the bile. As a consequence, affected individuals show very high levels of these non-cholesterol sterols, whereas cholesterol levels can be normal or just moderately increased. The patients suffer from tendon and tuberous xanthomas and premature athersclerosis and coronary artery disease (Bjorkhem and Boberg, 1995; Lee et al., 2001a). The genetic defect in Sitosterolemia was mapped to chromosome 2p21 (Patel et al., 1998) and recent reports identified mutations in two newly described adjacent half-size ATP-binding cassette transporters *ABCG5* (MIM# 605459) and *ABCG8* (MIM# 605460) (Berge et al., 2000; Lee et al., 2001). 32 different mutations and 24 polymorphisms have been published so far. We have analyzed the genomic DNA sequence of both ABC transporters of five Sitosterolemia patients and could identify a novel mutation in the *ABCG8* gene and report the first *ABCG5* mutation identified in an European Sitosterolemia patient.

## MATERIAL AND METHODS

We studied five unrelated Sitosterolemia patients and if blood samples were available, their family members. Genomic DNA was isolated from whole blood using the QIAamp® DNA Blood Midi Kit (QIAGEN, Hilden, Germany). Primers used for genomic amplification and sequencing of *ABCG5* and *ABCG8* are listed in Table 1. All exons of *ABCG5* and *ABCG8*, including all exon-intron boundaries as well as the promoter region, were amplified using the QIAGEN Taq PCR Core Kit on a Perkin Elmer Thermocycler under the following conditions: 2 min 94°C, 40 sec 94°C, 44 sec 55°C and 1 min 72°C for 35 cycles, and 5 min 72°C. Amplification products were purified using Amicon® Microcon®-PCR Centrifugal Filter Devices (Millipore, USA). The purified DNA fragments were sequenced on a ABI Prism Genetic Analyzer 310 (PE Biosystems, Foster City, USA) according to the manufacturer.

**Table 1: Oligonucleotides Used for Genomic Amplification and Sequencing of *ABCG5* and *ABCG8***

| ***ABCG8*** **Exon** | **Forward primer** | **Reverse primer** | **PCR Product** |
|---|---|---|---|
| **1** | ggc agg tag gcc gag gtg tc | ctg agg gaa gag aga aag gt | 253 bp |
| **2** | cct atg ttc tca gca gct tc | gaa ttt cct ggc tgt ccc tg | 213 bp |
| **3** | ctc tga acc att cag ctc tc | agg acc att ctg tat ccc ag | 263 bp |
| **4** | gag cag tgg ctg aca gcc tg | gca tgg aca ctg tag ctt ct | 363 bp |
| **5** | ggt cac aat gtg tcc agc cc | ctg gac aag gtc ttc acc ag | 312 bp |
| **6** | gca gct cct gtg gaa ccc ag | cat gtt ctt ccc cat cat tg | 546 bp |
| **7+8** | cac ctg tga gca ggt gcc ag | aag ggc tta atg tga tat ac | 460 bp |
| **9** | tat gga gac tgt gac att cc | gaa cac agc ttg gag gtg gc | 337 bp |
| **10** | gaa gca ctg tag att tat tc | cat cgg tga ctt cac atg ac | 194 bp |
| **11** | gca gtg aag gtg ctg gct tc | cca gtc aca tga gtc cta ac | 369 bp |
| **12+13** | cat gag aat atg agg gac ac | gag tgc agt tga agg gtc tg | 460 bp |
| **promoter** | cag ggc cag tgt ctt | gct ctg gga gcc tct | 386 bp |
| ***ABCG5*** **Exon** | **Forward primer** | **Reverse primer** | |
| **1** | ccc aac tga agc cac tct g | gtg aag aaa ggc agc aga gg | 290 bp |
| **2** | gca cag gta gga tca atg ctg g | caa tgt gga gtt taa ctc aag cc | 266 bp |
| **3** | cac aga ggg tct cgg gaa gc | ctc ggg cgt cag tgt agc c | 389 bp |
| **4** | gct tct cct acg tcc tgc ag | gaa gga atg ggc aag cgt acg | 290 bp |
| **5** | cat gtc ctc ccc agc cca tg | cca aag tat ctg cac aca cac | 278 bp |
| **6** | tgg gct ctg cac tac ctt aga | cct ggc cac tgg tac aaa | 274 bp |
| **7** | aag tgc atc gct acc ctt gt | ggt gtc atc cag gca gaa gt | 271 bp |
| **8+9** | cgt cag tgg ata ccc aaa gc | tta cag ctg gag aag gga gg | 578 bp |
| **10** | cta gcc ctc cct ttt tca gc | gca gag aac ttc acc ctg ga | 298 bp |
| **11** | att cac aga ggc aag tgc ag | cca cta tca gtt ctc tgg tat tcc t | 363 bp |
| **12+13** | cct gtt gga aaa tat aag gat tgc c | gct ttc act acc tgc taa tga g | 1266 bp |
| **13** | gac agc gct tgg taa ata ctt g | gct ttc act acc tgc taa tga g | 280 bp |

Sequencing was performed on both strands of DNA using the amplification primers. The results were analyzed online with the HUSAR software (www.genome.dkfz-heidelberg.de). Genomic deletions were excluded by

performing long expand genomic PCR reactions and agarose gel analysis of amplification products. To analyze the Leu195Gln mutation in 50 healthy, normolipidemic individuals we used a restriction enzyme assay. The wild type amplification product of exon 5 of *ABCG8* contains four restriction sites for Alu I, whereas the mutated form causes loss of an Alu I site and thus displays only three Alu I restriction sites.

## RESULTS

Table 2 provides an overview over the plasma cholesterol and sitosterol levels, the found mutations and the ethnicity of the analyzed patients.

**Table 2:Serum Lipoprotein Levels and Mutations in Analyzed Sitosterolemia Patients**

| Patient | Total Sterols (mg/dl) | LDL-Chol. (mg/dl) | HDL-Chol. (mg/dl) | Triglyc. (mg/dl) | beta-Sitost. (mg/dl) | Gene | Mutation Allele 1 | Mutation Allele 2 | Ethnicity |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 305 | 246 | 38 | 101 | 31.1 | *ABCG5* | Arg408X | - | Caucasian / German |
| **2** | 210 | 129 | 67 | 72 | 20.3 | *ABCG8* | Leu195Gln | - | Caucasian |
| **3** | 218 | 166 | 35 | 84 | 22.1 | *ABCG8* | Trp361X | Arg412X | Caucasian |
| **4** | 247 | 155 | 56 | 189 | 20.5 | *ABCG8* | Trp361X | Trp361X | Caucasian |
| **5** | 214 | 145 | 42 | 90 | 17.5 | *ABCG8* | Gly574Arg | Gly574Arg | Caucasian / Swiss |

Patient 1 was a man who suffered from a myocardial infarction at the age of 31 years. The nucleotide change (c.1362C>T) in exon 8 produces a premature stop codon (Arg408X).This mutation was heterozygous, however, a second mutation could not be identified.

In Patient 2, we detected a heterozygous missense mutation in the *ABCG8* gene. The mutation T>A is located in exon 5 at nt 584 and causes an amino acid change, Leu>Gln, at position 195. It was not detected in 50 normolipidemic individuals. No mutation was identified on the other chromosome.

In the remaining three patients we found exclusively mutations in the *ABCG8* gene. In patient 3 sequencing of the *ABCG8* gene revealed a compound heterozygous mutations in exon 7 (c.1083 G>A) and in exon 9 (c.1234 C>T). Both mutations generate stop codons at amino acid position 361 (Trp361X) and 412 (Arg412X). The two sons of the patient were both heterozygous for each mutation, and showed normal plasma total sterol and beta-sitosterol levels, as expected for carriers. Patient 4 was homozygous for Trp361X in the *ABCG8* gene and her parents were both heterozygous for the same mutation. Patient 5 carried a homozygous mutation in exon 11(c.1720 G>A) of the *ABCG8* gene resulting in an amino acid exchange at position 574 (Gly574Arg).

## DISCUSSION

The molecular basis of Sitosterolemia has been recently identified in mutations of two members of the subfamily G of ATP-binding cassette transporters (Berge et al., 2000; Lee et al., 2001b), *ABCG5* and *ABCG8*. Both molecules are highly homologous ABC transporters exclusively expressed in the liver and the small intestine and feeding of cholesterol-rich diet induces the expression of both genes (Berge et al., 2000). The human *ABCG5* and *ABCG8* genes are located nearby on chromosome 2p21 in a head-to-head orientation separated only by 374 bp. It is thus likely that both genes share a bidirectional promoter and are transcribed simultaneously. The encoded proteins sterolin-1 and sterolin-2 are half-size ABC transporters consisting of a hydrophilic nucleotide binding domain and six transmembrane segments (Fig. 1). Since a functional ABC protein is composed of twelve transmembrane domains and two ATP-binding cassettes, dimerization is a prerequisite for both half-size molecules. Based on the finding that mutations in either *ABCG5* or *ABCG8* cause Sitosterolemia and due to the selective expression pattern of the transporters, heterodimerization of sterolin-1 and sterolin-2 in order to function as gatekeepers for dietary sterol uptake and excretion is conceivable. However, *ABCG1* and *ABCG2*, two other members of this subfamily are also expressed in liver and intestine, and thus, heterodimerization of *ABCG5* and/or *ABCG8* with one of these transporters cannot be excluded (Schmitz el al., 2001).

In this report we expand the spectrum of *ABCG5* and *ABCG8* mutations identified in five European patients with Sitosterolemia. The total number of mutations in these two ABCG family members now comprises 32 (Fig.

1). As obvious from Figure 1, mutations in Sitosterolemia patients occur exclusively either in *ABCG5* or *ABCG8*, but never in both. Also, *ABCG8* mutations are more commmon in affected patients than *ABCG5* mutations. In both genes hotspots with clustering of mutations occurs either in the ATP-binding cassettes or in the transmembrane domains (Fig. 1).

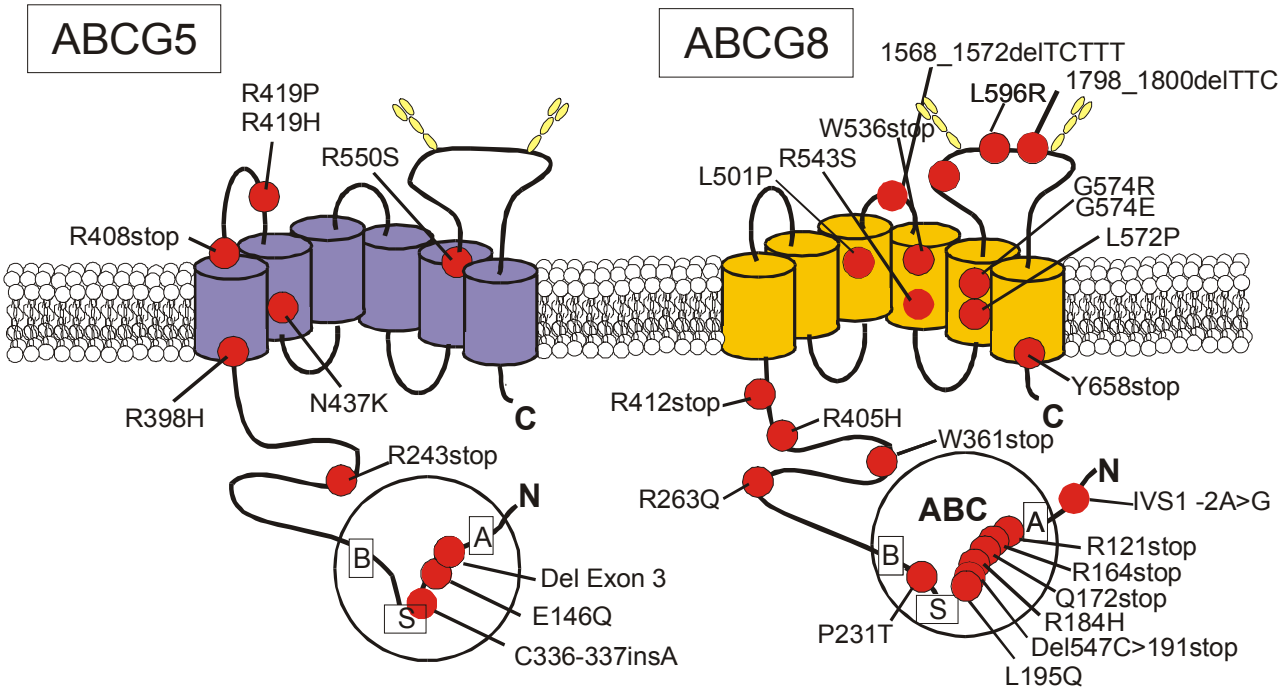


**Figure 1:** Putative topology of *ABCG5* and *ABCG8* with known sites of mutations causing Sitosterolemia, including the mutations reported in this paper.

A previously reported point mutation in *ABCG5*, Arg408X was identified in patient 1. The heterozygous transition in exon 8 (c.1362C>T) introduces a premature termination between the first and the second transmembrane domain of sterolin-1. If translated at all, the resulting truncated protein lacks the biggest part of the transmembrane domain and thus is unlikely to be functional. Since mutations in the *ABCG5* gene are usually found in Asian patients, with the exception of two cases with *ABCG5* mutations in White and African Americans, this is the first mutation in *ABCG5* reported in a patient of European origin.

Four other unrelated patients with Sitosterolemia all carried mutations in the *ABCG8* gene. A novel missense mutation producing a nonconservative amino acid change (Leu195Gln) was identified in patient 2. This alteration results in the substitution of a basic amino acid (Gln) for a hydrophobic amino acid (Leucine). Leucine at codon 195 is highly conserved among all five human ABCG transporters *ABCG1*, *ABCG2*, *ABCG4*, *ABCG5* and *ABCG8* (Fig. 2) and also in the corresponding mouse proteins (data not shown). This mutation is localized within the ATP-binding cassette between the Walker A motif and the Signature motif, an evolutionary conserved critical region for proper ABC transporter function. Therefore, it is very likely that the Leu195Gln substitution is the cause of Sitosterolemia in our patient. The causative nature of this novel mutation is additionally supported by the fact that we could not detect it in any of the 50 healthy, normolipidemic volunteers (i.e.100 normal control chromosomes). Sequencing of all exons of *ABCG5* and *ABCG8*, as well as the bidirectional promoter region failed to detect mutations of the second allele of patient 2. In addition long-expand PCR did not identify large genomic deletions in both genes. This suggests that a subtle mutation in intronic regions involved in transcription or translation may be affected.

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       201                          *                                Signature     250
ABCG1  Q..EKDEG.R REMVKEILTA L GLLSCANTR TGS.... .LS GGQR KRLAIA
ABCG2  ATTMTNHE.K NERINRVIEE L GLDKVADSK VGTQFIR GVS GGER KRTSIG
ABCG4  S..EKQEV.K KELVTEILTA L GLMSCSHTR TAL.... .LS GGQR KRLAIA
ABCG5  R..RGNPGSF QKKVEAVMAE L SLSHVADRL IGNYSLG GIS TGER RRVSIA
ABCG8  PRTFSQAQ.R DKRVEDVIAE L RLRQCADTR VGNMYVR GLS GGER RRVSIG
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**Figure 2:** Alignment of the human ABC transporters G1, G2, G4, G5 and G8. The amino acid change Leu195Gln in *ABCG8* found in patient 2 is located intracellularly between the Walker A and the Signature C-motif. This amino acid (*) is conserved among the human ABCG transporters.

We also have identified earlier described mutations on both alleles of the *ABCG8* gene in three patients. Patient 3 carried a compound heterozygous mutation resulting in a truncated protein (Trp361X and Arg412X), whereas patient 4 and patient 5 had homozygous mutations for Trp361X and Gly574Arg, respectively. Since so far only two Amish-Mennonite patients with the Gly547Arg mutation have been described (Lu et al., 2001), it is of special interest that the Swiss patient, we have analyzed, is the first Caucasian found carrying this mutation.

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