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Original Article



# Putative Pathogenic Variants of *ABCG5* and *ABCG8* of Sitosterolemia in Patients With Hyper-Low-Density Lipoprotein Cholesterolemia

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## ABSTRACT

**Objective:** Sitosterolemia is a rare autosomal recessive disease caused by the deleterious variants of adenosine 5'-triphosphate (ATP)-binding cassette sub-family G member 5 (*ABCG5*) or ATP-binding cassette sub-family G member 8 (*ABCG8*). There are only few data on the pathogenicity of *ABCG5* and *ABCG8*. This study aimed to propose a scheme for determining variant pathogenicity and to catalog the putative pathogenic variants in sitosterolemia.
**Methods:** This study enrolled 377 consecutive Japanese patients with hyper-low-density lipoprotein cholesterolemia (mean age: 46.5±19.8 years, with 192 men) who have targeted-sequenced data on *ABCG5* or *ABCG8* (among 21 Mendelian lipid genes for any dyslipidemias) and serum sitosterol levels at Kanazawa University Hospital from 2016 to 2021. Serum sitosterol levels were divided by 0.79 in patients treated with ezetimibe, accounting for the average reduction with this drug. *ABCG5* or *ABCG8* variants were defined as putative pathogenic if associated with serum sitosterol levels ≥5 μg/mL or homozygous if associated with serum sitosterol levels ≥10 μg/mL.
**Results:** Twenty-three *ABCG5* or *ABCG8* variants (16 missense, 2 nonsense, 2 frameshift, 2 deletion, and 1 splice mutation) were identified. Based on our definition, 11 putative pathogenic variants (median sitosterol level: 10.1 [6.5–17.1] μg/mL) were found in 36 individuals and 12 benign variants (median sitosterol: 3.5 [2.5–4.1] μg/mL) in 14 individuals.
**Conclusion:** The scheme proposed for assessing the pathogenicity of genetic variations (*ABCG5* and *ABCG8*) is useful. Using this scheme, 11 putative pathogenic, and 12 benign variants in *ABCG5* or *ABCG* were classified.

**Keywords:** ABCG5 protein, human; ABCG8 protein, human; Hyperlipoproteinemia type II; Sitosterols; Sitosterolemia

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**Conflict of Interest**
The authors have no conflicts of interest to declare.

**Data Availability Statement**
The datasets generated during and/or analyzed during the current study are not publicly available due to our regulations but may be available from the corresponding author on reasonable request.

**Author Contributions**
Conceptualization: Tada H; Data curation: Kojima N, Tada H, Nomura A, Usui S, Sakata K, Hayashi K, Nohara A, Inazu A, Kawashiri MA, Takamura M; Formal analysis: Kojima N, Tada H; Funding acquisition: Tada H; Investigation: Kojima N, Tada H; Methodology: Tada H; Resources: Tada H; Supervision: Nomura A, Usui S, Sakata K, Hayashi K, Kawashiri MA; Writing - original draft: Kojima N, Tada H, Nomura A, Usui S, Sakata K, Hayashi K, Nohara A, Inazu A, Kawashiri MA, Takamura M.

## INTRODUCTION

Sitosterolemia, caused by mutations in adenosine 5'-triphosphate (ATP)-binding cassette (ABC) sub-family G member 5 (*ABCG5*) or ABC sub-family G member 8 (*ABCG8*), is an extremely rare recessive lipid disorder.[1] Patients with sitosterolemia primarily exhibit cutaneous and tendon xanthomas associated with the increased absorption of high low-density lipoprotein cholesterol (LDL-C) and sitosterol and decreased excretion of sterols, including cholesterol, and sitosterol.[2]

A genetic study revealed that the prevalence of sitosterolemia is currently underestimated.[3] A few years back, we have established a standard measurement and reference values for serum sitosterol among general Japanese populations.[4] Advancements in genetics and the standardization of measuring serum sitosterol level could facilitate disease diagnosis. Currently, more genetic variants in *ABCG5* and *ABCG8* are identified via the so-called panel sequencing covering these genes, particularly among patients with hypercholesterolemia.[5-8] However, the pathogenicity of the genetic variants of *ABCG5* and *ABCG8* is still challenging to determine because there are only a few data regarding this issue. In fact, The American College of Medical Genetics and Genomics (ACMG) guidance for the interpretation of sequence variants is typically used for assessing the pathogenicity of the genetic variations.[9] However, it is almost impossible to determine the pathogenicity of the genetic variations of *ABCG5* and *ABCG8* using the ACMG criteria due to lack of ultimate functional analyses assessing serum sitosterol levels. A previous study has assessed the distribution of serum sitosterol levels according to the genetic status of *ABCG5* and *ABCG8*. Results found that serum sterol levels ≥5 μg/mL for the heterozygous variant and ≥10 μg/mL for the homozygous or compound heterozygous variant were the appropriate cut-off to determine the pathogenicity of these variants.[10]

The current study aimed to (1) propose a scheme to determine the pathogenicity of variants and (2) catalog putative pathogenic variants for sitosterolemia among Japanese patients with dyslipidemia by measuring their serum sitosterol levels.

## MATERIALS AND METHODS

### 1. Study participants

We retrospectively investigated 405 (mean age=46.8 years; with 218 men) consecutive Japanese participants with hyper LDL cholesterolemia (LDL-C level ≥140 mg/dL) who were assessed for serum sitosterol levels and the presence of *ABCG5* or *ABCG8* genetic variants at Kanazawa University Hospital between 2016 and 2021. Twenty-eight patients diagnosed with clinical FH were excluded from the analysis. Finally, we analyzed data from 377 patients (mean age=46.5 years, with 192 men).

### 2. Genetic analysis

Genomic DNA isolated from peripheral white blood cells according to standard procedures was used for polymerase chain reaction. The exome region of 21 dyslipidemia-related Mendelian genes, including *ABCG5*, and *ABCG8*, was sequenced.[11] We used a standard variant filtering focusing on variants that fulfilled (1) a minor allele frequency of <1% in the East Asian population and (2) those predicted as damaging by all 5 in silico software (SIFT, Polyphen2-HDIV, Polyphen2-HVAR, MutationTaster-2, and LRT).


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### 3. Determination of *ABCG5* and *ABCG8* pathogenicity

Putative pathogenic variants were determined by fulfilling either of the following: (1) median serum sitosterol level of ≥5 μg/mL (if the variants were heterozygous) and (2) a median serum sitosterol level of ≥10 μg/mL (if the variants were homozygous or compound heterozygous). Otherwise, the genetic variants were considered as benign.

### 4. Biochemical analysis

Blood samples were collected for assays after overnight fasting. The serum levels of total cholesterol, triglycerides, and high-density lipoprotein cholesterol were determined enzymatically. LDL-C levels were calculated using the Friedewald formula if the triglyceride levels were <400 mg/dL. Otherwise, it was determined enzymatically. The serum sitosterol levels were determined via high-sensitivity gas chromatography. Details regarding the measurements of serum sitosterol levels are described elsewhere.[4] The serum sitosterol level was divided by 0.79 in a patient treated with ezetimibe, which resulted in a 21% reduction in serum sitosterol levels, as published in 2004.[12] In this study, 122 participants used ezetimibe.

### 5. Statistical analysis

Continuous variables were expressed as mean ± standard deviation and categorical variables as counts and percentages. For values with a non-normal distribution, the median, and interquartile range were reported. The mean values of continuous variables were compared using the Student's *t*-test for independent data, and the median values were compared with the nonparametric Wilcoxon-Mann-Whitney rank-sum test. Categorical variables were compared using the chi-square test. A *p*-value of <0.05 was considered statistically significant.

### 6. Ethical considerations

This study was approved by the Ethics Committee of Kanazawa University (2018-285) and conducted in accordance with the Declaration of Helsinki (2008) of the World Medical Association. All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the 1975 Declaration of Helsinki, as revised in 2008, the Ethical Guidelines for Medical and Health Research Involving Human Subjects, and all other applicable laws and guidelines in Japan. All participants provided informed consent.

## RESULTS

### 1. Baseline characteristics of the participants

**Table 1** shows the baseline characteristics of participants. The mean age was 46.5 years, and 192 (50.9%) participants were men. The median sitosterol level was 2.7 μg/mL. The sitosterol level of the group with variants was significantly higher than that of the group without any variants (5.8 vs. 2.5 μg/mL, $p=1.9\times10^{-13}$). Other variables, including lipid-lowering agents, such as statins, and ezetimibe, did not significantly differ.

### 2. Baseline characteristics of the participants according to the presence of putative pathogenic variants

In the group with *ABCG5* or *ABCG8* variants, 36 individuals had putative pathogenic variants, and 11 did not (**Table 2**). The mean age of the group with putative pathogenic variants was 30.7 years, and 13 (46.4%) participants were male. The median sitosterol level of the group with putative pathogenic variants was 10.1 μg/mL. The sitosterol level of the group with


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**Table 1.** Characteristics of the participants

| Characteristics | All participants (n=377) | Participants with variant (+)* (n=47) | Participants with variant (–) (n=330) | *p*-value |
|---|---|---|---|---|
| Age (yr) | 46.5±19.8 | 39.5±24.6 | 47.6±18.8 | 0.01 |
| Male | 192 (50.9) | 25 (53.1) | 167 (50.6) | 0.74 |
| Sitosterol level (μg/mL) | | | | |
| Before treatment† | 2.7 (1.9–4.3) | 5.8 (3.7–10.7) | 2.5 (1.8–3.7) | $1.90\times10^{-13}$ |
| After treatment | 2.1 (1.6–3.9) | 4.8 (3.2–9.9) | 2.2 (1.6–3.6) | $2.10\times10^{-11}$ |
| TC level (mg/dL) | | | | |
| Before treatment | 260.3 (198–304.5) | 266 (202–306) | 258 (161–307) | 0.59 |
| After treatment | 208.5 (170.75–255.5) | 206 (182–254) | 209 (168–257) | 0.75 |
| Triglyceride level (mg/dL) | | | | |
| Before treatment | 106 (68–178) | 96 (60–129) | 109 (75–188) | 0.24 |
| After treatment | 97 (64.5–156.25) | 85 (61–124) | 99 (65–161) | 0.11 |
| HDL-C level (mg/dL) | | | | |
| Before treatment | 50 (40–63) | 51 (44–61) | 50 (40–64) | 0.33 |
| After treatment | 53 (43–65) | 54 (46.5–64) | 53 (42.7–65) | 0.55 |
| LDL-C level (mg/dL) | | | | |
| Before treatment | 164 (147–203) | 169 (144–205) | 163 (148–204) | 0.19 |
| After treatment | 104 (77–148) | 103 (89–154.5) | 104.5 (76–146.2) | 0.31 |
| Hypertension | 68 (18.0) | 6 (12.8) | 62 (18.8) | 0.42 |
| Diabetes | 36 (9.5) | 4 (8.5) | 32 (9.7) | 1.00 |
| Current smoking | 41 (10.9) | 5 (10.6) | 36 (10.9) | 1.00 |
| Coronary artery disease | 8 (2.1) | 1 (2.1) | 7 (2.1) | 1.00 |
| Statin | 236 (62.6) | 29 (61.7) | 207 (62.7) | 1.00 |
| Ezetimibe | 122 (32.4) | 12 (25.5) | 110 (33.3) | 0.37 |

Values are presented as mean ± standard deviation, number (%), or median (range).
TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
*Variant (+) indicates patients with any variants in adenosine 5'-triphosphate-binding cassette sub-family G member 5 or adenosine 5'-triphosphate-binding cassette sub-family G member 8.
†Adjusted if treated with ezetimibe.

**Table 2.** Baseline characteristics of the participants according to the existence of putative pathogenic variants

| Characteristics | Patients with variant (+)* (n=47) | Patients with putative pathogenic variant (n=36) | Patients with benign variant (n=11) | *p*-value |
|---|---|---|---|---|
| Age (yr) | 39.5±24.6 | 30.7±23.1 | 51.4±21.0 | 0.004 |
| Male | 25 (53.1) | 13 (46.4) | 12 (63.1) | 0.25 |
| Sitosterol level (before treatment†; μg/mL) | 5.8 (3.7–10.7) | 10.1 (6.5–17.1) | 3.5 (2.5–4.1) | $8.09\times10^{-9}$ |
| TC level (after treatment; mg/dL) | 206 (182–254) | 223.5 (180.7–264.2) | 201 (182–211.5) | 0.16 |
| Triglyceride level (after treatment; mg/dL) | 85 (61–124) | 69.5 (60.5–97.7) | 102 (66.5–138) | 0.17 |
| HDL-C level (after treatment; mg/dL) | 54 (46.5–64) | 57 (50.7–65.5) | 47 (41–54.5) | 0.01 |
| LDL-C level (after treatment; mg/dL) | 103 (89–154.5) | 135 (90.5–163.5) | 95 (86.5–115.5) | 0.11 |

Values are presented as mean ± standard deviation, number (%), or median (range).
TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
*Variant (+) indicates patients with any variants in adenosine 5'-triphosphate-binding cassette sub-family G member 5 or adenosine 5'-triphosphate-binding cassette sub-family G member 8.
†Adjusted if treated with ezetimibe.

putative pathogenic variants was significantly higher than that of the group without putative pathogenic variants (10.1 vs. 3.5 μg/mL, $p=8.09\times10^{-9}$).

## 3. Putative pathogenic variants in *ABCG5* or *ABCG8*

**Table 3** depicts the 11 putative pathogenic variants found in 36 patients. Five *ABCG5* variants were identified in 17 participants and 6 *ABCG8* variants in 19 participants. The sitosterol levels of these individuals ranged from 5.6 to 55.0 μg/mL. Among these variants, 7 were missense variants, 2 were nonsense variants, one was a frameshift variant, and one was a splice mutation. The most common variant was c.1256T>A (p.Ile419Asn) in *ABCG8*. All variants are extremely rare in the Genome Aggregation Database.[13] **Table 4** presents data on patients with homozygous or compound heterozygous *ABCG5* or *ABCG8* variant.


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**Table 3.** Summary of putative pathogenic variants

| Genes | Nucleotide change | Mutation type | Amino acid change | No. of patients | Serum sitosterol level (μg/mL) | Frequency* |
|---|---|---|---|---|---|---|
| *ABCG5* | c.433C>T | Missense | p.Arg145Cys | 1 | 6.6 | $3.95\times10^{-5}$ |
| | c.904+1G>A | Splice | | 1 | 40 | $6.57\times10^{-6}$ |
| | c.1166G>A | Missense | p.Arg389His | 8 | 5.8 (5.4–9.8) | $9.86\times10^{-5}$ |
| | c.1256G>A | Missense | p.Arg419His | 2 | 27.5 (15.9–39.0) | $6.57\times10^{-6}$ |
| | c.1673_1677del | Frameshift | p.Pro558GlnfsTer14 | 5 | 55.0 (7.1–59.4) | Not found |
| *ABCG8* | c.55G>C | Missense | p.Asp19His | 2 | 5.7 (3.9–7.5) | $6.44\times10^{-2}$ |
| | c.362G>A | Missense | p.Arg121Gln | 1 | 8.2 | $3.29\times10^{-5}$ |
| | c.647dup | Nonsense | p.Glu217Ter | 1 | 6.5 | Not found |
| | c.909T>G | Nonsense | p.Tyr303Ter | 1 | 5.6 | Not found |
| | c.1256T>A | Missense | p.Ile419Asn | 13 | 10.8 (9.2–24.5) | $6.57\times10^{-6}$ |
| | c.1295G>T | Missense | p.Gly432Val | 1 | 21.6 | Not found |

*ABCG5*, adenosine 5'-triphosphate-binding cassette sub-family G member 5; *ABCG8*, adenosine 5'-triphosphate-binding cassette sub-family G member 8.
*We used data on frequency score from the Genome Aggregation Database version 3.1.1.

**Table 4.** Compound heterozygous variants found in this study

| No. | Genes | Nucleotide change | Effect on protein | Sitosterol level (μg/mL) | TC level (mg/dL) | Triglyceride level (mg/dL) | HDL-C level (mg/dL) | LDL-C level (mg/dL) |
|---|---|---|---|---|---|---|---|---|
| 1 | *ABCG5* | c.1673_1677del | p.558GlnfsTer14 | 59.4 | 149 | 30 | 62 | 64 |
| | *ABCG8* | c.1256T>A | p.Ile419Asn | | | | | |
| 2 | *ABCG5* | c.1673_1677del | p.558GlnfsTer14 | 55.0 | 174 | 66 | 53 | 91 |
| | *ABCG8* | c.1256T>A | p.Ile419Asn | | | | | |
| 3 | *ABCG5* | c.1256G>A | p.Arg419His | 50.6 | 61 | 157 | 29 | 11 |
| | *ABCG5* | c.904+1G>A | | | | | | |
| 4 | *ABCG5* | c.1673_1677del | p.558GlnfsTer14 | 172.1 | 264 | 77 | 57 | 162 |
| | *ABCG5* | c.1256G>A | p.Arg419His | | | | | |

TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *ABCG5*, adenosine 5'-triphosphate-binding cassette sub-family G member 5; *ABCG8*, adenosine 5'-triphosphate-binding cassette sub-family G member 8.

## 4. Benign *ABCG5* or *ABCG8* variants

**Table 5** shows the 12 benign variants identified in 14 patients. Benign variants were evaluated based on the sitosterol level as stated before. The sitosterol levels of these individuals ranged from 1.5 to 4.4 μg/mL. Six *ABCG5* variants were identified in 8 participants and 6 *ABCG8* variants in 6 individuals. In these variants, 9 were missense variants, one was a frameshift variant, and 2 were deletion variants. The most common variant was c.1864A>G (p.Met622Val) in *ABCG5*. All variants are extremely rare in the Genome Aggregation Database.[13] Putative pathogenic variants and benign variants were summarized in **Fig. 1**.

**Table 5.** Summary of benign variants

| Genes | Nucleotide change | Mutation type | Amino acid change | Number of patients | Serum sitosterol level, μg/mL | Frequency* |
|---|---|---|---|---|---|---|
| *ABCG5* | c.362G>A | Missense | p.Arg121His | 1 | 1.7 | Not found |
| | c.406G>A | Missense | p.Asp136Asn | 1 | 2.7 | Not found |
| | c.1108_1118+2del | | | 1 | 4.4 | Not found |
| | c.1292C>T | Missense | p.Pro431Leu | 1 | 3.9 | Not found |
| | c.1493G>A | Missense | p.Arg498Gln | 1 | 2.3 | Not found |
| | c.1864A>G | Missense | p.Met622Val | 3 | 3.6 (3.2–9.6) | $4.77\times10^{-3}$ |
| *ABCG8* | c.94dup | Frameshift | p.Ser32LysfsTer2 | 1 | 2.9 | Not found |
| | c.605A>G | Missense | p.Asp202Gly | 1 | 2.4 | Not found |
| | c.980T>A | Missense | p.Ile327Asn | 1 | 1.5 | Not found |
| | c.1649C>T | Missense | p.Ala550Val | 1 | 3.3 | $1.97\times10^{-5}$ |
| | c.1832_1834del | | p.Arg611del | 1 | 3.5 | $3.29\times10^{-4}$ |
| | c.1858C>T | Missense | p.Leu620Phe | 1 | 3.8 | Not found |

*ABCG5*, adenosine 5'-triphosphate-binding cassette sub-family G member 5; *ABCG8*, adenosine 5'-triphosphate-binding cassette sub-family G member 8.
*We used data on frequency score from the Genome Aggregation Database Ver. 3.1.1.


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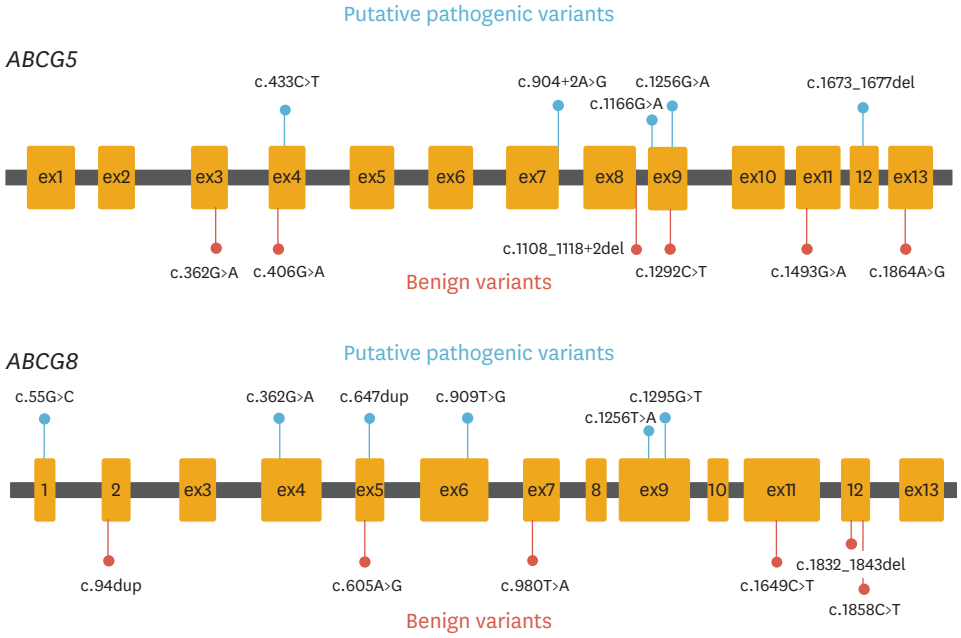


**Fig. 1.** Putative pathogenic variants and benign variants classified in this study. The upper panel shows putative pathogenic variants and benign variants in *ABCG5*. The lower panel depicts putative pathogenic variants and benign variants in *ABCG8*.
*ABCG5*, adenosine 5'-triphosphate-binding cassette sub-family G member 5; *ABCG8*, adenosine 5'-triphosphate-binding cassette sub-family G member 8.

## DISCUSSION

This study proposed a unique and simple scheme to determine the pathogenicity of the genetic variants of *ABCG5* and *ABCG8* for sitosterolemia. Results showed that serum sitosterol levels ≥10 μg/mL (if the variants were homozygous or compound heterozygous) or ≥5 μg/mL (if the variants were heterozygous) were pathogenic. In addition, we cataloged the putative pathogenic variants of *ABCG5* and *ABCG8* of sitosterolemia based on an ultimate functional testing of serum sitosterol levels (**Fig. 1**).

Recently, sitosterolemia has received significant attention for several reasons. First, this disease can be more common than previously considered. Historically, it had been described as extremely rare. However, according to recent population-wide genetic screening studies, this disease could affect at least 1 in 200,000 general populations, which is nearly the same as homozygous familial hypercholesterolemia (HoFH). Second, optimal medical therapy, and the prognosis of sitosterolemia may be different from those of HoFH. However, sitosterolemia has been considered a phenocopy of HoFH.[3] Dietary counseling and ezetimibe therapy were quite effective in reducing sitosterol and LDL-C levels in patients with sitosterolemia; nevertheless, these approaches have minimal effects on patients with HoFH.[14] Third, patients with a heterozygous variant of *ABCG5* can be at high risk for coronary heart disease based on increased LDL-C levels, despite the general conception of recessive disorder.[15] Indeed, sitosterolemia is a recessive disorder with consideration of the phenocopy of HoFH. That is, cutaneous, and tendon xanthomas, which is associated with extremely high LDL-C levels, were observed during childhood. However, ezetimibe is more effective in reducing LDL-C levels in carriers of *ABCG5* or *ABCG8* than in noncarriers. Therefore,


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identifying patients with inherited hypercholesterolemia and hypersitosterolemia caused by a heterozygous variant of *ABCG5* can lead to individualized treatment.[16]

A higher number of patients are undergoing genetic testing for various diseases. Thus, various genetic variants with unclear pathogenicity are identified.[17,18] This study proposed a useful scheme and identified the putative pathogenic variants of sitosterolemia, which can be helpful in genetic diagnosis in not only in Japan but also other countries worldwide. Notably, the scheme is reproducible anywhere, paving the way for further related research. We believe that the findings of this study can be considered as functional study *in vivo*. if the ACMG criteria are used for pathogenicity, either *in vivo*, or *in vitro* functional study can strongly support the pathogenicity. Accordingly, it is true that the pathogenicity of the variants cannot be determined by the scheme proposed in this study alone. Further, it can be used as an adjunct method to determine pathogenicity of a particular variant.

Importantly, the relatively high prevalence of pathogenic variants in *ABCG5* or *ABCG8* in this study population can be attributed to the inclusion criteria. That is, pathogenic variants in *ABCG5*, or *ABCG8* were associated with hyper LDL cholesterolemia.[16]

Another important implication of this study is that allele frequency alone cannot be used as a deterministic factor for the pathogenicity of genetic variants. In fact, all benign genetic variations were extremely rare. Accordingly, the rarity of the genetic variations should not be the deterministic factor of the pathogenicity of inherited diseases. However, it may be a useful markers of pathogenicity.

This study has several limitations. First, selection bias existed in the measurement of serum sitosterol levels and genetic analysis. In fact, we recruited patients with hyper LDL cholesterolemia (LDL-C level of ≥140 mg/dL) in this study. This should have led to a higher prevalence of pathogenic variants in *ABCG5* or *ABCG8*. In addition, we might have missed the variants with lesser effects on LDL-C levels. Second, the study included a small number of patients. It is challenging to enroll more patients without single or double mutations in *ABCG5* or *ABCG8* because of the rarity of this disorder. Third, we did not account for age, sex, and apolipoprotein E genotype, which are associated with sitosterol levels.[19] However, the degree of differences among the genotype groups of *ABCG5* or *ABCG8* may be significantly larger than those observed in a previous study. Accordingly, the essential message in this study could not be significantly affected by this limitation. Fourth, some patients were taking statins, which might have affected their sitosterol level. Fifth, there is no prior study focusing on genetic variants in *ABCG5* or *ABCG8* and serum sitosterol levels in particular. Therefore, it is actually impossible to compare with previous studies.

This study proposed a scheme useful for assessing the pathogenicity of the genetic variations of *ABCG5* and *ABCG8*. Using this scheme, 11 putative pathogenic, and 12 benign variants in *ABCG5* or *ABCG8* were classified.

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