Rare and Deleterious Mutations in ABCG5/ABCG8 Genes Contribute to Mimicking and Worsening of Familial Hypercholesterolemia Phenotype

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**Background:** A substantial proportion of patients clinically diagnosed as having familial hypercholesterolemia (FH) do not manifest causative mutation(s) in the FH genes such as LDLR, APOB, and PCSK9. We aimed to evaluate the effect of rare and deleterious mutation(s) in ABCG5/ABCG8 on hyper-low-density lipoprotein (LDL) cholesterolemia in individuals who meet the clinical criteria for FH.

**Methods and Results:** We compared the LDL cholesterol (LDL-C) values among 487 subjects with FH; the subjects were grouped according to the presence of mutation(s) in FH and ABCG5/ABCG8 genes. We identified 276 individuals with a deleterious mutation in a FH gene (57%, monogenic FH), but found no causative mutations in 156 individuals (32%, mutation-negative). A total of 37 individuals had deleterious mutations in ABCG5 or ABCG8, but not in FH genes (8%, ABCG5/ABCG8 mutation carriers). Among these, 3 individuals had sitosterolemia (0.6%) with double mutations. We also identified 18 individuals with deleterious mutations in an FH gene and ABCG5 or ABCG8 (4%, ABCG5/ABCG8-oligogenic FH). Subjects without mutations had significantly higher polygenic scores than those in any other groups. LDL-C levels in oligogenic FH subjects were significantly higher than in the monogenic FH subjects. Moreover, sitosterol/lathosterol levels were significantly affected by those mutations.

**Conclusions:** The results suggested that rare and deleterious mutations in ABCG5/ABCG8 contribute substantially to mimicking and exacerbation of the FH phenotype.

**Key Words:** ABCG5; ABCG8; Familial hypercholesterolemia; Low-density lipoprotein receptor; PCSK9

Familial hypercholesterolemia (FH; OMIM no. 143890) is characterized by the clinical triad of primary hyperlow-density lipoprotein (LDL) cholesterolemia, tendon xanthomas, and premature coronary artery disease (CAD). FH is considered to be caused mainly by deleterious mutations in genes associated with LDL metabolism such as the LDL receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin/kexin type 9 (PCSK9). However, a substantial number of individuals with clinical FH do not show causative mutations in the established FH genes. There are several potential explanations for this phenomenon: (1) novel genes associated with FH, (2) polygenic FH caused by accumulation of LDL-raising common single-nucleotide polymorphisms (SNPs), and (3) limitations of genetic analyses. On the other hand, a growing body of evidence suggests that rare and deleterious mutations in ABCG5/ABCG8 contribute to the development of the FH phenotype, especially among FH mutation-negative patients. We previously showed that concomitant mutations in “accessory” genes, including ABCG5 and ABCG8, contribute to worsening of the FH phenotype and we defined such a situation as “oligogenic FH”. According to The Exome Aggregation Consortium exome browser, 1 in 220 individuals has loss-of-function mutations in ABCG5 or ABCG8, indicating that a substantial number of individuals have deleterious mutation(s) in those genes. Based on this information, we aimed to evaluate the prevalence and clinical effect of mutations in ABCG5/ABCG8 on LDL cholesterol (LDL-C) and CAD among individuals with a clinical diagnosis of FH.

**Methods**

**Study Population**

We retrospectively investigated 543 subjects who met the Japanese clinical diagnostic criteria of FH at Kanazawa University Hospital from April 2014 to March 2017. We excluded 54 subjects for lack of lipid profile and/or genetic analysis. We also excluded 2 patients with homozygous or compound heterozygous FH. Thus, a total of 487 subjects...
criteria specified by the Japan Atherosclerosis Society: (1) LDL-C ≥ 180 mg/dL, (2) tendon xanthoma/xanthoma tuberosum, and (3) family history of FH or premature CAD among the patient’s 2nd-degree relatives.

Genetic Analysis
Genomic DNA was isolated from white blood cells in [45% men and 55% women, mean age=41 years, CAD=125 (26%)] remained in the current analysis. We reviewed the results of baseline examinations of each subject, which included a medical history review, physical examination, and blood analysis. Most of the subjects were inpatients referred to hospital, which made it possible to assess their fasting blood samples.

Clinical Diagnosis of FH
All subjects of this study fulfilled at least 2 of the 3 clinical criteria specified by the Japan Atherosclerosis Society: (1) LDL-C ≥ 180 mg/dL, (2) tendon xanthoma (tendon xanthoma on the backs of hands, elbows, knees, etc. or Achilles tendon hypertrophy: X-ray assessed Achilles tendon thickness ≥ 9 mm) or xanthoma tuberosum, and (3) family history of FH or premature CAD among the patient’s 2nd-degree relatives.

Figure 1. Effect of genetic status of familial hypercholesterolemia (FH) on low-density lipoprotein cholesterol (LDL-C) level. (A) We divided the subjects into 4 groups based on the presence/number of FH mutation(s) and ABCG5/ABCG8 genetic mutations (groups 1–4). (B) Boxplot shows LDL-C levels according to mutation status. (A, B) White, subjects without mutations; Pink, subjects with ABCG5/ABCG8 mutation(s); Light blue, subjects with a single mutation in conventional FH genes; Purple, subjects with a single mutation in conventional FH genes and an ABCG5/ABCG8 mutation. LDLR, low-density lipoprotein receptor.
Peripheral blood according to standard procedures. The isolated genomic DNA of FH subjects was used for polymerase chain reaction. We sequenced the exome region of 21 dyslipidemia-related Mendelian genes, including 3 FH genes (LDLR, APOB, and PCSK9), ABCG5, ABCG8, apolipoprotein E (APOE), and LDLR adaptor protein 1 (LRLRAP1). The pathogenicity of the variants was determined based on the allele frequency information obtained from ExAC Asian population data, in silico annotation tools, and Clinvar. Allele frequency <5% was defined as a rare mutation among Asian populations. Finally, we classified variants as pathogenic in the presence of supporting evidence based on the standard ACMG criteria (details described elsewhere). In addition, 4 SNPs validated for evaluation of polygenic cause for FH in East Asian patients were genotyped, and the weighted mean SNP scores were calculated based on the LDL-C-raising alleles. We divided the subjects into 4 groups based on mutations of FH and ABCG5/ABCG8 genes (Figure 1A).

**Ethical Considerations**
This study was approved by the Ethics Committee of Kanazawa University. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and the Helsinki Declaration of 1975, revised in 2008. Informed consent for genetic analyses was given by the FH subjects in this study.

**Biochemical Analysis**
Blood samples were drawn for assays after overnight fasting. The serum levels of total cholesterol, triglycerides (TGs), and high-density lipoprotein cholesterol were determined enzymatically (Quilagén, Sekisui Medical, Tokyo, Japan) using automated instrumentation based on assays previously described. LDL-C levels were calculated using the Friedewald formula if the TGs <400 mg/dL; otherwise, they were determined enzymatically. Serum levels of sitosterol and lathosterol were determined using gas-liquid chromatography-mass spectrometry. We assessed the data prior to the initiation of lipid-lowering therapies in all of the FH subjects.

**Clinical Evaluations**
Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the use of antihypertensive medication. The presence of diabetes was defined as previously described by the Japan Diabetes Society or the use of diabetes medication. CAD was defined as the presence of angina pectoris, myocardial infarction, or severe stenotic region(s) in the coronary artery, identified by either angiography or computed tomography.

**Statistical Analysis**
Categorical variables are expressed as percentages. Fisher’s exact test or chi-square test, whichever was most appropriate. Continuous variables with a normal distribution are presented as mean±SD, whereas median and interquartile ranges (IQR) are reported for values lacking a normal distribution. The mean values for continuous variables were compared using Student’s t-test for independent data, and median values were compared using nonparametric Wilcoxon Mann-Whitney rank sum test or chi-square test for categorical variables with Fisher’s post-hoc test. For each variable, two-way analysis of variance was used to determine significant differences among the variables. When differences were found, multiple comparisons among groups were performed by the Games-Howell test. Multivariate logistic analyses, including factors potentially associated with CAD, were used to assess the association between factors and outcomes. All statistical analyses were conducted using R statistical software, and P<0.05 was considered statistically significant.

**Results**
Characteristics of Study Subjects
The clinical characteristics of the FH subjects are shown in Table 1. The mean age was 41 years, and mean LDL-C level was 224 mg/dL. We identified 276 individuals with

### Table 1. Baseline Characteristics of the Subjects With FH Phenotype

| Variable              | All (n=487) | Group 1 (n=156) | Group 2 (n=37) | Group 3 (n=276) | Group 4 (n=18) |
|-----------------------|------------|----------------|---------------|----------------|---------------|
| Age (years)           | 41±20      | 44±19          | 43±23         | 38±20          | 42±16         |
| Male                  | 220 (45%)  | 79 (51%)       | 13 (35%)      | 121 (44%)      | 7 (39%)       |
| Hypertension          | 103 (21%)  | 36 (23%)       | 10 (27%)      | 52 (19%)       | 5 (28%)       |
| Diabetes              | 47 (10%)   | 22 (14%)       | 2 (5%)        | 13 (5%)        | 0 (0%)        |
| Smoking               | 119 (26%)  | 49 (31%)       | 11 (30%)      | 62 (22%)       | 5 (28%)       |
| Total cholesterol (mg/dL) | 304 [275–346] | 303 [271–339] | 290 [249–310] | 310 [278–352] | 370 [309–403] |
| Triglyceride (mg/dL)  | 110 [70–156] | 126 [84–183]† | 101 [66–174]† | 100 [66–145]§ | 99 [72–124]§  |
| HDL-C (mg/dL)         | 52 [44–63] | 52 [44–60]    | 56 [45–64]    | 52 [44–63]     | 55 [47–62]    |
| LDL-C (mg/dL)         | 224 [193–267] | 211 [188–255] | 199 [184–213] | 234 [201–276]§ | 266 [233–343]§ |
| CAD                   | 725 (19%)  | 269 (17%)     | 155 (56%)     | 216 (76%)      | 100 (56%)     |
| Carriers of APOE or LDLRAP1 mutation(s) (%) | 7 (1%) | 3 (1%) | 1 (0%) | 3 (1%) | 0 (0%) |

*Values were compared using nonparametric Wilcoxon rank sum test or chi-square test for categorical variables. Significant differences were found, multiple comparisons among groups were performed by the Games-Howell test. Multivariate logistic analyses, including factors potentially associated with CAD, were used to assess the association between factors and outcomes. All statistical analyses were conducted using R statistical software, and P<0.05 was considered statistically significant.

1. P<0.05 vs. group 2, group 3, and group 4; 2. P<0.05 vs. group 1 and group 2; 3. P<0.05 vs. group 1, group 2, and group 3; 4. P<0.05 vs. group 1, group 2, and group 3. APOE, apolipoprotein E; CAD, coronary artery disease; FH, familial hypercholesterolemia; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDLRAP1, LDL receptor adaptor protein 1.
Figure 2. Family tree of sitosterolemia (double mutations in ABCG5/ABCG8 genes); mutation carriers of ABCG5/ABCG8 confirmed to have a dominant pattern of cosegregation. (A, Left panel) The proband (red arrow) exhibited compound heterozygous mutations in ABCG5 (c.1673_1677del/p.Pro558GlnfsTer14 and c.1108_1118+2del). (A, Middle panel) The proband (red arrow) exhibited homozygous mutations in ABCG8 (c.1392_1403del/p.Leu456Pro). (A, Right panel) The proband (red arrow) exhibited homozygous mutations in ABCG8 (c.336_337insT/p.Leu456_Ile468del). (B, Left panel) Father and daughter exhibited heterozygous mutation in ABCG5 (c.831_849dup/p.Phe284SerfsTer54). (B, Middle panel) Mother and daughter exhibited heterozygous mutation in ABCG8 (c.55G>C/p.Asp19His). (B, Right panel) Father, daughter, and son exhibited heterozygous mutation in ABCG5 (c.831_849dup/p.Phe284SerfsTer54). HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Figure 3. Serum sterol levels among the subjects: (A) Sitosterol and (B) lathosterol. White, subjects without mutations; Pink, subjects with ABCG5/ABCG8 mutation(s); Light blue, subjects with a single mutation in conventional FH genes; Purple, subjects with a single mutation in conventional FH genes and an ABCG5/ABCG8 mutation.

* We excluded 3 individuals with double mutations (sitosterolemia) in this comparison.
monogenic FH who had a deleterious mutation in 1 FH gene (57%, monogenic FH, group 3), but no causative mutations were found in the remaining 156 individuals (32%, mutation-negative, group 1). A total of 37 individuals had deleterious mutations in \( \text{ABCG5} \) or \( \text{ABCG8} \), but not in FH genes (8%, \( \text{ABCG5/ABCG8} \) mutation carriers, group 2). Among these individuals, we found 3 with sitosterolemia (0.6%) who had double mutations. We also identified 18 individuals who had deleterious mutations in FH genes and \( \text{ABCG5} \) or \( \text{ABCG8} \) (4%, \( \text{ABCG5/ABCG8} \)-oligogenic FH, group 4).

The identified FH and \( \text{ABCG5/ABCG8} \) pathogenic genetic mutations are illustrated in Supplementary Tables 1, 2, and 3, respectively. In addition, we evaluated serum sitosterol levels among the individuals with heterozygous \( \text{ABCG5/ABCG8} \) genetic mutations (Supplementary Table 2) and found they were mildly elevated among heterozygous mutation carriers compared with the normal range. Moreover, serum TGs in the mutation-negative group (group 1) were significantly higher than in other groups (Table 1).

### Effect of Rare and Deleterious Mutations in \( \text{ABCG5/ABCG8} \) on LDL-C

LDL-C levels in the \( \text{ABCG5/ABCG8} \)-oligogenic FH individuals, who had a deleterious mutation in FH genes and \( \text{ABCG5} \) or \( \text{ABCG8} \), were significantly higher than in monogenic FH individuals (266 vs. 234 mg/dL, Table 1, Figure 1B). Additionally, LDL-C levels in the monogenic FH individuals were significantly higher than in the mutation-negative group (group 1) (234 vs. 211 mg/dL, Table 1, Figure 1B) and \( \text{ABCG5/ABCG8} \) mutation carriers (group 2) (234 vs. 199 mg/dL, Table 1, Figure 1B). There was no statistically significant difference between LDL-C levels in group 1 (mutation-negative) and group 2 (\( \text{ABCG5/ABCG8} \) mutation carrier) (Table 1, Figure 1B). On the other hand, the polygenic score for subjects with no FH mutations was significantly higher than that in the other groups (Table 1).

Moreover, we found 7 individuals with APOE or LDLRAP1 mutation (Supplementary Table 3). There was no significant difference in the prevalence of mutation carriers of APOE or LDLRAP1 among the 4 groups of subjects.

### Identification of Sitosterolemia (Double Mutations in \( \text{ABCG5/ABCG8} \))

Among the 37 individuals with mutation(s) in \( \text{ABCG5/ABCG8} \), 3 had sitosterolemia with both double rare and deleterious mutations in the proband. All the subjects had family members with the same mutation (mutation carriers) and exhibited significantly elevated LDL-C and sitosterol levels (Figure 2A).

### Identification of Carriers of Mutations in \( \text{ABCG5/ABCG8} \) and Pattern of Cosegregation

Among the 37 individuals with mutation(s) in \( \text{ABCG5/ABCG8} \), 3 had a dominant patterns of cosegregation within their families. In each family, the proband who met the clinical diagnosis of FH and other members with the same mutation exhibited hyper-LDL cholesterol and hyper-sitosterolemia (Figure 2B).

### Serum Sterol Levels According to Mutation Status

We compared the serum sterol levels (sitosterol and lathosterol) among the 4 groups of subjects (Figure 3). Interestingly, the serum sitosterol levels of the monogenic FH group (group 3) were slightly, but significantly lower than those of the mutation-negative group (group 1). In contrast, the serum lathosterol levels of the monogenic FH group (group 3) were significantly higher than those of the mutation-negative group (group 1). Also, we found that the serum sitosterol levels of \( \text{ABCG5/ABCG8} \) genetic mutation carriers (group 2) and \( \text{ABCG5/ABCG8} \)-oligogenic FH subjects (group 4) were significantly higher than those of the mutation-negative group (group 1) and monogenic FH group (group 3), as expected. In addition, we found that the serum lathosterol levels of \( \text{ABCG5/ABCG8} \) genetic mutation carriers (group 2) and \( \text{ABCG5/ABCG8} \)-oligogenic FH subjects (group 4) were significantly lower than those of the mutation-negative and monogenic FH groups (group 1 and group 3, respectively).

### Factors Associated With CAD

To clarify the factors associated with CAD in this study, we assessed the following, which may be associated with atherosclerotic disease, by multivariate logistic regression analysis: age (odds ratio (OR) = 1.10, 95% confidence interval (CI) = 1.07–1.13, \( P = 9.6 	imes 10^{-13} \)), male sex (OR = 2.38, 95% CI = 1.99–2.87, \( P = 0.044 \)), smoking (OR = 7.10, 95% CI = 3.45–15.87, \( P = 1.2 	imes 10^{-7} \)), LDL-C per 10 mg (OR = 1.05, 95% CI = 1.00–1.10, \( P = 0.046 \)), and FH mutation (OR = 1.95, 95% CI = 1.03–3.74, \( P = 0.0418 \)) were independently associated with CAD. \( \text{ABCG5/ABCG8} \) mutation status was not associated with CAD, probably because of a lack of statistical power (Table 2).

### Discussion

In the current study, we aimed to evaluate the prevalence and effect of mutations in \( \text{ABCG5/ABCG8} \) on LDL-C and CAD in patients with a clinical diagnosis of FH. Among the FH mutation-negative patients, we found 37 individuals (8%) with rare and deleterious mutation(s) in \( \text{ABCG5/ABCG8} \). In addition, we found 3 individuals with sitosterolemia and double mutations in \( \text{ABCG5/ABCG8} \) and at least 3 families with a dominant pattern of intrafamilial cosegregation. We also identified 18 individuals with significantly elevated LDL-C levels, who had an FH gene mutation and a rare/deleterious mutation in \( \text{ABCG5/ABCG8} \). Previously, we included APOE and LDLRAP1 in this concept, but in the present study we wanted to focus on

### Table 2. Factors Associated With CAD

| Variable | OR (95% CI) | P value |
|----------|-------------|---------|
| Age (years) | 1.10 (1.07–1.13) | 9.6×10⁻¹³ |
| Male | 2.38 (1.09–5.27) | 0.03 |
| Hypertension | 2.01 (1.02–3.99) | 0.044 |
| Diabetes | 2.20 (0.69–7.57) | 0.1958 |
| Smoking | 7.10 (3.45–18.57) | 1.2×10⁻⁷ |
| LDL-C (per 10 mg/dL) | 1.05 (1.00–1.10) | 0.046 |
| Triglycerides | 1.00 (0.99–1.00) | 0.2831 |
| HDL-C | 1.00 (0.99–1.00) | 0.51 |
| FH mutation | 1.95 (1.03–3.74) | 0.0418 |
| \( \text{ABCG5/ABCG8} \) mutation | 1.29 (0.30–2.01) | 0.23 |
Rare Mutations in FH Phenotype

ABCG5/ABCG8 because their contributions in terms of the number of variants and their effect on LDL-C levels seem to be quite large, as compared with APOE and LDLRAP1 mutations. In addition, we identified at least 3 independent families with a phenotype that could be defined as FH caused by ABCG5/ABCG8 mutation. Those observations motivated us to summarize the effect of ABCG5/ABCG8 on the FH phenotype.

FH is a common genetic cause of premature CAD because of lifelong elevated plasma LDL-C levels. Based on recent findings in Japan, the USA, and Europe, the prevalence of FH could be as high as 1 in 200 among the general population. However, many studies performed globally have indicated that genetic analyses of the established FH genes, namely, LDLR, APOB, and PCSK9, fail to identify pathogenic mutation(s) among a substantial proportion of FH patients. In this study, we confirmed that at least a portion of FH is caused by deleterious mutation(s) in ABCG5/ABCG8 and some individuals with double mutations in these genes should be diagnosed with sitosterolemia. The effect of mutations in ABCG5/ABCG8 may not be as large as that of LDLR, APOB, or PCSK9, but we suggest an assessment of the presence of such mutations, as it may lead to a genetic diagnosis and a better treatment strategy of using selective Niemann-Pick C1-like 1 inhibitor (ezetimibe), especially in individuals with sitosterolemia and double mutations in ABCG5/ABCG8.

Moreover, we found that serum sitosterol as well as lathosterol levels were significantly affected by mutation status of FH genes in addition to ABCG5/ABCG8. We believe this is a novel finding in the current study, and we speculate that it is based on a metabolic abnormality of FH in which cholesterol production is accelerated in addition to disturbance of cholesterol catabolism.

Study Limitations

First, it was a retrospective, cross-sectional observational analysis. However, the study contained one of the largest sample sizes evaluated for this issue in the Japanese population and could contribute to our understanding across ethnicities. In addition, our findings regarding the factors associated with CAD were consistent with results from previous studies. Second, we did not perform a full assessment of all the FH patients, which could lead to some biases. Third, we assessed structural genetic variations using the eXome Hidden Markov Model (XHMM) software instead of multiplex ligation-dependent probe amplification (MLPA). However, the structural variations identified using XHMM were well-validated with MLPA in another study. Fourth, mutations identified through the next-generation sequencer in this study were not validated using the Sanger sequencing technique. However, all subjects had ≥20 coverages in ≥98% of targeted exons. The median read depth for subjects was 242 (IQR, 153–342). Fifth, it could be debatable that the ACMG criteria of a disorder are applicable to a similar but different disease. In this regard, most of the ABCG5/ABCG8 mutations found in this study were classified as either (1) protein-truncating variants and/or (2) variants cosegregated with multiple individuals as hyper-LDL cholesterolemia. Accordingly, we believe that both situations are acceptable as “evidence” obtained from genetic analyses. Sixth, we did not assess the presence of fatty liver in this study, missing the chance to observe the association between fatty liver and ABCG5/ABCG8 mutation status. Seventh, determination of the pathogenicity of genetic variants may not be perfect in this study. However, we used a rather conservative strategy for this purpose via referencing multiple sources. Accordingly, we believe that misclassification should be minimal. Finally, we could not find a significant association between ABCG5/ABCG8 mutations and CAD in this study. However, we believe that this is likely because of our small sample size. Accordingly, further studies with a larger sample size are warranted to validate our results.

Conclusions

The current study results suggested that rare and deleterious mutations of ABCG5/ABCG8 contribute substantially to mimicking and exacerbating the phenotype of patients with FH.

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Disclosures

None.

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Supplementary Files
Please find supplementary file(s):
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