

Case Report

A Rare Coincidence of Sitosterolemia and Familial Mediterranean Fever Identified by Whole Exome Sequencing

Hayato Tada¹, Masa-aki Kawashiri¹, Hirofumi Okada¹, Saori Endo², Yuka Toyoshima², Tetsuo Konno¹, Atsushi Nohara¹, Akihiro Inazu³, Akira Takao², Hiroshi Mabuchi¹, Masakazu Yamagishi¹ and Kenshi Hayashi¹

¹Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

²Department of Pediatrics, Gifu Municipal Hospital, Gifu, Japan

³Department of Clinical Laboratory Science, Faculty of Health Sciences, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

Whole exome sequencing (WES) technologies have accelerated genetic studies of Mendelian disorders, yielding approximately 30% diagnostic success. We encountered a 13-year-old Japanese female initially diagnosed with familial hypercholesterolemia on the basis of clinical manifestations of severe hypercholesterolemia (initial LDL cholesterol=609 mg/dl at the age of one) and systemic intertriginous xanthomas with histories of recurrent self-limiting episodes of fever and arthritis. Both her phenotypes seemed to co-segregate in a recessive manner. We performed WES on this patient, who was considered a proband. Among 206,430 variants found in this individual, we found 18,220 nonsense, missense, or splice site variants, of which 3,087 were rare (minor allele frequency ≤ 0.01 or not reported) in 1000 Genome (Asian population). Filtering by assuming a recessive pattern of inheritance with the use of an *in silico* annotation prediction tool, we successfully narrowed down the candidates to the compound heterozygous mutations in the *ABCG5* gene (c.1256G>A or p.Arg419His/c.1763-1G>A [splice acceptor site]) and to the double-compound heterozygous mutations in the *MEFV* gene (c.329T>C/C or p.Leu110Pro/c.442G>C/C or p.Glu148Val). The patient was genetically diagnosed with sitosterolemia and familial Mediterranean fever using WES for the first time. Such a comprehensive approach is useful for identifying causative mutations for multiple unrelated inheritable diseases.

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Key words: Sitosterolemia, Familial Mediterranean fever, *ABCG5*, *ABCG8*, Whole exome sequencing

Introduction

Sitosterolemia (OMIM #210250) caused by mutations in either of two genes ATP-binding cassette (ABC) sub-family G members 5 and 8 (*ABCG5* and *ABCG8*) is an extremely rare autosomal recessive disorder of sterol metabolism characterized by increased

absorption and decreased biliary excretion of plant sterols and cholesterol, resulting in prominently elevated serum levels of plant sterols such as sitosterol and campesterol^{1, 2}. Subjects suffering from sitosterolemia primarily present tendinous and tuberous xanthomas and premature coronary atherosclerosis resembling familial hypercholesterolemia (FH)³. Familial Mediterranean fever (FMF, OMIM #249100), which is caused by mutations in the pyrin (*MEFV*) gene, is an autosomal recessive disorder characterized by recurrent attacks of fever and inflammation in the peritoneum, synovium, or pleura and is accompanied by pain^{4, 5}.

We encountered a Japanese female initially diagnosed with FH on the basis of clinical manifestations

Address for correspondence: Hayato Tada, Division of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, 920-8641, Japan.

Email: ht240z@sa3.so-net.ne.jp

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of severe hypercholesterolemia and systemic intertriginous xanthomas with histories of recurrent self-limiting episodes of fever and arthritis. Both phenotypes seemed to co-segregate in a recessive manner. We therefore tried to establish a comprehensive molecular diagnosis for this rare condition using whole exome sequencing (WES).

Case Report

Study Subjects

A 13-year-old Japanese female was referred to our lipid clinic due to her extremely high levels of LDL cholesterol, without any apparent secondary causes. Her LDL cholesterol at the age of one was 609 mg/dl when she was under breastfeeding. Both her parents showed no evidence of consanguineous marriage, and her younger sister was also included in this study.

Ethical Considerations

This study was approved by the Ethics Committee of Kanazawa University and conducted in accordance with the Declaration of Helsinki (2008) of the World Medical Association. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consents were obtained from all subjects for being included in the study.

Biochemical Analysis

Blood samples were drawn for assays after overnight fasting. Serum levels of total cholesterol, triglycerides, and HDL cholesterol were determined enzymatically⁶. Serum levels of sterols, including sitosterol, lathosterol, and campesterol, were determined using gas-liquid chromatography-mass spectrometry as previously described².

Exome Sequencing and Bioinformatics

Exome capture and sequencing were performed as previously described⁷. We applied three independent filters after standard variant quality controls in order to successfully discover a causal variant among the proband as previously described⁷. We filtered out the variants as 1) benign variants predicted by SnpEff, 2) minor allele frequency (MAF) >1% in an Asian population, and 3) unmatched segregation under the assumption of a recessive form of inheritance.

For the sample, paired-end reads were aligned using the Burrows-Wheeler Aligner on the human ref-

erence genome build hg19 using quality score calibration, soft clipping, and adapter trimming. Following the exclusion of PCR duplicate reads using Picard, insertion-deletions and single-nucleotide polymorphisms (SNPs) were identified using GATK^{8,9}. Variants (SNPs/indels) were filtered out on the basis of the Phred-scaled genotype quality score. Re-alignment was performed, and a calling algorithm was used to merge the output of the GATK UnifiedGenotyper. All samples were annotated using SnpEff version 3.6 to classify variants (e.g., missense, nonsense, splice site, synonymous, intronic, or stop gain/loss)¹⁰.

Functional filtering, missense, nonsense, and splice site variants were considered as candidate variants. The frequency filter was used to quantify the allele frequency estimates from the Asian cohort database in the 1000 Genomes Project, and a MAF >1% was used as the cut-off. Segregation pattern matching was defined as that the situation where the affected exhibited homozygous or compound heterozygous of alternative alleles in a particular gene. We also filtered out variants with the use of an *in silico* annotation prediction tool as described previously⁷. Following those evaluations, the putative variants identified by bioinformatics were confirmed using Sanger sequencing methods as previously described¹¹.

Characteristics of Study Subjects

The clinical characteristics of the study subjects are shown in **Table 1**. The proband (II.1) initially visited a hospital due to her intertriginous xanthomas in her legs at one year of age. At that time she was diagnosed with FH based on her having extremely high levels of LDL cholesterol as well as on physical findings. She was thought to have heterozygous FH, because her LDL cholesterol dramatically reduced to around 140 mg/dl with a small dose of a standard statin (5 mg pravastatin or 5 mg simvastatin) and because she was weaned from breastfeeding (**Fig. 1**). At the age of 13 she was referred to Kanazawa University Hospital to undergo a genetic diagnosis, because it was quite unusual to observe such a great reduction in LDL cholesterol in a patient with FH using such therapies.

At the time of genetic diagnosis, the patient had already exhibited Achilles' tendon thickness (**Fig. 2**), thickness in her carotid artery (**Fig. 3**), and moderate aortic regurgitation associated with aortic valve calcification (**Fig. 4**). Her father was taking statin (2.5 mg rosuvastatin) without any physical xanthomas or any family history of premature coronary artery disease. Her mother exhibited normolipidemia. Based on the recessive pattern of inheritance as well as the great

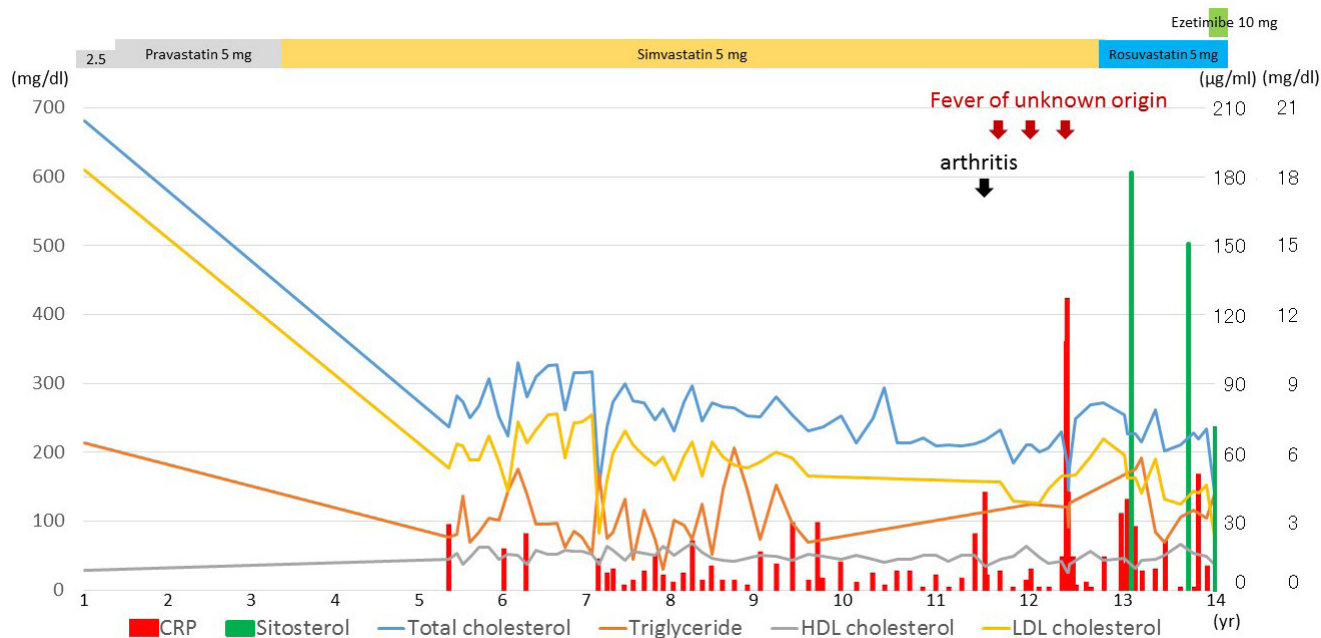
Table 1. Characteristics of the study subjects

Subject (gender)	I.1 (male)	I.2 (female)	II.1 (female)	II.2 (female)
<i>ABCG5</i> genotype	W/M1	W/M2	M1/M2	W/W
<i>MEFV</i> genotype	W/M1	W/M2	M1/M2	W/W
Age (yr)	51	47	1	6
Total cholesterol (mg/dl)	214*	202	681	137
Triglyceride (mg/dl)	123*	60	213	89
HDL cholesterol (mg/dl)	44*	45	29	39
LDL cholesterol (mg/dl)	145*	143	609	90
ApoA-I (mg/dl)	138*	124	96	120
ApoB (mg/dl)	116*	101	340	63
ApoE phenotype	3/3	3/3	3/3	3/3
CETP ($\mu\text{g/ml}$)	1.9*	1.9	3.1**	3.3
Sitosterol ($\mu\text{g/ml}$)	6*	7.1	182**	1.7
Lathosterol ($\mu\text{g/ml}$)	1.2*	1.4	0.6**	0.8
Campesterol ($\mu\text{g/ml}$)	13.1*	13.6	125**	3.5

ABCG5: ATP-binding cassette sub-family G member 5, *MEFV*: Mediterranean fever, CETP: cholesteryl ester transfer protein
ABCG5 genotype: W=wild type, M1=c.1256G>A, M2=c.1763-1G>A, *MEFV* genotype: W=wild type, M1=c.329 T>C/C, M2=c.442G>C/C

*Values measured when rosuvastatin was 2.5 mg.

**CETP, sitosterol, lathosterol, and campesterol were measured at the age of 13 (when rosuvastatin was 5 mg).

**Fig. 1.** Clinical course of the proband

X-axis indicates age. Line graphs on the Y-axis (left side) indicate total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol in mg/dl. Bar graphs on the Y-axis (right side) indicate CRP in mg/dl, and sitosterol in $\mu\text{g/ml}$.

reduction in LDL cholesterol, we suspected her condition to be sitosterolemia, and thus measured her serum sitosterol. Her initial serumal levels of sitosterol at the age of 13 was as high as 182 $\mu\text{g/ml}$, confirming

her clinical diagnosis as sitosterolemia. Her blood counts were within normal range. Her sitosterol levels could be reduced to 70 $\mu\text{g/ml}$ using 10 mg ezetimibe in accordance with a previous report²). We also

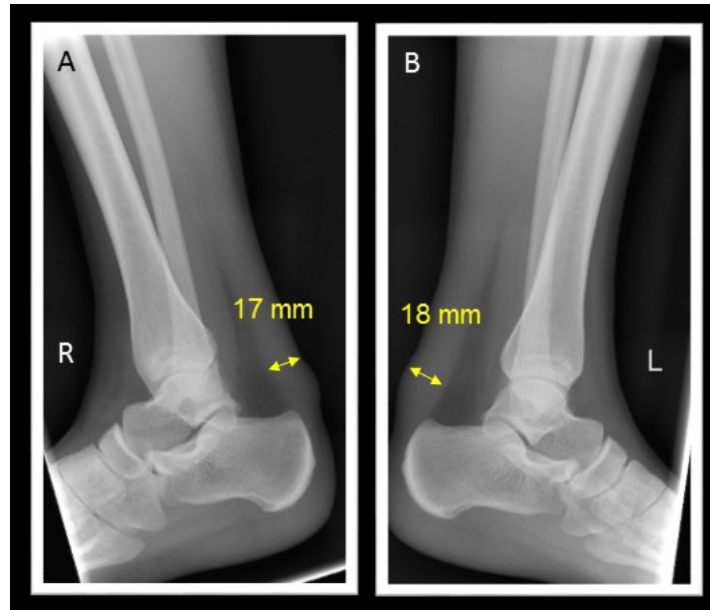


Fig. 2. X-ray for Achilles' tendon
A. Right. B. Left

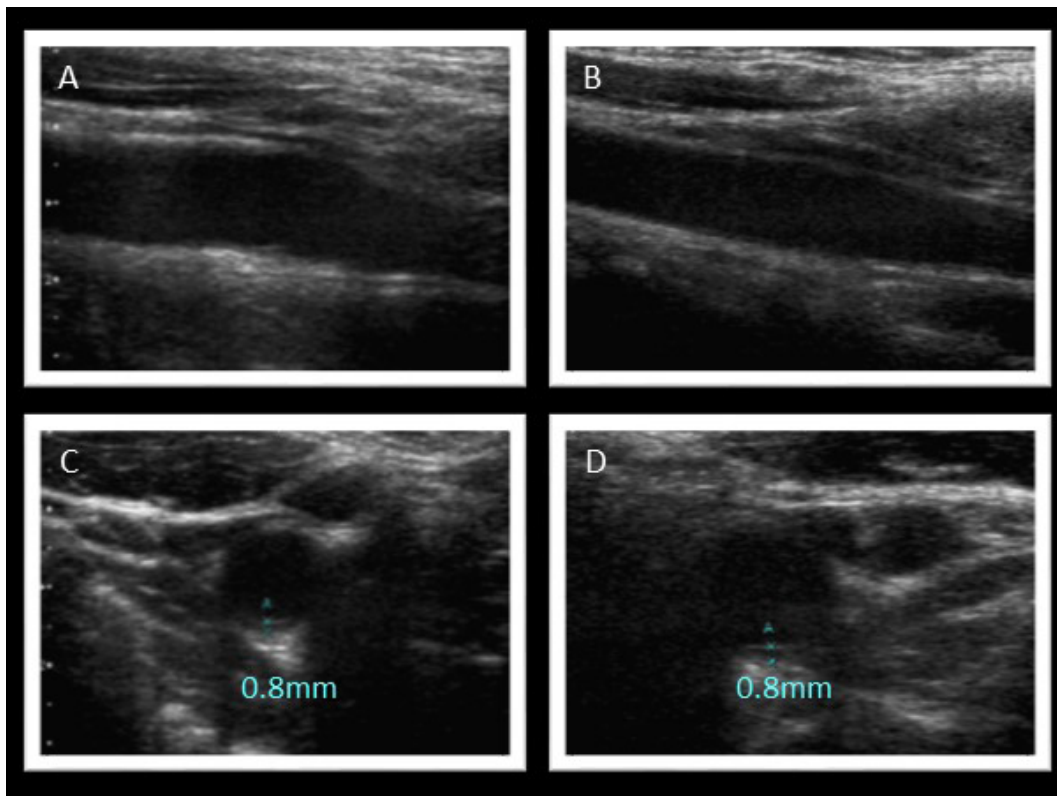


Fig. 3. Ultrasound images of carotid arteries
A. Long-axis view (right). B. Long-axis view (left). C. Short-axis view (right). D. Short-axis view (left). Bilateral carotid intima-media thickness was 0.8 mm.

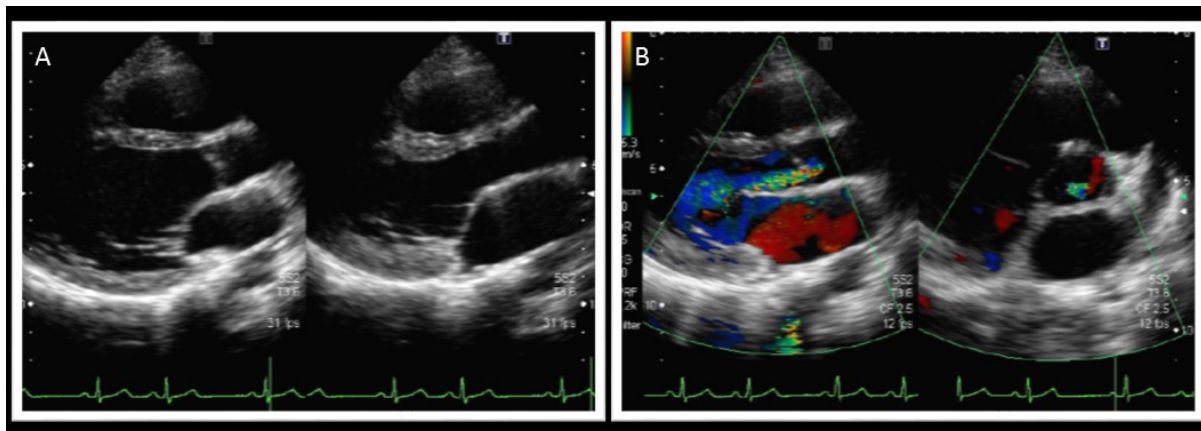


Fig. 4. Echocardiography

A. Long-axis view (plain). B. Long- and short-axis view (in color). Echocardiography revealed moderate aortic regurgitation associated with aortic valve calcification.

observed great reduction in her LDL cholesterol from 156 mg/dl to 76 mg/dl using 10 mg of ezetimibe. In addition, she had experienced several occurrences of high grade fever and arthritis associated with elevated C-reactive protein (**Fig. 1**).

Exome Sequencing and Bioinformatics Analysis

The proband underwent whole exome sequencing, and subsequent bioinformatics filtering. The mean depth was $99.9\times$ per base across the whole exome. The percentage of on-target reads was 84.6%. Also, the coverage rate of target coding lesions ($10\times$) was 99.0%.

The number of aligned variants in the proband that passed through standard quality control was 206,430. Of those, 18,220 were missense, nonsense, splice site, or frameshift variants. After removing “common” variants with MAF $>1\%$ using the Asian cohort in the 1000 genome project¹²⁾, 3,087 variants were detected. Subsequently, filtering against the segregation pattern while assuming the recessive form of inheritance with the use of *in silico* annotation prediction tool reduced the candidate variants to compound heterozygous mutations in the *ABCG5* gene (c.1256G $>$ A or p.Arg419His/c.1763-1G $>$ A [splice acceptor site]) on chromosome 2 and double compound heterozygous mutations in the *MEFV* gene (c.329 T $>$ C/C or p.Leu110Pro/c.442G $>$ C/C or p.Glu148Val) on chromosome 16. The former mutation in the *ABCG5* gene is one of the most common mutations among Asians with sitosterolemia¹³⁾, while the latter mutation in the *ABCG5* gene is considered novel. Meanwhile, both mutations in the *MEFV* gene identified in the proband are two of the most common mutations

among Japanese patients with FMF¹⁴⁾. The status of mutations in her family members (**Fig. 5A**) was determined by subsequent Sanger sequencing (**Fig. 5B-5E**). Finally, we diagnosed her as having sitosterolemia and FMF; this is considered to be a rare condition of sitosterolemia. We consider this to be the first time such a condition has been diagnosed using an exome-wide approach.

Discussion

Using a WES approach, we identified the first case of a rare coincidence of sitosterolemia and FMF exhibiting compound heterozygous mutations in the *ABCG5* gene and double compound heterozygous mutations in the *MEFV* gene. To the best of our knowledge, this is the first report of this coincidence. These findings allow us to draw several conclusions. First, the WES approach is useful to identify causative mutations in a family with a recessive form of inheritance, even when DNA of only one affected individual is available. Second, we can determine true causative mutations not from target re-sequencing, but from an exome-wide approach. Third, such a comprehensive approach is useful in identifying causative mutations for multiple unrelated inherited diseases.

In terms of mode of inheritance, her father exhibited mildly elevated levels of LDL cholesterol under statin therapy without any evidence of extreme hypercholesterolemia or cutaneous xanthomas. There was no deleterious mutation in the coding lesion of the LDL receptor or in the *PCSK9* gene in her father; this led us to the notion of recessive inheritance.

Thus far, only 100 or so patients have been diag-

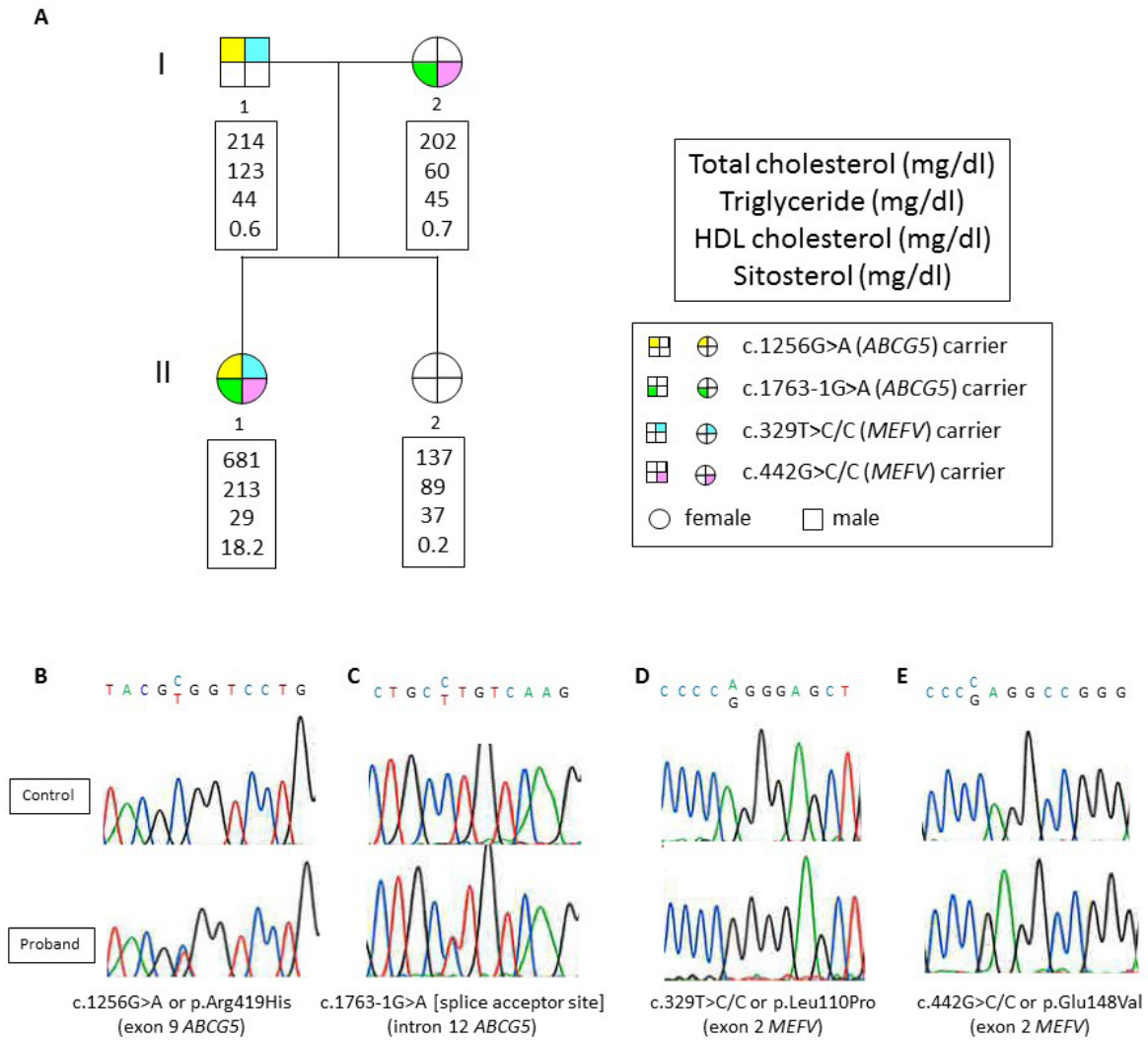


Fig. 5. Family tree and mutation validation

A. Yellow indicates the carrier of c.1256G>A or p.Arg419His mutation in the *ABCG5* gene. Green indicates the carrier of the c.1763-1G>A mutation in the *ABCG5* gene. Blue indicates the carrier of the c.329 T>C/C or p.Leu110Pro mutation in the *MEFV* gene. Pink indicates the carrier of the c.442G>C/C or p.Glu148Val mutation in the *MEFV* gene. B-E. Mutations were validated by Sanger sequencing.

nosed with sitosterolemia¹⁵). The clinical features of sitosterolemia includes xanthomas, premature atherosclerosis, and increased serum levels of plant sterols^{1, 2}. In addition, they may exhibit extreme hypercholesterolemia induced by breastfeeding in accordance with our previous findings in infantile cases with sitosterolemia². Separately, FMF, which is characterized by recurrent, self-limiting episodes of fever, affects more than 100,000 patients, primarily in the Mediterranean Basin¹⁶). The proband in this study was diagnosed with FH and was treated with statins; however, ezetimibe could be a much more reasonable therapy for her based on her recent diagnosis with sitosterol-

emia¹⁷⁻¹⁹).

Careful monitoring and additional therapeutic approaches are needed to reduce her serum levels of sitosterol because of the unfavorable combination of hypersitosterolemia and systemic inflammatory disease, both of which have been shown to be associated with an increased risk of cardiovascular disease.

We report a case exhibiting a rare coincidence of sitosterolemia and FMF identified using WES. Such a comprehensive approach is useful in identifying causative mutations for multiple unrelated inherited diseases.

Source of Finding

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None.

Conflict of Interest Statement

None.

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