Infantile Cases of Sitosterolaemia with Novel Mutations in the ABCG5 Gene: Extreme Hypercholesterolaemia is Exacerbated by Breastfeeding

Hayato Tada • Masa-aki Kawashiri • Mutsuko Takata •
Kunihiro Matsunami • Atsushi Imamura •
Misayo Matsuyama • Hirotake Sawada •
Hiroyuki Nunoi • Tetsuo Konno • Kenshi Hayashi •
Atsushi Nohara • Akihiro Inazu • Junji Kobayashi •
Hiroshi Mabuchi • Masakazu Yamagishi

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Abstract Few data exists regarding the clinical impact of breastfeeding in infantile sitosterolaemic cases. We report

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H. Tada (✉) • M.-a. Kawashiri • T. Konno • K. Hayashi •
M. Yamagishi
Division of Cardiovascular Medicine, Kanazawa University Graduate
School of Medicine, Kanazawa, Japan
e-mail: ht240z@sa3.so-net.ne.jp

M. Takata
Department of Internal Medicine, Komatsu Municipal Hospital,
Komatsu, Japan

K. Matsunami • A. Imamura
Department of Pediatrics, Gifu Prefectural General Medical Center,
Gifu, Japan

M. Matsuyama • H. Sawada • H. Nunoi
Division of Pediatrics, Department of Reproductive and
Developmental Medicine, Faculty of Medicine, University of
Miyazaki, Miyazaki, Japan

A. Nohara • H. Mabuchi
Department of Lipidology, Graduate School of Medical Science,
Kanazawa University, Kanazawa, Japan

A. Inazu
Department of Laboratory Science, Molecular Biochemistry and
Molecular Biology, Graduate School of Medical Science, Kanazawa
University, Kanazawa, Japan

J. Kobayashi
Department of General Medicine, Kanazawa Medical University,
Uchinada, Japan

four Japanese infantile cases of sitosterolaemia, an extremely rare inherited disease characterised by increased serum levels of plant sitosterol, presenting with severe hypercholesterolaemia and systemic xanthomas exacerbated by breastfeeding. In these four cases, genetic analyses were performed for low-density lipoprotein (LDL) receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9), LDL receptor adaptor protein 1 and ATP-binding cassette (ABC) subfamily G member 5 and 8 (ABCG5 and ABCG8) genes. We assessed their clinical manifestations, including responsiveness to a variety of treatments, especially to weaning from breastfeeding and use of ezetimibe. Two pairs of mutations in the ABCG5 gene in each case, including two novel mutations (c.130C>T or p.Ser44Ala and c.1813_1817delCTTTT or p.Pro558GlufsX14) and two known mutations (c.1306G>A or p.Arg389His and c.1336C>T or p.Arg446X), were identified. Significant reductions in cholesterol levels were obtained by means of weaning from breastfeeding alone. Substantial reductions in sitosterol levels, without any apparent side effects, were observed with ezetimibe. In conclusion, we have identified infantile Japanese sitosterolaemic subjects with extreme hypercholesterolaemia exacerbated by breastfeeding. Their unique response to weaning from breastfeeding, as well as to use of ezetimibe, could provide insights into the metabolic basis of sterols in humans.

Introduction

Sitosterolaemia (OMIM #210250) caused by mutations in either of two genes, ATP-binding cassette (ABC) subfamily G members 5 and 8 (ABCG5 and ABCG8), is an extremely rare autosomal recessive disorder of sterol metabolism characterised 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 (Shulman et al. 1976). Subjects suffering from sitosterolaemia present primarily with tendinous and tuberous xanthomas and premature coronary atherosclerosis resembling familial hypercholesterolaemia (FH: Salen et al. (2002), Bhattacharyya and Connor (1974)). Serum LDL cholesterol (LDL-C) levels tend to vary more in sitosterolaemia than in other genetic or nongenetic hyperlipidaemias. Notably, LDL-C can be elevated significantly in some cases with sitosterolaemia, especially in infantile cases, due to unknown mechanisms (Yoshida et al. 2000). Thus, those cases are sometimes misdiagnosed as homozygous FH.

We encountered four Japanese infantile cases initially diagnosed as homozygous FH based on their clinical manifestations of severe hypercholesterolaemia and systemic intertriginous xanthomas. However, those manifestations responded very well to a variety of treatments, especially weaning from breastfeeding, which would not be observed in homozygous FH. Therefore, the aims of this study were (1) determining their molecular diagnosis, including LDL receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDL receptor adaptor protein 1 (LDLRAP1) genes that have been described as causative genes of Japanese FH (Mabuchi et al. 2014) as well as ABCG5/8 genes as causes of Japanese sitosterolaemia (Tsubakio-Yamamoto et al. 2010), and (2) evaluating their responsiveness to a variety of treatments.

Materials and Methods

Study Subjects

The backgrounds of the study subjects are listed in Supplemental Tables 1, 2, and 3. All of the probands showed severe hypercholesterolaemia, and three of them exhibited intertriginous xanthomas, but no subjects showed tendon xanthomas. None of them were born from consanguineous marriages, and none of their parents exhibited any sign of physical xanthomas. In all the study subjects, we noted no evidence of secondary hypercholesterolaemia, such as thyroid insufficiency or nephritic syndrome. The families initially visited three different institutions (Kana-

zawa University Hospital, Gifu Prefectural General Medical Center and Miyazaki University Hospital). And the families from Gifu Prefectural General Medical Center and Miyazaki University Hospital were referred to Kanazawa University Hospital because of the (initial) clinical diagnosis of homozygous FH.

Ethical Considerations

This study was approved by the Ethics Committee of Kanazawa University and carried out 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 or their parents (if the subject was an infant) when they visited Kanazawa University Hospital for being included in the study.

Genetic Studies

Genomic DNA was isolated from peripheral white blood cells according to standard procedures and was used for polymerase chain reaction (PCR). Primers for the study were designed as previously reported (Noguchi et al. 2010); PCR products were purified by Microcon (Millipore Corp., Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions, using a dye terminator method, ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). We analysed ABCG5/ABCG8 genes as well as LDL receptor, PCSK9 and LDLRAP1 genes, as previously reported (Mabuchi et al. 2014). In order to precisely determine the deleted codons in Case 1, Case 2 and Case 4, we digested the PCR products using *Dra*I, which could recognise the 6-bp nucleotide TTAAAA and consequently make a 28-bp and an 85-bp fragment in the normal allele. Thus, we could discriminate the normal and abnormal alleles in exon 12 of the ABCG5 gene after electrophoresis through 2.5% polyacrylamide gels.

Biochemical Analysis

Blood samples were drawn for assays after overnight fasting. Serum levels of total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically (Allain et al. 1974; Sugiura et al. 1977; Kajikawa et al. 1981). Serum levels of LDL-C were derived from the Friedewald's formula (Friedewald

et al. 1972). Serum cholesteryl ester transfer protein (CETP) levels were determined by a specific enzyme-linked immunosorbent assay (Kiyohara et al. 1998). Serum levels of sterol, including those of sitosterol, lathosterol and campesterol, were determined using gas–liquid chromatography–mass spectrometry (Ahmida et al. 2006).

Results

Genetic Analysis (Fig. 1, Supplemental Fig. 1)

No mutation was identified in the three FH genes (LDL receptor, PCSK9 and LDLRAP1) amongst any of the four sitosterolaemic cases.

In Case 1, direct sequencing analysis for exon 12 of ABCG5 gene showed abnormal overlapping after nucleotide position 1813, suggesting deletion of several nucleotides in this region (Fig. 1a). To clarify the supposed overlapping sequence, we attempted to digest the PCR products with *Dra*I and found no fragmentation of PCR products that would have suggested the existence of an abnormal sequence (Fig. 1b). After direct sequencing of the non-fragmented PCR products, we could confirm a novel mutation of a 5-nucleotide deletion (c.1813_1817delCTTTT or p.Pro558GlufsX14) causing a premature termination at codon 571 (Fig. 1c, d). In addition, we identified another mutation in exon 9 (c.1306G>A or p.Arg389His, Fig. 1e), which is one of the most common mutations in Asian sitosterolaemic cases (Park et al. 2014). We confirmed the same mutations in Case 2 (the younger sister of Case 1). In Case 3, another novel substitution mutation in exon 1 of the ABCG5 gene (c.130T>C or p.Ser44Ala, Fig. 1f) and the same mutation in exon 9 as observed in Cases 1 and 2 (c.1306G>A or p.Arg389His, Fig. 1e) were identified by direct sequencing analysis, despite no apparent evidence of a familial relationship with Case 1 and Case 2. In Case 4, the same deletion mutation (c.1813_1817delCTTTT or p.Pro558-GlufsX14) was detected in the ABCG5 gene, as observed in Case 1, despite no apparent evidence of a familial relationship with Case 1. In addition, another mutation was found in exon 10 of ABCG5 (c.1336T>C or p.Arg446X, Fig. 1g). The latter is also one of the most common mutations in Asian sitosterolaemic cases (Wang et al. 2014). No other mutation was found in either the ABCG5 or the ABCG8 gene in any of the four cases.

Clinical Courses and Interventions (Fig. 2)

The decisions on the treatments of the four cases were made based on their personalities, the policies of each institution and the preferences of the parents, providing us

an opportunity to observe responsiveness to a variety of treatments.

The original serum TC, LDL-C and sitosterol levels of Case 1 were 523 mg/dl, 407 mg/dl and 101 µg/ml, respectively. These levels were reduced to 151 mg/dl, 81 mg/dl and 46 µg/ml, respectively, with 10 mg ezetimibe daily (gradually increased from 2 mg daily) and a low-cholesterol (<200 mg daily) and low-plant-sterol diet (avoiding vegetable oils, nuts and cereals), after weaning (Fig. 2a). Intertriginous xanthomas (Supplemental Fig. 2a, b) completely regressed in association with the reduction of these sterol levels (Supplemental Fig. 2c, d).

In the initial blood test, the serum levels of sitosterol in Case 2 were lower than those of Case 1 (Case 2's elder sister), whereas the serum levels of TC were similar for Cases 1 and 2. Weaning from breastfeeding was effective in reducing the TC levels of Case 2, but it increased her sitosterol levels; this increase was probably also associated with increased sitosterol intake. However, additional treatment with ezetimibe further reduced her TC and sitosterol levels (Fig. 2b). In this case, ezetimibe was introduced at 10 mg daily because this dose was well tolerated in Case 1 (Case 2's elder sister). In Case 3, the original serum TC, LDL-C and sitosterol levels were as high as 870 mg/dl, 796 mg/dl and 80 µg/ml, respectively. In a clinical course similar to that of Case 1, these levels dramatically reduced to 228 mg/dl, 157 mg/dl and 64.5 µg/ml, respectively, with administration of 10 mg of ezetimibe daily and a low-cholesterol (<200 mg daily) and low-plant-sterol diet (avoiding vegetable oils, nuts and cereals) after weaning (Fig. 2c). Ezetimibe was introduced at 10 mg daily in this case, based on discussions with her parents. Her intertriginous xanthomas (Supplemental Fig. 3) also successfully regressed (data not shown). In a similar way to that of Cases 2 and 3, in Case 4, weaning itself resulted in a great reduction in serum TC and LDL-C levels (from 756 mg/dl and 589 mg/dl to 326 mg/dl and 115 µg/ml, respectively) (Fig. 2d). Interestingly, his intertriginous xanthomas (Supplemental Fig. 4) completely disappeared at the age of five (data not shown). Ezetimibe at 10 mg daily, rather than colestimide, was effective in reducing serum sitosterol levels.

It is noteworthy that whilst weaning itself was effective in reducing TC and LDL-C levels and in promoting the regression of intertriginous xanthomas, it was not sufficient to reduce serum sitosterol levels (Table 1). Although the impact on the degree of reduction was different in each case, ezetimibe was effective in reducing serum sitosterol levels in all four cases.

Although several papers have suggested the association between sitosterolaemia and haematologic abnormalities, such as macrothrombocytopenia, haemolytic anaemia and splenomegaly (Park et al. 2014; Wang et al. 2014), none of our cases exhibited any abnormal haematological manifestations.

Fig. 1 DNA analysis for ABCG5 gene. (a) DNA sequence data of control and the proband, the latter of which showed an abnormal gap of several nucleotides in the coding region of exon 12. (b) Restriction enzyme analysis using DraI. Deletion of nucleotides in this region protected from fragmentation by this enzyme. (c) Sequencing of the non-fragmented PCR product revealed a novel mutation of a five-nucleotide deletion in exon 12 (c.1813_1817delCTTTT). (d) Blue-coloured nucleotides indicate those deleted in the proband. This

deletion mutation caused a premature termination at codon 571. (e) DNA sequence data of the carrier which showed G to A substitution in exon 9 of the ABCG5 gene (c.1306G>A). (f) DNA sequence data of the carrier which showed T to C substitution in exon 1 of the ABCG5 gene (c.130T>C). (g) DNA sequence data of the carrier which showed T to C substitution in exon 10 of the ABCG5 gene (c.1336T>C)

Clinical and Laboratory Characteristics of Infantile Cases with Sitosterolaemia (Table 1)

We have summarised clinical and laboratory characteristics of infantile cases with sitosterolaemia of ours and those from the literature (Niu et al. 2010; Park et al. 2014; Rios

et al. 2010) in Table 1, focusing on the changes in clinical manifestations before and after the weaning. Most of the infantile cases with sitosterolaemia exhibited intertriginous xanthomas associated with extremely high LDL cholesterolaemia during breastfeeding, which decreased dramatically after weaning.

Fig. 2 Clinical courses of the cases. *Open circle* indicates sitosterol and *closed triangle* indicates total cholesterol. *Closed square* indicates triglyceride. *Closed rhombus* indicates high-density lipoprotein cho-

lesterol. *Cross mark* indicates low-density lipoprotein cholesterol. Clinical courses of Cases 1 to 4 (a–d)

Discussion

The main findings of the current study are as follows: (1) we identified novel ABCG5 gene mutations (c.1813_1817delCTTTT or p.Pro558GlufsX14 and c.130T>C or p.Ser44Ala) in Japanese cases with sitosterolaemia; (2) all four infantile cases with sitosterolaemia exhibited severe hypercholesterolaemia, and three of them showed intertriginous xanthomas resembling homozygous FH; (3) weaning from breastfeeding was very effective in reducing serum cholesterol levels; and (4) ezetimibe, not colestimide, was effective in reducing serum sitosterol levels.

Interestingly, all four cases, treated in three different institutions, had been clinically misdiagnosed as homozygous FH due to severe hypercholesterolaemia and/or intertriginous xanthomas. Several subjects with sitosterolaemia, especially infantile cases, have been described as exhibiting clinical manifestations resembling homozygous FH. Thus, they were sometimes diagnosed as having “pseudo-homozygous FH” in the past (Yoshida et al. 2000). However, all four cases showed great reductions in

serum cholesterol levels immediately after weaning, which would not have been observed in homozygous FH. Rios et al. reported an 11-month-old girl with compound heterozygous sitosterolaemia due to ABCG5 gene mutations who showed extreme hypercholesterolaemia (serum TC level, 1,023 mg/dl) (Rios et al. 2010). Her cholesterol levels also dramatically decreased after weaning, as with our cases. Park et al. also reported a similar case to ours, in which a 15-month-old girl whose severe hypercholesterolaemia (serum LDL-C level, 540 mg/dl) was also complicated by intertriginous xanthomas during breastfeeding; her hypercholesterolaemia disappeared two months after the introduction of a low-fat/low-cholesterol diet and cholestyramine. She was diagnosed with compound heterozygous sitosterolaemia due to an ABCG5 gene mutation (Park et al. 2014). Our findings of greatly reduced serum cholesterol levels seen after weaning in four infantile sitosterolaemic cases reinforce the previous findings (Table 1).

However, the mechanism by which serum cholesterol levels increase during breastfeeding in sitosterolaemia is still unclear. Stigmasterol, the other plant sterol that

Table 1 Summary of the clinical and laboratory characteristics of the infantile cases of sitosterolaemia reported herein plus others gleaned from the literature

Age (sex)	Ethnicity	Percutaneous xanthomas		Diet		TC (mg/dl)		LDL-C (mg/dl)		Sitosterol (µg/ml)	Mutated gene (variants)	First Author (Reference)
		Before	After	Before	After	Before	After	Before	After			
3 months (F)	Chinese	NA	NA	Breast milk	NA	402	NA	304	NA	71	ABCG5 (c.1306G>A/c.1336C>T)	Niu et al. (2010)
18 months (F)	Chinese	Present	Disappear	NA	NA	705 ^a	152 ^b	565 ^a	71 ^b	37.9 ^c	ABCG5 (c.1306G>A/c.1336C>T)	Niu et al. (2010)
23 months (F)	Chinese	Present	Disappear	NA	NA	640 ^a	223 ^c	519 ^a	NA	70.7 ^e	ABCG5 (c.1306G>A/c.1306G>A)	Niu et al. (2010)
11 months (F)	Romanian	Present	NA	Breast milk	NA	1023	154	837	NA	23.7	ABCG5 (c.47C>T/c.1336C>T)	Rios et al. (2010)
15 months (F)	Korean	Present	Disappear	Breast milk 80%	NA	675	184	540	118	NA	ABCG5 (c.1336C>T/c.904 + 1G>A)	Park et al. (2014)
18 months (F)	Japanese	Present	Disappear	Breast milk 30%	Japanese diet ^e	523	151	407	81	101	ABCG5 (c.1306G>A/c.1813_1817delCTTTT)	Tada (Present Case 1)
10 months (F)	Japanese	Not present	Not present	Breast milk 100%	Japanese diet ^e	555	237	NA	145	33	ABCG5 (c.1306G>A/c.1813_1817delCTTTT)	Tada (Present Case 2)
13 months (F)	Japanese	Present	Disappear	Breast milk 100%	Japanese diet ^e	870	228	796	157	80	ABCG5 (c.1306G>A/c.130C>T)	Tada (Present Case 3)
24 months (M)	Japanese	Present	Disappear	Breast milk 100%	Japanese diet ^e	756	326	589	200	115	ABCG5 (c.1336C>T/c.1813_1817delCTTTT)	Tada (Present Case 4)

TC total cholesterol, LDL-C low-density lipoprotein cholesterol, *Before* before weaning (during breastfeeding), *After* after weaning, *NA* not available

^a It is not clear whether those values were affected by breastfeeding

^b Measured under cholestyramine 1 g/day

^c Measured under ezetimibe 10 mg/day

^d Measured under low-saturated-fat/low-cholesterol diet

^e Rice-based traditional Japanese food

accumulates in sitosterolaemia, inhibits the processing of a transcription factor, sterol regulatory element binding protein-2 which stimulates the transcription of hepatic LDLR (Yang et al. 2004). Accordingly, the transcription of hepatic LDLR is assumed to be inhibited, resulting in an increase in circulatory cholesterol. Breast milk contains around 100 mg/dl of sitosterol, which is independent of plant sterol intake (Laitinen et al. 2009). Thus, the infantile consumption of sitosterol is estimated at around 1,000 mg/day if an infant ingests 1,000 ml of breast milk per day; this amount of sitosterol intake is close to that of an adult but greater in terms of proportion with body weight. The accumulation of non-cholesterol sterols contributes to the very low cholesterol biosynthesis in cases with sitosterolaemia (Yang et al. 2006), leading us to speculate that severe hypercholesterolaemia observed in infantile sitosterolaemia during breastfeeding reflects the reduced catabolism of LDL or excretion of cholesterol into the bile, rather than an increase in cholesterol biosynthesis. Compared with the dietary intake of cholesterol by adults, infants ingest about 3 to 4 times the amount of cholesterol per kg body weight through breast milk (Ohta et al. 2002; Ohlsson 2010), or the high content of saturated fatty acids in breast milk (Ohlsson 2010), which may explain the extreme hypercholesterolaemia of infants with sitosterolaemia. Other possible factors could be increased absorption and retention of sterols induced by ABCG5 deficiency itself.

Niu et al. described the effectiveness of ezetimibe in reducing cholesterol levels in sitosterolaemic cases, including infantile cases (Niu et al. 2010); we noted good results with ezetimibe in the present study as well; however, our serial assessment of the clinical courses of the current cases revealed that a reduced intake of cholesterol (weaning from breastfeeding) should be considered as an important cause of the greatly reduced cholesterol levels. Although weaning was effective in reducing serum cholesterol levels and regressing intertriginous xanthomas, it was not sufficient to reduce serum levels of sitosterol. Ezetimibe, not colestimide, effectively reduced sitosterol levels by ~50%, although there is a lack of evidence for its long-term safety, especially in children. We therefore suggest weaning from breastfeeding and a restriction of plant sterols or the introduction of ezetimibe for the treatment of infantile sitosterolaemia, rather than the introduction of bile acid-sequestering resins.

A limitation of this study is that we could not elucidate the exact mechanism of the reduction of cholesterol after weaning from breastfeeding. However, it is difficult to conduct a crossover clinical trial from breastfeeding to weaning. Kinetic studies using stable isotopes could reveal

a detailed record of abnormalities in lipid metabolism (Tada et al. 2012), but they are difficult to perform in infantile cases.

In conclusion, we identified two novel mutations in the ABCG5 gene in Japanese infantile sitosterolaemic cases, all of whom exhibited great reductions in serum cholesterol levels by means of weaning from breastfeeding. These novel mutations should add new support to the molecular heterogeneity of sitosterolaemia in the Asian population. Additionally, their unique response to weaning from breastfeeding, as well as to use of ezetimibe, could provide insights about the metabolic basis of sterols in humans.

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Take-Home Message

Extreme hypercholesterolaemia is exacerbated by breastfeeding in the infantile case with sitosterolaemia.

Compliance with Ethics Guidelines

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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 patients or their parents (if the subject was an infant) when they visited Kanazawa University Hospital for being included in the study.

Author Contributions

Hayato Tada: designed research and performed research and wrote paper. Masa-aki Kawashiri: designed research and wrote paper. Mutsuko Takata: collected data and drafted paper. Kunihiro Matsunami: collected data and drafted paper. Atsushi Imamura: collected data and drafted paper. Misayo Matsuyama: collected data and drafted paper. Hirotake Sawada: collected data and drafted paper. Hirayuki Nunoi: collected data and drafted paper. Tetsuo Konno: collected data and drafted paper. Kenshi Hayashi: collected data and drafted paper. Atsushi Nohara: collected data and drafted paper. Akihiro Inazu: collected data and drafted paper. Junji Kobayashi: collected data and drafted paper. Hiroshi Mabuchi: collected data and drafted paper. Masakazu Yamagishi: designed research and wrote paper.

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