Clinical and molecular genetic analysis of a family with sitosterolemia and co-existing erythrocyte and platelet abnormalities

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We describe the clinical, biochemical and molecular genetic features of a Chinese family with sitosterolemia, mainly manifested by hematologic abnormalities. The clinical features of three patients were analyzed. Their plasma sterol levels were measured, and *ABCG5* and *ABCG8* genes sequenced to search for the causative mutation. The main clinical features of these patients were hemolysis and macrothrombocytopenia; they had increased plasma sitosterol but maintained normal cholesterol levels. Sequence analysis revealed a novel Gln22X nonsense mutation in exon 1 or *ABCG5*. Our results suggest that blood cells could be a target for the toxic effect of plasma phytosterols; the coexisting hematologic abnormalities might represent a specific subtype of sitosterolemia.

Key words: sitosterolemia, hemolysis, platelet, gene, mutation.

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Sitosterolemia (MIM number 210250), also known as phytosterolemia, is a rare autosomal recessive inherited disorder of lipoprotein metabolism characterized by increased levels of plasma phytosterols including sitosterol, stigmasterol and campesterol, with sitosterol being the most abundant. Patients have increased intestinal absorption and decreased biliary excretion of phytosterols. This disease was first described in 1974.[1] The homozygosity mapping study limited the disease locus to human chromosome 2p21 in 1998.[2] Berge *et al.*[3] identified two genes responsible for sitosterolemia. One is the ATP-binding cassette, subfamily G, member 5 (*ABCG5*), the other is *ABCG8*. Mutations in either *ABCG5* or *ABCG8* may result in sitosterolemia. Patients with sitosterolemia mainly present with tendon and tuberous xanthomas, arthralgia and arthritis, premature coronary and aortic atherosclerosis, while hemolysis and/or thrombocytopenia are seen occasionally. In 2005 Rees *et al.*[4] first reported that stomatocytic hemolysis and macrothrombocytopenia were a hematologic presentation of sitosterolemia. Recently, we found a Chinese family with sitosterolemia; all the three affected patients in this family had co-existing abnormal erythrocytes of various shapes and large platelets, besides xanthomas. We describe here the clinical, biochemical and molecular genetic features of this family.

Design and Methods

Patients

The three affected patients, aged 25, 24 and 23 years, are siblings from a Chinese family. Their parents are first cousins. All three patients had almost the same case history. At the age of 3 to 4 years, they had the evidence of hemolysis with reticulocytosis, very low hemoglobin levels (2.7-5.0 g/dL), and splenomegaly. They all underwent splenectomy 10 years ago. Their anemia was markedly improved after the surgical intervention. Since the age of 18 years they had noticed enlarging tendon and tuberous xanthomas that began on elbows and knees, and then hips. None of them had clinically obvious evidence of atherosclerosis or liver function abnormality. The second sibling had frequent episodes of spontaneous gingival bleeding, menorrhagia and occasional epistaxis. In addition, she suffered from gallstones. All the patients had an otherwise normal physical development. The routine hematologic indices and lipid levels of the patients and their parents are shown in Table 1. Two of the three affected patients had high white blood cell counts. Their blood films showed various abnormally shaped erythrocyte, and very large platelets, some of which were as large as the red blood cells (Figure 1). Their platelet volume distribution curves were displaced right-

Table 1. Routine hematologic indices and plasma lipid in a family with sitosterolemia.

Subject	Hematology				Lipid						
	Hb (g/dL)	Retics (%)	Plt ($\times 10^9$/L)	WBC ($\times 10^9$/L)	TC mmol/L	TG mmol/L	HDL-C mmol/L	LDL- C mmol/L	APO-A g/L	APO-B g/L	LP (a) mg/L
Patient 1	110.0	0.5	361.0	7.40	5.32	0.98	1.00	4.00	1.00	1.57	58.0
Patient 2	100.0	1.9	16.0 (56)	27.50	5.69	0.90	1.10	3.90	1.10	1.65	122.0
Patient 3	126.0	1.4	14.0 (64)	20.10	3.48	0.48	0.93	2.30	1.05	0.93	68.0
Patients' father	120.0	0.6	287	6.7	5.44	1.31	1.64	4.02	1.53	1.59	359.0
Patients' mother	112.0	0.7	168	5.4	4.96	1.14	1.40	3.16	1.35	1.06	57.0
Normal range	110.0-150.0	0.5-0.15	100.0-300.0	4.00-10.00	2.90-5.71	0.35-2.30	0.90-1.81	2.07-3.36	1.00-1.72	0.60-1.14	0.0-300

This table shows the data of the three affected patients after splenectomy. Hematological and lipid tests were performed by routine clinical laboratory methods using automated analyzers. The automated analyzer tends to underestimate platelet count in the presence of large platelets, while the optical detection method (results in parentheses) is adaptable. Hb: hemoglobin; Retics: reticulocytes; WBC: white blood cell; Plt, platelet count. TC: total cholesterol; TG: triglycerides; HDL-C, high density lipoprotein cholesterol; LDL- C, low density lipoprotein cholesterol; APO-A: apolipoprotein-A: APO-B,apolipoprotein-B; LP(a), lipoprotein(a).

ward. The patients' bleeding time (11, 12 and 15 min) was longer than the normal range (4-8 min). The platelets could aggregate normally in response to collagen and ADP, but failed to agglutinate in the presence of 1.2 mg/mL ristocetin. Platelet membrane glycoproteins (GP)IIb/IIIa and GPIb were normal. Bone marrow biopsy specimens showed no marrow blast hyperplasia with normal myeloid and megakaryocytic series. The patients' red blood cells showed a greater osmotic fragility than normal cells. Hemolysis began at concentrations of 0.52 to 0.56 percent of saline solution, and was complete at 0.36-0.40 percent. The results of other routine hematologic tests to identify the cause of hemolysis, including Coombs' test, Ham's test, glucose-6-phosphate dehydrogenase, glucose phosphate isomerase and pyruvate kinase activity, were either negative or within normal ranges. Physical examination of the patients' parents revealed no abnormalities' and their red cell and platelet indices were normal. All individuals, including 70 healthy volunteers, gave their informed consent prior to the investigators.

High-performance liquid chromatography (HPLC) for sterol assay

Fasting blood samples were collected into test-tubes without anticoagulants from the affected patients, their parents, two other family members and ten normal volunteers. Sitosterol, cholestanol and stigmasterol standards were purchased from Sigma (St. Louis, MO, USA). Serum sterols were quantified by using HPLC, as described by Kasama *et al.*[5]

DNA isolation and sequence analysis

Citrate-anticoagulant-preserved blood samples were collected from the patients and their family members as well as 70 normal volunteers. Genomic DNA was isolated from whole blood using a PUREGENE DNA purification kit (Gentra Systems, Minneapolis, MN, USA). Exons 1-13 of *ABCG8* (AC108476) and exons 1-5 and 7-13 of *ABCG5* (AC108476) were amplified using primers reported by Rees *et al.*,[4] and exon 6 of *ABCG5* amplified using primers reported by Wang *et al.*[6] Polymerase chain reaction (PCR) amplification was carried out as described by Wang *et al.*[7] Amplified DNA fragments of the three patients and their parents were purified and subjected to direct cycle sequence analysis on an ABI PRISM 377 DNA Sequencer (Applera, Foster, CA, USA).

Allele-specific restriction enzyme analysis (ASRA)

The nucleotide substitution C18802T creates a new recognition site for restriction enzyme BfaI (New England BioLabs, Beverly, MA, USA), which will cleave

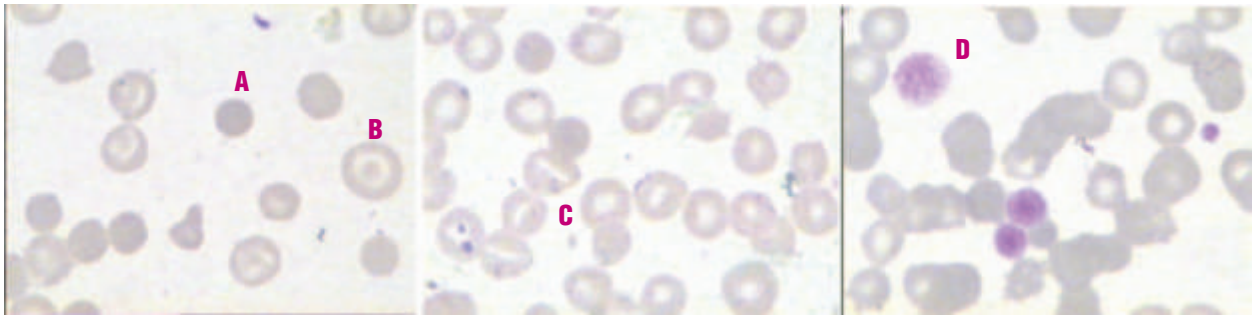

Figure 1. Abnormal erythrocyte shapes and large platelet on Wright Giemsa-stained blood films of patients with sitosterolemia. Spherocyte (A), target cell (B), stomatocyte (C) and large platelet (D) were observed on the peripheral blood smear.

Table 2. Plasma sterol levels of homozygotes and heterozygotes in a family with sitosterolemia. The three affected patients were homozygous. Their parents and two relatives were heterozygous.

Subject	*Cholestanol (mg/dL)*	*Sitosterol (mg/dL)*	*stigmasterol (mg/dL)*
Patient 1	1.53	27.6	13.54
Patient 2	1.33	48.52	43.87
Patient 3	0.92	35.98	21.75
Patient's father	0.42	6.18	3.08
Patient's mother	0.30	2.40	2.35
Patient's relative 1	0.46	2.00	2.29
Patient's relative 2	0.41	12.5	4.49
Control subjects(n=10)	0.12±0.05	1.12±0.22	3.31±1.12

**Mean±standard deviation.*

the 293bp PCR product of mutant exon 1 of ABCG5 and its flanking regions into two fragments. PCR products (3 μL) were digested with the specific enzyme, and then electrophoresed on 3% agarose gels and stained with ethidium bromide.

Results and Discussion

Plasma concentrations of sitosterol, stigmasterol and cholestanol were markedly elevated in the affected patients. Their parents and two other family members showed slight elevations of plasma sitosterol and cholestanol levels compared with normal subjects, but their stigmasterol levels were normal. In addition, two affected patients and their father had increased LDL-cholesterol and Apo-B levels (Table 2).

DNA sequencing revealed a novel nonsense mutation in *ABCG5*: C→T (18802) transition in the first position of codon 22 encoding a premature stop codon instead of the normal Gln. The three affected patients were shown to be homozygous, whereas their parents were heterozygous for this mutation. The sequencing of genomic DNA from affected patients showed no mutations in the coding regions of *ABCG8*.

The patients'samples digested with restriction enzyme BfaI produced 159bp and 134bp fragments, also indicating the homozygosity of the substitution, while samples form their parents and two other family members produced 293bp, 159bp and 134bp fragments indicating the heterozygosity of the substitution. On the other hand, the samples from the remaining family members as well as 70 normal controls showed only one 293bp fragment.

The three affected patients described here had some significant differences from most previously reported patients. The main clinical manifestation of most reported patients is xanthomas. However our patients had more severe hemolysis, abnormally shaped erythrocytes and large platelets, besides xanthomas. Moreover hemolysis was the initial presentation of their disease. The reason for the severe hemolysis remains unclear. It could be related to an excess of phytosterols in the plasma. Some phytosterols can incorporate into erythrocyte membranes,[1,8,9] which results in increased red blood cell fragility, thus exacerbating the development of hemolysis. In addition, the second sibling, in whom the abnormal erythrocyte shapes and large platelets were most obvious, had the lowest platelet count and highest plasma phytosterol levels among the three affected patients. In contrast, the first sibling, in whom morphologic abnormalities of erythrocytes and platelets were least obvious, had a normal platelet count and her plasma phytosterol levels, which were still significantly higher than those of normal subjects, were the lowest among the three affected patients. These findings suggest a direct association between hematologic abnormalities and increased plasma phytosterol levels. In an *in vivo* study, feeding phytosterol-enriched diets to SHRSP (stroke-prone spontaneously hypertensive) rats' which are important animal models of sitosterolemia[10] resulted in a large incorporation of phytosterols, particularly campesterol and sitosterol, into erythrocytes, low erythrocyte deformability, defective erythrocyte membrane morphology, low platelet counts and large platelets similar to those seen in sitosterolemia.[11] Therefore, sitosterolemia should be considered in the differential diagnosis of inherited hemolytic anemia and/or macrothrombocytopenia, including heterozygous forms of Bernard-Soulier syndrome. The exact mechanism by which the platelets of patients only failed to agglutinate in response to ristocetin but aggregated normally in response to ADP remains to be evaluated in the future. It is possible that this phenomenon is related to abnormal sitosterol metabolism.

The most important biochemical abnormality in sitosterolemia is the increase in plasma phytosterol levels. In the three patients reported here, the plasma sitosterol and stigmasterol levels were obviously higher than those in healthy volunteers, confirming the diagnosis of sitosterolemia. Sitosterolemia is often associated with premature atherosclerosis. Although our very young patients have not yet presented with this complication, their elevated phytosterols, cholestanol, LDL cholesterol and apoB imply that they will have an increased risk of atherosclerosis.

Sitosterolemia is caused by homozygosity or compound heterozygosity for a mutation in *ABCG5* and *ABCG8* genes. In the present study, we found one nonsense mutation, Gln22X in exon 1 *ABCG5*. The three affected patients were homozygous for this mutation, whereas their parents and two relatives were heterozygous, suggesting an autosomal recessive trait of inheritance. Interestingly, the three affected patients are the

only children in this family. The Gln22X mutation in *ABCG5* reported in this study is a novel genetic defect of sitosterolemia, which, to our knowledge, has not been described previously. We hypothesize that the more severe hemolysis in this family is related to *ABCG5* exon 1 nonsense mutation, which predicted complete loss of the protein product. In addition, it might also be the result of an environmental factor: because the three affected patients come from a rural area in China, their diet contains more vegetable.

It has been proposed that patients with sitosterolemia might respond to a low-phytosterol diet and lipid-lowering drugs. In this family, the second sibling, who was the most ill, was treated with a low-phytosterol diet in combination with Lipanthyl (200 mg/day). After 50 days of this therapy, her plasma sitosterol and stigmasterol levels decreased from 48.52 to 28.5 mg/dL and from 43.87 to 22.22 mg/dL, respectively. At the same time, her bleeding stopped, although, her erythrocyte and platelet abnormatilities did not improve significantly. Her elevated white blood cell count has returned to the normal range. After three months, the tuberous xanthomata in her hip had regressed partially, while her tendinous xanthomata regressed at a slower rate. These findings indicate that this therapy was effective in this patient. In summary, our findings suggest that blood cells could be a target for the toxic effect of plasma phytosterols in some patients, and that the co-existing hematologic abnormalities might represent a specific subtype of sitosterolemia, which should be included in the differential diagnosis of inherited hemolytic anemia and/or macrothrombocytopenia. The novel nonsense mutation, Gln22X, in *ABCG5* reported in this study could be useful for investigating the molecular basis of sitosterolemia with erythrocyte and platelet abnormalities.

Y-HS and Z-YW contributed to the conception and design of the study and analysis/interpretation of the data, drafted the article and approved the final version to be published; H-YY, L-JC, FL and XB were involved in collecting the samples and their phenotypic analysis. C-GR gave advice about clinical and laboratory research work. The authors would like to thank Jian Jin (Jiangnan University, Jiangsu, China) for his help with HPLC analysis. The authors declare that they have no potential conflicts of interest.

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