Short Communication

Hereditary spherocytosis is associated with elevated C3 levels in dried blood spots used for newborn screening

Hideki Matsumoto[1,2]*, Hideo Sasai[1,2,3], Mai Mori[1,2], Hiroki Otsuka[1,2], Tomohiro Hori[1], Norio Kawamoto[1,2], Hironori Kobayashi[4], Hidenori Ohnishi[1,2,5]

[1]Department of Pediatrics, Graduate School of Medicine, Gifu University, 1−1 Yanagido, Gifu, Gifu 501−1194, Japan
[2]Division of Neonatal Intensive Care Unit, Gifu University Hospital, 1−1 Yanagido, Gifu, Gifu 501−1194, Japan
[3]Department of Early Diagnosis and Preventive Medicine for Rare Intractable Pediatric Diseases, Graduate School of Medicine, Gifu University, 1−1 Yanagido, Gifu, Gifu 501−1194, Japan
[4]Laboratories Division, Shimane University Hospital, 89−1 Enya, Izumo, Shimane 693−8501, Japan
[5]Laboratory of Intractable and Rare Diseases, Graduate School of Medicine, Gifu University, 1−1 Yanagido, Gifu, Gifu 501−1194, Japan

Abstract High propionylcarnitine (C3) levels and a high C3/acetylcarnitine (C2) ratio are used in newborn screening to indicate propionic acidemia and methylmalonic acidemia. Hyperbilirubinemia and maternal vitamin B12 deficiency are other factors that can cause high C3 levels during newborn screening, but hereditary spherocytosis (HS) has recently been identified as an important differential disorder. We experienced two cases of HS that were diagnosed through detailed examinations of high C3 levels. In Case 1, C3 level and C3/C2 ratio in the dried blood spots on day 4 after birth were 6.28 nmol/mL (cutoff value 3.60 nmol/mL) and 0.25 (cutoff value 0.25), respectively. In Case 2, C3 level and C3/C2 ratio in the dried blood spots on day 5 after birth were 9.32 nmol/mL and 0.42, respectively. In both cases, C3 levels in serum were not elevated, and a diagnosis of HS was confirmed at an early stage by a peripheral blood hemogram, thus avoiding unnecessary testing and treatment for suspected organic acidemia. When performing a detailed examination in the case of high C3 levels and a high C3/C2 ratio, it is important to confirm possible HS using a peripheral blood hemogram and to test for inherited metabolic diseases.

Key words: hereditary spherocytosis, propionylcarnitine, dried blood spots, liquid-chromatography tandem mass spectrometry, newborn screening

Introduction

In Japan, high propionylcarnitine (C3) levels and high C3/acetylcarnitine (C2) ratios in dried blood spots (DBSs) are used in newborn screening (NBS) to detect propionic acidemia (PA) and methylmalonic acidemia (MMA)[1)], which require prompt detailed examination. Other known factors can contribute to high C3 levels besides inherited metabolic diseases (IMDs), such as hyperbilirubinemia[2)] and maternal vitamin B12 deficiency[3)]. In 2021, van Dooijeweert et al. reported that acylcarnitine analysis of DBSs derived from patients with hereditary spherocytosis (HS) showed elevated C3 levels[4)]. We have experienced two cases of HS diagnosed on the basis of high C3 levels, and this report describes the clinical course and laboratory data of each case.

Case Presentation

Case 1: The patient was delivered vaginally at 38 weeks and 4 days of gestation, and his birth weight was 2660 g (−1.1 standard deviation). His parents were non-consanguineous, and he had no family history of IMD or HS. He was the

* **Corresponding author**
Hideki Matsumoto
Department of Pediatrics, Graduate School of Medicine, Gifu University, 1−1 Yanagido, Gifu, Gifu 501−1194, Japan
Tel: +81−58−230−6386, Fax: +81−58−230−6387
E-mail: hmatsumoto-gif@umin.ac.jp
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second child of his parents. He was treated with phototherapy for jaundice on days 2, 6, and 10 after birth. An NBS sample was collected on day 4 after birth, and acylcarnitine analysis of the DBSs showed a C3 level of 6.28 nmol/mL (cutoff value 3.60 nmol/mL) and a C3/C2 ratio of 0.25 (cutoff value 0.25) (Table 1). He was transferred to our hospital on day 13 after birth for a detailed examination. Blood tests showed no metabolic acidosis or hyperammonemia, his vitamin B12 level was 405 pg/mL (reference range 180 to 914), his total homocysteine level was 5.2 nmol/mL (reference range 5.3 to 15.2), and plasma amino acid analysis showed no abnormalities. Serum acylcarnitine analysis showed a C3 level of 0.75 nmol/mL (reference $<$2.5 nmol/mL) and a C3/C2 ratio of 0.07 (reference $<$0.2) (Table 1), and urinary organic acid analysis showed mild increases in methylcitrate and 3-hydroxypropionic acid. Urinary organic acid analysis at 14-month-old showed no increases in methylcitrate and 3-hydroxypropionic acid. The clinical course is shown in Fig. 1A. Because of anemia, erythrocyte transfusions were performed on day 13 and day 52 after birth. A peripheral blood hemogram showed numerous spherical erythrocytes. Genetic testing showed no abnormalities in the genes responsible for PA or MMA (including *PCCA*, *PCCB*, *MMUT*, *ABCD4*, *HCFC1*, *LMBRD1*, *MMAA*, *MMAB*, *MMACHC*, and *MMADHC*). Genetic testing revealed a heterozygotic c.1602+1 G>A variant in the *ANK1* gene, which was unreported. Familial analysis showed no variants in the parents; therefore, this was considered a *de novo* variant. We determined the variant to be pathogenic on the basis of the American College of Medical Genetics and Genomics guidelines[5)] (PVS1+PS2+PM2+PP3+PP4). Finally, the patient was diagnosed with HS. The patient is now 18 months old and showed no metabolic crisis at the time of infection, and he has no evidence of progressive anemia and normal growth and development. No recurrence of a high C3 level in DBSs was observed after the neonatal period.

Table 1. Acylcarnitine analysis results of Case 1

Acylcarnitine	DBSs at day 4	Cut-off	Serum at day 13	Reference
C0	53.23	(≤9.00)	45.81	(20–60)
C2	28.49		11.45	(10–40)
C3	6.28	(≥3.60)	0.75	(≤2.5)
C4	0.46		0.25	(≤1.0)
C5	0.33	(≥1.00)	0.33	(≤0.5)
C5:1	0.02		0.01	(≤0.02)
C5-DC	0.18	(≥0.29)	0.06	(≤0.25)
C5-OH	0.21	(≥1.00)	0.06	(≤0.15)
C6	0.07		0.07	(≤0.2)
C8	0.09	(≥0.30)	0.06	(≤0.3)
C10	0.10	(≥0.40)	0.09	(≤0.3)
C12	0.16	(≥0.40)	0.09	(≤0.2)
C14	0.34		0.03	(≤0.15)
C14:1	0.14	(≥0.40)	0.06	(≤0.2)
C16	1.97	(≥6.00)	0.04	(≤0.2)
C16-OH	0.009	(≥0.050)	$<$0.01	(≤0.03)
C18	0.66		0.04	(≤0.3)
C18:1	1.39		0.09	(≤0.4)
C18:1-OH	0.016	(≥0.050)	$<$0.01	(≤0.05)
C3/C2	0.25	(≥0.25)	0.07	(≤0.2)

Unit: nmol/mL, DBSs: dried blood spots, C0: free carnitine, C2: acetylcarnitine, C3: propionylcarnitine, C4: butyrylcarnitine, C4-OH: 3-hydroxybutyrylcarnitine, C5: isovalerylcarnitine, C5:1: tiglylcarnitine, C5-DC: glutarylcarnitine, C5-OH: 3-hydroxyisovalerylcarnitine, C6: hexanoylcarnitine, C8: octanoylcarnitine, C8:1: octenoylcarnitine, C10: decanoylcarnitine, C10:1: decenoylcarnitine, C12: dodecanoylcarnitine, C12:1: dodecenoylcarnitine, C14: tetradecanoylcarnitine, C14:1: tetradecenoylcarnitine, C16: palmitoylcarnitine, C16-OH: hydroxy-hexadecanoylcarnitine, C18: stearoylcarnitine, C18:1: oleylcarnitine, C18:2: linoleylcarnitine, C18-OH: hydroxy-octadecanoylcarnitine, C18:1-OH: hydroxy-octadecenoylcarnitine, Abnormal date are underlined.

Case 2: The patient was delivered vaginally at 39 weeks and 4 days of gestation, with a birth weight of 3330 g (+1.1 standard deviations). Her parents were non-consanguineous, and she had no family history of IMD, but her mother was diagnosed with HS and had been splenectomized because of this disease. She was treated with phototherapy for jaundice from day 1 to day 4 and on day 6 after birth. A peripheral blood hemogram showed numerous spherical erythrocytes; therefore, HS was suspected on the basis of family history. An NBS sample was collected on day 5 after birth, and acylcarnitine analysis of the DBSs showed a C3 level of 9.32 nmol/mL (cutoff value 3.60 nmol/mL) and a C3/C2 ratio of 0.42 (cutoff value 0.25) (Table 2). The patient was transferred to our hospital on day 12 after birth for a detailed examination. Blood tests showed no metabolic acidosis or hyperammonemia, her vitamin B12 level was 272 pg/mL, her total homocysteine level was 5.5 nmol/mL, and plasma amino acid analysis results were normal. Serum acylcarnitine analysis showed a C3 level of 0.55 nmol/mL (reference $<$2.5 nmol/mL) and a C3/C2 ratio of 0.08 (reference $<$0.2) (Table 2). Urine organic acid analysis showed no abnormal findings. The clinical course is

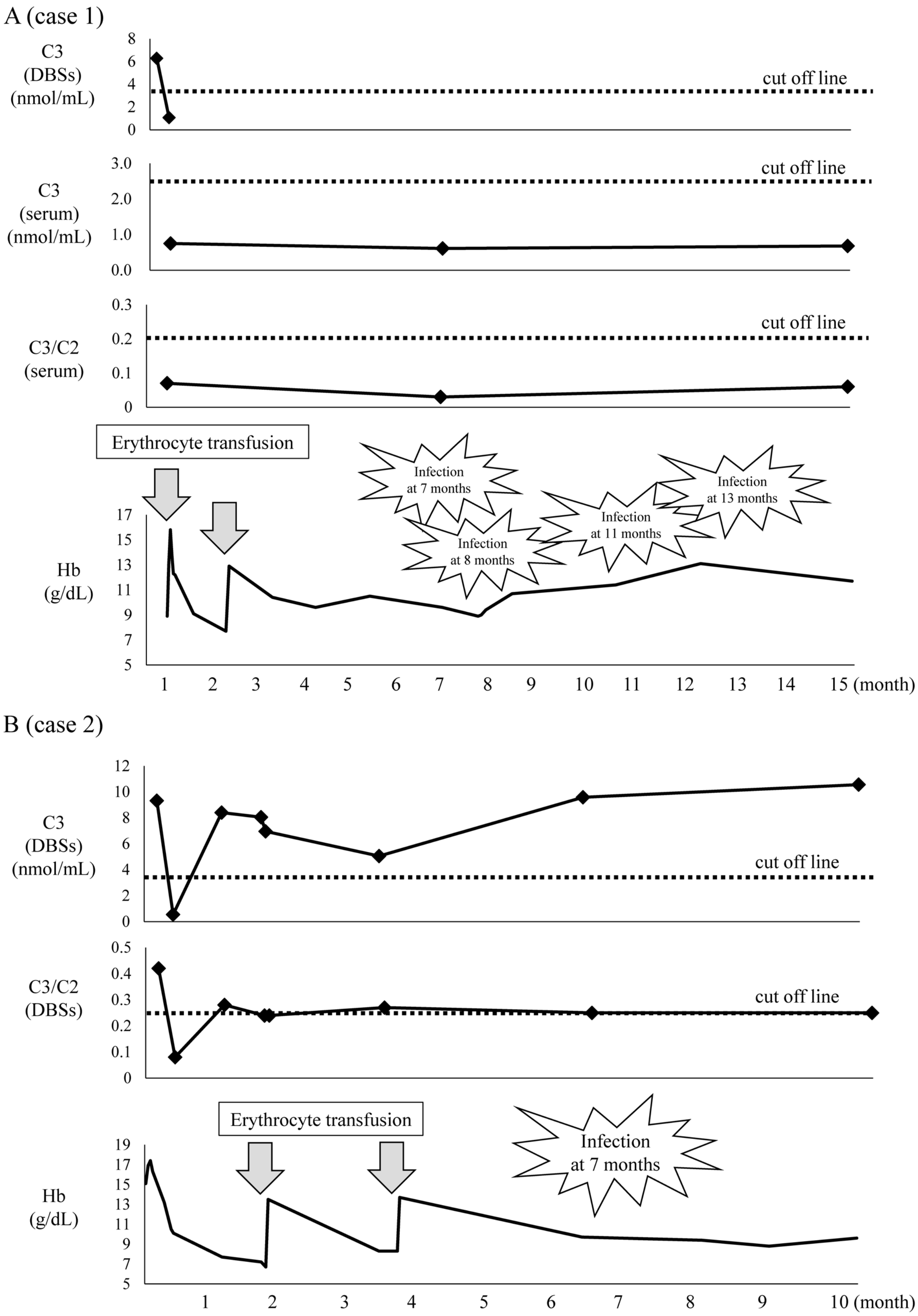

Fig. 1. Changes in C3 level, C3/C2 ratio, and hemoglobin levels over the clinical course. A, The clinical course of Case 1. B, The clinical course of Case 2. C3: propionylcarnitine, C2: acetylcarnitine, Hb: hemoglobin, DBSs: dried blood spots.

Table 2. Acylcarnitine analysis results of Case 2

Acylcarnitine	DBSs at day 5	Cut-off	Serum at day 12	Reference	DBSs at 10 mo	Reference	Serum at 10 mo	Reference
C0	34.34	(≤9.00)	70.78	(20−60)	75.21	(20−60)	43.77	(20−60)
C2	23.90		6.91	(10−40)	45.00	(4.5−45)	6.40	(10−40)
C3	9.32	(≥3.60)	0.55	(≤2.5)	11.90	(≤3.5)	0.70	(≤2.5)
C4	0.47		0.16	(≤1.0)	0.28	(≤0.8)	0.14	(≤1.0)
C5	0.35	(≥1.00)	0.18	(≤0.5)	0.21	(≤0.8)	0.09	(≤0.5)
C5:1	0.01		0.01	(≤0.02)	0.01	(≤0.05)	<0.01	(≤0.02)
C5-DC	0.19	(≥0.29)	0.01	(≤0.25)	0.10	(≤0.25)	0.02	(≤0.25)
C5-OH	0.35	(≥1.00)	0.03	(≤0.15)	0.48	(≤1.0)	0.03	(≤0.15)
C6	0.08		0.04	(≤0.2)	0.07	(≤0.15)	0.04	(≤0.2)
C8	0.07	(≥0.30)	0.04	(≤0.3)	0.05	(≤0.3)	0.05	(≤0.3)
C10	0.06	(≥0.40)	0.06	(≤0.3)	0.06	(≤0.4)	0.07	(≤0.3)
C12	0.13	(≥0.40)	0.06	(≤0.2)	0.08	(≤0.4)	0.06	(≤0.2)
C14	0.27		0.03	(≤0.15)	0.31	(≤0.4)	0.04	(≤0.15)
C14:1	0.10	(≥0.40)	0.05	(≤0.2)	0.05	(≤0.3)	0.04	(≤0.2)
C16	1.84	(≥6.00)	0.08	(≤0.2)	2.35	(0.5−3.0)	0.20	(≤0.2)
C16-OH	0.017	(≥0.050)	<0.01	(≤0.03)	0.01	(≤0.08)	<0.01	(≤0.03)
C18	0.32		0.02	(≤0.3)	0.93	(0.2−2.0)	0.07	(≤0.3)
C18:1	1.11		0.06	(≤0.4)	2.08	(0.37−2.8)	0.22	(≤0.4)
C18:1-OH	0.015	(≥0.050)	<0.01	(≤0.05)	0.02	(≤0.05)	<0.01	(≤0.05)
C3/C2	0.42	(≥0.25)	0.08	(≤0.2)	0.26	(≤0.2)	0.11	(≤0.2)

Unit: nmol/mL, mo: months, Abnormal date are underlined.

shown in Fig. 1B. Because of anemia, erythrocyte transfusions were performed on day 52 and day 109 after birth. Genetic testing revealed no abnormalities in the genes responsible for PA or MMA (same as in Case 1). Genetic testing revealed a heterozygotic c.856C>T (p.Arg286Ter) variant in the *ANK1* gene. This variant was reported as pathogenic[6], and therefore the patient was diagnosed with HS. Familial analysis showed no variant in the father, but her mother had the same variant. The patient had persistently high C3 levels and C3/C2 ratios until 10 months of age only when tested using the DBSs. A discrepancy between the serum and the DBSs acylcarnitine analysis was observed at 10 months (Table 2). The patient is now 12 months old, and her growth and development are within the normal range.

Materials and Methods

Sample preparation for serum acylcarnitine analysis

Serum acylcarnitine analysis was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample preparation was performed using NeoSMAAT® AC (SEKISUI Medical Co., Tokyo, Japan), a standardized acylcarnitine extraction reagent designed to ensure consistent quantification for clinical testing. The kit used for acylcarnitine analysis included standard compounds for C0, C2, C3, C4, C5, C5-OH, C5-DC, C8, C10, C12, C14, C14:1, C16, and C18. Additionally, the kit contained stable isotope-labeled internal standards for each of these analytes to ensure accurate quantification. Quantification of hydroxylated (−OH) and unsaturated acylcarnitine species was performed using internal standards consisting of structurally similar acylcarnitines, following the method used for DBSs analysis.

A 5 μL aliquot of human serum was mixed with 5 μL of 1% formic acid in ethanol and 5 μL of internal standard solution. Then, 200 μL of ethanol was added, and the mixture was vortexed for 10 s before centrifugation at 10,000 rpm at 4°C for 5 min. The resulting supernatant was carefully transferred to a separate container and evaporated under a 40°C nitrogen stream for 60 min. The residue was reconstituted in 50 μL of 0.5% formic acid in acetonitrile, followed by vortexing for 30 s. Subsequently, 50 μL of acetonitrile was added, and the sample was vortexed again for 30 s. The final mixture was centrifuged at 2000 rpm at 4°C for 3 min, and the entire supernatant was transferred to a high-performance liquid chromatography plate for LC-MS/MS analysis. The injection volume was 10 μL.

For calibration standard (CAL) samples, a minimum five-point calibration curve required for quantification was constructed by incorporating a portion of the quality control samples into the CAL series. The quality control and CAL samples were prepared using 5% fat-free human serum albumin in phosphate-buffered saline as the matrix, and commercial acylcarnitine mixtures were used when available. The processing steps, including ethanol precipitation, centrifugation, and nitrogen evaporation, were identical to those used for clinical samples.

LC-MS/MS analysis

Samples were measured using a Nexera MP System equipped with an SIL-30ACMP Multi-Plate auto-sampler and an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Co. Kyoto, Japan) with a positive electrospray ionization source. Chromatographic separation was achieved using a Triart Diol-HILIC column (2.0×100 mm, 1.9 μm, YMC, Japan) maintained at 40°C. The mobile phase consisted of 0.05% formic acid in water (Phase A) and 0.05% formic acid in acetonitrile (Phase B), with a gradient elution mode at a flow rate of 0.2 to 0.3 mL/minute over a 10-min runtime. The mass spectrometer was operated in positive electrospray ionization mode with multiple reaction monitoring for each acylcarnitine quantification.

Results

Case 1: The results of acylcarnitine analysis of serum at day 13 and DBSs at day4 using LC-MS/MS were shown in Table 1. The results of serum showed no C3 elevation and high C3/C2 ratio.

Case 2: The results of acylcarnitine analysis of serum at day 12, 10-month-old and DBSs at day 5, 10-month-old using LC-MS/MS were shown in Table 2. The specimens at 10-month-old were collected at the same time. The results of serum showed no C3 elevation and high C3/C2 ratio.

Discussion

High C3 levels and C3/C2 ratios detected by acylcarnitine analysis of DBSs are used to screen for MMA and PA in Japan[1)]. There is no relationship between the degree of C3 level or C3/C2 ratio elevation and the severity of the disease[7)]. Therefore, all cases in which the C3 and/or C3/C2 values exceed the cutoff require prompt diagnosis and careful follow-up.

There are two known causes of falsely high C3 and C3/C2 levels in DBSs analysis, hyperbilirubinemia[2)] and vitamin B12 deficiency in newborns resulting from maternal vitamin B12 deficiency[3)]. Elevated C3 has been reported in cases of neonatal hyperbilirubinemia, but the detailed mechanism is unclear. The vitamin B12 levels in neonates correlate with maternal vitamin B12 levels. Mitochondrial methylmalonyl-CoA mutase (MUT) requires vitamin B12 as a coenzyme, and when vitamin B12 is deficient, the enzyme activity of MUT decreases. Decreased MUT enzyme activity induces increases in methylmalonic acid and propionyl CoA, which is further converted to C3, and finally the C3 level increases[3)].

In 2021, van Dooijeweert et al. analyzed DBSs from HS patients and reported that several polyamines and acylcarnitines including C3 and 2,3-diphosphoglycerid acid were significantly elevated in DBSs from HS patients[4)]. Among them, C3 was suggested to be strongly correlated with red blood cell hydration and deformability. When comparing the acylcarnitine analyses of Case 1 and Case 2, the C3 value was higher in Case 2 than in Case 1. In Case 1, hemolytic findings were relatively mild, and C3 elevation was observed in the DBSs only on day 4. In contrast, in Case 2, hemolytic findings were relatively severe, and C3 elevation in the DBSs persisted beyond the neonatal period. A significant increase in C3 was also reported in the DBSs analysis of pyruvate kinase deficiency (#OMIM 266200)[8)], an inherited hemolytic disease that causes hemolytic anemia similar to HS, suggesting that C3 may be elevated in patients with hemolytic anemia in general. The detailed mechanism by which C3 is elevated in HS is unknown. It is also unclear whether the C3 peak detected in DBSs analysis of HS patients using LC-MS/MS is only C3 or complex of C3 and another substance. In 2019, Haijes et al. reported that direct-infusion high-resolution mass spectrometry (DI-HRMS) can correctly measure both DBSs and plasma C3 levels in patients with PA[9)]. van Dooijeweert et al. also measured C3 in DBSs of HS patients using DI-HRMS[4)]. We measured high C3 levels in DBSs of patients with HS using LC-MS/MS; however, C3 levels in serum were very low, even in specimens collected at the same time. The limitation of this report is that we have not been able to identify the substance responsible for the high C3 levels in DBSs. Some reports have shown that carnitine is involved in the stabilization of erythrocyte membranes[10], but the detailed mechanism is still unclear and further studies are needed.

HS is a hemolytic anemia disease caused by extravascular hemolysis because of the destruction and phagocytosis of erythrocytes in the spleen caused by defects in the genes encoding erythrocyte membrane proteins (alpha spectrin, beta spectrin, ankyrin, band 3, and protein 4.2)[11]. The frequency of this disease in Japan is estimated to be 1 in 50,000 to 100,000 persons. Ankyrin is an erythrocyte membrane protein that is encoded by the *ANK1* gene, which is inherited in an autosomal dominant manner. Laboratory findings for this condition include hyperbilirubinemia with a predominance of indirect bilirubin, high lactate dehydrogenase, increased reticulocytes, increased urinary urobilinogen, and polychromatic erythroblasts, which reflect hemolysis, as well as small spherical erythrocytes in peripheral blood hemograms. However, it should be noted that approximately one-third of HS patients showed no spherical erythrocytes in peripheral blood hemograms in the neonatal period[11].

We recognized the necessity of considering HS as another differential diagnosis for C3 and C3/C2 elevations through these two cases. When conducting a detailed examination of high C3 and C3/C2 levels, it is important to test for IMDs and to repeatedly check peripheral blood hemograms. Early diagnosis of HS is important to avoid unnecessary testing and treatment for suspected organic acidemia.

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Conflict of Interest

Hideo Sasai has received an endowed chair funded by Gifu Research Center for Public Health. The other authors declare no conflict of interest.

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