Research Paper

# A case of $N^5$, $N^{10}$-methylenetetrahydrofolate reductase deficiency found by a pilot newborn screening for hypomethioninemia

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**Abstract** Current newborn screening (NBS) in Japan detects homocystinuria type 1 by elevated levels of methionine (Met), which is insufficient to detect homocystinuria types 2 and 3 that are associated with hypomethioninemia. We have conducted a pilot NBS for inborn errors of cobalamin metabolism in Hiroshima area of Japan since 2018, using the ratio of propionylcarnitine (C3) to Met (C3/Met) and low Met level as markers. We found a newborn female with low Met (9.54 $\mu$mol/L, cutoff $<10$ $\mu$mol/L) without elevated C3 or C3/Met in a dried blood specimen collected 4 days post-birth. Second-tier tests revealed slightly elevated total homocysteine (tHcy) (15.7 $\mu$mol/L, cutoff $\geq 5.0$ $\mu$mol/L) but normal methylmalonic acid (MMA) levels. At 20 days of age, plasma tHcy rose to 52.0 $\mu$mol/L, with slightly elevated MMA (3.9 $\mu$mol/L, cutoff $\geq 1.0$ $\mu$mol/L) and decreased serum vitamin $B_{12}$ ($VB_{12}$: 146 pg/mL, reference range 197-771 pg/mL). Gene panel analysis of related disorders detected two *MTHFR* variants: p.G149V (c. 447delinsTT) and p.P65L (c.193T>C). 5,10-methylenetetrahydrofolate reductase (MTHFR) activity in lymphocytes and in a dried blood specimen were 56.27 $\mu$U/mg protein (8.40% of mean control value) and 50.19 mmol/min/L (11.5% of mean control value), respectively. On the basis of a diagnosis of MTHFR deficiency associated with mild $VB_{12}$ deficiency, we initiated betaine and mecobalamin administration. The successful detection of this mild case of MTHFR deficiency suggests a good sensitivity of our pilot NBS for the disease, while the combination of second-tier tHcy and MMA measurements is required to retain an appropriate level of specificity.

**Key words:** disorders of cobalamin metabolism, homocystinuria, hypomethioninemia, methylmalonic acidemia, newborn screening

## Introduction

Current newborn screening (NBS) in Japan identifies methylmalonic acidemia and propionic acidemia by elevated levels of propionylcarnitine (C3), and homocystinuria (HCU) type 1 caused by cystathionine $\beta$-synthase (CBS) deficiency through elevated levels of methionine (Met) in dried blood specimens. However, C3 measurement is not always sufficiently sensitive to detect methylmalonic acidemia caused by defects in the pathway of adenosylcobalamin

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synthesis. In HCU type 2 caused by defects in methylcobalamin synthesis and HCU type 3 caused by 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency, Met levels decrease due to disturbed remethylation of homocysteine. Because the prognosis of these diseases can be greatly improved by starting specific medication as soon as possible after birth[1–5], we have been conducting a pilot NBS for inborn errors of cobalamin metabolism in Hiroshima area of Japan since 2018, using C3/Met and low Met levels as first-tier markers, and total homocysteine (tHcy) and methylmalonic acid (MMA) as second-tier markers. In this report, we present our successful identification of an asymptomatic newborn with MTHFR deficiency.

## Materials and Methods

*Research subjects*

Newborns from 10 major hospitals in Hiroshima area were enrolled into the pilot newborn screening (NBS) program following the provision of written informed consent for study participation from their parents. Dried blood specimens (DBS) for NBS were collected on the four or five days of old and analyzed at Hiroshima City Medical Association Clinical Laboratory.

*Screening tests*

First-tier screening tests used the following indices: index 1, C3 $\geq$3.6 $\mu$mol/L and C3/acetylcarnitine (C2) $\geq$0.23 (current NBS indices); index 2, C3/Met $\geq$0.25; and index 3, Met $<$10 $\mu$mol/L. Samples that met one or more of the three indices were transported to the National Center for Child Health and Development for the second-tier measurement of MMA (cutoff $\geq$1.0 $\mu$mol/L) and tHcy (cutoff $\geq$5.0 $\mu$mol/L). Newborns with elevated MMA and/or tHcy levels were further examined in the Department of Pediatrics, Hiroshima University Hospital.

*Biochemical and genetic analysis*

The analysis of amino acids and acylcarnitines in DBS was performed using an LCMS-8045 tandem mass spectrometer (Shimadzu, Kyoto, Japan) according to a previously described flow injection method[6] with some modification. The second-tier measurement of MMA and tHcy in DBS was performed using liquid chromatography–mass spectrometry (LCMS-8050, Shimadzu) according to previously described methods[7]. To confirm the diagnosis, panel analysis of genes engaged in cobalamin metabolism (*ABCD4*, *HCFC1*, *LMBRD1*, *MMAA*, *MMAB*, *MMACHC*, *MMADHC*, *MTR*, *MTRR*, *MTHFR*), methylmalonyl-CoA mutase (*MUT*) and propionyl-CoA carboxylase (*PCCA*, *PCCB*) was performed using next-generation sequencing with the MiSeq Sequencing System (Illumina, San Diego, CA, USA) at Kazusa DNA Research Institute (Chiba, Japan).

## Results

*Overall findings of the pilot NBS*

Between April 2019 and September 2024, 21,105 out of a total of 103,602 newborns born in Hiroshima area were enrolled in the pilot NBS. The total number of cases positive for the first-tier tests was 267 (1.27%): 14 (0.06%) for index 1, 21 (0.10%) for index 2, and 232 (1.10%) for index 3. Some of these subjects were positive for two of the three indices: 6 (0.03%) for indices 1 and 2, and 24 (0.11%) for indices 2 and 3 (Table 1). In the second-tier tests, MMA was elevated in five cases, and tHcy was elevated in one case. These six cases were examined at Hiroshima University Hospital (Table 2). A diagnosis of methylmalonyl-CoA mutase deficiency was confirmed in two (cases 3 and 4), and that of MTHFR deficiency in one (case 6). Diagnoses for the other three (cases 1, 2, and 5) were not revealed by the gene panel analysis.

To evaluate whether positive rates for the three indices increased among low-birth-weight infants, we compared distribution of the values in DBS of infants with a birth weight $<$2,000 g with that of mature infants born between

**Table 1. Overall results of the pilot screening from April 2019 to September 2024**

| | All newborns in Hiroshima area N=103,602 | Newborns enrolled in this study N=21,105 |
|---|---|---|
| C3 $\geq$3.6 $\mu$mol/L & C3/C2 $\geq$0.23 [+C3/Met $\geq$0.25] | 302 (0.29%) [16] | 14 (0.06%) [6] |
| C3/Met $\geq$0.25* | 96 (0.09%) | 21 (0.10%) |
| Met $<$10 $\mu$mol/L [+C3/Met $\geq$0.25] | 530 (0.51%) [34] | 232 (1.10%) [24] |
| Total | 928 (0.90%) | 267 (1.27%) |
| MMA $\geq$1.0 $\mu$mol/L | — | 5 |
| tHcy $\geq$5.0 $\mu$mol/L | — | 1 |

*Number (rate) of C3/Met-positive cases without elevation of C3, C3/C2 or decrease of Met.

**Table 2. Positive findings in the second-tier tests and the diagnoses**

| | C3 & C3/C2 | Met | C3/Met | MMA | tHcy | Diagnosis |
|---|---|---|---|---|---|---|
| Cutoff | ≥3.6 & ≥0.23 | <10.0 | ≥ 0.25 | ≥1.0 | ≥5.0 | |
| Case 1 | 2.87/0.14 | 7.89 | 0.36 | 1.2 | 1.0 | VUS: compound heterozygous variant of *PCCA* |
| Case 2 | 4.46/0.23 | 22.79 | 0.20 | 1.0 | 4.8 | VUS: compound heterozygous variant of *PCCA* (Siblings also showed a mild increase in MMA.) |
| Case 3 | 11.14/0.71 | 33.03 | 0.34 | 337.7 | 2.8 | MUT deficiency |
| Case 4 | 11.26/0.64 | 15.27 | 0.74 | 559.5 | 2.9 | MUT deficiency |
| Case 5 | 3.94/0.23 | 20.96 | 0.04 | 1.0 | 2.4 | VUS: homozygous variant of *LMBRD1* |
| Case 6 | 1.39/0.08 | 9.54 | 0.14 | <1.0 | 15.7 | MTHFR deficiency |

**Table 3. Correlation between low birth weight and positive findings in the first-tier tests, methionine, phenylalanine and Met/Phe levels**

| | Birth Weight ≥2,000 g | Birth Weight <2,000 g | P |
|---|---|---|---|
| n (%) | 18,080 (97.9%) | 385 (2.1%) | — |
| Birth Weight (g) | 3,026±237 | 1,670±86.5 | <0.001 |
| C3≥3.6 μmol/L& C3/C2 ≥0.23 [+C3/Met ≥0.25] n (%) | 13 (0.07%) [4] | 0 | — |
| C3/Met ≥0.25 n (%) | 13 (0.07%) | 2 (0.52%) | <0.001 |
| Met <10 μmol/L [+C3/Met ≥0.25] n (%) | 32 (0.17%) [2] | 37 (9.61%) | <0.001 |
| Met (μmol/L) | 21.66±0.85 | 16.83±9.25 | <0.001 |
| Phe (μmol/L) | 44.76±10.94 | 46.23±9.54 | <0.001 |
| Met/Phe | 0.48±0.11 | 0.36±0.08 | <0.001 |

No association with low birth weight was found for index 1 (C3 ↑ & C3/C2 ↑ ), but the frequency of low birth weight infants increased for indices 2 (C3/Met ↑ ) and 3 (Met ↓ ). There was a tendency for low methionine levels in infants with a birth weight <2,000 g. Phenylalanine levels were higher for the low birth weight infants.

April 2022 and September 2023. We also compared levels of phenylalanine (Phe) between the two groups, because Met/Phe ratio could reportedly be a better marker for the diseases showing hypomethioninemia[4,8,9]. As a result, positive rates were higher in the low-birth-weight infants than in the mature infants for index 2 (0.52% versus 0.07%) and index 3 (9.61% versus 0.17%), while there was no significant difference for index 1. Distribution of Met levels was significantly lower in the low-birth-weight infants than in the mature infants (16.83±9.25 versus 21.66±0.85, mean±SD), while distribution of Phe levels was higher in the low-birth-weight infants than in the mature infants (46.23±9.54 versus 44.76±10.94, mean±SD) (Table 3).

## Case Report

A baby girl (Case 6 shown in Table 2) born as the third child of healthy nonconsanguineous parents at 39 weeks' gestation with a birth weight of 2,420 g showed low Met level (9.54 μmol/L) in the pilot NBS test. Second-tier tests revealed slightly elevated tHcy level (15.7 μmol/L) but normal MMA level. At the first examination on the 20th day after birth, she was healthy and had been mostly breastfed. Her levels of plasma tHcy and serum MMA were elevated (52.0 μmol/L, cutoff ≥ 10.0 μmol/L; 3.9 μmol/L, cutoff ≥1.0 μmol/L, respectively) (Table 4). Because her serum vitamin $B_{12}$ ($VB_{12}$) level was slightly low (146 pg/mL; reference range, 197–771 pg/mL), we suspected maternal $VB_{12}$ deficiency, but her mother's serum $VB_{12}$ level was normal. These biochemical findings persisted until the age of 47 days, when we started the administration of methylcobalamin (250 μg/day).

At the age of 2 months, her serum MMA level was nor-

**Table 4. Clinical course of case 6. After methylcobalamin medication under suspicion of a vitamin B12 deficiency, serum MMA levels normalized but tHcy levels remained elevated. After betaine administration, plasma tHcy levels decreased.**

| DBS | day 4 | day 20 | | day 20 | day 47 | 2M | 3M | 5M |
|---|---|---|---|---|---|---|---|---|
| tHcy (≥ 5.0 $\mu$mol/L) | 15.7 | 17.7 | Plasma tHcy (≥ 10.0 $\mu$mol/L) | 52.0 | 45.5 | 20.4 | 25.1 | 33.4 |
| MMA (≥ 1.0 $\mu$mol/L) | <1.0 | 1.7 | Serum MMA (≥ 1.0 $\mu$mol/L) | 3.9 | 3.2 | <1.0 | <1.0 | <1.0 |
| Met (<10 $\mu$mol/L) | 9.54 | 22.5 | Plasma Met (<10.0 $\mu$mol/L) | 28.6 | 28.2 | 35.0 | 18.5 | 21.5 |
| | | | SerumVB12 (197−771 pg/mL) | 146 | 141 | 5,618 | 7,590 | 6,467 |
| | | | Serum folic acid (5.1−11.7 nmol/L) | 7.6 | 7.8 | 7.6 | 7.1 | 5.8 |

methylcobalamin

betaine

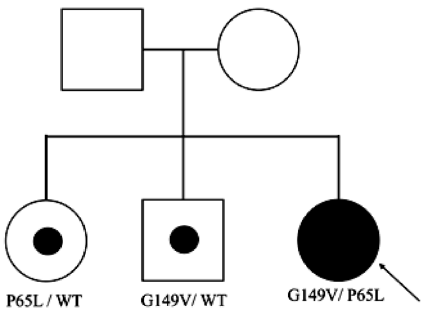


**Fig. 1. Pedigree chart of case 6 that was confirmed to have MTHFR deficiency.**
●: affected individual, ⊙ and ⊡: carriers.

mal, but plasma tHcy level remained slightly high (20.4 $\mu$mol/L). Gene panel analysis detected two missense *MTHFR* variants: p.P65L and p.G149V. Her sister was heterozygous for p.P65L, and her brother for p.G149V (Fig. 1) which was previously reported as a variant of the infantile onset type[10]. p.P65L is not registered in the Human Gene Mutation Database, but in silico analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) suggested pathogenicity. Her MTHFR enzyme activity was 56.3 $\mu$U/mg protein in lymphocytes (8.4% of mean control value), 19.6 $\mu$U/mg protein in lymphoblast extracts (5.0% of mean control value), and 50.2 mmol/min/L in DBS (11.5% of mean control value). On the basis of these findings, we diagnosed late-onset MTHFR deficiency, and initiated betaine administration (100 mg/kg/day) at the age of 9 months. At the age of 10 months, she showed normal growth and development, with marker metabolite levels of tHcy 18.9 $\mu$mol/L and Met 30.5 $\mu$mol/L.

## Discussion

HCU is classified into three forms. HCU type 1 is caused by cystathionine synthase deficiency, in which conversion of homocysteine to cystathionine is impaired and remethylation to methionine increases. HCU type 2 is caused by defects in the synthesis of methylcobalamin, which is a methyl donor in the reaction of methionine synthase. HCU type 3 results from MTHFR deficiency, in which the formation of 5′-methyltetrahydrofolate is impaired, causing methylcobalamin deficiency. Because the supply of Met from the remethylation of homocysteine is reduced in HCU types 2 and 3, they cannot be detected by the current NBS. In these forms of homocystinuria, betaine (N,N,N-trimethylglycine) can serve as another methyl donor instead of methylcobalamin as it is a substrate for betaine−homocysteine methyltransferase (BHMT). Though the physiological function of BHMT cannot compensate for methionine synthase which requires methylcobalamin, the pharmacological dosage of betaine can be sufficient to reduce tHcy and increase Met levels in the blood. Met is converted into S-adenosylmethionine (SAM), which is an important methyl donor for various methylation reactions. Therefore, maintaining normal levels of plasma Met is essential in preventing severe damage to the central nervous system caused by SAM deficiency, especially during infancy and childhood[1]. Because it has been shown that the early introduction of betaine therapy can suppress the symptoms of homocystinuria caused by remethylation defects[1–4], a highly preventive effect of NBS is expected.

MTHFR deficiency is known to present with a wide range of phenotypes. In severe cases, hydrocephalus, respiratory disorders, and epilepsy appear in the neonatal period[11]. The disease prognosis can be greatly improved by the earliest introduction of betaine and other supplements, including folic acid, vitamin $B_6$, vitamin $B_{12}$, methionine and $N^5$-methyltetrahydrofolate. Among these therapeutics, betaine has been shown to be most effective in improving psychomotor development[4]. Prior to the present study, we treated two siblings with MTHFR deficiency. The proband presented with severe symptoms from the neonatal period. The diagnosis was confirmed at the age of 4 months. Betaine therapy was started and her condition improved remarkably, but severe neurological sequelae remained. The younger sibling was proved to be affected with the same disease soon after birth. Betaine therapy was started on the 7th day of life, and he remained asymptomatic until the last examination at the age of 6 years. Met levels in their newborn DBS were 6.6 and 8.7 μmol/L, respectively[1].

In the late-onset form of MTHFR deficiency, progressive polyneuropathy, cognitive impairment, seizures, and leukoencephalopathy become more pronounced after school age. However, it is often difficult to diagnose the disease because these symptoms are not very specific[12]. MTHFR enzyme activity in skin fibroblasts is estimated to be 10–20% in late-onset MTHFR deficiency[12]. The disease cannot be detected during the neonatal period using the current NBS in Japan, but in several countries, newborns presumed to have the late-onset form of disease were successfully found by detecting slightly elevated homocysteine levels in DBS[13].

In several studies on NBS for disorders of cobalamin metabolism[4,8,9], low Met levels with a cutoff of 8–11 μmol/L were used in the first-tier test. In our preliminary retrospective study on newborn DBS comparing performance of Met cutoff set at 9.0 μmol/L and 10.0 μmol/L, the positive rate increased significantly from 0.05% for the former to 0.12% for the latter[1]. Referring to the NBS data of the above-mentioned siblings, we set the Met cutoff for hypomethioninemia at 10 μmol/L in our prospective pilot NBS to ensure sufficient sensitivity.

In the current study, 267 (1.27%) of 21,105 enrolled newborns became positive in the first-tier tests, which reduced to six by the second-tier measurement of MMA and tHcy, representing a false positive rate in the first-tier tests of 97.8%. As second-tier tests are not financially supported in the current NBS program by the local governments, alternative ways to reduce the false positive rate in the first-tier tests must be found. Low-birth-weight infants and preterm infants were previously reported to show low Met levels in their blood[9], and similar tendencies were observed in our study (Table 3). In several countries where similar screening has been carried out, other markers such as ratio of Met/Phe are adopted in the first-tier tests. In our study, we observed a tendency for high Phe levels in infants with a birth weight <2,000 g. Based on these data, it is suggested that Met/Phe would not help to reduce the positive rate in NBS for hypomethioninemia. In the practice of current NBS in Japan, all newborns with a birth weight below 2,000 g are retested after their weight reaches 2,000 g. In our preliminary retrospective study, all the low-birth-weight infants who were assigned as positive for hypomethioninemia (Met <10 μmol/L) became negative in the retest after the above-mentioned weight gain. As a practical solution, infants with a birth weight <2,000 g who are positive for hypomethioninemia should be retested after their weight reaches 2,000 g so long as they remain free from symptoms indicative of remethylation disorders. When clinical changes are noticed, further evaluation should be started immediately. This scheme will enable to rule out most of false-positive results in the first-tier NBS without requiring the second-tier measurement of MMA and tHcy.

## Conclusions

We conducted a pilot NBS program for disorders of cobalamin metabolism. The successful detection of a mild case of MTHFR deficiency suggests good sensitivity of C3, C3/C2, C3/Met, and low Met markers in combination, keeping appropriate specificity with second-tier measurements of tHcy and MMA. High false-positive rate for low Met that were mostly due to transient hypomethioninemia of low-birth-weight infants can also be resolved by retest after enough weight gain.

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## Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the ethics committee of Hiroshima University (E2018-1522, 12 Dec 2020).

## Informed Consent Statement

Informed consent was obtained from the parents of all the subjects enrolled in this study.

## Conflicts of Interest

The authors declare no conflict of interest.

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