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CASE REPORT

Congenital methemoglobinemia type II in a 5-year-old boy

Elizabeth A. Mannino[1], Thomas Pluim[2], Jacob Wessler[3], Megan T. Cho[4], Jane Juusola[4] & Samantha A. Schrier Vergano[5,6]

[1]Eastern Virginia Medical School, Norfolk, Virginia
[2]Division of Pediatric Critical Care Medicine, Naval Medical Center Portsmouth, Portsmouth, Virginia
[3]Division of Pediatric Hematology/Oncology, Naval Medical Center Portsmouth, Portsmouth, Virginia
[4]GeneDx, Gaithersburg, Maryland
[5]Division of Medical Genetics and Metabolism, Children's Hospital of The King's Daughters, Norfolk, Virginia
[6]Department of Pediatrics, Eastern Virginia Medical School, Norfolk, Virginia

Correspondence
Samantha A. Schrier Vergano, Division of Medical Genetics and Metabolism, Children's Hospital of The King's Daughters, Norfolk, VA 23507. Tel: 757-668-9723; Fax: 757-668-9724; E-mail: Samantha.vergano@chkd.org

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Key Clinical Message

Congenital Methemoglobinemia is a rare neurologic condition which can mimic other diseases such as epilepsy syndromes and leukodystrophies. The responsible gene, *CYB5R3*, is not typically included on commonly order neurologic and epilepsy panels. We recommend that laboratories include this gene on these tests which often precede larger-scale genetic studies.

Keywords

Cyanosis, *CYB5R3*, developmental delay, leukodystrophy, methemoglobin reductase, methemoglobinemia type II, microcephaly.

Introduction

Congenital methemoglobinemia (RCM) (MIM #250800) is a rare, autosomal recessive condition associated with alterations in the *CYB5R3* gene, located on chromosome 22q13 and containing nine exons and eight introns [3]. Mutations in the *CYB5R3* gene ultimately lead to decreased or nearly absent activity of the NADH-cytochrome b5 reductase enzyme. Hemoglobin M disease and cytochrome b5 reductase deficiency are alternate names for inherited methemoglobinemia type II, each reflecting decreased or lack of the cytochrome b5 reductase enzyme and the associated clinical symptoms.

Determining the severity of the enzyme deficiency is one of the primary diagnostic indicators for RCM [21]. NADH-cytochrome b5 reductase is present in an

erythrocytic form as well as a membrane-bound form on mitochondrial and endoplasmic reticulum membranes and is responsible for methemoglobin reduction [13, 15]. There are two main forms of RCM (Types I and II) that can develop based on the type of mutation in the *CYB5R3* gene and the resulting protein change. Type I RCM has been found to be associated with enzyme deficiency localized only to the erythrocytes, while Type II RCM involves enzyme deficiency in all tissues [6]. The localization of the NADH-cytochrome b5 reductase enzyme deficiency to red blood cells in Type I RCM leads to a less severe form of RCM characterized by cyanosis without neurologic impairment. This form can also be treated more easily than Type II RCM [1]. Leroux et al. [15] demonstrated that cytochrome b5 reductase was deficient in cells from a variety of tissues, including red blood cells, leukocytes, muscle, liver, and fibroblasts in a patient with methemoglobinemia and mental retardation. Essentially, this study demonstrated the first correlation between patients with methemoglobinemia associated with neurologic impairment and an enzyme deficiency in all cell types. It was proposed that the deficiency of cytochrome b5 reductase in all cell types leads to abnormalities with fatty acid metabolism, which then leads to neurologic impairments in these patients [15].

We report here a male proband who initially presented to genetics at 6 months of age with concerns of a movement disorder. Subsequent visits to genetics revealed acquired microcephaly, developmental delay, oral aversion, seizures, dystonia, and white matter changes on brain imaging. After an extensive workup, including exome sequencing, his diagnosis remained unknown. After an acute illness at around the age of 3 years for nonhypoxic cyanosis, the concern for methemoglobinemia was raised. Reanalysis of exome sequencing and further testing revealed a previously reported pathogenic nonsense variant [1] and a partial intronic deletion (in trans). To our knowledge, this report introduces the first genetically confirmed case of RCM type II in the United States. In addition, this case introduces another novel deletion in the *CYB5R3* gene that has not yet been reported to cause either type of RCM. We report the proband's diagnostic odyssey in order to elucidate the genetic and clinical features of RCM type II in order to assist clinicians with similar patient presentations. A brief review of common epilepsy and neurologic panels do not appear to have *CYB5R3* on these tests. We would recommend that this gene be included in some of these frequently ordered panels as they are often a prerequisite to more expensive and broad tests such as exome sequencing.

Clinical Report

The proband (Fig. 1) was initially referred to medical genetics at 6 months of age due to a possible movement disorder. He was the product of a full-term gestation born to his 30-year-old G3P1-2 mother after a relatively uncomplicated pregnancy. At birth, the proband weighed 3.4 kg (75th centile), with a length of 50 cm (75th centile), and had a head circumference of 36.5 cm (>90th centile). Although the patient was discharged at 2 days of age, he was readmitted at 8 days of life due to feeding difficulty and frequent vomiting. At 2 months of age, the feeding difficulties continued and were accompanied by poor weight gain, lack of a social smile, and hypotonia. He was admitted to the hospital again at the age of 3 months because of frequent vomiting and failure to thrive. At this point, his head circumference was below

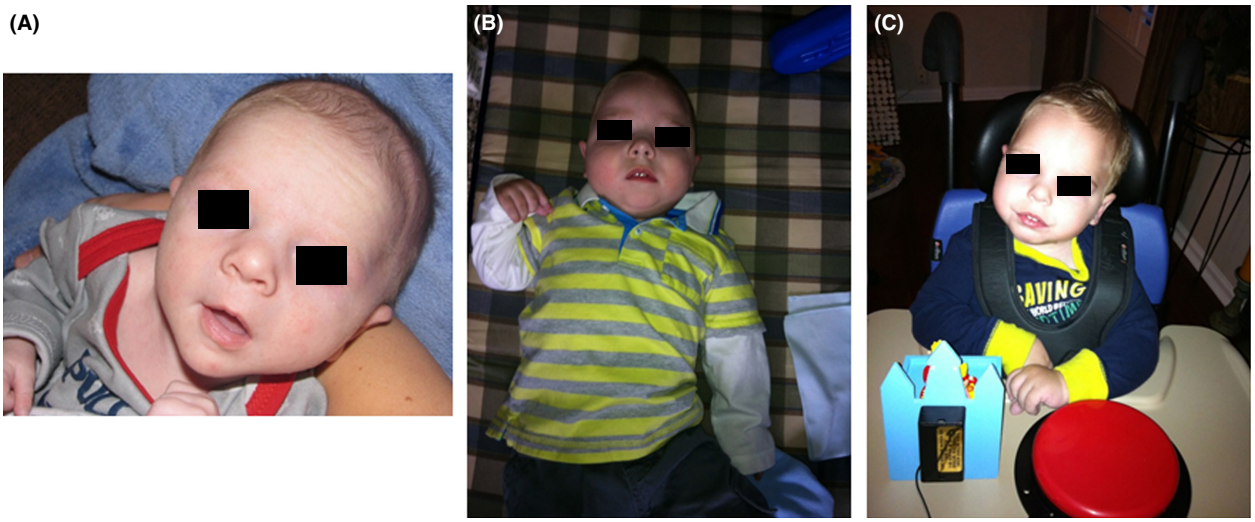

Figure 1. The proband at ages 1 month (A), 13 months (B), and 2 years (C). Note progressive microcephaly.

the third percentile. Nasogastric feeds were initiated. At 5 months, a brain MRI was obtained and showed prominent extra-axial fluid spaces and supratentorial sulci, enlarged lateral and third ventricles, and decreased white matter volume. A gastrostomy tube was placed at 6 months and was eventually converted to a gastrojejunostomy tube at 7 months. In addition, the proband had staring spells, hyperkinetic movements, spasticity of the lower extremities, and dystonic movements. Torticollis was also noted at this time. By 10 months, the proband was completely averse to any oral stimulation, including sucking on a pacifier.

At 12 months, the proband was diagnosed with dysmotility and visceral hyperalgesia. A repeat brain MRI performed at 13 months noted white matter atrophy and further volume loss. At 17 months of age, he was diagnosed with infantile spasm-type seizures based on abnormal EEG findings, which included disorganization, discontinuity, electrodecrement, and multifocal and generalized spikes and spike-wave discharges. He was diagnosed with Lennox–Gastaut Syndrome due to the frequency of spastic movements. His seizures were controlled with a ketogenic diet which was introduced at age 28 months.

When he was seen by medical genetics at 3 years and 5 months, he was having four to ten seizures a day that lasted 5–10 min each. In addition, he was not meeting any developmental milestones other than following faces and turning to sounds. He was able to roll and sit by 12 months, but he lost these abilities, along with head control, when the seizures began at 17 months.

Shortly prior to that evaluation in clinic, at 34 months, the proband was hospitalized due to a 2-week period of nonhypoxic cyanosis. At one point, during a blood draw, the intensivist caring for the proband noted that his blood appeared "chocolate-colored." This raised concern for possible methemoglobinemia. Methemoglobin reductase levels were ordered and found to be deficient (<2.6 U/g, normal 6.6–13.3 U/g).

At 3 years and 5 months, the proband was seen at the National Institutes of Health Clinical Center. A brain MRI (Fig. 2) performed at the hospital where he was seen previously was reviewed and showed progressive degeneration of the brain, with both cerebral and cerebellar atrophy, in addition to ventriculomegaly and enlargement of the subarachnoid spaces. Additionally, white matter hypomyelination was present. Developmentally, he was felt to function at the level of a 6 months old. Range of

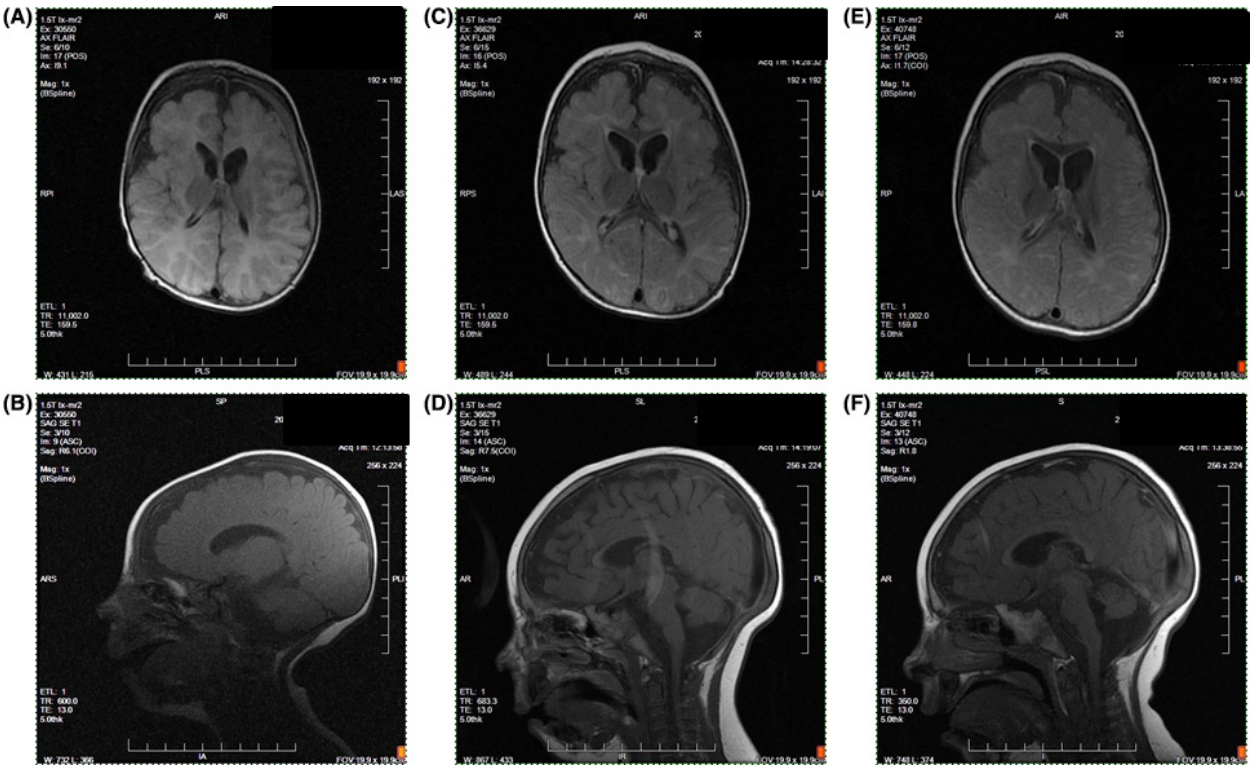

Figure 2. Axial (top) and sagittal (bottom) MRI views of the proband's brain performed at 5 months of age (A, B), 13 months of age (C, D), and 19 months of age (E, F) demonstrating diffuse, progressive cerebral white matter volume loss.

motion was full, but limb strength and tone were described as fluctuating in the upper limbs, trunk, and lower limbs. There was decreased muscle mass, appendicular hypertonia, and axial hypotonia. The proband was also noted to have uncontrolled and rather spastic movements, which were described as myoclonic jerks. In addition to these muscular abnormalities, the proband also had an anterior rib prominence on the left side, dextroscoliotic curvature of the midthoracic region, and levoscoliotic curvature of the upper lumbar region. Ophthalmologic examinations revealed delayed visual maturation, cortical visual impairment, and hyperopic astigmatism. He was reported to have normal hearing, but reduced middle ear system mobility bilaterally.

Up until this point, extensive genetic testing had been completed and included whole-exome sequencing, an epilepsy panel, metabolic studies, and mitochondrial genome panels. The epilepsy and mitochondrial panels returned negative. Whole-exome sequencing was performed at 14 months of age prior to the episode of cyanosis and was initially nondiagnostic.

The proband's clinical diagnosis including cyanosis, "chocolate-colored" blood, and neurologic symptoms, along with negative genetic testing results led physicians to perform follow-up targeted analysis of the *CYB5R3* gene at age 34 months. These results ultimately were felt to be consistent with the diagnosis of RCM Type II.

Genomic DNA was extracted from whole blood from the proband, mother, and father. Exome sequencing (ES) was performed on exon targets isolated by capture using the Agilent SureSelect Human All Exon V4 (50 Mb) kit (Agilent Technologies, Santa Clara, CA). The sequencing methodology and variant interpretation protocol have been previously described [27]. Data were later reanalyzed after being initiated as part of routine clinical care.

The exome sequencing initially performed at 14 months identified three maternally inherited heterozygous variants in *SUMF1*, *BRAT1*, and *SUCLG2*. Exome sequencing at 30 months uncovered a paternally inherited *KCNA1* variant. Reanalysis of exome sequencing data after the episode of nonhypoxic cyanosis revealed a previously reported nonsense variant in the *CYB5R3* gene (p.R160X). A follow-up deletion/duplication assay revealed a heterozygous partial deletion of intron 1 of the *CYB5R3* gene. This deletion is based on the NM−_000398.6 transcript. The deletion includes noncoding exons in the following transcripts: NM_001129819, NM_007326, and NM_001171661. This deletion also includes coding exon 1 in the NM_001171660 transcript. Samples from the mother and father were analyzed for this deletion, and the mother was found to be heterozygous for this partial deletion of intron 1 in the *CYB5R3* gene. The father was negative for the partial deletion. To our knowledge, this deletion has not been reported previously as a cause for either form of RCM.

Discussion

We report here a proband with microcephaly, seizures, dystonia, and white matter changes who ultimately was diagnosed with RCM II after a long diagnostic workup and a relatively incidental finding of the classic "chocolate" appearance to his blood after an episode of nonhypoxic cyanosis. To our knowledge, this is the only published case of RCM Type II from the United States, as well as the first report of this particular combination of a mutation and partial deletion in the *CYB5R3* gene leading to disease. The child's phenotype of microcephaly, seizures, spastic movements, and developmental delay is consistent with many of the neurologic impairments that are characteristic of RCM type II. His early presentation of feeding difficulty, with subsequent development axial hypotonia and appendicular hypertonia, is also consistent with other cases of this condition. Brain MRIs showed progression of white matter volume loss, continued CSF space enlargement, and cerebral and cerebellar atrophy. He was able to achieve some developmental milestones, but eventually lost these abilities after his seizures began. The proband was also diagnosed with infantile spasm-type seizures and eventually began to have cyanotic episodes. Based on his clinical features and "chocolate-colored" blood, he was finally diagnosed with RCM type II and underwent genetic analysis of the *CYB5R3* gene which confirmed his diagnosis. The treatment for acute methemoglobinemia is with methylene blue, which our proband received after the initial diagnosis [4]. Treatment for the remainder of the neurologic abnormalities seen in Type II is predominately supportive in nature.

The differential diagnosis of neurologic regression in children can be broad, but may include leukodystrophies (X-linked adrenoleukodystrophy, metachromatic leukodystrophy), lysosomal or other storage disorders (Krabbe, neuronal ceroid lipofuscinosis, and mucopolysaccharidosis), and a multitude of other syndromes associated which may be associated with microcephaly, seizures, and abnormal movements. Although his relatively nonspecific symptoms made the ultimate diagnosis difficult, a brief review of commonly ordered neurologic panels does not appear to have the *CYBR53* gene included. We would recommend that this gene be incorporated into some of these panels to help reduce the almost inevitably long diagnostic process.

The variants that were identified in the proband's initial exome sequencing analysis were not felt to be contributory to the proband's phenotype. The three variants

inherited from his healthy, unaffected mother were all autosomal recessive and heterozygous in inheritance. The p.G222R *SUMF1* variant has not yet been reported as a disease-causing variant. However, pathogenic variants in *SUMF1* have been reported to cause multiple sulfatase deficiency, which is an autosomal recessive condition that involves many of the clinical features seen in the proband. These features include developmental delay, neurologic abnormalities, ocular abnormalities, and seizures [5]. While this was considered in the differential, extensive metabolic studies carried out when the proband was seen initially, including acylcarnitine profiles, plasma amino acids, urine organic acids, ammonia levels, lysosomal enzyme studies, and urine oligosaccharides and glycosaminoglycans all returned normal.

The p.L321R *BRAT1* variant has also not been reported as a disease-causing variant. Pathogenic variants in the *BRAT1* gene have been reported to be associated with neonatal rigidity, seizures, microcephaly, and lack of developmental progress [23]. Finally, the maternally inherited heterozygous p.N374S variant in the *SUCLG2* has not been reported as a disease-causing variant. Pathogenic variants in the *SUCLG1* and *SUCLG2* have been reported to cause infantile lactic acidosis along with other mitochondrial abnormalities [24]. However, the proband never had any episodes of lactic acidosis or elevated lactate, and due to the zygosity and mode of inheritance of these variants, we do not believe that any of these variants contributed to the phenotype of the proband. Finally, the heterozygous p.Q20H variant in the *KCNA1* gene, inherited from his healthy father, has also not been reported as a disease-causing mutation. Mutations in the *KCNA1* gene have been found to be associated with episodic ataxia [2]. It was inherited in an autosomal dominant manner and therefore is not thought to be associated with the proband's phenotype.

Table 1. Mutations leading to RCM type II

Mutation type	Codon change	Amino acid change	Exon/Intron	Publication
Missense/Nonsense	c.129C > A	Tyr42Term	Exon 2	[17]
Missense/Nonsense	Unknown	Lys111Met	Exon 4	[6]
Missense/Nonsense	c.287C > A	Pro95His	Exon 4	[17]
Missense/Nonsense	c.247C > T	Arg83Ter	Exon 4	[10]
Missense/Nonsense	c.229C > T	Gln77Ter	Exon 4	[1]
Missense/Nonsense	Unknown	Gly76Ser	Boundary of Exon 4 and Exon 5	[20] in a type I/[6]
Missense/Nonsense	Unknown	Leu131Pro	Exon 5	[6]
Missense/Nonsense	c. 382T > C	Ser127Pro	Exon 5	[12]
Missense/Nonsense	c. 379A > G	Met127Val	Exon 5	[13]
Missense/Nonsense	c.478C > T	Arg160Ter	Exon 6	[1]
Missense/Nonsense	c.608G > A	Cys203Tyr	Exon 7	[16]–first reported in Wang 1999 in RCM Type I
Missense/Nonsense	c.610T > C	Cys204Arg	Exon 7	[30]
Missense/Nonsense	c.705G > A	Trp235Term	Exon 8	[16]
Missense/Nonsense	A > G (position unknown)	Arg240Gly	Exon 8	[29]
Missense/Nonsense	c.655C > T	Arg219Ter	Exon 8	[30]
Missense/Nonsense	c.708G > A	Trp236Ter	Exon 8	[31, 32]
Missense/Nonsense	c.757G > A	Val253Met	Exon 9	[13]
Missense/Nonsense	c.173C > G	Arg58Pro	Unknown	[22]
Splice site	IVS 2 ds + 1 G > A	Unknown	Intron 2	[6]
Splice site	IVS 4 as -2 A > G	Loss of exon5	Intron 4	[13]
Splice site	IVS 5 ds + 2 T > C	Mis-splicing of exon 5	Intron 5	[33]
Splice Site	IVS 5 ds + 8 G > C	Loss of exon 5	Intron 5	[30]; also reported in [8]
Splice site	IVS 5 as -2 A > C	Loss of exon 6	Intron 5	[19]
Splice site	IVS 5 -2 A > C	Loss of exon 6	Intron 5	[18]
Splice site	IVS 8 as -1 G > T	Mis-splicing of exon 9	Intron 8	[26]
Deletion	c.215delG	Gly72AlafsTer	Exon 3	[11]
Deletion	Unknown	Lys173-Ser174-Val175del	Exon 6	[31, 32] MGM
Deletion	c.815_817del	Met272del	Exon 9	[30]
Deletion	c.895_897del	Phe298del	Exon 9	[25, 28]
Deletion	c.562_564del	Leu188del	Unknown	[22]
Small insertion/Deletion	c. 882_884delinsAA	Thr295fsTer	Exon 9	[14]
Gross deletion	c.22 1320_633 + 1224del	Unknown	Exons 2–7	[9]

Table 2. Clinical features in 35 genetically confirmed cases of RCM type II

Complications	Heterozygous				Homozygous				
	Proband (termination–deletion)	Missense–Missense	Missense–deletion, termination, or splice site	Termination–Termination	Missense	Termination	Deletion	Splice site	Total
General									
Complicated pregnancy	100% (1/1)	0% (0/2)	42.86% (3/7)	0% (0/1)	0% (0/7)	33.33% (1/3)	0% (0/5)	11.11% (1/9)	17.14% (6/35)
DD/ID	100% (1/1)	50% (1/2)	42.86% (3/7)	100% (1/1)	71.43% (5/7)	100% (3/3)	80% (4/5)	7/9	71.43% (25/35)
FTT	100% (1/1)	0% (0/2)	0% (0/7)	100% (1/1)	28.57% (2/7)	0% (0/3)	20% (1/5)	22.22% (2/9)	20% (7/35)
Short stature	100% (1/1)	0% (0/2)	14.29% (1/7)	100% (1/1)	14.29% (1/7)	0% (0/3)	20% (1/5)	0% (0/9)	14.29% (5/35)
Cyanosis	100% (1/1)	100% (2/2)	100% (7/7)	100% (1/1)	57.14% (4/7)	0% (0/3)	100% (5/5)	55.55% (5/9)	71.43% (25/35)
Gastrointestinal									
Feeding difficulty	100% (1/1)	100% (2/2)	14.29% (1/7)	100% (1/1)	0% (0/7)	0% (0/3)	20% (1/5)	33.33% (3/9)	22.86% (8/35)
Feeding tube	100% (1/1)	50% (1/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	8.57% (3/35)
GI dysmotility	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Musculoskeletal									
Hypotonia	100% (1/1)	50% (1/2)	0% (0/7)	0% (0/1)	14.29% (1/7)	0% (0/3)	60% (3/5)	44.44% (4/9)	28.57% (10/35)
Hypertonia	100% (1/1)	50% (1/2)	14.29% (1/7)	100% (1/1)	0% (0/7)	33.33% (1/3)	40% (2/5)	11.11% (1/9)	22.86% (8/35)
Scoliosis	100% (1/1)	50% (1/2)	0% (0/7)	100% (1/1)	0% (0/7)	0% (0/3)	20% (1/5)	0% (0/9)	11.43% (4/35)
Torticollis	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	20% (1/5)	0% (0/9)	5.71% (2/35)
Decreased muscle mass	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	20% (1/5)	0% (0/9)	5.71% (2/35)
Jerking movements	100% (1/1)	50% (1/2)	57.14% (4/7)	100% (1/1)	42.86% (3/7)	66.66% (2/3)	60% (3/5)	77.77% (7/9)	62.86% (22/35)
Quadriparesis/tetraparesis		0% (0/2)	42.86% (3/7)	100% (1/1)	14.29% (1/7)	0% (0/3)	20% (1/5)	11.11% (1/9)	20% (7/35)
Ear, Nose, and Throat									
Reduced middle ear mobility	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Neurologic									
Seizures	100% (1/1)	0% (0/2)	0% (0/7)	100% (1/1)	14.29% (1/7)	0% (0/3)	0% (0/5)	11.11% (1/9)	11.43% (4/35)
Staring spells	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Microcephaly	100% (1/1)	100% (2/2)	57.14% (4/7)	100% (1/1)	57.14% (4/7)	66.66% (2/3)	60% (3/5)	88.88% (8/9)	71.43% (25/35)
Cerebellar/cerebral atrophy	100% (1/1)	0% (0/2)	0% (0/7)	100% (1/1)	42.86% (3/7)	33.33% (1/3)	0% (0/5)	22.2% (2/9)	22.86% (8/35)
Enlarged ventricle	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
White matter loss	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Delayed myelination	100% (1/1)	0% (0/2)	14.29% (1/7)	100% (1/1)	57.14% (4/7)	33.33% (1/3)	0% (0/5)	11.11% (1/9)	21.71% (9/35)
Enlarged CSF or subarachnoid spaces	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	14.29% (1/7)	0% (0/3)	0% (0/5)	0% (0/9)	5.71% (2/35)
Abnormal EEG	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	14.29% (1/7)	0% (0/3)	0% (0/5)	11.11% (1/9)	8.57% (3/35)
Diffuse brain atrophy	0%(0/1)	0% (0/2)	0% (0/7)	0% (0/1)	28.57% (2/7)	33.33% (1/3)	0% (0/5)	11.11% (1/9)	14.29% (5/35)
Corpus callosum abnormality	0%(0/1)	0% (0/2)	14.29% (1/7)	0% (0/1)	14.29% (1/7)	0% (0/3)	0% (0/5)0	0% (0/9)	5.71% (2/35)
General neurologic issue	0%(0/1)	0% (0/2)	28.57% (2/7)	0% (0/1)	0% (0/7)	0% (0/3)	60% (3/5	0% (0/9)	14.29% (5/35)
Hypoplasia of basal ganglia	0%(0/1)	0% (0/2)	0% (0/7)	100% (1/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Cortical atrophy	0%(0/1)	0% (0/2)	14.29% (1/7)	100% (1/1)	0% (0/7)	0% (0/3)	20% (1/5)	11.11% (1/9)	11.43% (4/35)
Encephalopathy	0%(0/1)	0% (0/2)	42.86% (3/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	8.57% (3/35)

(*Continued*)

Table 2. (Continued).

Complications	Heterozygous				Homozygous				
	Proband (termination–deletion)	Missense–Missense	Missense–deletion, termination, or splice site	Termination–Termination	Missense	Termination	Deletion	Splice site	Total
Heart defect	0%(0/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	20% (1/5)	0% (0/9)	2.86% (1/35)
Ophthalmology									
Delayed visual maturation	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Cortical visual impairment	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Hyperopic astigmatism	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)
Strabismus	0%(0/1)	50% (1/2)	14.29% (1/7)	0% (0/1)	28.57% (2/7)	0% (0/3)	20% (1/5)	44.44% (4/9)	21.71% (9/35)
Other diagnosis	100% (1/1)	0% (0/2)	0% (0/7)	0% (0/1)	0% (0/7)	0% (0/3)	0% (0/5)	0% (0/9)	2.86% (1/35)

Many of the phenotypic features of the proband and the previously reported patient with the p.R160X pathogenic variant are similar. These patients both have different mutations on their second allele; however, they have many of the same phenotypic features. These include developmental delay and intellectual disability, short stature, failure to thrive, cyanosis, use of a feeding tube, hypertonia, spastic movements, scoliosis, and seizures. Both of these patients also had similar brain abnormalities including microcephaly, cerebellar/cerebral atrophy, and delayed myelination. Although these patients have many common clinical features, it seems that the proband that we report has a more severe phenotype than the patient reported by Aalfs et al. in 2000. We propose that the novel deletion in combination with the p.R160X pathogenic variant in our proband may have contributed to this increased severity compared to the [1] patient, who had two missense mutations. Deletions, splice site, and terminations appear to create the most severe methemoglobinemia phenotypes leading to type II rather than type I because of the significantly reduced methemoglobin reductase activity.

We have compiled the 34 published cases of RCM type II confirmed by genetic analysis (Table 1) and analyzed the clinical features of each case according to mutation type (Table 2). Many patients with RCM type II and their respective mutations have been reported in the literature. Some of the variants that have been discovered in RCM type II patients cause the severe type II form of the disease if homozygous in the patient, but cause type I RCM if the patient is heterozygous for the variant [9]. The most recent reviews of pathogenic variants in the *CYB5R3* gene leading to RCM Types I and II were published in 2008. Since that time, three novel missense mutations and an additional four novel deletions leading to clinical features of RCM type II have been published (Table 1). The cases that have been published since the 2008 reviews of RCM type II all report clinical features that are consistent with our proband and the previously reported cases of RCM type II. These features include developmental delay, cyanosis, feeding difficulty, use of a feeding tube, failure to thrive, spastic limb movements, hypotonia, microcephaly, and changes in the brain including cerebellar and/or cerebral atrophy and delayed myelination. However, there does not seem to be a clear correlation between the type/severity of mutation and the severity of the phenotypic features that are manifested (Table 2).

One of the aspects to consider when evaluating these patients is methemoglobin reductase levels. All of the cases compiled for this report either reported an actual methemoglobin reductase level, the percentage of activity of the normal enzyme activity, or were described as

"reduced" or "deficient." Out of the 35 cases, there were five cases that had methemoglobin reductase activity levels that were within or slightly higher than the normal range (6.6–13.3 U/g Hb). The five cases with normal or slightly elevated methemoglobin reductase activity levels included one heterozygous missense–nonsense mutation, two homozygous missense mutations, a homozygous nonsense mutation, and a nine nucleotide deletion. The remaining cases had severely reduced levels/activity of methemoglobin reductase. We attempted to determine whether there was a correlation between the severity of the mutation and the level of methemoglobin reductase. This analysis showed that 9 of 30 cases with abnormal methemoglobin reductase activity contained homozygous splice site variant, 11 of 30 cases with either a homozygous missense variant, nonsense variant, or deletion, 6 of 30 cases were heterozygous for a missense variant and either a deletion, termination, or splice site variant, 2 of 30 cases were missense heterozygous, and 2 of 30 cases had two different nonsense variants or a heterozygous nonsense and deletion. There seems to be a wide variety of variants reported in these cases, which suggests that a particular variant type does not necessarily lead to decreased methemoglobin levels and activity.

This report introduces the first genetically confirmed case of RCM type II in the United States and details a previously unreported combination of causative genetic changes in the *CYB5R3* gene. Although the condition may not be the ideal condition for inclusion on newborn screening, we would recommend that the *CYB5R3* gene be included on common neurologic testing such as epilepsy, microcephaly, or leukodystrophy panels. To our knowledge, this gene is not readily available for analysis in these types of multigene panels that are often ordered prior to more large-scale analyses such as whole-exome sequencing. Earlier detection and recognition of the condition may shorten the often prolonged diagnostic odyssey for the relatively nonspecific signs and symptoms that are seen in RCM II.

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Authorship

SSV, TP, and JW: involved in patient management. MTC and JJ: provided molecular analysis of samples. EM, TP, JW, MTC, JJ, and SSV: provided editing and review of the manuscript. EM, MTC, JJ, and SSV: wrote the manuscript.

Conflict of Interest

None declared

References

1. Aalfs, C. M., G. B. Salieb-Beugelaar, R. J. A. Wanders, M. M. A. M. Mannens, and F. A. Wijburg. 2000. A case of methemoglobinemia type II due to NADH-cytochrome b5 reductase deficiency: determination of the molecular basis. Hum. Mut. 16:18–22.
2. Browne, D. L., E. R. Brunt, R. C. Griggs, J. G. Nutt, S. T. Gancher, E. A. Smith, et al. 1995. Identification of two new *KCNA1* mutations in episodic ataxia/myokymia families. Hum. Mol. Genet. 4:1671–1672.
3. Bull, P. C., E. A. Shephard, S. Povey, I. Santiseteban, and I. R. Phillips. 1988. Cloning and chromosomal mapping of human cytochrome b5 reductase (DIA1). Ann. Hum. Genet. 52:263–268.
4. Cooper, M. S., M. Randall, M. Rowell, M. Charlton, A. Greenway, and C. Barnes. 2016. Congenital methemoglobinemia type II–clinical improvement with short-term Methylene Blue treatment. Pediatr. Blood Cancer 63:558–560.
5. Cosmo, M. P., S. Pepe, G. Arenti, C. Settembre, I. Annunziata, R. Wade-Martins, et al. 2004. Molecular and functional analysis of *SUMF1* mutations in multiple s sulfatase deficiency. Hum. Mutat. 23:576–581.
6. Ewenczyk, C., A. Leroux, A. Roubergue, V. Laugel, A. Afenjar, M. Saudubray, et al. 2008. Recessive hereditary methaemoglobinaemia type II: delineation of the clinical spectrum. Brain 131:760–771.
7. Fusco, C., G. Soncini, D. Frattini, E. Della Giustina, C. Vercellati, E. Fermo, et al. 2011. Cerebellar atrophy in a child with hereditary methemoglobinemia type II. Brain Dev. 33:357–360.
8. Galeeva, N. M., S. A. Nenasheva, S. Kleymenova, and A. V. Polyakov. 2012. Novel large deletion c.221320_633 + 1224del in the *CYB5R3* gene from patients with hereditary methemoglobinemia. Genetika 48:1148–1157.
9. Higasa, K., J. Manabe, T. Yubisui, H. Sumimoto, P. Pung-Amritt, V. S. Tanphaichitr, et al. 1998. Molecular basis of hereditary methaemoglobinaemia, types I and II: two novel mutations in NADH-cytochrome b5 reductase gene. Br. J. Haematol. 103:922–930.
10. Hudspeth, M. P., S. Joseph, and K. R. Holden. 2010. A novel mutation in type II methemoglobinemia. J. Child Neurol. 25:91–93.

11. Kobayashi, Y., Y. Fukumaki, T. Yubisui, J. Inoue, and Y. Sakaki. 1990. Serine-proline replacement at residue 127 of NADH-cytochrome b5 reductase causes hereditary methemoglobinemia, generalized type. Blood 75:1408–1413.
12. Kugler, W., A. Pekrun, P. Laspe, B. Erdlenbruch, and M. Lakomek. 2001. Molecular basis of recessive congenital methemoglobinemia, types I and II: exon skipping and three novel missense mutations in the NADH cytochrome b5 reductase (diaphorase1) gene. Hum. Mutat. 17:348.
13. Leroux, A., F. Leturcq, N. Deburgrave, and M. F. Szajnert. 2005. Prenatal diagnosis of recessive congenital methaemoglobinaemia type II: novel mutation in the NADH-cytochrome b5 reductase gene leading to stop codon read-through. Eur. J. Haematol. 74:389–395.
14. Leroux, A., C. Junien, J. Kaplan, and J. Bamberger. 1975. Generalised deficiency of cytochrome b5 reductase in congenital methaemoglobinaemia with mental retardation. Nature 258:619–620.
15. Kedar, P. S., P. Warang, K. Ghosh, and R. B. Colah. 2011. Severe mental retardation and recessive congenital methemoglobinemia in three Indian patients: compound heterozygous for NADH-cytochrome b5 reductase gene mutations. Am. J. Hematol. 86:327–329.
16. Manabe, J., R. Arya, H. Sumimoto, T. Yubisui, A. J. Bellingham, D. M. Layton, et al. 1996. Two novel mutations in the reduced nicotinamide adenine dinucleotide (NADH)- cytochrome b5 reductase gene of a patient with generalized type, hereditary methemoglobinemia. Blood 88:3208–3215.
17. Maran, J., Y. Guan, C. Ou, and J. T. Prchal. 2005. Heterogeneity of the molecular biology of methemoglobinemia: a study of eight consecutive patients. Haematologica 90:687–689.
18. Owen, E. P., J. Berens, A. M. Marinaki, H. Ipp, and E. H. Harley. 1997. Recessive congenital methaemoglobinaemia type II, a new mutation which causes incorrect splicing in the NADH-cytochrome b5 reductase gene. J. Inher. Metab. Dis. 20:610.
19. Percy, M. J., L. J. Crowley, D. Roper, T. J. Vulliamy, D. M. Layton, and M. J. Barber. 2006. Identification and characterization of the novel FAD-binding lobe G75S mutation in cytochrome b5 reductase: an aid to determine recessive congenital methemoglobinemia status in an infant. Blood Cells Mol. Dis. 36:81–90.
20. Percy, M. J., and T. R. Lappin. 2008. Recessive congenital methaemoglobinaemia: cytochrome b5 reductase deficiency. Br. J. Haematol. 141:298–308.
21. Percy, M. J., C. Barnes, G. Crighton, R. J. Leventer, R. Wynn, and T. R. Lappin. 2012. Methemoglobin reductase deficiency: novel mutation is associated with a disease phenotype of intermediate severity. J. Pediatr. Hematol. Oncol. 34:457–460.
22. Puffenberger, E. G., R. N. Jinks, C. Sougnez, K. Cibulskis, R. A. Willert, N. P. Achilly, et al. 2012. Genetic mapping and exome sequencing identify variants associated with five novel diseases. PLoS ONE 7:e28936.
23. Sakamoto, O., T. Ohera, K. Murayama, A. Ohtake, H. Harashima, D. Abukama, et al. 2011. Neonatal lactic acidosis with methylmalonic aciduria due to novel mutations in the *SUCLG1* gene. Pediatr. Int. 53:921–925.
24. Shirabe, K., Y. Fujimoto, T. Yuisui, and M. Takeshita. 1994. An in-frame deletion of codon 298 of the NADH-cytochrome b5 reductase gene results in hereditary methemoglobinemia type II (generalized type). A functional implication for the role of the COOH-terminal region of the enzyme. J. Biol. Chem. 269:5952–5957.
25. Shirabe, K., M. T. Landi, M. Takeshita, G. Uziel, E. Fedrizzi, and N. Borgese. 1995. A novel point mutation in a 3′ splice site of the NADH cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NADH-dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. Am. J. Hum. Genet. 57:302–310.
26. Tanaka, A. J., M. T. Cho, F. Millan, J. Juusola, J. Retterer, C. Joshi, et al. 2015. Mutations in *SPATA5* Are Associated with Microcephaly, Intellectual Disability, Seizures, and Hearing Loss. Am. J. Hum. Genet. 97:457–464.
27. Takeshita, M., T. Matsuki, K. Tanishima, T. Yubisui, Y. Yoneyama, K. Kurata, et al. 1982. Alteration of NADH-diaphorase and cytochrome b5 reductase activities of erythrocytes, platelets, and leucocytes in hereditary methaemoglobinaemia with and without mental retardation. J. Med. Genet. 19:204–209.
28. Toelle, S. P., E. Boltshauser, E. Mossner, K. Zurbriggen, and S. Eber. 2004. Severe neurological impairment in hereditary methaemoglobinaemia type 2. Eur. J. Pediatr. 163:207–209.
29. Vieira, L. M., J. C. Kaplan, A. Kahn, and A. Leroux. 1995. Four new mutations in the NADH-cytochrome b5 reductase gene from patients with recessive congenital methemoglobinemia type II. Blood 85:2254–2262.
30. Warang, P. P., P. S. Kedar, C. Shanmukaiah, K. Ghosh, and R. B. Colah. 2015. Report clinical spectrum and molecular basis of recessive congenital methemoglobinemia in India. Clin. Genet. 87:62–67.
31. Warang, P., P. Kedar, S. Sivanandam, K. Jothilakshmi, R. Sumathi, and R. Colah. 2015. A novel nine base deletion mutation in NADH-cytochrome b5 reductase gene in an Indian family with recessive congenital methemoglobinemia type II. Mol. Genet. Metab. Rep. 5:44–47.
32. Yilmaz, D., O. Cogulu, F. Ozkinay, and K. Kavakli. 2005. A novel mutation in the *DIA1* gene in a patient with methemoglobinemia type II. Am. J. Med. Genet. Part A 133A:101–102.
33. Wang, Y., Y. S. Wu, P. Z. Zheng, W. X. Yang, G. A. Fang, Y. C. Tang, F. Xie, et al. 2000. A novel mutation in the NADH-cytochrome b5 reductase gene of a Chinese patient with recessive congenital methemoglobinemia. Blood. 95 (10):3250–3255.