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Sensitivity and specificity of decreased CSF asialotransferrin for eIF2B-related disorder

A. Vanderver, MD, **Y. Hathout, PhD**, **J. Maletkovic, MD**, **E.S. Gordon, MS**, **M. Mintz, PhD**, **M. Timmons, MD**, **E.P. Hoffman, PhD**, **L. Horzinski**, **F. Niel**, **A. Fogli, MD**, **O. Boespflug-Tanguy, MD**, and **R. Schiffmann, MD**

Children's National Medical Center (A.V., Y.H., J.M., E.S.G., M.M., E.P.H.), Children's Research Institute, Center for Genetic Medicine, Washington, DC; Developmental and Metabolic Neurology Branch (DMNB) (M.T., R.S.), National Institute of Neurologic Disorders and Stroke (NINDS)/ National Institutes of Health (NIH), Bethesda, MD; INSERM (L.H., A.F., O.B.-T.), Faculté deMédecine, Clermont-Ferrand; CHU (F.N.), Biochimie Médicale, Clermont-Ferrand; and CHU (O.B.-T.), Génétique Médicale, Clermont-Ferrand, France

Abstract

Objective—This is a study estimating diagnostic accuracy of CSF asialotransferrin to transferrin ratio measurement in eIF2B related disorders by using clinical evaluation and *EIF2B* mutation analysis as the reference standard. eIF2B-related disorder is a relatively common leukodystrophy with broad phenotypic variation that is caused by mutations in any of the five *EIF2B* genes. There is a need for a simple and clinically valid screening tool for physicians evaluating patients with an unclassified leukodystrophy.

Methods—CSF two-dimensional gel (2DG) electrophoresis analyses to measure asialotransferrin to transferrin ratios were performed in 60 subjects including 6 patients with documented *EIF2B* gene mutations, patients with other types of leukodystrophy, and patients with no leukodystrophy.

Results—All six patients with mutation proven eIF2B-related disease showed low to nearly undetectable amounts of asialotransferrin in their CSF when compared to 54 unaffected controls by CSF 2DG analyses in this study. eIF2B-like patients, with clinically similar presentations but no mutations in *EIF2B1-5*, were distinguished from patients with mutations in *EIF2B1-5* by this biomarker. Patients with mutations in *EIF2B1-5* had asialotransferrin/transferrin ratio levels significantly different from the group as a whole ($p < 0.001$). Using 8% asialotransferrin/ transferrin ratio as a cutoff, this biomarker has a 100% sensitivity (95% CI = 52–100%) and 94% specificity (95% CI = 84–99%).

Conclusion—Decreased asialotransferrin/transferrin ratio in the CSF of patients with eIF2B-related disorder is highly sensitive and specific. This rapid (<48 hours) and inexpensive diagnostic

Address correspondence and reprint requests to Dr. Adeline Vanderver, Children's National Medical Center, 111 Michigan Ave., NW, Washington, DC 20010, avanderv@cnmc.org.

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tool for eIF2B-related disorders has the potential to identify patients with likely eIF2B-related disorder for mutation analysis.

eIF2B-related disorders were initially described as childhood onset ataxia with CNS hypomyelination (CACH),[1] vanishing white matter disease (VWM),[2] or myelinopathia centralis diffusa[3] based on clinical and MRI criteria. eIF2B-related disorders are an autosomal recessive leukoencephalopathy currently diagnosed based on clinical phenotype and MRI pattern recognition confirmed by mutation analysis of the five *EIF2B* genes.[4–6] In many cases, the clinical phenotype is readily recognizable, with childhood onset of a progressive spastic ataxia with variable seizures, optic atrophy, and late dementia. Episodic deterioration may be seen after fevers, mild head trauma, or fright,[7] and may result in coma or even death. MRI typically demonstrates rarefaction and cystic degeneration of white matter best seen on fluid-attenuated inversion recovery images (FLAIR). White matter signal is replaced with CSF isointense signal, hence the appellation VWM.[2,8]

However, eIF2B-related disorders are proving to have a much broader phenotype than originally reported.[9,10] The use of MRI or neuropathologic criteria to select patients with undetermined leukodystrophies for *EIF2B1-5* gene analysis demonstrated the wide clinical spectrum of *EIF2B1-5*-mutated patients: from severe infantile cases such as Cree leukoencephalopathy[11] and congenital forms with rapid death,[12] to adult-onset forms with slow neurologic progression[13–15] and ovarian failure such as ovarioleukodystrophy.[16,17] Neonatal cases have been seen with severe encephalopathy and extraneurologic features such as cataracts, kidney hypoplasia, and ovarian dysgenesis.[12,18] Rare patients with a peripheral neuropathy have been described.[19,20] While MR images are often diagnostic,[21] asymptomatic patients, early infantile patients, and patients early in the course of the disease[2,15,22] may be hard to recognize. In addition, non-eIF2B related disorders can sometimes mimic the radiologic features of eIF2B-related disorders.[23,24]

eIF2B, composed of five subunits (eIF2Bα, eIF2Bβ, eIF2Bγ, eIF2Bδ, eIF2Bε), is encoded by five different genes (*EIF2B1–5*) with a total of 57 exons, making gene sequencing expensive and time consuming. Mutations have been found in all five genes and in multiple different exons.[6,25] The great number of individual mutations has confounded efforts at simplifying mutation analysis in suspected eIF2B-related cases, requiring in many cases the sequencing of all five genes to exclude a diagnosis.

Efforts to establish a screening tool to streamline the diagnosis of eIF2B-related disorders have included CSF glycine levels,[26] measuring guanine nucleotide exchange factor activity of eIF2B in lymphoblasts (GEF activity),[27] and most recently, decreased CSF asialotransferrin to transferrin ratio.[28] Detection of CSF asialotransferrin to transferrin ratio by 2DG analysis has the advantage of being technologically simple, rapid (<48 hours), inexpensive, and highly sensitive. In this study, we focus on establishing the diagnostic accuracy of 2 DG analysis of CSF asialotransferrin to transferrin ratio for the diagnosis of eIF2B-related disorders. Current diagnosis is based on clinical evaluation followed by confirmation of eIF2B mutation, and this approach will be used as the reference standard.

METHODS

Sample collection

All CSF samples were collected in accordance with an Institutional Review Board approved protocol at Children's National Medical Center and collaborating institutions. Affected and disease control samples were obtained at Children's National Medical Center, the National Institute of Neurological Disorders and Stroke/NIH, and Clermont-Ferrand's University Hospital. All available samples from patients with mutations in the genes *EIF2B1–5* were included.[6] All leukodystrophy control samples[23] and neurologic disease control samples[10] were obtained from patients referred to the above tertiary care centers for evaluation of a leukodystrophy of unknown cause. Non-neurologic disease control samples[15] were obtained from Children's National Medical Center. Only excess CSF drawn for other clinical or research purposes was used for these analyses. Patients, with the exception of the nonneurologic disease control samples, were clinically evaluated at INSERM UMR384/ Clermont-Ferrand's University Hospital or the National Institute of Neurological Disorders and Stroke/NIH and patients believed to have a leukodystrophy compatible with eIF2B-related disorder were selected for *EIF2B* mutation analysis.[12] Confirmation of *EIF2B1–5* mutation status was performed at Children's National Medical Center and at INSERM UMR384/Clermont-Ferrand's University Hospital prior to or concomitant with CSF studies. This approach to diagnosis has been the reference standard in eIF2B-related disorder. CSF samples were blinded at INSERM UMR384/Clermont-Ferrand's University Hospital, Children's National Medical Center, or the National Institute of Neurological Disorders and Stroke/NIH by a person not performing the CSF studies and received by A.V. for this analysis between April and November 2006. CSF samples were studied in 60 subjects (figure 1): 6 patients with documented *EIF2B1–5* mutations; 6 patients with eIF2B-like presentation but no *EIF2B* mutations; 23 patients with other leukodystrophies (2 with Alexander disease, 3 with idiopathic hypomyelination, 7 with Pelizaeus-Merzbacher disease, 1 with X-linked adrenoleukodystrophy, 1 with Coats disease, 1 with a megalencephalic leukoencephalopathy with Cysts–like disorder, and 8 with an undetermined leukodystrophy); 10 patients with other neurologic abnormalities (1 with progressive cerebellar atrophy, 1 with spinal cord lesion, 1 with hereditary spastic paraplegia, one with Coffin Lowry syndrome, one with mitochondrial complex I deficiency, 1 with Gaucher disease type 3, 1 with X-linked mental retardation, 3 with encephalopathy of unknown origin); and 15 patients with non-neurologic reasons for lumbar puncture. In seven of these subjects, including two patients with mutations in *EIF2B1–5*, samples had been included in an unblinded fashion for the previous analysis,[28] but were used in a blinded fashion in this study. Samples were collected in a standardized fashion, tested for blood contamination, and stored at −80°C until analysis.

Two-dimensional gel electrophoresis

Protein concentration was measured in each CSF sample using the Bio- Rad protein assay reagent (Bio-Rad, Hercules, CA). Typically, CSF samples showed a protein concentration ranging from 0.16 to 0.4 mg/mL. Aliquots containing 100 μg of total protein were taken from each sample and processed for 2DG analysis as follows[28,29]: samples were desalted against 10 mM Tris HCl pH 7 using p6 Bio-Spin columns (Bio-Rad). Each solution was

then dried by centrifugation under vacuum. A total of 180 μL of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mmol DTT, and 0.5% ampholyte pH 3–10) was added to the dry sample to solubilize and denature the proteins. First dimension electro-focusing was performed on IPG strips (11 cm, pH 3–10) using a Bio-Rad electro-focusing chamber operated as follows: 12 hours rehydration, 250 V for 15 minutes, 1,000 V for 1 hour, and 10,000 V for 4 hours. The second dimension SDS-PGE was performed on criterion Tris-HCl (8 to 16% or 10%) pre-cast gels (Bio-Rad). Protein spots were visualized using Bio-Safe Coomassie stain (Bio-Rad). The gel was then scanned on a GS800 densitometer (Bio-Rad) and archived as a TIFF file. Asialotransferrin and transferrin isoforms (figure 2) were identified based on previous mass spectrometry analyses.[28] The resulting gels arrays were compared using a 2DG image analysis software, PDQuest (Bio-Rad), and the volume and intensity of spots of interest were determined using Quantity One software (Bio-Rad). All 2DG analyses were performed by J.M. and A.V. who were blinded to the results of *EIF2B1-5* mutation sequencing and clinical characteristics of the patients studied.

Data analysis

Initially, 30 samples, including seven from patients with mutations in *EIF2B1-5*, had been analyzed with prior knowledge of *EIF2B* mutation status (Vanderver, Schiffmann et al., 2005). For the purposes of this validation study, an additional 60 samples were run with the investigator blinded to the *EIF2B* mutation status, including the number of samples with *EIF2B* mutations. This study was powered to detect a difference of greater than 20% between affected and unaffected individuals. In seven cases, blinded samples had also been analyzed in the initial data set, including two samples with *EIF2B* mutations. In addition, serial analyses were performed in 25 samples at different time points to establish intra-sample reproducibility. If multiple gels were performed on a single sample, the results were averaged. In no case of a patient with a mutation in *EIF2B1–5* did individual results cross the cutoff boundary for reclassification prior to averaging. A ratio of 8% of CSF asialotransferrin relative to total transferrin has been identified as distinguishing between affected and unaffected samples based on the previous test data set (Vanderver, Schiffmann et al., 2005). Ratios were compared using a Fisher exact test. Sensitivity and specificity were calculated and 95% CIs were determined for each of them. Likelihood ratios were also calculated using STATA software. A log transformed Pearson's correlation was calculated to determine the reproducibility of repeated gel analysis and to look for correlations between percentage of asialotransferrin and age at time of lumbar puncture and clinical features in affected patients.

RESULTS

An initial test data set of 30 samples (including 7 patients with documented mutations in *EIF2B1–5*, 3 patients with clinical pictures consistent with eIF2B-related disorder but no mutation, 5 patients with other leukoencephalopathies, and 15 patients with other reasons for lumbar puncture) identified a decrease in the ratio of asialotransferrin/transferrin isoforms seen in subjects with mutations in *EIF2B1–5*.[28] Based on this data set, samples with asialotransferrin/transferrin ratio of less than or equal to 8% were considered to have

possible mutations in *EIF2B1–5*, while samples with greater than 8% were considered to be unaffected (figure 2).

A subsequent validation data set of 60 blinded samples obtained from the National Institute of Neurological Disorders and Stroke/NIH[36] and INSERM UMR384[24] led to the correct identification of all six samples from patients with *EIF2B1–5* mutations. Of importance, six patients with clinical phenotypes consistent with eIF2B-related disorders, but no documented mutations in *EIF2B1–5*, were also screened in a blinded fashion. In one of these eIF2B-like patients, the asialotransferrin/transferrin ratio was 7.98%; however, repeat measurements demonstrated higher ratios (mean 8.64, SD 0.704), and allowed for correct identification of the patient as a control. All the other eIF2B-like patients were appropriately classified as controls. In addition, only two other controls (one patient with an undetermined leukodystrophy, and one patient with a mutation in the *GFAP* gene) had percentages of asialotransferrin/ transferrin in the range of 5–8% and were classified as possibly affected.

Fisher exact test of the combined test and validation data sets was performed on the 60 blinded samples. Patients with *EIF2B1–5* mutations had asialotransferrin/transferrin ratio levels significantly different from the group as a whole (Fisher exact test, $p < 0.001$) (table 1). Using 8% asialotransferrin/ transferrin ratio as a cutoff, this biomarker has a 100% sensitivity (95% CI = 52– 100%) and 94% specificity (95% CI = 84–99%). The likelihood ratio (LR) of mutation in *EIF2B1–5* with a positive CSF test result as defined by less than 8% asialotransferrin/transferrin ratio was significant (LR = 18; 95% CI = 5.99– 54.06).

Further analysis was performed on subgroups of patients defined as having mutations in *EIF2B1-5* (6 patients), eIF2B-like without mutations (6 patients), and general controls (48 patients). The CSF asialotransferrin/transferrin ratio was significantly different in eIF2B-mutated and eIF2B-like patients (Fisher exact test, $p \leq 0.01$). This subgroup was too small to identify valid sensitivity and specificity. CSF asialotransferrin/transferrin ratio was not significantly different in eIF2B-like patients without *EIF2B* mutations and the remainder of the controls (Fisher exact test, $p = 0.302$).

Internal validity was measured by analysis of CSF samples from the same patients at several days to several month intervals. Log transformed Pearson correlation calculations suggest high intra-sample reproducibility (Pearson r = 0.883). From a qualitative perspective, no patient with mutations in *EIF2B1-5* had a pre-average asialotransferrin/ transferrin ratio of more than 8%. Asialotransferrin/transferrin ratio did not appear to correlate with age at onset, length of disease prior to lumbar puncture used for analysis, age at death, or constellation of symptoms in patients with eIF2B-related disorders. Gender and age at time of lumbar puncture did not correlate with asialotransferrin/transferrin ratio in the control patient population.

DISCUSSION

In our sample group, CSF asialotransferrin to transferrin ratios correctly identified all patients with mutations in *EIF2B1–5*. Six out of six patients with mutation proven eIF2Brelated disease showed low to nearly undetectable amounts of asialotransferrin in their

CSF when compared to 54 unaffected controls by CSF 2DG analysis. Patients with nearly identical clinical and radiologic presentations (figure 3) but no mutations in *EIF2B1–5* were identified as controls. Of note, the patient shown in figure 3, with eIF2B-like phenotype, was one of the original patients described with CACH[1] (patient 4) who later was demonstrated not to have mutations in *EIF2B1–5*. The only two patients with asialotransferrin/transferrin ratios of 6–8% who were not affected with eIF2B-related disorder had clearly distinct radiologic and clinical presentations. In addition, serial 2DG analyses demonstrated good intra-sample reproducibility, even for samples stored for several months at −80°C.

Samples from patients with eIF2B-related disorder included patients with missense and frame shift mutations in *EIF2B2*, *EIF2B4*, and *EIF2B5*, with the greatest number of patients with *EIF2B5* mutations (table 2). Asialotransferrin to total transferrin ratio did not appear to correlate with age at onset, length of disease prior to lumbar puncture used for analysis, age at death, or constellation of symptoms in patients affected by eIF2B-related disorders. Specifically, patients with adult onset of symptoms had percentages of asialotransferrin similar to patients with early onset of symptoms. Of note, in this analysis, we did not have any patients with neonatal or very early onset disease. Further analyses will be necessary to confirm these findings in these subgroups.

This decrease in CSF asialotransferrin to transferrin ratio has, to our knowledge, not been described in any other disorders. Of note, an increase in CSF asialotransferrin to transferrin ratio has been reported in patients with congenital disorders of glycosylation since these disorders have been recognized.[30] There is increasing evidence that transferrin plays a role in oligodendrocyte maturation and homeostasis,[31–37] in addition to its known role in iron metabolism and oxidative stress.[38] Thus, decreased asialotransferrin to transferrin ratio in the CSF may simply be a manifestation of disturbed protein homeostasis within the CNS as a result of *EIF2B* mutations, or it may play a role in the pathophysiology of the disorder.

In serum, transferrin originates from the liver and exists as sialotransferrin, unless a pathologic condition occurs such as congenital disorders of glycosylation or alcoholism.[39] In the CSF, both asialotransferrin and sialotransferrin are found.[28,40] It is presumed that sialotransferrin may be found in CSF due to a small transfer of serum-derived transferrin across the blood–brain–CSF barriers. It has been suggested that asialo-forms could result from partial metabolic degradation of the serum sialotransferrin transferred to the brain compartment.[41] Others have suggested de novo synthesis of asialotransferrin by oligodendrocytes and astrocytes.[40,42] In the brain of patients with *EIF2B* mutations, oligodendrocytes have been reported with a foamy aspect consistent with the presence of material rich in glycoproteins.[43] Increasing evidence supports an exaggerated ER stress response with abnormal activation of the unfolded protein response in *EIF2B*-mutated cells with abnormal protein synthesis and cellular homeostasis[20,44–48] in particular in oligodendrocytes and astrocytes of the white matter from affected patients.[46] Hence, we hypothesize that the decrease of CSF asialotransferrin to transferrin ratio in patients with eIF2B-related disorders may be due to reduced de novo secretion from these cells.

The expanding phenotype and hope for possible therapeutic strategies makes novel diagnostic approaches valuable in eIF2B-related disorders. Although clinical characteristics and radiologic features may be suggestive, the difficulty and cost of diagnosis by DNA sequencing make a rapid, inexpensive screening tool attractive for clinical practice. Decreased asialotransferrin/transferrin ratio is a novel, clinically useful biomarker for eIF2B-related disorder. Its high sensitivity and specificity makes it useful as a screening tool prior to *EIF2B1–5* mutation analysis in selected cases. In addition, its technological simplicity, rapidity (less than 48 hours for CSF asialotransferrin/ transferrin ratio determination vs several weeks to months for *EIF2B1-5* mutation analysis), and low cost (less than $30 for supplies and reagents) make it an excellent candidate for clinical use. Although testing of CSF is an invasive procedure, precedent exists for using CSF as a screening tool for more specific analysis, such as in the neurotransmitter disorders.[49] CSF asialotransferrin/transferring ratio determination could be used as a screening tool in cases with suggestive MRI manifestations prior to *EIF2B* mutation analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GLOSSARY

2DG	two-dimensional gel
CACH	hood onset ataxia with CNS hypomyelination
FLAIR	fluid-attenuated inversion recovery
LR	likelihood ratio
VWM	vanishing white matter

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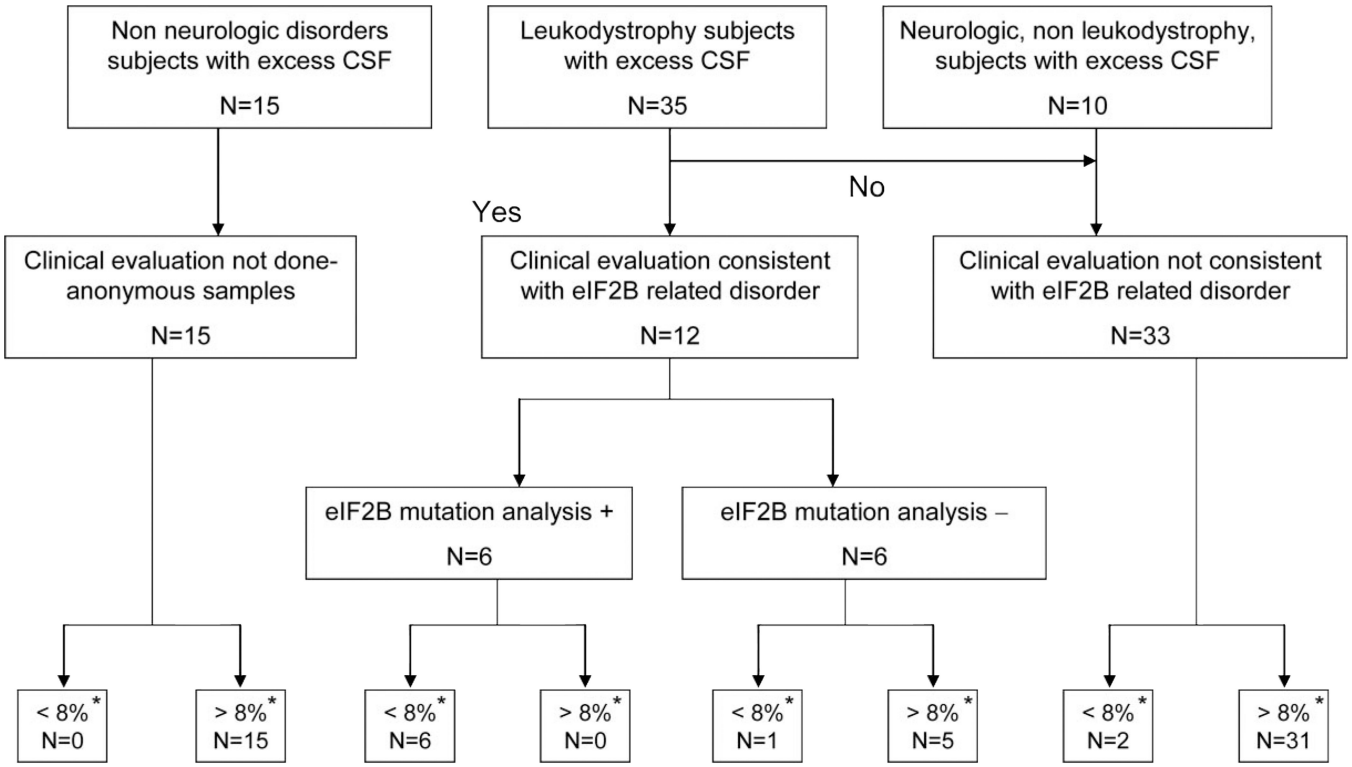

Figure 1.

Flow diagram of CSF asialotransferrin/transferrin ratio diagnostic accuracy study.

* Asialotransferrin/transferrin ratio

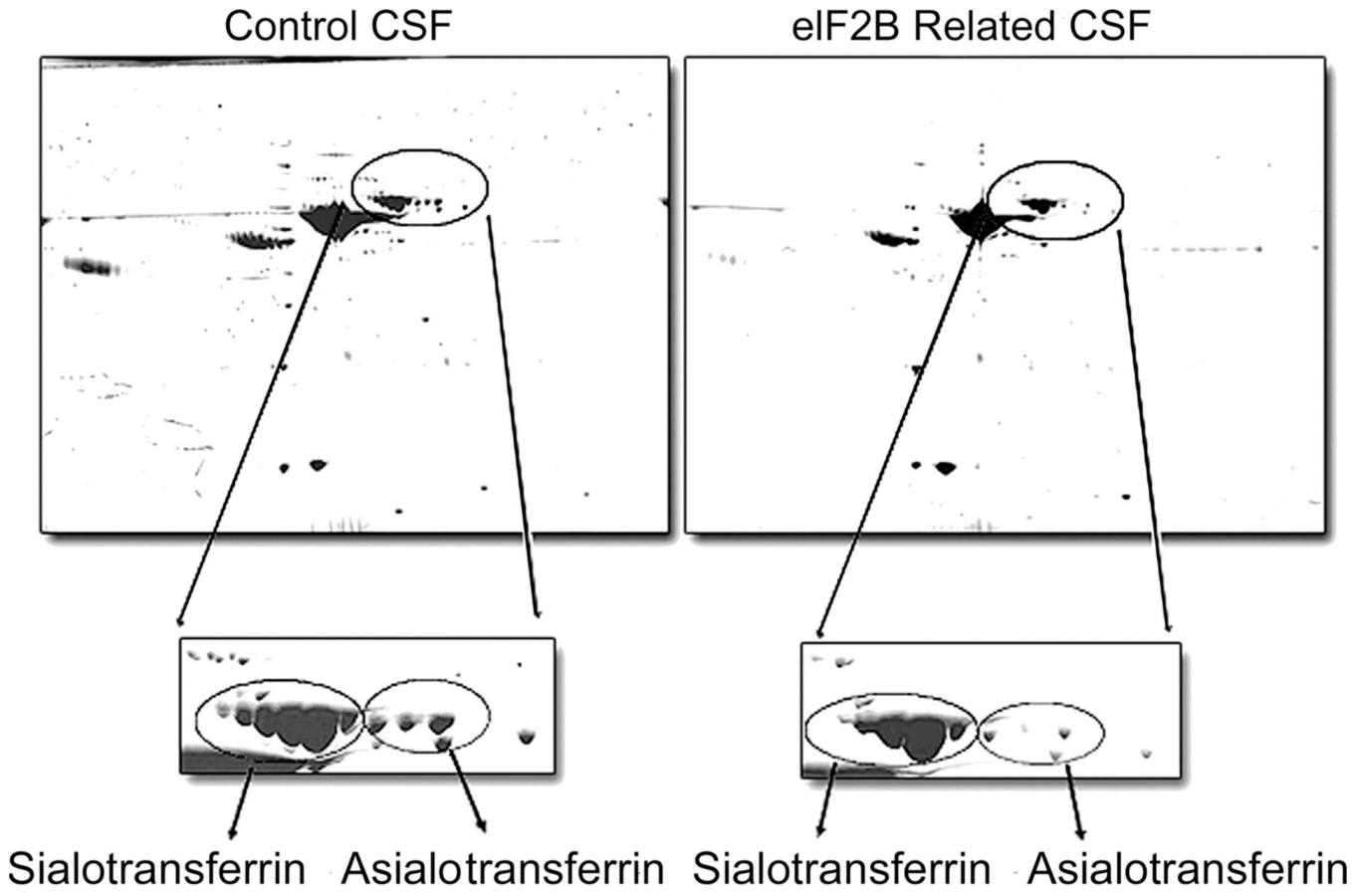

Figure 2.

Scanned image of 2DG electrophoresis of control CSF (left) and *EIF2B*-mutated CSF (right) Box corresponds to transferrin isoforms in each sample type. Note the decreased asialotransferrin isoforms relative to the total transferrin isoforms in *EIF2B*-mutated sample relative to the control sample. This patient (DMN 99.31) has mutations in *EIF2B5* (Y343C/I385V).

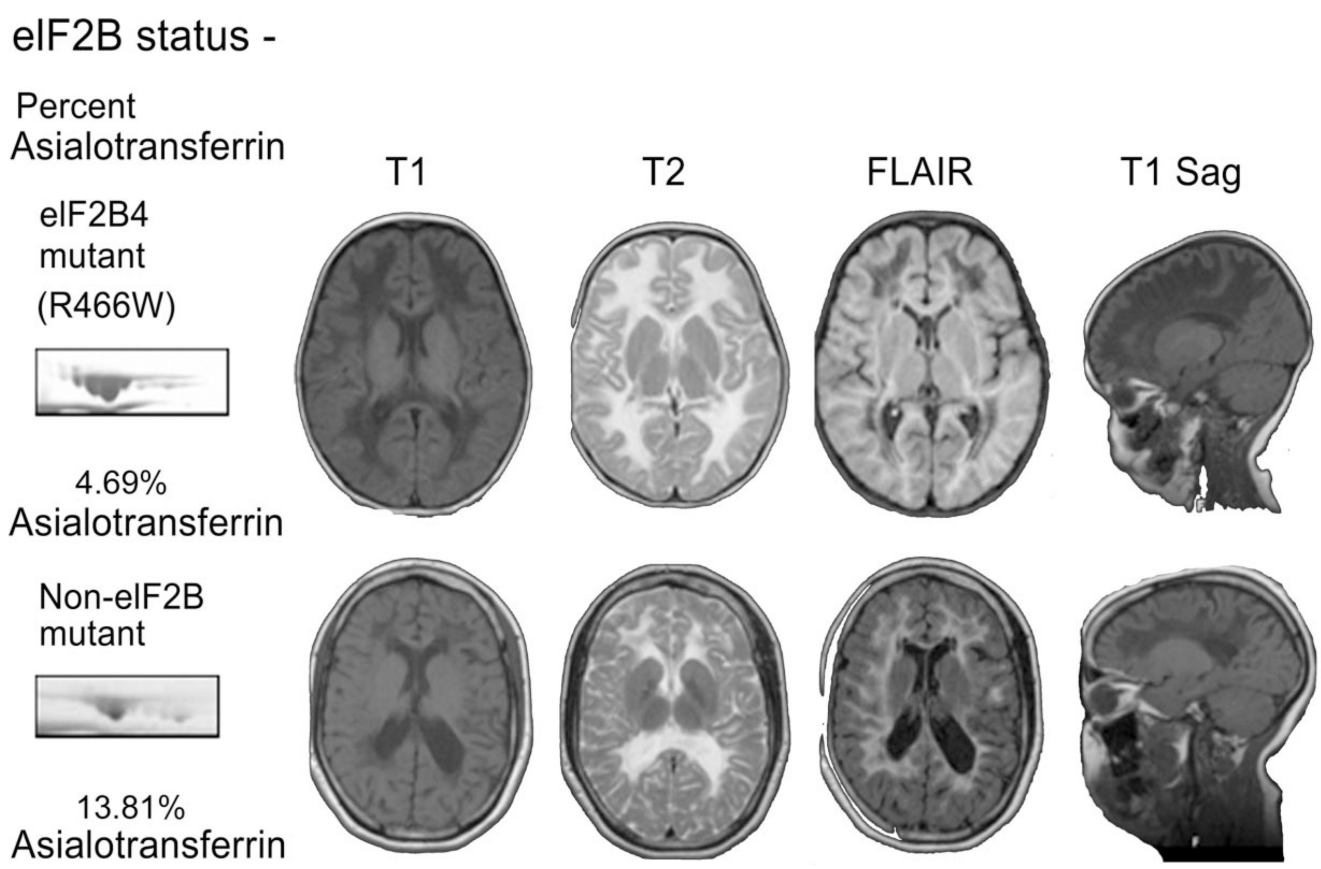

Figure 3.

Patients with *EIF2B4* (R466W homozygous) mutation and with eIF2B-like clinical and radiologic presentation, but without eIF2B mutations

Top: Patient with *EIF2B4* (R466W homozygous) mutation. MRI reveals T2 signal intensity similar to CSF, fluid-attenuated inversion recovery (FLAIR) images consistent with rarefaction of involved white matter, and striae on T1 sagittal images. Asialotransferrin to transferrin ratio is less than 8%. Bottom: Patient with eIF2B-like clinical and radiologic presentation, but without eIF2B mutations. MRI reveals T2 signal intensity similar to CSF, FLAIR images consistent with rarefaction of involved white matter, and striae on T1 sagittal images. Asialotransferrin to transferrin ratio is more than 8%.

Table 1

Percent of asialotransferrin to total transferrin in studied samples

	Sample type				
	***EIF2B* mutated**	***eIF2B*-like, no mutation**	**Other leukodystrophy**	**Other neurologic**	**Control non-neurologic**
Range	0.93–7.77	7.98–28.92	5.57–48.97	8.63–26.98	10.72–30.32
Mean	4.51	16.46	15.82	18.87	18.98
SD	2.18	8.14	9.01	8.30	6.66

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Table 2

Clinical characteristics and mutation analysis relative to percentage of asialotransferrin in CSF in eIF2B mutated patients and eIF2B-like patients

Patient	% Asialotransferrin	Nucleotide changes	Amino acid change	Gene	Age at disease onset	Age ay death	Symptoms	GEF activity
DMN 03.01	1.46	1069C>T/1069C>T	R357W/R357W	*EIF2B4*	2 y	NA	Motor decline with febrile illness, epilepsy	NA
DMN 91.46	2.11	338G>A/1884G>A	R113H/W628X	*EIF2B5*	18 mo	6 y	Progressive ataxia after febrile illness	NA
DMN 96.115	3.71	512C>T/607–612delInsTG	S171F/M203fs	*EIF2B2*	10 y	NA	Scoliosis, gait disorder	NA
G648-2*	4.11	638A>G/638A>G	E213G/E213G	*EIF2B2*	7 y	NA	Progressive ataxia, behavior disturbance	64 ± 4
DMN 99.31	4.36	1028A>G/1153A>G	Y343C/I385V	*EIF2B5*	13 mo	5 y	Neurologic deterioration after febrile illnesses	NA
DMN 01.45*	4.52	338G>A/splicing site exon 10	R113H/splicing site exon 10	*EIF2B5*	2 y	NA	Progressive ataxia after fall	70 ± 1
DMN 91.55	4.65	338G>A/805C>T	R113H/R269X	*EIF2B5*	18 mo	5 y	Progressive ataxia, acute coma resulting in death	NA
DMN 05.06	4.69	1399C>T/1399C>T	R466W/R466W	*EIF2B4*	15 mo	NA	Neurologic deterioration after febrile illness	NA
DMN 01.11*	5.50	338G>A/338G>A	R113H/R113H	*EIF2B5*	4 y	NA	Hemiparesis after fall	77.5 ± 2.5
DMN 02.02	7.77	338G>A/806G>T	R113H/R269L	*EIF2B5*	2 y	NA	Progressive ataxia w/o provoking event	NA
DMNL 99.001	4.8	47C>A/338G>A	A16D/R113H	*EIF2B5*	3 y	NA	Progressive ataxia after mild head trauma	NA
NA	13.81	None	None	None	5 y	NA	Progressive ataxia w/o provoking event	NA
NA	20.78	None	None	None	3 y	NA	Progressive ataxia w/o provoking event	NA
NA	14.69	None	None	None	12 y	NA	Progressive ataxia w/o provoking event	NA
NA	8.64	None	None	None	4 y	NA	Progressive ataxia w/o provoking event	103.8 ± 2.8
NA	8.60	None	None	None	37 y	NA	Progressive ataxia w/o provoking event	99 ± 3
NA	22.42	None	None	None	3 y	NA	Progressive ataxia w/o provoking event	111.3 ± 3.7

*Patients previously included in Fogli et al., 2004.[27]

GEF = guanine nucleotide exchange activity, measured in lymphoblasts from affected patients; NA = not available or still alive; fs = frameshift.