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Short communication

Critical issues for the proper diagnosis of Metachromatic Leukodystrophy

Laura Lorioli [a,b,c,d], Martina Cesani [a], Stefano Regis [e], Francesco Morena [f], Serena Grossi [e], Francesca Fumagalli [b,g], Serena Acquati [a], Daniela Redaelli [a], Antonella Pini [h], Maria Sessa [b,g], Sabata Martino [f], Mirella Filocamo [e], Alessandra Biffi [a,b,c,*]

[a] San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
[b] HSR-TIGET Pediatric Clinical Research Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
[c] Pediatric Immunohematology, San Raffaele Scientific Institute, Milan, Italy
[d] Vita Salute San Raffaele University, Milan, Italy
[e] "Centro di Diagnostica Genetica e Biochimica delle Malattie Metaboliche", Istituto G. Gaslini, Genova, Italy
[f] Department of Experimental Medicine and Biochemical Sciences, Sect. Biochemistry and Molecular Biology, University of Perugia, Italy
[g] Neurology Unit, Department of Neurology, San Raffaele Scientific Institute, Milan, Italy
[h] Child Neuropsychiatric Unit, IRCCS Istituto Scienze Neurologiche, AUSL Bologna, Bologna, Italy

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ABSTRACT

Metachromatic Leukodystrophy is a lysosomal storage disorder caused by Arylsulfatase A deficiency. Diagnosis is usually performed by measurement of enzymatic activity and/or characterization of the gene mutations. Here we describe a family case in which the determination of enzyme activity alone did not allow diagnosis of the pre-symptomatic sibling of the index case. Only combination of gene sequencing with thorough biochemical analysis allowed the correct diagnosis of the sibling, who was promptly directed to treatment.

1. Introduction

Metachromatic Leukodystrophy (MLD; MIM# 250100) is a rare inherited lysosomal storage disorder caused by the deficiency of Arylsulfatase A (ARSA; E.C. 3.1.6.8). The disease manifests itself with a broad spectrum of clinical variants characterized by progressive demyelination and neurodegeneration in the central and peripheral nervous systems (Von Figura et al., 2001). Patients' diagnosis is usually performed by biochemical evaluation of the residual enzymatic ARSA activity (Baum et al., 1950b) and/or molecular characterization of the *ARSA* gene (MIM# 607574) through complete sequencing of the 8-exon-encoding region and exon–intron boundaries (GenBank accession numbers: NC_000022.9; NM_000487.4 and NP_000478.2). Measurements of sulfatide excretion in urines are also recommended as part of the diagnostic investigations (Philippart et al., 1971b; Von Figura et al., 2001). Residual enzyme activity does not correlate with the severity of the clinical manifestations, while a genotype–phenotype correlation has been established (Biffi et al., 2008; Polten et al., 1991).

Here we describe a family case in which the determination of the residual enzyme activity alone was not exhaustive in providing a definitive diagnosis of MLD in a pre-symptomatic sibling of the index case and of MLD carrier in the mother.

2. Materials and methods

2.1. Patients

The index case (Sib1), born to non-consanguineous parents of Italian origin, was evaluated at 34 months of age for recent appearance of motor and cognitive impairment. Her younger sister (Sib2), aged 10 months at the time, did not show any symptoms of the disease. Following ethical guidelines, cell and/or nucleic acid samples were obtained for analysis and storage with the individuals' (and/or a family member's) written informed consent. The consent was sought using a form approved by the local Ethics Committee.

2.2. ARSA activity determination with p-nitrocatechol sulfate (p-NCS)

ARSA residual activity was determined in homogenates of leukocytes from all family members, as described (Baum et al., 1950a). Briefly,

Abbreviation: ARSA, Arylsulfatase A; ARSB, Arylsulfatase B; DEAE Cellulose chromatography, Diethylaminoethyl Cellulose chromatography; HD, Healthy Donor; MLD, Metachromatic Leukodystrophy; MRI, Magnetic Resonance Imaging; 4-MUS, 4-methyl-umbellipheryl-sulfate; p-NCS, p-nitrocatechol sulfate; PD allele, PseudoDeficiency allele; SapB, Saposin B; Sib, Sibling.

* Corresponding author at: HSR-TIGET, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy.
E-mail address: biffi.alessandra@hsr.it (A. Biffi).

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cells were resuspended in sodium acetate trihydrate 0.05 M (500.000 cells in 25 μl). Samples were then sonicated and centrifuged and the supernatant was collected for ARSA activity and protein quantification (Bradford assay—Biorad #500-0006). ARSA activity was detected with p-nitrocatechol sulfate (p-NCS) as substrate, loading a maximum concentration of protein extract of 0.3 μg/μl diluted in sodium acetate trihydrate 0.05 M.

2.3. Urine sulfatide determination

Sulfatide excretion was evaluated in 24-h urine collections by thin layer chromatography as described (Philippart et al., 1971a).

2.4. DEAE-cellulose chromatography and ARSA activity determination

Leukocytes were harvested, washed in phosphate-buffered saline, and resuspended in 10 mM TRIS/HCl buffer, pH 7.5, containing 0.1% (v/v) Nonidet NP40 detergent (Sigma-Aldrich). Cell lysates were subjected to three rounds of sonication at 4 °C. Proteins were measured by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

500000 cells were loaded into Diethylaminoethyl Cellulose (DEAE) chromatography columns and enzyme activity retained by the column was eluted in fractions, as described (Biffi et al, Science, 2013) (Martino et al., 2005).

ARSA activity was then assayed on the fractions by using the artificial fluorogenic substrate 4-methyl-umbellipheryl-sulfate (4-MUS), in the presence and absence of 125 μM $AgNO_3$ (a specific ARSA inhibitor—ARSA activity is calculated by subtracting the value obtained in the presence of $AgNO_3$, corresponding to Arylsulfatase B activity, from that measured in the absence of the inhibitor, corresponding to ARSA + ARSB activity), and the natural substrate sulfatide, as described (Biffi et al, Science, 2013) (Chang et al., 1981a; Christomanou and Sandhoff, 1977a; Martino et al., 2005).

2.5. Molecular studies

Genomic DNA was extracted from cultured fibroblasts or peripheral blood leukocytes using QIAmp DNA blood mini kit according to the manufacturer's protocol (Qiagen).

Total RNA was extracted from cell lines using Rneasy mini Kit (Qiagen). First-strand cDNAs were synthesized by Advantage RT-for-PCR Kit (Clontech) using random hexamer primers.

The products from PCR and/or RT-PCR were purified and directly sequenced using an ABI 377 DNA automated sequencer with dye terminator cycle sequencing kits (Life Technologies).

3. Results and discussion

Sib1 is the index case of the family we are describing. She presented the first symptoms of the disease at 18–19 months of age, with motor and cognitive regression; brain MRI performed 10 months after symptom onset documented a demyelinating pattern suggestive of MLD. ARSA activity, tested by the artificial substrate para-N-cathecol-sulfate (p-NCS), was consistent with MLD diagnosis, being ≤15% of the normal values (Fig. 1A). Molecular analysis revealed compound heterozygosity for the known *ARSA* alleles [c.459+1G>A; p.W193C; p.T391S] and [c.849-1G>A; *96A>G; p.W193C;p.T391S] (Fig. 1A) (Biffi et al., 2008; Regis et al., 2004).

In light of this diagnosis, a comprehensive evaluation was planned on the asymptomatic younger Sib2, aged 10 months at the time. As expected at that age, brain MRI was not informative for diagnostic purposes, because the physiological process of myelination was not yet completed. While ARSA activity on leukocytes, tested by p-NCS-based assay, resulted equal to 50% of normal values, molecular analysis revealed the presence of both the mutant alleles identified in Sib1 at two separate testings (Fig. 1A). The presence of disease-associated mutations on the ARSA gene of both siblings rendered not necessary to investigate the SapB gene sequence. To complete the diagnostic work up, sulfatide excretion was evaluated in a 24-h urine collection (Philippart et al., 1971b; Polten et al., 1991) and resulted abnormal (Fig. 1A). In addition, molecular testing extended to the parents revealed their carrier status. However, ARSA activity tested by p-NCS-based assay in the mother was much higher than expected (Fig. 1A). Indeed, she carries the so-called "allele I" [c.459+1G>A; p.W193C; p.T391S], which has always been found in association with the severe late-infantile form of the disease and demonstrated not to encode for functional ARSA polypeptides(Polten et al., 1991).

To better interpret these results more detailed biochemical investigations were performed. An alternative ARSA assay, based on the use of the artificial substrate 4-methyl-umbelliferyl-sulfate (4-MUS) in the presence and absence of $AgNO_3$ (a specific ARSA inhibitor)(Chang et al., 1981b; Christomanou and Sandhoff, 1977b), did not generate supportive data (Fig. 1A). On the contrary, DEAE-cellulose chromatography that allows detection and isolation of ARSA(Martino et al., 2005) was informative, as the tested samples showed distinct patterns as compared to healthy donors (HDs) and canonical MLD patients (Fig. 1B). ARSA activity of the paternal sample had only a slight variation of the elution point by chromatography compared to HDs (Fig. 1B) and the eluted ARSA peak showed a sulfatide hydrolytic activity similar to that of HDs (2.65 vs 3.30 nmol/0.003 mU)(Fig. 1A), indicating that the allele [c.849-1G>A; *96A>G; p.W193C; p.T391S] has low influence on the protein chromatographic characteristics. The maternal genotype was instead associated to a more complex scenario characterized by the presence of two peaks of activity eluted by chromatography (Fig. 1). The first peak (I), eluted at 0.088 M NaCl, was poorly sensitive to $AgNO_3$ and was absent in HDs, in the carrier-father's chromatogram and in classical MLD profiles (Fig. 1). Surprisingly, peak (I), despite differing from what expected as canonical ARSA enzyme profile, was able to hydrolyze the sulfatide (1.62 ± 0.7 nmol)(Fig. 1A). The second peak (II) was eluted at the same NaCl concentration as ARSA in HDs (0.15 M) and had a sulfatide hydrolytic activity comparable to HDs (3.11 ± 0.2 nmol), thus could be considered *bona fide* representative of ARSA enzyme. The samples retrieved from the two siblings displayed a unique peak of activity eluted at the same salt concentration of the maternal peak (I), and showing a similar sulfatide hydrolytic activity (1.6 ± 0.1 and 1.3 ± 0.3 nmol, respectively). Of note, no peak corresponding to *bona fide* ARSA activity was retrieved from the siblings, consistently with their diagnosis of late-infantile MLD.

Family chromatographic peaks eluted by the gradient of NaCl were also tested toward 4-MUS and p-NCS at 0 °C for 15 h (Lee-Vaupel and Conzelmann, 1987; Rip and Gordon, 1998). The activity of all peaks was preserved at 0 °C, thus excluding the ARSB contribution (Fig. 1A).

The isolated peak (I) is not associated to the pseudodeficiency status, since it is present in the mother, who does not have the PD allele, while it is absent in the father, who carries the PD allele in heterozygosis (Fig. 1A). Moreover, the presence of this peak does not seem to exert a protective effect on the clinical outcome: indeed, Sib 1 shows the typical disease course expected by her severe mutations. Although additional studies are needed to characterize the enzyme activity isolated as peak (I) by chromatography, our findings as well as recent reports (Wiegmann et al., 2013) suggest the existence of other enzymes with sulfatase-like activity, which may affect the diagnostic potential of routinely employed ARSA activity assays.

4. Conclusion

The diagnostic workup for Metachromatic Leukodystrophy is usually subsequent to the appearance of the first symptoms of the disease in the