



Short Communication

Sixteen novel mutations in the arylsulfatase A gene causing metachromatic leukodystrophy

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ARTICLE INFO

Article history:

Accepted 17 August 2013

Available online 31 August 2013

Keywords:

Arylsulfatase A

Metachromatic leukodystrophy

Lysosomal storage diseases

MLD

Sulfatides

Genotype–phenotype correlations

ABSTRACT

Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused mainly by mutations in the arylsulfatase A (ARSA) gene. In this manuscript we report sixteen novel mutations identified in the ARSA gene of fifteen unrelated patients affected with MLD. Of these 16 mutations nine were missense mutations (p.L11Q, p.S44P, p.L81P, p.R84L, p.V177D, p.P284S, p.R288S, p.G301R, p.P425S), three were nonsense mutations (p.Q51X, p.Y149X, p.C156X), three were frame shift mutations (c.28delG, c.105C→A+106_124dup, c.189delC) and one was a splice-site mutation (c.1102-2A>G). In addition, three previously reported mutations were identified on an allelic background different from the one in the original reports. Two mutations, p.G309S and p.E312D, were identified on the background of the so-called pseudodeficiency (Pd) allele while previously they were reported alone. On the other hand, mutation p.R311X was identified in two unrelated patients not in cis with the Pd mutations, as previously reported.

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1. Introduction

Metachromatic leukodystrophy (MLD) is a neurodegenerative lysosomal storage disease caused, in the majority of cases, by the deficiency of arylsulfatase A (ARSA, E.C. 3.1.6.1) activity resulting in accumulation of sulfatides in the nervous system. The disease is transmitted as an autosomal recessive trait and it is one of the most common among the lysosomal storage diseases with an estimated frequency of 1.45:100,000 births (Giugliani, 2012). In our Lysosomal Diseases Testing Laboratory nearly 800 cases of MLD have been diagnosed in samples from patients with neurological symptoms. Metachromatic leukodystrophy is characterized by progressive demyelination resulting in a wide range of neurologic symptoms. Three forms of the disease are recognized according to the age of onset: late infantile, juvenile and adult forms. The late infantile form is the most common, accounting for 50–60% of all cases, with onset between one and three years of age. The most frequently reported symptoms are loss of acquired skills and difficulty walking. Patients with the juvenile form of MLD present between 4 and 14 years of age, while in the adult form the onset is after puberty. In the juvenile cases the most commonly reported signs of the disease are decreased school performance, gait abnormality, weakness, and abnormal behavior while in the adults the presentation can be neuromuscular or behavioral.

A diagnosis of MLD can be reached by detecting low ARSA enzymatic activity in several tissues (like leukocytes or fibroblast) together with excess excretion of sulfatides in the urine. Due to the high frequency of the so-called pseudodeficiency (Pd) allele (Gieselmann et al, 1989) the identification of low ARSA activity alone is not diagnostic of MLD. Individuals with two copies of the Pd allele can have the ARSA activity in the same range of MLD patients, but they are not affected with the disease. Also, they do not excrete sulfatides in urine. However, it should be noted that some disease-causing mutations have been found in cis with the Pd allele (Gieselmann et al, 1991; Rafi et al, 2003). On the other hand, some individuals with a deficiency of saposin B, an activator protein needed for the proper functioning of arylsulfatase A (Inui et al, 1983), have a normal ARSA enzymatic activity but are affected with MLD, as confirmed by the presence of sulfatides in urine.

Since the cloning of the ARSA cDNA (Stein et al, 1989) and gene (Kreysing et al, 1990) more than 150 mutations have been reported (The Human Gene Mutation Database). Two mutations are identified quite frequently: the 459+1G>A change, a splice-site mutation, is associated with late-infantile onset of the disease and is commonly referred to as “the common late infantile (LI) mutation” and the p.P426L, a missense mutation, is associated with a later onset of the disease and is commonly called “the common adult mutation” (Polten et al, 1991). Patients who are compound heterozygotes for these two mutations have a juvenile form of the disease. While an initial diagnosis of MLD is based on finding low ARSA activity using a synthetic substrate in leukocytes or fibroblasts, the residual activity measured is not predictive of the age of onset or clinical course. In general we can hypothesize that a mutation that results in very low ARSA activity in vivo would indicate an early onset depending on the mutation in the second allele. While mutations that have some

Abbreviations: ARSA, arylsulfatase A; DD, developmental delay; LI, late infantile; MLD, metachromatic leukodystrophy; MRI, magnetic resonance imaging; Pd, pseudodeficiency; PCR, polymerase chain reaction; WM, white matter.

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residual activity in vivo are predicted to result in a later-onset form of MLD. In many cases the assignment of mutation severity can be based on the combination of alleles found and the clinical presentations of patients.

In our laboratory, once the diagnosis of MLD is confirmed by the detection of sulfatides in urine, mutation analysis of the ARSA gene is performed if requested by the patient's family and physician. In this manuscript we report 16 novel mutations identified in the ARSA gene of patients with metachromatic leukodystrophy. In addition we describe three previously reported mutations in an allelic background different from the one originally reported.

2. Materials and methods

2.1. Patients

Blood samples from 16 of the 19 patients reported in this manuscript and cultured skin fibroblasts from patient # 15 were sent to our laboratory to be screened for a lysosomal storage disorder (Table 1). The screen resulted in a low ARSA value, indicating a possible diagnosis of MLD (Table 1). A urine sample was then requested to evaluate the presence of excess sulfatides to confirm the MLD diagnosis (Table 1). Patients # 8 and # 18 were previously diagnosed elsewhere but samples were sent here for mutation analysis. For patient # 18 we only received a DNA sample for sequencing, therefore no ARSA activity or sulfatides values are available, while for patient # 8 we received both DNA and urine to confirm the diagnosis. No urine sample was received for patient # 15. Blood samples from parents were requested for all diagnosed patients.

In Table 1, the clinical characteristics of the patients described in this report are summarized. The information was provided to us by the physician requesting the tests: some descriptions reported a detailed record of the presenting symptoms while others contained minimal information. Age of onset was established based on the age at the

time of diagnosis, clinical information and history of initial symptoms provided by the patient's caregivers.

All procedures were in compliance with institutional guidelines and testing was performed with the consent of the patient's parents.

2.2. Determination of arylsulfatase A activity and urine sulfatides

Leukocytes were isolated from whole blood (Wenger and Williams, 1991) and ARSA activity was measured in leukocyte or fibroblast sonicates using p-nitrocatechol sulfate substrate (Baum et al, 1959).

Presence of excess sulfatides in urine was detected as previously described (Rafi et al, 2003).

2.3. DNA extraction and molecular analysis

DNA was extracted from the remaining sonicated samples using a DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions. The DNA was then utilized to screen for the common LI mutation, the common adult mutation (p.P426L) and the Pd allele, using a rapid detection method based on PCR amplification followed by restriction digestion (Ben-Yoseph and Mitchell, 1994; Chabás et al., 1993).

For sequence, the full length ARSA gene was amplified in two overlapping fragments using a GeneAmp XL PCR Kit (Applied Biosystem). The fragments were respectively 1980 bp and 1400 bp in size. The first one spanned from 100 bp before the initial ATG to the beginning of intron 6 and the second fragment spanned from the end of intron 4 to 140 bp beyond the polyadenylation signal. The primers used to amplify the first fragment were: sense primer (ctc agg gaa ggg cgg cgc) and antisense primer (ggg cca agg atc tgg gat ca); for fragment II: sense primer (cca tgg ctc atg agc gcc tcc) and antisense primer (cag tt cct cat tcg tac cac ag).

The PCR products were then purified using a QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions. Sequencing of the ARSA gene was performed at the Kimmel Cancer Center Molecular Analysis Laboratory at Thomas Jefferson University using the BigDye Terminator v3.1 cycle sequencing kit with the ABI 3730xl

Table 1
Clinical and biochemical findings in patients included in this study.

Patient #	Age at onset	Signs and symptoms	ARSA activity ^a	Sulfatide Excretion ^b	Ethnicity
1	2.5 y	Spasticity, loss of gross motor milestones, cognitive problems, hypertonia	11.2	+	Hispanic, 2 more siblings with same genotype
2	3 y	Regression of milestones since 1 year of age after high fever, increased tone, spasticity, rigidity, diffuse WM disease on MRI	13.0	+	From Kenya
3	2 y	Ataxia, motor regression, increased T2 periventricular WM signal on MRI	8.1	+	Unknown
4	2.5 y	Axial hypotonia, legs extended, hand fisted, swallowing dysfunction with increased choking and gagging, unable to sit, nystagmus, MRI c/w leukodystrophy, nonverbal, irritable	12.5	+	African American
5	2 y	Altered mental status	10.3	+	Adopted from Ethiopia
6	2 y	Difficulty walking with recent rapid regression of skills	2.7	+	Unknown
7	2 y	Hypotonia and developmental regression, spasticity, unable to sit unassisted, slurred speech, tight heel cords	5.8	+	Caucasian
8	2.0 y	Motor, speech, and cognitive regression starting at 2 years of age, opisthotic posturing, hypertonia, atrophy of muscle, cachectic, diffuse leukodystrophy on MRI	NA	+	Arabic
9	2.5 y	Progressive gait abnormalities, spasticity, dysarthria, dysmetria, dysphagia, MRI showing WM changes	4.8	+	Caucasian
10	6.5 y	Cognitive regression in the past 3 months, increasing dystaxia, loss of bowel/bladder function, generalized hypotonia, MRI c/w leukodystrophy	7.1	+	Unknown
11	12 y	Developmental delays	7.9	+	Caucasian
12	2.5 y	DD, difficulty walking, genu valgus, decreased balance and coordination, speech delay, staring episodes	9.2	+	Caucasian
13	2.5 y	Wide based gait, unsteady, speech delay, absent reflexes, MRI c/w leukodystrophy	15.4	+	Chinese
14	15 y	Progressive cognitive decline since 9th grade, decrease in IQ, WM changes on MRI. Gait, muscle stretch reflexes, cerebellar testing all normal. No problems with coordination or loss of strength	4.1	+	Caucasian
15	11 y	MRI showing diffuse abnormal WM and thin corpus callosum, loss of cerebral volume, kyphoscoliosis, microcephaly	8.3	NA	From Saudi Arabia
16	2.5 y	Nystagmus noted at 9 months, hypotonia, regression of milestones, legs extended, tremors, spasticity of lower limbs, muscle wasting	5.9	+	Unknown
17	2 y	Lack of coordination	0	+	Caucasian
18	12 y	Behavioral problems, dropping IQ, abnormal CT	NA	NA	Unknown
19	3 y	Progressive gait ataxia, mild speech delay, mild hypotonia, MRI showing WM changes	1.2	+	Caucasian

Abbreviations: y = years; NA = not available; WM = white matter; DD = developmental delay; and c/w = consistent with.

^a ARSA activity expressed as nmol/h/mg protein. Normal mean = 70; Affected range 0–16.

^b Presence (+) or absence (–) of excess sulfatides in urine.

DNA analyzer (Applied Biosystem). The results were provided as chromatograms. If some areas of the sequence were not clear (high background noise, peaks not well resolved, intensity of the peaks too low) or even a single base pair was unclear, new sequencing reactions were prepared using different sense or antisense primers.

Also, when available, parental DNA was analyzed to confirm the presence of the mutations found in the patient and to establish, in cases where more than two changes were found, on which allele they occurred.

The mutation database HGMD-professional version 2013.1 (accessed at <http://www.hgmd.cf.ac.uk>) was consulted to verify that the novel mutations identified were not previously reported. In addition, the exome variant database (<http://evs.gs.washington.edu/EVS/>) was consulted to rule out that these novel mutations were polymorphisms.

Novel mutations were also analyzed *in silico* using the program PolyPhen2 (accessed at <http://genetics.bwh.harvard.edu/pph2/>) and the program Provean (Protein Variation Effect Analyzer, accessed at <http://provean.jcvi.org>) to predict the effect on the ARSA protein.

3. Results and discussion

Currently more than 70 patients with MLD have been characterized molecularly in our laboratory: 16 previously unreported mutations have been identified in 15 unrelated patients. All the other patients had previously reported mutations. These new mutations are described in this manuscript (Table 2). Also, three previously reported mutations were identified on a different allelic background than in the previous reports (Table 3). With the exception of three patients being homozygous for new mutations, and one (# 8) having two different new mutations, all the others are compound heterozygous with a second mutation previously reported (Table 2). Of the 19 patients described, the majority had the late infantile form of the disease (see age of onset in Table 1) and only 5 (patients # 10, 11, 14, 15 and 18) had the juvenile form. Six of the 19 patients had the common LI mutation as the second allele and two had the common adult mutation as the second allele. Of the 16 new mutations reported, nine were missense mutations, three were nonsense, three were frame shift mutations and one was a splice-site

mutation. To evaluate the effect of the novel mutations on the protein activity, the mutations reported in this paper were analyzed *in silico* using two different programs, PolyPhen2 and Provean. With the PolyPhen2 program only missense mutations could be analyzed, utilizing a scale between zero (benign mutation) and one (probably damaging). With this program eight of the reported missense mutations were predicted to be “probably damaging” with scores equal or very close to 1.00 indicating high confidence (Table 2). The p.Y39F missense mutation had a lower score (0.788) that was indicated as “possibly damaging”. This mutation was found in cis with p.Y149X. Of these two novel mutations p.Y149X was considered to be the disease-causing mutation with p.Y39F being regarded as an additional change of uncertain significance. With the Protean program missense and nonsense mutations were analyzed: variants with a score equal to or below −2.5 are considered “deleterious”, while variants with a score above −2.5 are considered “neutral”. Of the novel missense mutations analyzed six were indicated as “deleterious” by this program, while p.Y39F, p.V177D and p. R288S were considered “neutral” (Table 2). In addition, of the 3 nonsense mutations reported two were indicated as “deleterious” and p.Q51X was considered “neutral”. Mutation c.32T>A was predicted to be a “benign” mutation by both programs (see patient # 2).

- The c.28delG mutation found in patient # 1 is located in the leader sequence. However, this deletion results in a frame shift in exon 1 and it is therefore predicted to be severe. This mutation was identified in a family of Hispanic origin with three affected siblings with the same genotype. The youngest of the three is a 30-month-old presenting with spasticity, cognitive problems and loss of motor milestones (Table 1). It is not clear at what age the other two affected siblings started to show signs of the disease, but both were diagnosed around 7 years of age. The second mutation identified in this family is p.E382K. This mutation was reported by Barth et al (1993) and found homozygous in a juvenile MLD patient in cis with the Pd allele. However, in our patient this mutation is not associated with the Pd allele. Other investigators identified this mutation in a typical late infantile patient (Shotelersuk et al, 2004) and

Table 2
Summary of mutations found in patients reported in this study.

Patient #	Allele I			Probability of deleterious mutation ^a		Comment	Allele II		
	cDNA position (gene position)	Location	Effect	PolyPhen2	Provean		cDNA position (gene position)	Location	Effect
1	28delG (28)	Ex1	GGCC > GCC, A10fs	NA	NA	Homozygous	1144G>A (2133)	Ex7	E382K
2	32T>A	Ex1	L11Q	0.12	−1.638				
3	105C>A+106_124dup (105)	Ex1	D35E + P42fs	NA	NA		1277C>T (2381)+ Pd	Ex8	P426L
4	130T>C (130)	Ex1	S44P	1.00	−2.856		459+1G>A	Int2	Loss of splice donor site
5	151C>T (151)	Ex1	Q51X	NA	−2.251	Homozygous			
6	189delC (189)	Ex1	TACG > TAG, Y63X	NA	−6.918		740T>C (1077)	Ex4	F247S
7	242T>C (391)	Ex2	L81P	1.00	−3.188		459+1G>A	Int2	Loss of splice donor site
8 ^b	251G>T (400)	Ex2	R84L	1.00	−6.710	Both mutations on same allele	1273C>T (2377)	Ex8	P425S
9	116A>T (116)+447C>G (596)	Ex1 + Ex2	Y39F + Y149X	0.788 (Y39F)	−2.042 (Y39F) −6.156 (Y149X)		634G>C (897)	Ex3	A212P
10	468C>A (731)	Ex3	C156X	NA	−6.711		1277C>T (2381)	Ex8	P426L
11	530T>A (793)	Ex3	V177D	1.00	−1.894		459+1G>A	Ex8	Loss of splice donor site
12	850C>T (1499)	Ex5	P284S	1.00	−7.482	Homozygous	635C>T (898)	Ex3	A212V
13	862C>A (1511)	Ex5	R288S	0.999	−1.945		730C>T (1067)	Ex4	R244C
14	901G>A (1550)	Ex5	G301R	1.0	−7.504		536T>G (799)	EX3	I179S
15	1102-2A>G	Int6	Loss of splice acceptor site	NA	NA	Homozygous			
8 ^b	1273C>T (2377)	EX8	P425S	1.0	−6.038		251G>T (400)	Ex2	R84L

New mutations are listed in bold. NA = not applicable.

^a Scores obtained by *in silico* analysis of the mutations using the program PolyPhen2 and the program Provean.

^b This patient has two new mutations.

Table 3
Mutations previously reported on a different allelic background.

Patient #	Mutations previously found on a different background						Second mutation			
	cDNA position (gene position)	Location	Effect	Probability of deleterious mutation ^a		Presence of Pd mutation in cis	Comment	cDNA position (gene position)	Location	Effect
				PolyPhen2	Provean					
16	925G>A (1574)	Ex5	G309S	1.00	−5.928	+	Previously reported without Pd	459+1G>A	Ex8	Loss of splice donor site
17	931C>T (1580)	Ex5	R311X	NA	−5.928	−	Previously reported on Pd allele	459+1G>A	Ex8	Loss of splice donor site
18	931C>T (1580)	Ex5	R311X	NA	−5.928	−	Previously reported on Pd allele	536T>G (799)	Ex3	I179S
19	936G>C (1585)	Ex5	E312D	0.983	−2.114	+	Previously reported without Pd and G>T change	459+1G>A	Ex8	Loss of splice donor site

NA = not applicable.

^a Scores obtained by *in silico* analysis of the mutations using the program PolyPhen2 and the program Provean.

described that the pathogenic effect is not due to the amino acid substitution but to the disruption of a potential exonic splicing enhancer resulting in a complete exon 7 skipping.

- Patient # 2 from Kenya was found to be homozygous for a c.32T>A mutation (p.L11Q). *In silico* analysis gives a “neutral” score with both programs used predicting a “benign” effect on the protein activity. This mutation results in a substitution in the leader sequence of a leucine with a glutamine at position 11. No other changes were identified in this patient after repeated sequencing of the ARSA gene. In addition, one copy of this mutation was confirmed to be present in both parents. The father's ARSA gene was also sequenced completely and no other changes were identified. The patient has an enzymatic activity for the arylsulfatase A in the affected range, excretes sulfatides and has a clinical presentation consistent with late infantile MLD (Table 1). The ARSA activities for both parents are in the carrier range. The 18-amino acid signal peptide for the ARSA gene has the typical structure of a leader sequence: the core of the peptide contains a long stretch of 12 hydrophobic amino acids. Mutation p.L11Q is located in the middle of this structure and introduces a charged amino acid that disrupts the hydrophobic core. The hydrophobic core in signal peptides is required for secreted proteins to be efficiently translocated across the endoplasmic reticulum (von Heijne, 1985). We hypothesize that this mutation is disease-causing due to the fact that the ARSA protein is inefficiently processed. Mutations in the signal peptides of other proteins have been reported as the cause of the disease due to the deficiency of that specific protein (Arnold et al, 1990; Rosenblum et al, 1996; Zschenker et al, 2001).
- The duplication of 19 base pairs starting at cDNA position 106 proceeded by a substitution c.105C>A (patient # 3) also results in a frame shift beginning at amino acid position 42. This mutation is therefore predicted to be “severe”. This mutation was found in trans with the common adult mutation which is in cis with the Pd allele. As reported before (Regis et al, 2002), the common adult mutation in cis with the Pd mutation results in a more severe phenotype than the adult mutation alone. Patient # 3 in fact presented at 2 years of age with a typical late infantile form of the disease (Table 1).
- Mutation c.130T>C found in patient # 4 results in a substitution of a serine at position 44 with a proline. This mutation in an African American patient was found together with the common LI mutation (Table 2). The patient had the typical late infantile presentation (Table 1) indicating that this new mutation is indeed severe as predicted by both *in silico* analysis programs.
- Mutation c.151C>T (patient # 5) results in a premature stop codon in exon 1 (p.Q51X). This mutation was found homozygous in a patient from Ethiopia with an early presentation of the disease. This is consistent with the expected severity of this mutation. The Provean program predicts this mutation to have a “neutral” effect on the protein

activity. This seems highly unlikely and raises concerns about the reliability of such programs.

- The deletion of a C at position 189, found in patient # 6, results in a premature stop codon (p.Y63X). This mutation was identified in a 2-year-old patient (Table 1) presenting with difficulty walking and rapid regression of skills. The second mutation, p.F247S, was previously reported in a juvenile patient in trans with the common adult mutation (Olkovich et al, 2003). The early onset and the rapid course of the disease in this patient indicate that not only the new reported deletion is severe (as predicted by the Provean program) but that also p.F247S must produce a protein with very little residual activity.
- Mutation c.242T>C (p.L81P) was identified in a patient with a typical late infantile presentation (Table 1, # 7) in trans with the common LI mutation. Based on the clinical presentation this new mutation is therefore severe. Both *in silico* analysis programs predict a “deleterious” effect on the protein.
- Patient # 8 was a patient of Arabic origin who was diagnosed at 4.5 years of age. He started to show signs of regression at 2 years of age and at the time of diagnosis the stage of disease was very advanced (Table 1). Two previously unreported mutations were identified in this patient: c.251G>T (p.R84L) in exon 2 and c.1273C>T (p.P425S) in exon 8. Both *in silico* analysis resulted in a score predicting a “damaging” effect for both mutations indicating high confidence in the deleterious outcome on the ARSA protein activity. Also one copy of the Pd allele was detected in this patient. Parental DNA was not available, therefore it was not possible to establish which of the two mutations was in cis with the Pd mutation. It is interesting to note that different mutations have been reported on the same nucleotides, resulting in a different amino acid substitution: c.251G>A (p.R84Q) (Kappler et al, 1992) and c.1273C>A (p.P425H) (Marcão et al, 2003). Mutation p.R84Q has been reported with and without the Pd allele (Coulter-Mackie and Gagnier, 2003; Rafi et al, 2003).
- Patient # 9 presented at 2.5 years of age with characteristic symptoms of late infantile MLD. Mutation analysis showed the presence of two new mutations in cis: p.Y39F in exon 1 and p.Y149X in exon 2. The disease-causing mutation was considered to be p.Y149X since it creates a premature stop codon, with p.Y39F being regarded as a change of unknown significance. This is also in agreement with the results of the *in silico* analysis (Table 2). The second mutation identified in this patient (p.A212P) was previously reported and is considered a severe mutation (Grossi et al, 2008). This is in agreement with the clinical picture of this patient.
- Mutation c.468C>A (p.C156X) was found in a 6.5-year-old patient (patient # 10) having the common adult mutation on the other allele. This new mutation is expected to be severe since it creates a premature stop codon in exon 3, as also predicted by the Provean program. Very recently, mutation p.C156X was identified in our laboratory homozygous in a typical late infantile patient of Hispanic origin

presenting at 2 years of age with regression of milestones, hypotonia and evidence of white matter disease (ARSA activity = 8.6 nmol/h/mg protein and presence of sulfatides in the urine). The juvenile onset in patient #10 is explained by the presence of the p.P426L mutation in trans with p.C156X.

- Mutation c.530T>A (p.V177D) was identified in a 12-year-old patient (# 11). The provided clinical history for this patient was minimal, with the major complaint being “developmental delays”. The second mutation is the common LI mutation, indicating that p.V177D is a milder mutation able to mitigate the effect of a severe mutation and to result in a juvenile form of the disease. The PolyPhen2 program predicts this mutation to be “damaging” with a score of 1 while according to the Provean program this mutation is “neutral”.
- Mutation c.850C>T (p.P284S) was identified in a 3-year-old patient (# 12) in trans with a previously reported severe mutation (p.A212V) (Barth et al, 1993; Coulter-Mackie et al, 1997). *In silico* analysis predicts a damaging mutation with both program used, and the clinical picture of this patient confirms the severity of the change.
- Mutation c.862C>A resulting in a substitution of an arginine with a serine at position 288 was identified in a 2.5-year-old patient (# 13) of Chinese ancestry. It is interesting to note that a mutation of the C at position c.862 with a T, resulting in a substitution of the arginine with a cysteine, has been reported before and was also identified in a Chinese patient (Gieselmann et al, 1994; Wang et al, 2007). The second mutation identified in this patient, p.R244C, was reported before (Draghia et al, 1997) and it is considered severe. The PolyPhen2 program predicts mutation p.R288S to be “damaging”, while according to the Provean program this mutation is “neutral”.
- Mutation c.901G>A (p.G301R) was identified in a juvenile/adult patient (# 14) with symptoms presenting around 15 years of age. The second mutation is a known later-onset mutation, p.I179S (Fluharty et al, 1991), characterized mostly by cognitive decline more than motor involvement, consistent with the disease presentation in this patient. *In silico* analysis with both programs predicts the new mutation p.G301S to be “damaging”, but no prediction of severity can be done for this mutation.
- The only splice-site mutation reported in this manuscript (1102-2A>G) was found homozygous in an 11-year-old patient from Saudi Arabia (# 15). The effect of this mutation is a loss of a splice acceptor site in intron 6 probably resulting in abnormal splicing. Since this mutation results in a juvenile form of the disease, some residual activity may be present.

In Table 3 are reported three mutations previously identified on a different allelic background.

Two of the mutations reported in this table, c.925G>A (p.G309S) and c.936G>C (p.E312D) were found in cis with the Pd allele while before they were not reported to be on the Pd allele (Hermann et al, 2000 and Kreysing et al, 1993).

The c.925G>A mutation on the Pd allele was identified in a late infantile patient (# 16) in trans with the common late infantile mutation. The previous report also identified this mutation in a late infantile patient (Kreysing et al, 1993).

The c.936G>C mutation was reported before with a G>T change still resulting in a substitution of a glutamine into asparagine (p.E312D) (Hermann et al, 2000). The patient described by Hermann et al presented at 13 years of age with an unusual slow progression of the disease. The authors attributed the milder form of the disease to the p.E312D mutation since the second mutation was a known severe mutation found previously in a late infantile patient. In our patient (# 19) the p.E312D mutation was in cis with the Pd allele and the second mutation was the common late infantile. This patient presented with symptoms at 3 years of age with progressive gait ataxia, mild hypertonia, white

matter changes on MRI and mild speech delay. His phenotype is clearly more severe than the one described by Hermann et al (2000), indicating that probably the presence of the Pd allele increases the pathogenicity of the mutation. This is not surprising since the association of one otherwise mild mutation with the Pd mutation is being shown to result in a more severe clinical phenotype like in the case of the common adult mutation (Regis et al, 2002). However, other factors, such as genetic background and environment, could also be responsible for the more severe phenotype of this patient. The PolyPhen2 program predicts mutation p.E312D to be “damaging” while according to the Provean program this mutation is “neutral”. The third mutation reported in Table 3 (p.R311X) was identified in two unrelated patients and in both of them it was not in cis with the Pd allele, as was found previously (Rafi et al, 2003).

In patient # 17 the p.R311X was in trans with the common late infantile mutation and the patient had the typical late infantile presentation of the disease as expected from two severe mutations.

In patient # 18 the p.R311X mutation was instead associated with the known late-onset mutation p.I179 (Fluharty et al, 1991) on the other allele and the patient presented around 12 years of age with mostly behavioral problems.

4. Conclusions

In this manuscript we report 16 novel mutations associated with metachromatic leukodystrophy. For the purpose of genotype-phenotype correlation, we tried to classify, when possible, these mutations as “severe” or “mild” based on the patient’s clinical presentation and age of onset and considering the “severity” of the second mutation, when it was known. *In silico* analysis of novel mutations was of limited value in predicting the severity of a mutation. In addition, the two different methods employed were not in agreement regarding the possible damaging effect of a mutation in three of the reported cases. It is important to have mutations identified in more patients to assist in patient management. This information will be useful for both families and physicians caring for these patients. Very recent preliminary results from a hematopoietic stem cell gene therapy clinical trial involving MLD patients have been published (Biffi et al, 2013). As approaches to treatment become more available it will be important to have more mutations characterized.

Conflict of interest

The authors declare they have no conflicting interest.

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