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ORIGINAL RESEARCH

Four novel *ARSA* gene mutations with pathogenic impacts on metachromatic leukodystrophy: a bioinformatics approach to predict pathogenic mutations

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Abstract: Metachromatic leukodystrophy (MLD) disorder is a rare lysosomal storage disorder that leads to severe neurological symptoms and an early death. MLD occurs due to the deficiency of enzyme arylsulfatase A (ARSA) in leukocytes, and patients with MLD excrete sulfatide in their urine. In this study, the *ARSA* gene in 12 non-consanguineous MLD patients and 40 healthy individuals was examined using polymerase chain reaction sequencing. Furthermore, the structural and functional effects of new mutations on *ARSA* were analyzed using SIFT (sorting intolerant from tolerant), I-Mutant 2, and PolyPhen bioinformatics software. Here, 4 new pathogenic homozygous mutations c.585G>T, c.661T>A, c.849C>G, and c.911A>G were detected. The consequence of this study has extended the genotypic spectrum of MLD patients, paving way to a more effective method for carrier detection and genetic counseling.

Keywords: psychomotor regression, demyelinating, gait abnormality and impairment, metachromatic leukodystrophy (MLD), behavioral disturbances

Introduction

Metachromatic leukodystrophy (MLD) is a rare lysosomal storage disorder, caused by deficiency of the enzyme arylsulfatase A (ARSA, E.C. 3.1.6.1) with a frequency of approximately 1 per 40,000 worldwide.[1] ARSA catalyzes initiative step of the metabolic pathway, sphingolipid 3′-o-sulfogalactosylceramide, known as sulfatide. Sulfatide is especially abundant in the myelin sheath of the nervous system.[2] Mutations in the *ARSA* gene (Figure 1) (GenBank accession number NP_000478) could lead to a deficiency in ARSA activity, leading to accumulation of sulfatide, especially in the nervous system.[3,4] This phenomenon causes a progressive demyelination that leads to different neurological symptoms including ataxia, an initially flaccid and later spastic paresis, optic atrophy, and dementia.[5]

To date, there is no effective treatment for MLD. However, bone marrow transplantation and stem cell therapy can be beneficial for patients with juvenile- and adult-onset forms in the early stages of the disease. In addition, gene and enzyme replacement therapies for the treatment of MLD have shown promising outcomes in mice models.[6–11]

ARSA deficiency is divided into 3 clinical subtypes: late-infantile (50%–60%), juvenile (20%–30%), and adult (15%–20%). The disorder course may range from 3 to 10 years or more in the late-infantile type and up to 20 years in the juvenile and adult types.[9]

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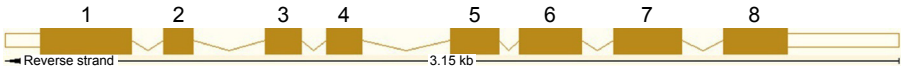

Figure 1 The *ARSA* gene maps to chromosome 22q13 covers 3.2 kb of genomic DNA and includes 8 exons.

Typical magnetic resonance imaging (MRI) alterations in MLD have been explained in the literature. T2-weighted MRI (T2W MRI) of the brain in MLD patients typically indicates butterfly-shaped confluent white matter hyperintensities with early involvement of the corpus callosum.[12] In addition, there is elevated white matter involvement, including U-fibers and cerebellar white matter, as well as cerebral atrophy with progression of the MLD disease.[13–16] In this study, the *ARSA* gene was examined in individuals who met the proposed clinical criteria for MLD in order to identify the pathogenic impact of the associated mutations in MLD.

Materials and methods

Patient collection and ethical statement

In this study, 12 Iranian non-consanguineous MLD patients, with a mean age of 3.5 years, were diagnosed between January 2009 and November 2012. Clinical characteristics of MLD patients are summarized in Table 1. Blood samples from 12 MLD patients and 40 healthy individuals were obtained from the Special Medical Center (SMC), Tehran, Iran. Written informed consent for genetic study and molecular analysis and consent to publish results was obtained from the healthy controls, patients, and parents on behalf of their children. The clinical ethics committee of SMC specifically approved this study (Approval No ML-41-1224) in December 2012. The exclusion criteria for healthy individuals were any history of familial and sporadic cancers, hereditary and non-hereditary metabolic disorders, and nuclear and mitochondrial DNA-associated disorders.

Enzymatic ARSA activity assay

ARSA activity was determined in leukocytes using p-nitrocatechol sulfate as described previously by Molzer et al.[17]

Neuroimaging (MRI) analysis

T2-weighted spin-echo sequences of the brain were carried out using a Siemens Magnetom Avanto 1.5 Tesla MRI (Munich, Germany). Images were evaluated in a blind fashion by a neuroradiologist.

DNA extraction and polymerase chain reaction (PCR)

The genomic DNA was extracted from the blood samples of MLD patients by the QIAamp DNA Micro Kit (Qiagen #56304). PCR primers[18] for amplification of exons 1–8 of the *ARSA* gene are as indicated in Table 2. Briefly, PCR was carried out in final volumes of 25 µL, containing 100–200 ng of total genomic DNA, 10 pmol of forward and reverse primers, 2.5 mM of $MgCl_2$, 200 mM of each

Table 1 Clinical and biochemical features of 12 Iranian MLD patients

Patient no	Age (years)	Initial manifestation	Age of onset (years)	Neuropathy	Seizures	Urine sulfatides	ARSA enzyme activity (%)
P1	8	Gait abnormality	5	Demyelinating	+	>20†	3
P2	5.5	Behavioral disturbances	4	Demyelinating	–	>20	2.8
P3	2.5	Psychomotor regression	2	ND	–	>20	0
P4	2	Psychomotor regression	1	ND	+	>20	1.1
P5	4	Psychomotor regression	2	Demyelinating	–	>20	2.5
P6	2	Psychomotor regression	1.5	DTR: absent	–	>20	1.5
P7	2	Psychomotor regression	1.5	DTR: absent	–	>20	1.2
P8	2.5	Psychomotor regression	1	ND	–	>20	2
P9	6	Gait impairment	4.5	Demyelinating	–	>20	2.8
P10	6	Behavioral	4.5	Demyelinating	–	>20	4
P11	3	Gait	2.5	Demyelinating	–	>20	2.5
P12	4	Psychomotor regression	3	ND	–	>20	2.8

Note: †Times higher compared to normal.
Abbreviations: MLD, metachromatic leukodystrophy; ND, not determined; DTR, deep tendon reflexes.

Table 2 PCR primers for amplification of *ARSA* gene in MLD patients

Primers	Exon	Primer sequence (5′–3′)	Amplicon size (bp)	TM (°C)
1	1	F: TCGGGGAGTATTTGGGTC R: GCAATCCATTGGGAGGAAAG	405	57
2	1*–2	F: TTGCCCGTCCGCCCAACATCGTG R: CCCTGGTCACAGCCACCGTCGCAAG	737	68
3	2*–4	F: GATTTCTAGGCATCCCGTACTC R: CCCTCACCCACTATGTTCTTG	706	62
4	5–7*	F: GCCAAGAACATAGTGGGTGAGG R: GGTAGAAGAAGAGAGACTGCCGAG	860	62
5	7–8	F: GCAAGAAGCGGTGCACGTCC R: CCACGACACCAGGGTTCAAATCC	916	63

Note: *Due to the large size of exons 1, 2, and 7, they are divided into 2 overlapping fragments.
Abbreviations: TM, temperature; PCR, polymerase chain reaction; MLD, metachromatic leukodystrophy; F, forward; R, reverse.

deoxyribonucleoside triphosphates (dNTP), and 1 U of super Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The PCR mixture was cycled for 35 times at 95°C for 1 minute; annealing temperature was based on temperature (TM) (°C) of forward and reverse primers (Table 2). The PCR products were examined on 2% agarose gel electrophoresis (Figure 2) in 0.5× Tris-borate-EDTA (TBE) buffer at 110 V for 50 minutes, and then stained with 0.002 mg/mL EtBr solution and visualized using ultraviolet light.

DNA sequencing and bioinformatics analysis

The PCR products were sequenced by forward or reverse primers on an ABI 3700 sequencer (Kosar Company, Tehran, Iran) and the results were compared using Finch TV program and were then analyzed on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Moreover, the target sequence of patient was compared with normal reference sequence and *ARSA* gene mutations in exons and the splicing sites of the introns were identified. The functional and structural impacts of identified novel mutations in *ARSA* gene were assessed using in silico prediction algorithms including SIFT,[19] PolyPhen-2,[20] and I-Mutant 2.0 (http://folding.biofold.org/i-mutant/i-mutant2.0.html).

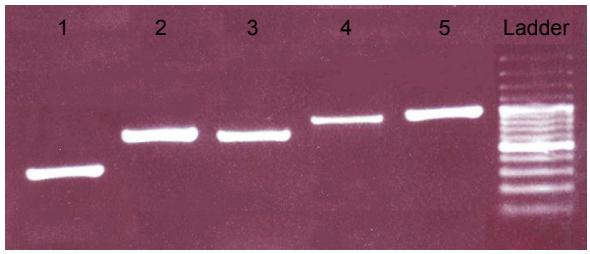

Figure 2 Agarose gel electrophoresis of PCR product. The presence of PCR products was confirmed by analyzing the products on a 2% agarose gel. From left: lane 1: exon 1 (405 bp), lane 2: exon 1–2 (737 bp), lane 3: exon 2*–4 (706 bp), lane 4: exon 5–7* (860 bp), lane 5: exon 7–8 (916 bp), lane 6: DNA ladder (Thermo Scientific Gene Ruler 100 bp #SM0241/2/3).
Abbreviation: PCR, polymerase chain reaction.

Statistical analysis

The chi-square test was used with Statistical Package for the Social Sciences (version 13) to examine the association between patient and healthy control samples, whereas *P*-value <0.05 was considered statistically significant.

Results

At T2W MRI, MLD patients demonstrated symmetric confluent areas of high signal intensity in the periventricular white matter (Figure 3: arrows in A–C) with sparing of the subcortical U-fibers (Figure 3: arrows in A). No enhancement is evident in computed tomography or MRI. The tigroid (Figure 3: arrows in D) and leopard skin (Figure 3: arrows in E and F) patterns of demyelination, suggesting sparing of the perivascular white matter, are identified in the periventricular white matter and centrum semiovale. The corpus callosum, corticospinal, and internal capsule tracts are also frequently involved. The cerebellar white matter may appear hyperintense at T2W MRI. In the later step of MLD, corticosubcortical atrophy is often ascertained, particularly after involvement of the subcortical white matter.

Eight exons of the *ARSA* gene were examined in 12 patients with MLD symptoms. The c.100G>A homozygous mutation in exon 1, c.661T>A homozygous mutation in exon 3, c.739G>A homozygous mutation in exon 4, c.827C>T homozygous mutation in exon 4, c.911A>G homozygous mutation in exon 5, c.931G>A homozygous mutation in exon 5, c.849C>G homozygous mutation in

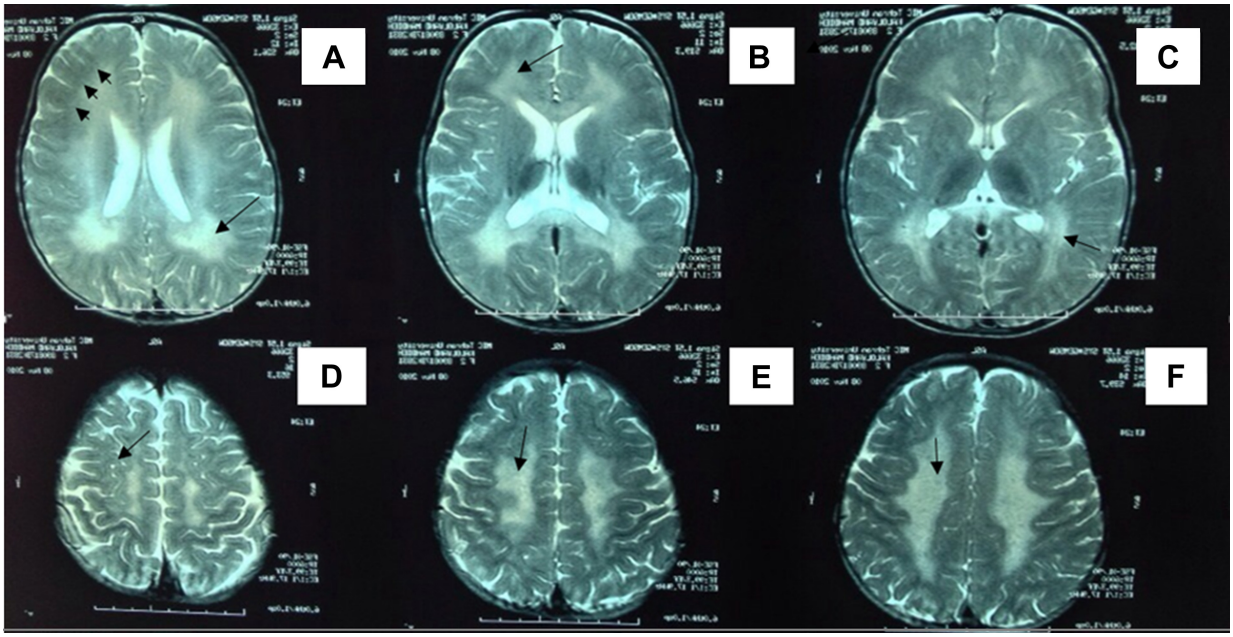

Figure 3 Axial T2-weighted brain MRI from patient P9.
Notes: The area with arrows represents of high signal intensity in the periventricular white matter (**A**–**C**) with sparing of the subcortical U-fibers (arrows), the tigroid (**D**) and leopard skin patterns of demyelination (**E** and **F**).
Abbreviations: MRI, magnetic resonance imaging; P9, patient 9.

exon 4, A>G 96 relative to termination codon homozygous mutation, W195C homozygous mutation in exon 3, and c.978+1G>A homozygous mutation in intron 5 were detected as presented in Table 3. The 4 new mutations c.585G>T, c.661T>A, c.849C>G, and c.911A>G were significantly ($P<0.05$) identified in 4 patients (Table 4 and Figure 4A–D). The possible structural and functional effects of identified new mutations in *ARSA* were examined using the bioinformatics SIFT, PolyPhen, and I-Mutant 2.0 software. Here, SIFT outcomes showed that W195C, F221I, D283E, and K340R mutations were determined as deleterious with scores of −0.734, −5.852, −3.908, and −2.931, respectively. I-Mutant analysis, based on the free energy change value (sign of DDG), demonstrated that p.W195C, p.F221I, p.D283E, and p.K340R mutations decreased protein stability. According to the PolyPhen score, the c.585G>T, c.661T>A, c.849C>G, and c.911A>G mutations were determined as probably damaging the protein structure and function with scores of 0.994, 1.000, 1.000 and 1.000, respectively (Table 4).

Table 3 Mutations in the *ARSA* gene in Iranian MLD patients

Nucleotide change	Amino acid change	Location	Mutation type	Status	References
c.100G>A	p.G34S	Exon 1	Missense	Homo (1)*	21
c.585G>T	**p.W195C**	Exon 3	Missense	Homo (1)	Not reported
c.661T>A	**p.F221I**	Exon 3	Missense	Homo (1)	Not reported
c.736C>T	p.R246C	Exon 4	Missense	Homo (1)	22
c.739G>A	p.G247R	Exon 4	Missense	Homo (1)	23
c.827C>T	p.T276M	Exon 4	Missense	Homo (1)	24
c.849C>G	**p.D283E**	Exon 4	Missense	Homo (1)	Not reported
c.911A>G	**p.K304R**	Exon 5	Missense	Homo (1)	Not reported
c.931G>A	p.G311S	Exon 5	Missense	Homo (2)	25
G>A	c.978+1G>A	–	Splicing	Homo (1)	27
A>G	A>G 96 relative to termination codon	–	Regulatory	Homo (1)	26

Notes: *The number in parentheses in the status column shows the number of patients; new mutations are shown in bold.
Abbreviation: MLD, metachromatic leukodystrophy.

Table 4 Statistical and bioinformatics analysis of 4 novel pathogenic mutations

Patient no	Novel mutations	Statistical analysis			Bioinformatics analysis		
		P-value	PolyPhen 2		SIFT		I-Mutant 2.0
			Prediction	Score	Prediction	Score	Prediction (DDG)
P12	W195C	<0.05	Probably damaging	0.994	Deleterious	−0.734	Decrease stability
P2	F221I	<0.05	Probably damaging	1	Deleterious	−5.852	Decrease stability
P8	D283E	<0.05	Probably damaging	1	Deleterious	−3.908	Decrease stability
P6	K304R	<0.05	Probably damaging	1	Deleterious	−2.931	Decrease stability

Notes: Novel pathogenic mutations were examined using 3 computational methods to assess the structural and functional impacts of novel amino acid changes: PolyPhen 2 (benign or damaging), SIFT (tolerated or deleterious), and I-Mutant 2.0 (increase or decrease stability). PolyPhen prediction score: benign (≤0.5) and probably damaging (0.5<). SIFT prediction score: tolerated (≥0.05) and deleterious (≤0.05). I-Mutant 2.0 prediction: Free energy change value (DDG): decreasing or increasing protein stability.
Abbreviations: SIFT, sorting intolerant from tolerant; DDG, the predicted free energy change value.

Discussion

In this study, 4 novel pathogenic mutations including c.585G>T, c.661T>A, c.849C>G, and c.911A>G in the *ARSA* gene were identified among 12 unrelated Iranian MLD patients. The previous reported mutations including the c.100G>A mutation in patient 1 (P1), c.736C>T mutation in P3, c.739G>A mutation in P4, c.827C>T mutation in P5, c.931G>A mutation in P7 and P9, A>G 96 relative to the termination codon in P15, and c.978+1G>A mutation in P11 that were reported by Gort et al,[21] Gieselmann et al,[22] Hasegawa et al,[23] Harvey et al,[24] Kreysing et al,[25] Gieselmann et al,[26] and Eng et al,[27] respectively.

Hence, additional research is required to affirm the biological role of these pathogenic mutations and confirm the in silico bioinformatics findings that have shown possible effects on protein structure and/or function in the absence of the ARSA enzyme.

The *ARSA* gene has 8 exons, which are located on chromosome 22 (22q13.33).[25] At present, more than 150 mutations were identified in the *ARSA* gene according to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ARSA) (Figure 5).

MLD is a heterogeneous disease with 3 frequent defective alleles including a missense mutation that leads to a Ilel79Ser substitution, a splice donor-site mutation at the exon 2/intron 2 border, and a missense mutation that causes a Pro246Leu substitution, which account for 12%, 25% (among European patients), and 25% of all defective alleles, respectively.[28,29] Other mutant alleles were reported in only a few or single patients.[30]

Conclusion

The result of this research has broadened the genotypic spectrum of Iranian patients with MLD, paving way to a more

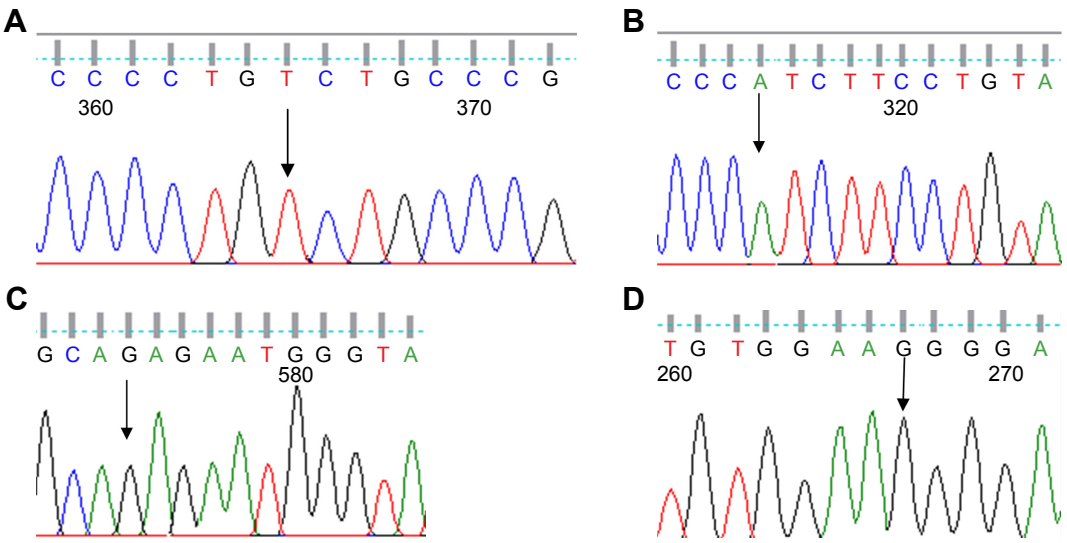

Figure 4 DNA sequencing result of the *ARSA* gene. (**A**) Sequence with a new G>T homo W195C mutation (patient 12). (**B**) Sequence with a new T>A homo F221I mutation (patient 2). (**C**) Sequence with a new C>G homo D283E mutation (patient 8). (**D**) Sequence with a new A>G homo K304R mutation (patient 6).

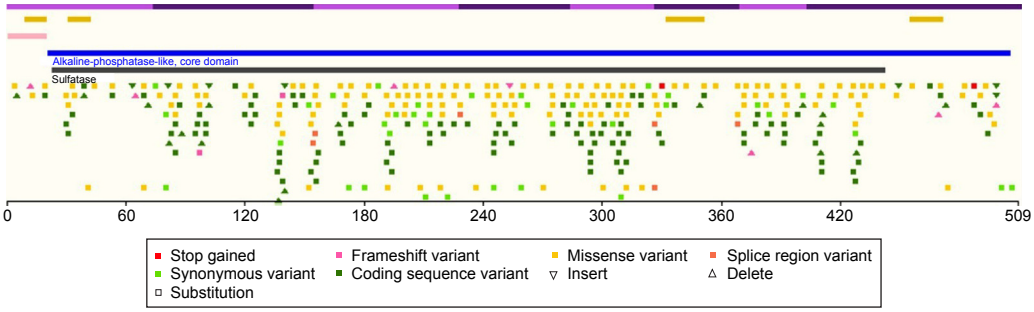

Figure 5 Mutations identified in the *ARSA* gene. (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ARSA).
Note: Reproduced from HGMD® [database on the Internet]. Cardiff, UK: Cardiff University. Available from: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=ARSA. Accessed April 4, 2017.[31]

effective method for career detection, genetic diagnosis, and counseling of Iranian patients with MLD disorder.

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Author contributions

MH conceived and designed the experiments. OA, EK, and SD performed the experiments and contributed to reagents/materials/analysis tools. FA, GYM, and MT analyzed the data. BK wrote the manuscript, contributed to the discussion, and reviewed the manuscript. All the authors read and approved the final manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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