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Original article

Prevalent *MLC1* mutation causing autosomal recessive megalencephalic leukoencephalopathy in consanguineous Palestinian families

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Abstract

Background: Recessive forms of megalencephalic leukoencephalopathy with subcortical cysts (MLC, OMIM 604004) is a rare early-onset leukodystrophy that presents with macrocephaly, seizures, slowly progressive gross motor deterioration, and MRI evidence of diffuse symmetric white matter swelling and subcortical cysts in the anterior temporal and frontoparietal regions. Later in the disease course, significant spasticity and ataxia develop, which may be accompanied by intellectual deterioration. This disease is caused mostly by biallelic pathogenic variants in the *MLC1* gene.

Methods: In this study, we analysed the clinical and molecular architecture of 6 individuals, belonging to 4 unrelated consanguineous Palestinian families, presenting with consistent MLC features. We sequenced the entire coding and flanking intronic regions of the *MLC1* gene.

Results: In all recruited individuals, we detected one recurrent homozygous splice donor mutation NM_015166.4: c.423 + 1G > A. All parents were heterozygous carriers. The mutation abolishes a highly conserved splice site in humans and other species. *In silico* splice predictors suggested the loss of a canonical splice donor site (CADD score 33.0. SpliceAI: 0.980). The c.423 + 1G > A variant is rare; it was detected in only 4 heterozygous carriers in gnomAD.

Conclusion: In this study, we identified a recurrent *MLC1* variant (c.423 + 1G > A) as the cause of MLC among a group of Palestinian patients originating from a particular region of the country. Cost-effective studies should be performed to evaluate the implementation of carrier screening in adults originating from this region. Our findings have the potential to contribute to improved genetic diagnosis and carrier testing for individuals within this population and the wider community.

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Keywords: Megalencephalic leukoencephalopathy with subcortical cysts; Seizure; Developmental regression; Autosomal recessive; Splice donor mutation

1. Introduction

Megalencephalic leukoencephalopathy with subcortical cyst (MLC, OMIM 604004) is a rare, autosomal recessive neurodegenerative disorder, characterized by infantile onset macrocephaly (head circumference above the 97th percentile) and later onset epilepsy and motor regression usually initiated or exacerbated by head trauma [1,2]. The disease is progressive and results in loss of previously gained motor and cognitive skills, and the development of ataxia, spasticity, and intellectual disability [3,4]. Biallelic pathogenic mutations in the *MLC1* or *HEPACAM* (also known as *GLIALCAM*) genes are responsible for most cases of MLC, with more than 75% of described cases attributed to *MLC1* gene variants. Magnetic Resonance Imaging (MRI) is diagnostic and is characterized by diffuse white matter signal abnormalities with mildly swollen appearance of the cerebral hemispheres, and the presence of large subcortical cysts in the anterior temporal lobes bilaterally in all patients [5]. Frontoparietal cysts can be variably present, but cysts were never detected in the occipital lobes [2,4]. With a consistent clinical presentation of MLC disease, high variation in the severity and the progression of symptoms is reported [1,6,7].

MLC1 encodes a membrane protein almost exclusively expressed in astrocytes in the central nervous system [8,9]. MLC1 function is not fully understood; Emerging evidence suggests that MLC1 and HEPACAM, the two molecules implicated in MLC pathogenesis, form a functional unit and interact at the astrocyte end-feet contacting the blood–brain barrier and the pial membrane where they may regulate ion fluxes involved in ion homeostasis and handling of osmotic stress [10,11]. Brain biopsies from *MLC1* patients contained liquid vacuoles in myelin sheaths, and reactive and stressed astrocytes with vacuoles and swelling in their end-feet [12,13]. Reduction of MLC1 expression in cultured astrocytes resulted in the appearance of intracellular vacuoles [11].

MLC1 pathogenic variants were first identified in 2001 [2]. Since then, several biallelic loss of function and missense variants were described. These variants were distributed all over the protein, affecting protein stability and membrane localization, and resulting in premature degradation [3,13,14]. In this study, we report on the clinical, neuroimaging, and genetic features in the first MLC patients described from Palestine.

2. Material and methods

2.1. Patients

Patients were selected based on clinical criteria including infantile-onset macrocephaly, relatively normal initial motor and cognitive milestones, slow deterioration of motor followed by cognitive function and normal metabolic studies. The diagnosis was confirmed by MRI findings. Six patients were enrolled from four unrelated families. Informed consents for detailed clinical phenotyping, genetic studies, and publication of the results were obtained from the enrolled individuals and/or their legal guardians (ethical approval; the Palestinian Health Research Council PHRC/HC/518/19). Blood samples were collected from six living patients and their parents for DNA extraction using standard procedures. Direct sequencing of the *MLC1* exons and flanking intronic regions was performed using BigDye Terminator cycle sequencing and as previously described [2].

3. Results

3.1. Clinical presentation

6 patients belonging to four unrelated families, living in eastern-southern villages of Nablus/ Palestine were identified. All patients are products of consanguineous union, with parents being first cousins, double first cousins, or first and second cousins. Patients' Ages ranged from 5 to 26 years at the latest evaluation.

Family (i) had two affected children, a brother, and a sister (patients IV:2 and IV:4, Fig. 1, and Table 1). Patient IV:2 was evaluated at the age of 16 years. She was noticed at 4 months to have macrocephaly. She had mild to moderate developmental delay in the first 2 years; she sat without support at 11 months, walked independently but with a slow unbalanced gait, and frequent falls at 20 months. She was able to say her first words at 11 months. At the age of 4 years, she started to develop recurrent episodes of epileptic seizures that were resistant to treatment by several antiepileptic drugs. In parallel, she started to show signs of slowly progressive motor, cognitive and speech regression; articulation was completely lost at the age of 5 years, she stopped attending school in the 2nd grade due to difficulty in walking and was completely bedridden at the age of 8 years. On physical examination, her head circumference measured 62 cm (+6.90 SD). She had upper

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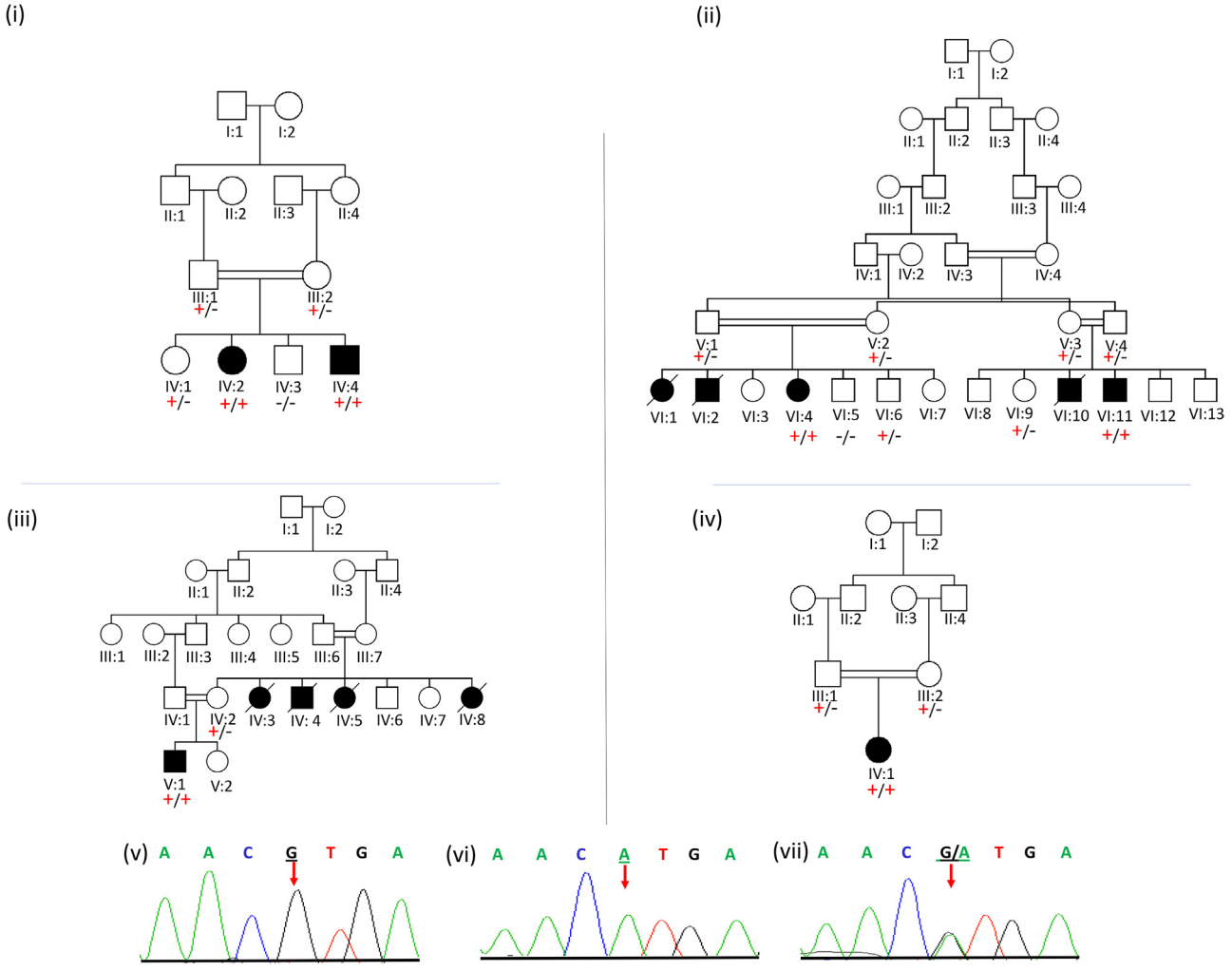

Fig. 1. Pedigrees and genetic analysis of four MLC families. Simplified pedigrees of the families investigated (i, ii, iii, iv), demonstrating segregation of the *MLC1* variant ['+': Chr22:50518346C > T, NM_015166.4: c.423 + 1G > A '−': wild type]. (v, vi, vii); Electropherogram showing the DNA sequence at the position of *MLC1* c.423 + 1G/A in a homozygous affected individual (v), an individual with WT genotype (vi), and a heterozygous carrier (vii).

motor neuron signs in the upper and lower limbs, with severe peripheral spasticity, brisk reflexes, and clonus.

The brother, Patient IV:4 was evaluated at the age of 8.5 years. He was noticed at the age of 1 year to have macrocephaly. His developmental milestones were up to age in the first 2 years of life; head control was achieved at five months, sitting without support at 7 months, walking independently, but with frequent falls, at the age of 16 months, and he was able to say his first words at the age of 1 year. He had fluent speech at the age of 3 years. He attended school and had mild learning difficulties. His examination is noticeable for macrocephaly (head circumference was 59 cm, +4.87 SD), ataxic gait, upper and lower limb hypertonia, spasticity, and hyperreflexia.

In family (ii), 2 patients belonging to two related nuclear families (individuals VI:4, VI:11, family ii, Fig. 1, and Table 1) had macrocephaly in the first year of life. Additional three patients, patients VI:1, VI:2, and VI:10, (family ii, Fig. 1), were deceased at the time of clinical evaluation. The family reports consistent clinical features of the deceased patients with patients VI:4 and VI:11, who were evaluated at the age of 26 and 25 years, respectively.

Patient VI:11 was noticed to have macrocephaly at the age of 5 months. Head control was possible at four months, sitting without support at six months, walking independently at the age of two years, but with a slow unbalanced gait and frequent falls. Developmental regression started at the age of four years including motor, cognitive, and speech regression, and was accompanied by epileptic seizures. The patient attended school to 2nd grade only. Examination revealed macrocephaly, (head circumference 64 cm, +6.21 SD), severe spasticity, hyperreflexia, and positive Babinski sign.

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Table 1
A comparison of clinical features of affected individuals homozygous for the c.423 + 1G > A in the MLC1 gene.

Patient	Family i IV:2	Family i IV:4	Family ii VI:4	Family ii VI:11	Family iii V:1	Family iv IV:1
Genotype	+/+	+/+	+/+	+/+	+/+	+/+
Ethnicity	Palestinian	Palestinian	Palestinian	Palestinian	Palestinian	Palestinian
Sex	F	M	F	M	M	F
Age at phenotyping	16Y	8.5Y	25Y	26Y	11 mo	6Y
OFC (cm) [SD] at birth	NA	NA	NA	NA	34 (−0.40 SD)	33 (−1.41 SD)
OFC (cm) [SD] at examination	62 (+6.90 SD)	59 (+4.87 SD)	65 (+9.60 SD)	64 (+6.21 SD)	63 (+9.63 SD)	59.5 (+6.1 SD)
Onset of macrocephaly	4 mo	12 mo	6 mo	4 mo	3 mo	2 mo
Axial Hypotonia	+	+	–	+	–	–
Walking independently	20 mo	16 mo	18 mo	24 mo	20 mo	24 mo
Developmental delay	mild	–	–	mild	severe	mild
Seizure after head trauma	+	+	+	+	+	+
Loss of consciousness after head trauma	+	+	+	+	+	–
Onset of motor deterioration	4Y	7Y	8Y	10Y	2.5Y	3Y
Onset of first seizure	4Y	7Y	4Y	10Y	2Y	3Y
Seizure severity	++	+	+	++	+++	+
Seizure control	Resistant to multiple antiepileptic drugs	yes	yes	yes	Resistant to multiple antiepileptic drugs	yes
Seizure improved	at 8 yrs	–	yes	no	no	–
Onset of cognitive deterioration	5 yrs	No	No	10 yrs	3 years	No
Schooling	Up to the 2nd grade	at 3rd grade	Up to the 9th grade	Up to the 3rd grade	No	N/A
Gait	Not ambulatory	Ataxic gait	scissoring gait	On wheelchair	Not ambulatory	Ataxic gait
Ataxia	NA	–	–	NA	+	+
Dysmetria	NA	+	+	NA	+	N/A
Spasticity	++	+	++	+	++	++
Hyperreflexia	+	+	+	+	+	++
Clonus	+	–	–	+	+	++
Speech	vocalization	fluent	Dysarthria	dysarthria	vocalization	Slow slurred

Abbreviations: F, female; M, male; mo, months; NA, not available; OFC, occipitofrontal circumference; SD, standard deviation scores; Y, years; the (+) and (−) symbols indicate the presence or absence of a feature in an affected subject respectively.
OFC SD-scores were calculated using a Microsoft Excel add-in to access growth references based on the LMS method [15] using a reference European population [16].

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Patient VI:4 is a double cousin to patients VI:11. She was first noticed to have a large head at 6 months. Her developmental milestones were appropriate for age in the first year of life, she smiled at 3 months, head control was possible at four months, sat without support at six months, and said her first words at 8 months. She was able to walk without support at 18 months, but her knees and thighs pressed against each other and had frequent falls. Epileptic seizures started at the age of 4 years and were well controlled by antiepileptic treatment. The patient developed good verbal communication skills and attended school until the 9th grade when it became very difficult for her to walk to school. On physical examination, her head circumference was 65 cm (+9.60 SD). She was freely ambulatory but had a scissoring gait with limb spasticity and brisk reflexes and lower limb clonus and a positive Babinski sign.

In family (iii), family history is remarkable for several children who died in early childhood due to large head size, motor delay, and severe seizure disorder. The only living child is patient V:1 who was born to parents who are first and second cousins. At birth, he was admitted to the neonatal intensive care unit due to respiratory distress, and anal atresia, which was successfully operated. Since infancy, he had recurrent episodes of apnea, and cyanosis requiring hospitalization. He was noted at the age of 6 months to have a large head, head circumference of 46 cm at 6 months (+1.01 SD). Developmental milestones were delayed; he walked unsteadily at 20 months, only spoke few simple words, and had limited social interaction with caregivers or other children. The ability to walk independently was lost at 28 months and he had recurrent episodes of generalized tonic-clonic seizures that were resistant to multiple antiepileptic drug therapy. Brain MRI (no images) was reported to show severe white matter changes and bilateral temporal cysts.

Family (iv) had one affected female child (individual IV:1, Fig. 1, Table 1) who was first evaluated at 4 months due to large head circumference. Head circumference at birth was 34 cm (−0.79 SD). At 4 months, head circumference measured 52 cm (+7.76 SD). When re-evaluated at the age of 6 years, she lost the ability to walk independently due to lower limb spasticity. She had an ataxic gait, slow slurred speech, and seizures that were controlled by antiepileptic treatment.

3.2. Imaging

Magnetic resonance imaging of the brain showed classical MLC abnormalities in all examined individuals. In axial T2 sequence, high-intensity white matter signals involving both cerebral hemispheres, the subcortical U-fibers (* in Fig. 2A, B), and to a lesser extent, the internal capsule (arrow Fig. 2A) and corpus callosum (arrow in Fig. 2C, arrowhead in 2D) were noted. T1 Coronal sections revealed multiple cysts in the subcortical frontal, parietal regions (not shown), and temporal lobes (* in Fig. 2E). Notably, the cysts were more prominent in younger individuals, whose images were recorded at early childhood, and then tended to decrease with age. Exaggerated cerebrospinal fluid spaces at the frontal lobes and enlarged anterior lateral and third ventricles were more prominent with advancing age, mostly due to atrophic changes (arrowhead in Fig. 2B, C). In addition, evidence of mild atrophy in cerebellar hemispheres, pons, and dilated fourth ventricle were more prominent with time with continued disease progression (arrowheads in Fig. 2F).

3.3. Genetic findings

We suspected the diagnosis of the recessive form of MLC in our patients based on typical clinical presentation, MRI findings, and inheritance pattern. Direct sequencing of the entire coding and flanking regions of the *MLC1* gene (MIM number 605908) in all affected individuals and available family members revealed a recurrent homozygous splice donor mutation Chr22:50 518346C > T, NM_015166.4: c.423 + 1G > A, (Fig. 1, (v)). All parents were heterozygous carriers, (Fig. 1, (vii)). Healthy siblings had a wild type genotype or were heterozygous carriers. The mutation is located at the 5′ end of intron 4 and is expected to cause loss of a canonical splice donor site (Fig. 3C, D). Computational analysis of this base substitution predicts the abolition of this splice donor site; it has a Combined Annotation Dependent Depletion (CADD) score of 33 (CADD v1.6, [17]), and Δ score of 0.98 in SpliceAI deep learning prediction tool (maximum score 1, [18]) and an ESEfinder score 7.459, (cut-off score 6.6, [19]). Conservation analysis across 100 vertebrate species using PhyloP algorithm in UCSC browser revealed high conservation of the splice site variant (PhyloP score: 6.66) (Fig. 3D, E).

4. Discussion

Patients with recessive mutations in *MLC1* have a consistent clinical phenotype [4]. The major features are macrocephaly, hypotonia, and mildly delayed motor skills evident in the first year of life. Motor regression and seizure disorder is prominent in early childhood and is usually associated with a history of trauma. The disease is progressive and results in ataxia, spasticity, and cognitive decline in adolescence or early adulthood. Here, we report the first Palestinian patient group with MLC due to a homozygous splice donor mutation (c.423 + 1G > A) in the *MLC1* gene. The diagnosis of MLC was based on the typical clinical and radiological phenotype, and the mode of inheritance.

Although the clinical and radiological features were consistent among all described patients, variability in

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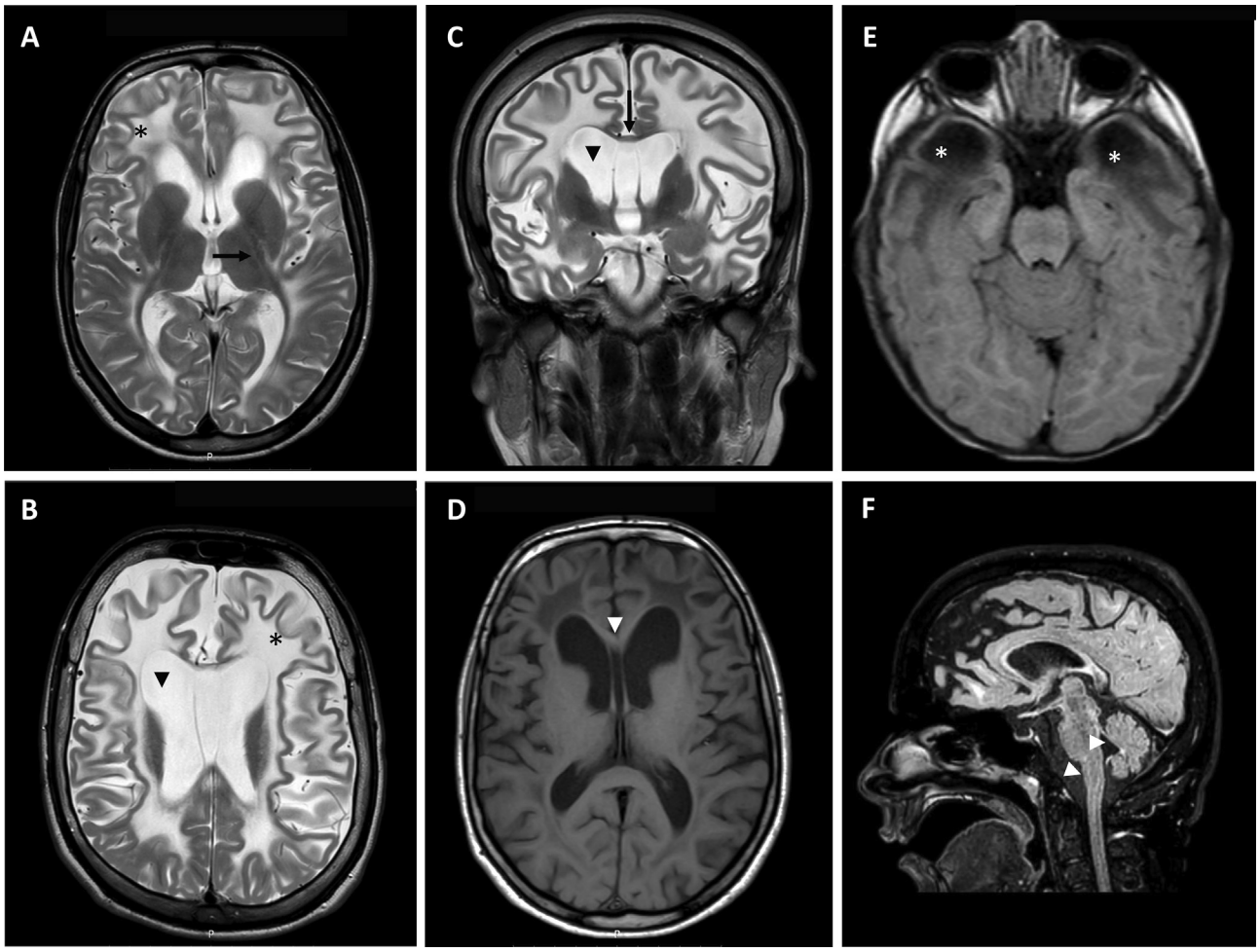

Fig. 2. Brain magnetic resonance imaging in MLC patients. MRI for patients from family ii, patient IV:4 (A, D, F), and patient IV:11, (B, C) at the age of 20 and 25 years respectively. MRI for patient VI:1, family (vi) (E) at 4 months of age. Axial T2 sequence shows high-intensity white matter signals involving both cerebral hemispheres, the sub cortical U-fibers (* in figure A, B), and to a lesser extent the internal capsule (arrow in A) and corpus callosum (arrow in C, arrowhead in D). Coronal T1 sections revealed multiple cysts in the subcortical temporal region (* in figure E). Exaggerated cerebrospinal fluid spaces at the frontal lobes and enlarged anterior lateral and third ventricles were more prominent with advancing age (arrow in B). Mild atrophy in cerebellar hemispheres, pons, and dilated fourth ventricle were more prominent with time and continued disease progression (arrowheads in F).

the severity of symptoms and rapidity of disease progression is noted. For example, the severe phenotype observed in patient V:1 (Family iii, Table 1), is probably due to the presence of perinatal stress, and duodenal atresia. In addition, patient IV:4 (Family i, Table 1 and Fig. 1), showed a milder phenotype in comparison to his sister, patient IV:1. He was ambulatory at 8 years, and performed well at school, while his sister was bedridden and showed symptoms of cognitive decline at this age.

Clinical and genetic heterogeneity is already described in MLC. Hamilton et al. 2018 have shown that this variability is mainly attributed to differences in the mutated gene and mode of inheritance; classical slowly progressive MLC is due to recessive mutations in the *MLC1* gene [2] or *HEPACAM* (MIM 613925) gene [13]. Another remitting MLC phenotype is attributed to dominant *HEPACAM* mutations (MIM 613926) [20]. In this study, we describe a large group of MLC patients having the same *MLC1* mutation and showing evidence of phenotypic heterogeneity among affected individuals. No systematic study on survival among patients with *MLC1* has been reported yet. In our studied families, several cases of mortality are reported between 25 and 30 years. A closer follow up to correlate survival rate to disease severity is needed.

The reported patients belong to 5 nuclear families, living in 4 different villages. The villages are in proximity. All patients are born to consanguineous first cousins or double cousins' parents, and consanguineous marriage is commonly practiced in the families described in the study. According to families, several other individuals died of a similar phenotype in the past decade.

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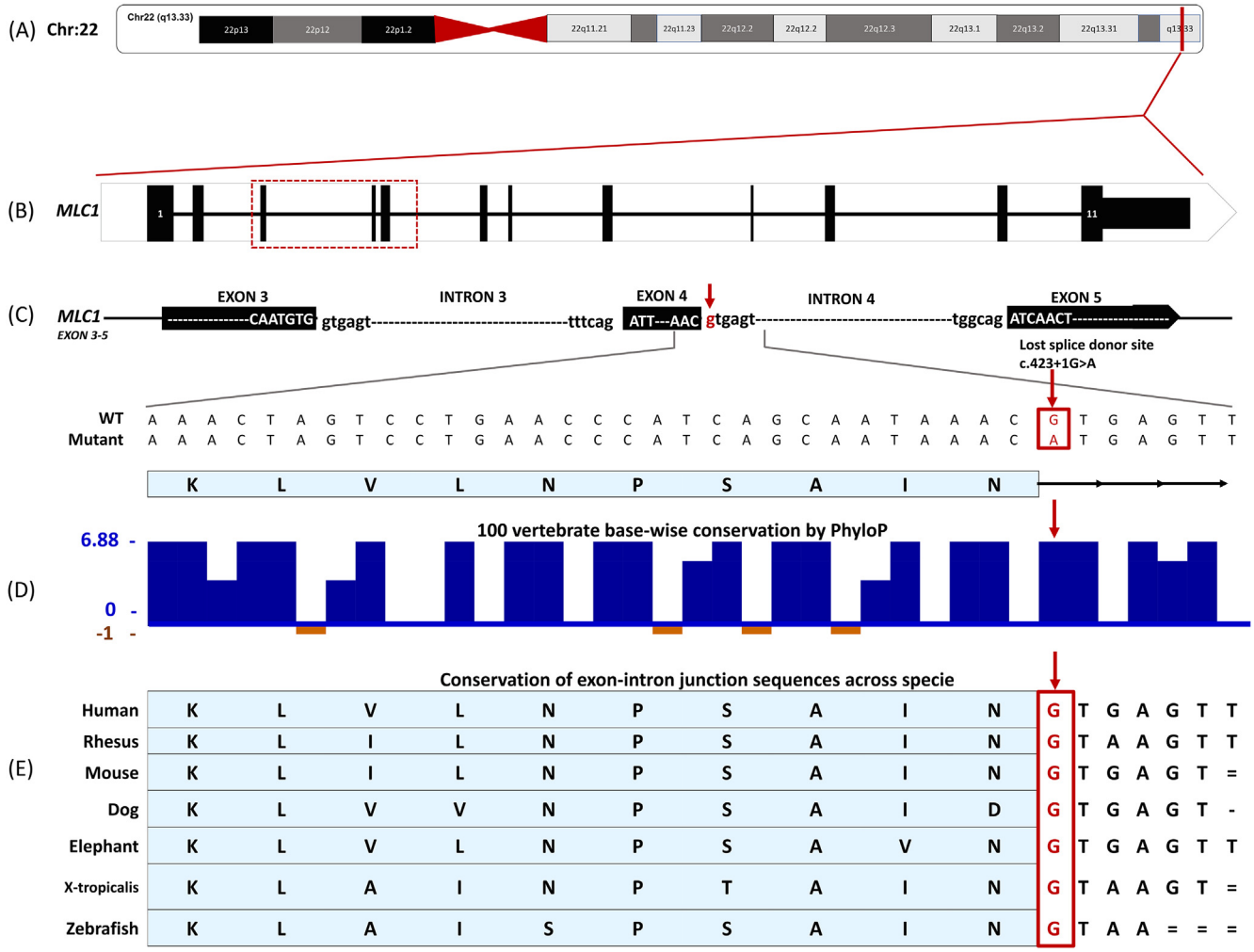

Fig. 3. *MLC1* splice site mutation. A simplified chromosome (A) and gene diagram (B) showing exon–intron organisation of the *MLC1* gene (NM_015166.4), and the location of splice site mutation with a zoomed in view of the mutated residue to depict the conservation of splice site across species (C, D). The mutation is located at the 5′ end of intron 4 and is expected to cause loss of a canonical splice donor site (C). The site of the mutation is highly conserved among humans and other vertebrates as depicted using PhyloP algorithm in UCSC conservation track across 100 vertebrate species (D, E).

The identification of *MLC1* c.423 + 1G > A variant in multiple families with consistent disease phenotype from the 4 nearby villages suggest a high frequency of this mutation in this region. Based on the present findings, putative prenatal testing, and genetic counselling for adults in reproductive age living in this area is of great importance. The c.423 + 1G > A variant is rare; it is absent in 150 in-house whole exome or whole genome sequencing samples. Additionally, it was detected in only 4 heterozygous carriers in gnomAD (v3.1.1. Allele frequency 0.00002628, December 2021), two of them of Middle Eastern origin (v3.1.1. Allele frequency, 0.006329, December 2021). MLC due to Homozygous c.423 + 1G > A in the *MLC1* gene was previously reported in a patient of a North-African origin [21]. In addition, a recent study of 100 families with recessive forms of neurodevelopmental disorders among the Jordanian population identified the same c423 + 1G > A variant as a cause of seizures, global developmental delay, and multifocal hyperintensity of cerebral white matter on brain MRI [22]. Altogether, these findings may suggest a geographical distribution of the *MLC1* c.423 + 1G > A variant to close Mediterranean countries. Determination of the ancestry of the affected population using genome-wide SNP data analysis and ancestry clustering may be needed to decide on the best screening approach in this region.

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Conflict of interest disclosures

The authors declare no competing interests.

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