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DATA REPORT

MLC1 mutations in Japanese patients with megalencephalic leukoencephalopathy with subcortical cysts

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is an autosomal recessive neurological disorder manifesting early onset macrocephaly and delayed-onset neurological deterioration. Characteristic radiological findings revealed by brain magnetic resonance imaging are the most important factors for obtaining a clinical diagnosis. In this study, we analyzed the causative gene, *MLC1*, in seven unrelated Japanese patients. The most common mutation in our study was p.S93L; this mutation was observed in 11 alleles (78.6%). The second most common mutation, p.A275D, was observed in two alleles (14.3%). A novel single-nucleotide deletion, c.578delG (p.V194Sfs*2), was identified in one allele. As the clinical severities of patients with MLC were variable even among those sharing identical genotypes, this condition may be modified by environmental factors, modifier genes or epigenetic factors.

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC, MIM #604004) is an autosomal recessive neurological disorder first described by van der Knaap *et al.*[1] that is characterized by early onset macrocephaly and delayed-onset neurological deterioration.[1] Patients with MLC show macrocephaly during the first year of life, followed by slowly progressive deterioration of motor functions with ataxia and spasticity. In such cases, brain magnetic resonance imaging (MRI) shows diffuse signal abnormality in the white matter of the cerebral hemisphere, and subcortical cysts are often observed in the anterior-temporal region and/or in the front-parietal area. The causative gene, *MLC1* (MIM #605908), was first identified in 2001 and maps to chromosome band 22q13.33; this gene contains 12 exons.[2] *MLC1* mutations have been observed in 75% of patients with MLC.[3] Herein, we report the result of an ongoing study to obtain a genetic diagnosis for *MLC1*.

This study was approved by the Ethics Committee of Tokyo Women's Medical University. After obtaining written informed consent from patients or their families, blood samples were obtained from patients with a clinical diagnosis of MLC. The diagnosis was made based on previously defined criteria.[1,4] Genomic DNA was extracted from blood samples using the QIAamp DNA Extraction Kit (QIAGEN, Hamburg, Germany). Parental samples were also obtained to determine inheritance. All exons of *MLC1* were genotyped using standard Sanger sequencing.

We identified three types of *MLC1* mutations in seven unrelated Japanese patients with MLC (Table 1). Eleven alleles contained the c.278C>T (p.S93L) missense mutation (Supplementary Figure 1) in exon 4 (78.6%), and all patients had p.S93L in either of the alleles (Table 1). Patients 1–4 showed homozygous patterns of p.S93L, and the remaining three patients were compound heterozygous for the mutation and one of other mutations. This mutation has been previously reported in individuals from Japan, Turkey and Finland.[2,5,6] Tsujino *et al.*[6] reported that p.S93L is a common mutation in Japanese patients with MLC, with 85.7% of patients and 71.4% alleles showing this mutation.[6] This finding was also confirmed in the present study. Although we did not analyze parental origins in patients 1–4 due to lack of parental samples, parental consanguinities in patients 1–3 and the high frequency of p.S93L suggest that these patients would be homozygous for p.S93L.

The second most common mutation, c.824C>A (p.A275D) in exon 10 (Supplementary Figure 1), was observed in two alleles (14.3%) from patients 5 and 7 (Table 1). Compound heterozygosity was confirmed in these two patients because p.S93L and p.A275D were identified in either of the parents independently. Although this mutation was reported previously by Montagna *et al.*,[7] this is the first report in Japanese patients.

A single-nucleotide deletion, c.578delG (p.V194Sfs*2) (Supplementary Figure 1), was identified in exon 7 in patient 6. To the best of our knowledge, this is a novel mutation. The mother of patient 6 was a carrier of p.S93L (heterozygous of p.S93L); however, the origin of c.578delG was unknown due to lack of a sample from his father. This deletion causes a frameshift mutation and creates a premature termination codon, leading to nonsense-mediated decay. As a consequence, this likely leads to a loss of function of *MLC1*. Such single-nucleotide deletions are rare in *MLC1*.

In the present study, all patients showed brain MRI abnormalities consistent with diffuse white matter abnormalities (Figure 1). Although most of the patients showed detectable subcortical cysts, particularly in anterior-temporal regions (Figure 1), some were too small to be detected in the regular images. The MLC

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Table 1. Summaries of the clinical information of the patients and the results of mutation analyses

Patient	*Gender*	*Present age (y/m)*	*Onset age (y/m)*	*First symptom*	*Present status*	*Consanguinity*	*Mutations*	*Parental origins*	*Provoking event*	*Macrocephaly/ OFC*[a] *(measured age)*	*Seizures (onset age) (y/m)*
1	F	55 y	1 y 10 m	Seizure	Bedridden	+	S93L/S93L	Not confirmed	Head trauma at 18 m	+ 58.5 cm (55 y)	+ (22 m)
2	F	51 y	41 y	Cognitive impairment	Intellectual disability Spasticity	+	S93L/S93L	Not confirmed	–	– 58 cm (51 y)	+ (47 y)
3	F	36 y	1 y 6 m	Macrocephaly	Bedridden	+	S93L/S93L	Not confirmed	Head trauma at 2 y	+ 54 cm (1 y) 62 cm (15 y)	+ (2 y)
4	F	31 y	1 y 3 m	Seizure	Intellectual disability	–	S93L/S93L	Not confirmed	Fever at 15 m	+ NI	+ NI
5	F	18 y	2y	Motor disability	Inability to walk	–	S93L/A275D	Confirmed	–	– 58 cm (18 y)	–
6	M	11 m	11 m	Macrocephaly	NA	–	S93L/V194Sfs*2	Not confirmed	–	+ 50.3 cm (11 m; +2.9 s.d.)	–
7	F	9 m	9 m	Macrocephaly	NA	–	S93L/A275D	Confirmed	–	+ 49.5 cm (9 m; +2.5 s.d.)	–

Abbreviations: F, female; M, male; m, months; NI, no detailed information; s.d., standard deviation; y, years.
[a]Mean OFC for adult Japanese females is 54.6 cm (the third percentile = 52.0 cm, the 97th percentile = 58.1 cm).

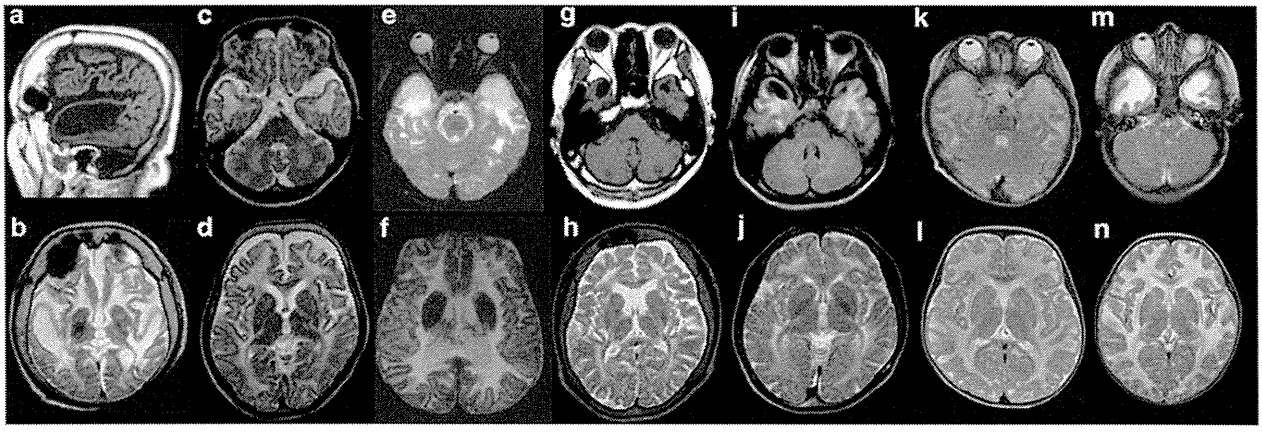

Figure 1. Brain magnetic resonance imaging findings. (**a** and **b**) Patient 1 examined at 55 years of age. The T1-weighted sagittal image (**a**) shows a subcortical cyst in the anterior-temporal region, and diffuse volume loss of the cerebrum is noted (**b**). (**c** and **d**) Patient 2 at 51 years of age. Although subcortical cysts are too small to be detected in the anterior-temporal regions (**c**), mild volume loss of the cerebrum is detectable (**d**). (**e** and **f**) Patient 3 at 16 years of age. (**g** and **h**) and patient 4 at 30 years of age. The T1-weighted axial image (**g**) shows subcortical cysts in the anterior-temporal regions. (**i** and **j**) Patient 5 at 18 years of age. An asymmetric subcortical cyst in the right anterior-temporal regions is noted in the T1-weighted axial image (**i**). (**k** and **l**) Patient 6 at 11 months of age. (**m** and **n**) patient 7 at 9 months of age. The T2-weighted axial images indicate high intensity in the white matter in all patients (**b**–**f**, **h** and **j**–**n**). Subcortical cysts in the anterior-temporal regions are detectable in some of the T2-weighted axial images (**e** and **m**).

patients could be classified into two groups according to age, that is, infant patients and patients older than the teenage years. Two infant patients were diagnosed with MLC as a result of examination for macrocephaly; no neurological findings were observed in either of these two patients at the time of genetic diagnosis.

In comparison, the older patients showed impaired motor and/or intellectual disability and required supports for daily life. Macrocephaly was observed in only two of the five older patients (2/5). The clinical courses and prognoses of these patients were variable. The onset of MLC in the four patients with the p.S93L homozygous mutation was variable, ranging from 15 months to 41 years. Patient 1, who was homozygous for p.S93L, was severely impaired and bedridden from the age of 4. On the other hand, patient 2 showed only mild cognitive and memory deficit and suffered seizures followed by weakness of the extremities at 47 years of age. This type of patient with a good long-term prognosis was also reported by Koyama *et al.*[8] Therefore, this may not necessarily indicate that p.S93L is the mutation causing severe neurological prognosis.

In MLC patients, provoked events are often observed after high fever and head trauma. We observed such provoked events in three patients (Table 1). Because the clinical severities of patients with MLC varied even among patients sharing identical genotypes, disease prognosis may be modified by environmental factors including fever, head trauma, unknown modifier genes, and epigenetic factors.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.515, http://dx.doi.org/10.6084/m9.figshare.hgv.517,

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COMPETING INTERESTS

The authors declare no conflict of interest.

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[Originall Article]

Mutations in the genes encoding eukaryotic translation initiation factor 2B in Japanese patients with vanishing white matter disease

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Abstract

Objective: Vanishing white matter disease (VWM) is a chronic, progressive leukoencephalopathy associated with episodes of rapid deterioration following minor stress events such as head traumas or infectious disorders. The white matter of the patients with VWM exibits characteristic radiological findings.

Method: The genes encoding all five subunits of eukaryotic translation initiation factor 2B (EIF2B) were analyzed in patients, who were tentatively diagnosed with VWM, by Sanger sequencing.

Results: Seven mutations were identified in the genes encoding the subunits 1, 2, 4, and 5 of EIF2B. Among them, one mutation (p.V83E) in the subunit 2 (*EIF2B2*) was recurrently identified in three alleles, indicating the most common mutation in Japanese patients with VWM. Two patients were homozygous, and the other four patients were compound heterozygous.

Conclusion: All patients showed white matter abnormalities with various degrees. One patient showed manifestations of end-stage VWM disease. Some patients showed late onset and slow progression associated with brain magnetic resonance imaging displaying T2 high intensity only in the deep white matter. There was clinical heterogeneity among patients with VWM.

Keywords: Vanishing white matter disease (VWM); eukaryotic translation initiation factor 2B (EIF2B); leukoencephalopathy; mutation

1. Introduction

Childhood ataxia with central hypomyelination (CACH) or leukoencephalopathy with vanishing white matter (VWM; MIM #603896) is a chronic, progressive leukoencephalopathy associated with episodes of rapid deterioration following minor stress events such as head trauma or infectious disorders [1-3]. Patients with VWM show abnormal radiological findings in the brain; i.e., the cerebral white matter appears progressively diffuse and symmetrical abnormalities such as rarefaction and cysts. VWM is an autosomal recessive disease caused by mutations in any of the genes encoding the subunits of eukaryotic translation initiation factor 2B (EIF2B), which is a GTP exchange protein essential for protein synthesis [4, 5]. Until now, many disease-causing mutations have been identified [6-16].

In this study, we report on the results of our on-going study to obtain genetic diagnosis for Japanese patients with VWM.

2. Materials and methods

2.1. Patients and samples

This study was approved by the ethics committee at the Tokyo Women's Medical University. After obtaining written informed consents from patients or their families, blood samples were obtained from patients. Patients were recruited under candidate diagnosis of VWM as defined by previously proposed diagnostic criteria by van der Knaap et al.[17]. In the early stages of VWM, white matter involvements may not fulfill the criteria. Therefore, patients who did not show full manifestations but were tentatively diagnosed as VWM were also included in this study. Genomic DNAs were extracted from blood samples using the QIAamp DNA Mini Kit (QIAGEN, Hamburg,

Germany). Parental samples were also obtained to confirm inheritance patterns.

2.2. Molecular analysis

All exons of the genes encoding the five subunits of EIF2B (*EIF2B1*, *EIF2B2*, *EIF2B3*, *EIF2B4*, and *EIF2B5*) were genotyped using standard Sanger sequencing. Information on the primers used for this study can be obtained upon request. When heterozygous or homozygous variations were identified in patients, inherited patterns were analyzed using corresponding parental samples. PCR products were subcloned into the pGEM® T-vector (Promega, Madison, WI) to identify the allelic locations of the mutation as described previously [18]. Subsequently, nucleotide sequences of inserted fragments were analyzed in both directions. When a *de novo* mutation was suspected, the biological relationship between the patient and the corresponding parental samples was confirmed by microsatellite marker analysis using the Linkage Mapping Set (Life Technologies, Foster City, CA) as described previously [19].

The identified non-synonymous variants were tested for mutational effects using damaging predication scores obtained from the SIFT [20] (http://sift.jcvi.org/), PolyPhen-2 [21] (http://genetics.bwh.harvard.edu/pph2/), and Combined Annotation-Dependent Depletion (CADD) [22] (http://cadd.gs.washington.edu/info) in accordance with methods reported elsewhere [23]. Interspecies amino acids conservation was checked using the UCSC Genome Bioinformatics Site (https://genome.ucsc.edu/).

3. Results

3.1. Pathogenic mutations

We analyzed a total of 22 patients. Among them, we identified mutations in the

genes encoding the EIF2B subunits in six patients including four unrelated individuals and two siblings. All identified mutations were missense mutations (Supplemental Figure 1 and 2). The results of the molecular analyses in accordance with the clinical information of the patients are summarized in Table 1. Patients 1 and 2 showed homozygous mutations in *EIF2B1* and *EIF2B2*, respectively. Patient 2 was homozygous for p.V83E in *EIF2B2*, and patient 3 had compound heterozygous mutations associated with p.V83E in *EIF2B2*. The other patients showed compound heterozygous mutations in either of *EIF2B2*, *EIF2B4* and *EIF2B5*. Parental origins of all mutations other than p.M305I were confirmed (Table 1). Predicted scores for the deleterious effects of mutations provided by SIFT, Polyphen-2, and CADD are included in Table 1.

Both p.M305I and p.I385T were identified in exon 7 of *EIF2B5* in patient 6. To assess the allelic locations of these two mutations, PCR products were subcloned into the plasmid vector. At least 10 clones were isolated and sequenced. Consequently, all clones showed one of the mutations, indicating that the two mutations were located on the independent alleles. Although p.I385T was identified in the patient's mother, p.M305I was not identified in both parents. We confirmed the biological relationship between patient 6 and his parents by linkage analysis (data not shown).

3.2. Patient reports

Patient 1 is a 61-year-old female, whose parents were cousins. Initial neurological symptoms with gait disturbance were first observed at 29 years of age. At that time, her intellectual quotient was evaluated to be 66. Motor incoordination and spasticity were also noted. Routine laboratory examinations of blood, urine, and cerebrospinal fluid (CSF) showed normal results. Enzyme activities including arylsulfatase A, β-hexosaminidase A, β-mannosidase, and α-fucosidase were within the

normal limits. The motor nerve conduction velocity of the left peroneal nerve was 32 m/sec, which indicated a delay. Cranial computed tomography showed diffusely distributed low-absorption in the white matter (no more detailed information). Her neurological deterioration progressed, and she is now bedridden. Brain magnetic resonance imaging (MRI) performed at 60 years of age showed diffuse high intensity in the T2-weighted images (Figure 1).

Patient 2 is an 8-year-old girl. Language developmental delay was noted at 3 years of age. At 7 years, she was admitted to the hospital due to drowsiness after an infectious disease of mycoplasma pneumonia. Due to frequent seizures and respiratory failure, intubation was performed. Brain MRI showed diffuse T2 high intensity in the white matter (Figure 1). Routine laboratory examinations of blood, urine, and CSF showed no abnormality. Her neurological findings have gradually improved. At present, she can speak simple sentences and can walk unassisted.

Patient 3, a baby boy, was born with a weight of 2,878 g (25^{th}~50^{th} centile), a length of 49.4 cm (50^{th}~75^{th} centile), and occipitofrontal circumference (OFC) of 33.0 cm (25^{th}~50^{th} centile) at 41 weeks of gestation. At 6 months of age, he showed postnatal growth delay with a weight of 6.2 kg (<3^{rd} centile), a length of 63.4 cm (3^{rd}~10^{th} centile), and OFC of 42 cm (10^{th}~25^{th} centile). Although he showed normal development until 8 months of age, he suddenly displayed drowsiness and poor sucking after an infectious disorder causing high fever, and was admitted to the hospital. At that time, the brain MRI showed diffuse T2 high intensity in the white matter (Figure 1). Routine laboratory examinations of blood, urine, and CSF, including lactate and pyruvate, showed no abnormality. Screening tests for metabolic disorders of amino acids and very long chain fatty acids also appeared normal. Thereafter, he showed spasticity and severe

developmental delay.

Patient 4 is a 22-year-old male, first born from non-consanguineous parents. At the age of 13 years, he started to show epileptic seizures. At that time, brain MRI showed diffuse T2 high intensity in the white matter. Screening tests for metabolic disorders of amino acids and very long chain fatty acids showed normal patterns in patient 4. Enzyme activity of arylsulfatase A and β-galactosidase as well as peripheral nerve conduction velocities, were all within the normal limit. At present, he only shows mild ataxia. Patient 5, the 19-year-old younger brother of patient 4, is the third born among three siblings; his elder sister (the second born) is healthy. Patient 5 also showed a clinical course similar to that of patient 4; he showed epileptic seizures and brain MRI abnormality at age 13 years. When he was 16 years old, a traumatic accident triggered disease progression; he showed prolonged delirium, and then muscular weakness in his left side. Routine laboratory examinations of blood, urine, and CSF showed no abnormality in this sibling case.

Patient 6 is a 3-year and 5-month-old boy. There was no remarkable family or past history. At 13 months, he showed transient drowsiness and gait disturbance two weeks after a febrile convulsion. Brain MRI showed T2 high intensity in the white matter (Figure 1). After 2 years of age, he easily dropped due to ataxic gait. At present, his height is 95.1 cm (25^{th}~50^{th} centile), weight is 15.9 kg (75^{th}~90^{th} centile), and OFC is 51.8 cm (90^{th}~97^{th} centile). He cannot stand alone due to spasticity in his lower extremities. Compared to motor development, his cognitive development was within the normal limit. Screening tests for metabolic disorders of amino acids and very long chain fatty acids showed normal patterns. Enzyme activities including arylsulfatase A, β -hexosaminidase A, β -galactosidase, galactosylceramidase were within the normal

limits. There were no mutations in the glial fibrillary acidic protein gene (*GFAP*) nor the megalencephalic leukoencephalopathy with subcortical cysts 1 gene (*MLC1*).

4. Discussion

In this study, a molecular diagnosis of VWM was established in six patients from five families (Table 1). All of the identified mutations are depicted in the primary structures of EIF2B genes together with previously reported mutations (Figure 2) [4-16]. The damage prediction scores of the identified mutations were calculated and summarized in Table 1. Although the some of the predictions from SIFT and Polyphen-2 appeared benign or tolerated, all CADD scores (PHRED-like) were higher than 15. Thus, mutations identified in this study likely to have pathogenic effects.

In patient 6, p.I385T was inherited from his mother; however, p.M305I was not identified either parent. Therefore, p.M305I was suspected to be of *de novo* origin in the paternally derived allele. The UCSC Genome Bioinformatics Site displays the different single nucleotide polymorphisms (SNPs) in the same residues, p.M305L (rs200143780) and p.I385V (rs113994073) (Supplemental Figure 2). The p.I385V is registered as a "flagged" SNP and has previously been reported as pathogenic [24] (Figure 2). Compared to this, p.M305L is not shown as a "flagged" in the database; however, the minor allele frequency (MAF) of p.M305L is reported to be as low as 0.050% (1/2,000). Because VWM is caused by an autosomal recessive trait, existence of only one individual with heterozygosity of p.M305L among 1,000 normal populations may suggest that this individual is a healthy carrier of this possibly disease-causing variant. Therefore, existence of the different SNP of this residue (p.M305L) in the database does not deny pathogenesis of p.M305I. These findings indicate that these two variants are

likely deleterious.

Among the 10 alleles present in the five families, three alleles (30%) shared the p.V85E mutation in *EIF2B2*. Previously, p.V85E has been identified in Japanese patients [25]. We also reported this mutation in a patient with VWM, which was unmasked by a microdeletion of the homologous allele [26]. Therefore, this mutation is most common in the Japanese population. Because p.V85E has also identified in a Chinese patient [13], this variant may be common in individuals of east Asian origin. Other than p.V85E in *EIF2B2*, p.R357Q in *EIF2B4*, registered as rs113994033, was recurrently identified in the literature [8]. The other five mutations identified in this study were novel and have not been reported previously (Table 1). Due to the limited number of patients, we were unable to identify any genotype–phenotype correlation.

In this study, patient 1 at 61 years of age presented clinical manifestations of the end stage of VWM, with completely vanishing white matter. We are unable to distinguish the border of periventricular zones in the brain MRI for this patient. Although patient 1 is now bedridden with no response or motor activity, the onset of her neurological symptoms began at age 29. Therefore, compared to the other patients, this patient showed later onset of disease and slower disease progression. The sibling case of patient 4 and 5 also showed late onset and slow progression, and only started to exhibit neurological symptoms after adolescence. In the early stage of VWM, brain MRI may not necessarily show diffuse cerebral white matter abnormalities and rarefaction or cystic degeneration [27]. Therefore, the brain MRI of patient 4 and 5, showing abnormal T2 high intensity only in the deep white matter, is suggestive of an early disease stage.

The other three patients (patient 2, 3, and 6) showed typical, diffuse white matter abnormalities in MRI. They started to show neurological symptoms during early

infancy, and their disease occurrences were triggered by environmental factors (high fever due to infections) and were followed by episodes of acute deterioration associated with disturbed consciousness and seizures. These provocations have been frequently observed in VWM patients [11].

Because EIF2B is involved in regulating the first steps of protein synthesis and is ubiquitously expressed, it is unclear why EIF2B alterations cause a brain-specific disease [28, 29]. Although many mutations identified in patients with VWM showed reduced EIF2B activities [30], basal activities *per se* do not explain the disease severity. Rather, the decreased EIF2B activity might impair the cellular stress response and improperly activate the unfolded protein response (UPR) leading to the endoplasmic reticulum (ER) stress [31]. The ER load in astrocytes and oligodendrocytes is possibly higher than in other cell types, rendering them vulnerable to conditions that predispose to ER stress [32, 33]. This is the probable reason for disease provocations after environmental stress factors in patients with EIF2B alterations.

In this study, we recruited 22 patients who showed mimicking clinical manifestations of VWM. Among them, only six patients had genomic mutations in EIF2B genes. The final diagnosis of the other 16 patients is unknown at present. This would be challenges to be overcome in our future.

Conflict of interest

The authors have no conflict of interests to declare.

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