

## ARTICLE

# Whole-genome sequencing identifies a novel *ABCB7* gene mutation for X-linked congenital cerebellar ataxia in a large family of Mongolian ancestry

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X-linked congenital cerebellar ataxia is a heterogeneous nonprogressive neurodevelopmental disorder with onset in early childhood. We searched for a genetic cause of this condition, previously reported in a Buryat pedigree of Mongolian ancestry from southeastern Russia. Using whole-genome sequencing on Illumina HiSeq 2000 platform, we found a missense mutation in the *ABCB7* (ABC-binding cassette transporter B7) gene, encoding a mitochondrial transporter, involved in heme synthesis and previously associated with sideroblastic anemia and ataxia. The mutation resulting in a substitution of a highly conserved glycine to serine in position 682 is apparently a major causative factor of the cerebellar hypoplasia/atrophy found in affected individuals of a Buryat family who had no evidence of sideroblastic anemia. Moreover, in these affected men we also found the genetic defects in two other genes closely linked to *ABCB7* on chromosome X: a deletion of a genomic region harboring the second exon of copper-transporter gene (*ATP7A*) and a complete deletion of *PGAM4* (phosphoglycerate mutase family member 4) retrogene located in the intronic region of the *ATP7A* gene. Despite the deletion, eliminating the first of six metal-binding domains in *ATP7A*, no signs for Menkes disease or occipital horn syndrome associated with *ATP7A* mutations were found in male carriers. The role of the *PGAM4* gene has been previously implicated in human reproduction, but our data indicate that its complete loss does not disrupt male fertility. Our finding links cerebellar pathology to the genetic defect in *ABCB7* and *ATP7A* structural variant inherited as X-linked trait, and further reveals the genetic heterogeneity of X-linked cerebellar disorders.

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## INTRODUCTION

### X-linked congenital cerebellar ataxias

X-linked congenital cerebellar ataxia is a heterogeneous nonprogressive disease that occurs generally in the first years of life and is characterized by developmental delay and difficulties in coordination owing to hypoplasia/atrophy of the cerebellum. Most of the congenital cerebellar hypoplasia and atrophy cases, such as oligophrenin-1 syndrome (OMIM: 300486) or MICPCH/CASK syndrome (calcium/calmodulin-dependent serine protein kinase (membrane-associated guanylate kinase family (MAGUK) family); OMIM: 300749), are considered to be X-chromosome-associated intellectual disability pathologies.<sup>1,2</sup> Loss of function of oligophrenin-1 (*OPHN1*) gene leads to cerebellar hypoplasia and frontotemporal atrophy,<sup>1</sup> and to hippocampal alterations<sup>3</sup> by affecting Rho GTPase-dependent signaling required in cell migration and morphogenesis, synapse maturation and plasticity of neurons. Similarly, the dysfunction of *CASK* gene, which encodes the calcium/calmodulin-dependent serine protein kinase (MAGUK family), is associated with ataxia, mental retardation and microcephaly.<sup>2</sup> The cerebellar dysfunctions are also linked to ion exchange abnormalities. In one familial case, the cause of the disease was a Ca<sup>2+</sup> imbalance as a result of mutation in the *ATP2B3* gene, an

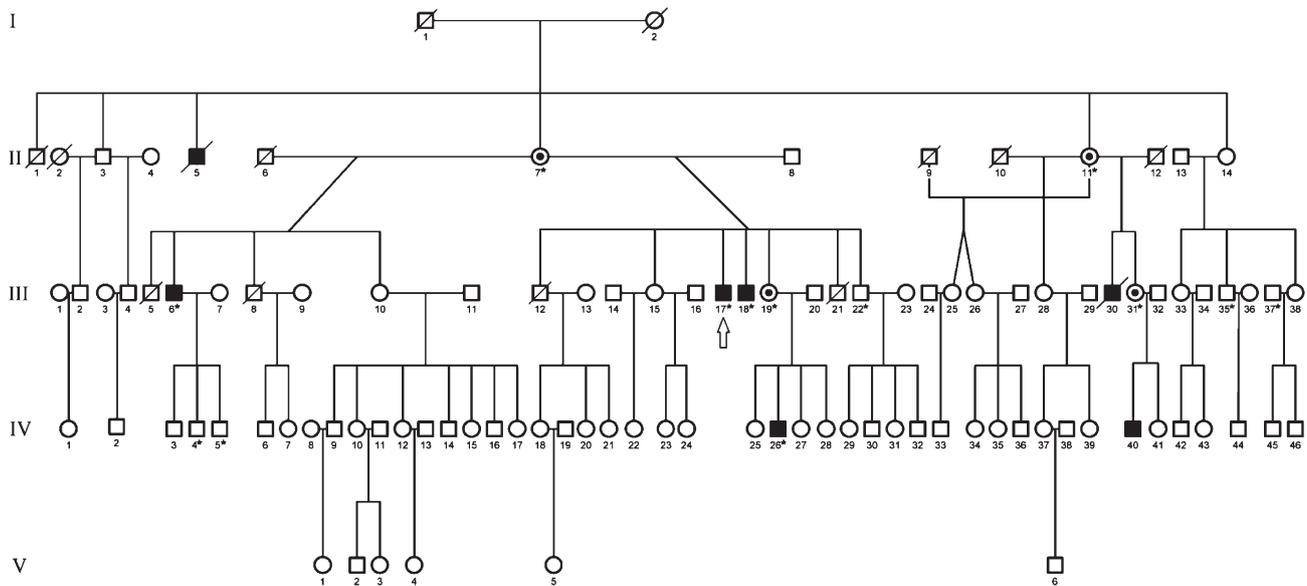
ATPase transmembrane transporter that moves Ca<sup>2+</sup> ions out of the cell against concentration gradients.<sup>4</sup> In another case, the X-linked spinocerebellar ataxia was linked to a nonsynonymous variant in the *GJB1* gene encoding protein connexin 32, which supports ions and transfer of small molecules by forming gap junction channels between cells.<sup>5</sup> Here, we report the case of inherited neurological pathology linked to genetic defects in genes for metal-ion transporters in a large Buryat pedigree (Figure 1).<sup>6</sup>

### Clinical description

Magnetic resonance imaging (MRI) scans revealed hypoplasia of cerebellar hemispheres and vermis in affected males from the Buryat pedigree. Common neurological symptoms for all affected family members were development delay, difficulties in speech and coordination, limb and truncal ataxia and dysarthria (Supplementary Table 1). The patients examined were not able to sit without support any time before 15 months of age, or to walk independently before 7 years of age and to speak their first words before 4 years of age. For the majority of patients, nystagmus, ophthalmoplegia and increased tendon reflexes were observed. There were no signs of memory or cognitive impairment in all the patients from this pedigree.<sup>6</sup>

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**Figure 1** Genealogy of the Buryat family. The whole-genome sequencing was performed for the patient designated by an arrow; solid symbols indicate affected individuals; dotted circles – obligate heterozygous carrier females; open symbols – unaffected individuals; slashed symbols – deceased subjects; asterisks – individuals who were used for genotyping.<sup>6</sup>

Features of sideroblastic anemia or copper disorder were not detected. Hematological tests performed for the patient (III-18) with X-linked ataxia from the Buryat pedigree showed no erythropoietic cell abnormalities or accumulation of iron granules. Hemoglobin values averaged 149 g/l (normal range, 130–180 g/l), color index averaged 1.0 (normal range, 0.80–1.05), the erythrocyte sedimentation test averaged 3 mm/h (normal range, 0–15 mm/h) and white blood cell count was generally normal. All biochemical tests were normal, except for a slightly elevated level of bilirubin, 14.5 mg/l (normal range, 5–12 mg/l), and the urine analysis was unremarkable.<sup>6</sup>

## MATERIALS AND METHODS

Blood samples of all the subjects were collected previously with appropriate informed consent and these data have already been reported.<sup>6</sup> No consanguinity was found to be present in the pedigree (Figure 1). A genomic library was made from 2 µg DNA sample of patient III-17 following the protocol for Paired-End DNA Sample Prep Kit (Illumina, San Diego, CA, USA). High-throughput genome sequencing was performed on Illumina HiSeq 2000 platform with at least 14-fold coverage depth of the genome GRCh37. The genome analysis was performed using 'ngs-pipeline' software designed by our group <http://rogaevlab.ru/ngs-pipeline>. The identified rare variants were submitted to NCBI ClinVar and are available at [www.ncbi.nlm.nih.gov/clinvar/?LinkName=orgtrack\\_clinvar&from\\_uid=505407](http://www.ncbi.nlm.nih.gov/clinvar/?LinkName=orgtrack_clinvar&from_uid=505407).

Paternity was determined by STR (short tandem repeat) analysis using home-made STR markers and PowerPlex 16 System (Promega, Madison, WI, USA).

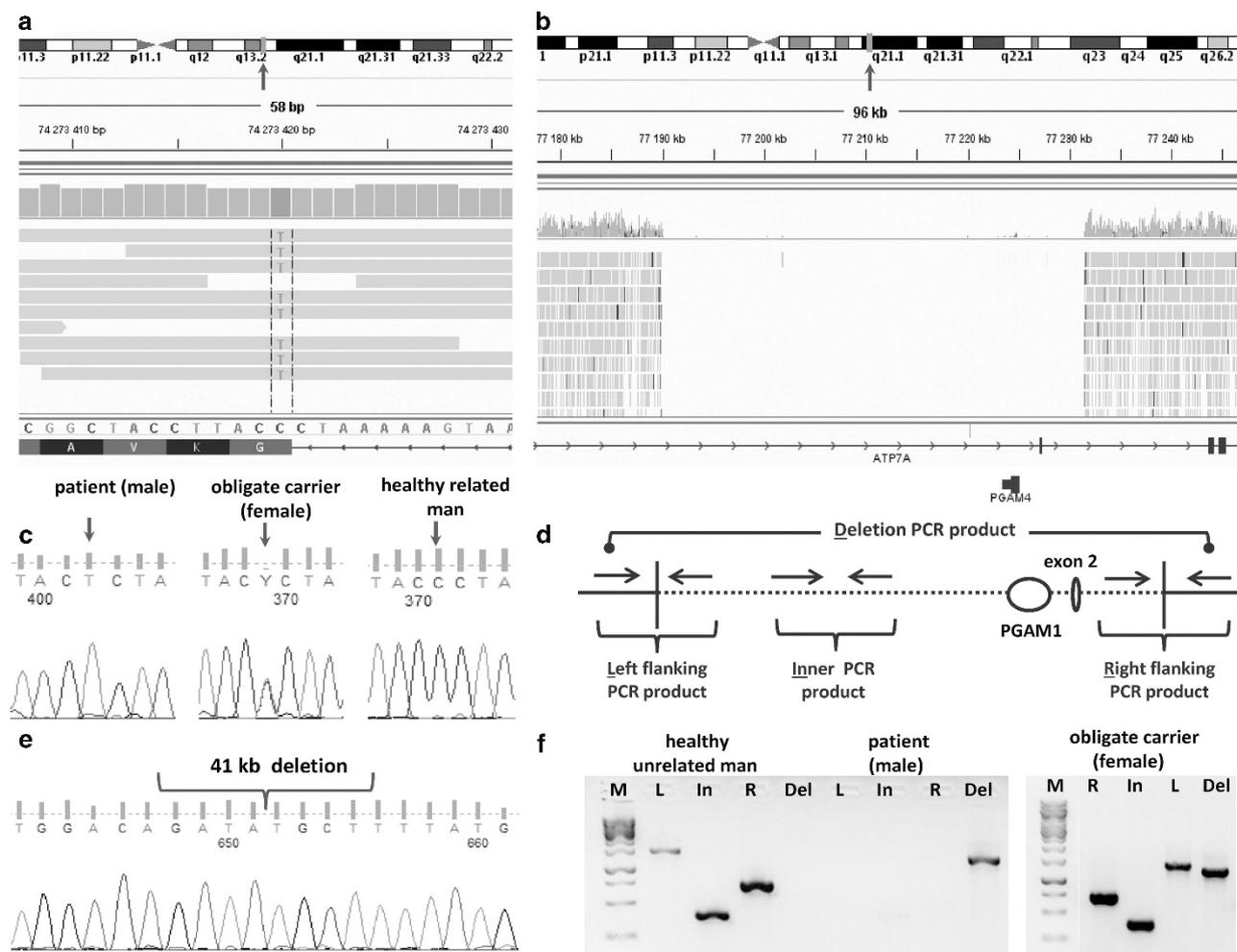
## RESULTS

### Whole-genome sequencing

Using pairwise linkage analysis and haplotype reconstruction, the gene responsible for the disorder was mapped to a 38-cM interval on chromosome Xp11.21-q24, flanked by the STR markers DXS991 and DXS1001. Multipoint linkage analysis showed the robust linkage (maximal lod score of 4.66) at the DXS1059 (Xq23).<sup>6</sup> We next performed whole-genome sequencing and analysis of one male patient (III-17; Figure 2) from the Buryat family, with the described symptoms generating 542 million short reads using our bioinformatics pipeline (<http://rogaevlab.ru/ngs-pipeline>). The raw read sequences are

freely available through the NCBI Sequence Read Archive under the accession number SRP049620 and from the website <http://rogaevlab.ru/ataxia>. Using these primary sequencing data, we reconstructed the complete mitochondrial genome of this person by assigning it to haplogroup C4b,<sup>7</sup> which is commonly found in native populations of southern Siberia<sup>8,9</sup> (Supplementary Table 2). Assembly sequence of complete mitochondrial genome is available at the NCBI GenBank under the accession number KR153486. Analysis of Y-chromosome sequences linked it to haplogroup N1c1a1a\*,<sup>10</sup> a subgroup of N1c1 of Asian origin,<sup>11</sup> which is frequent in Buryat and Mongolian populations<sup>12</sup> (Supplementary Table 3). In total, 4.2 million variants, including 3.7 million single-nucleotide polymorphisms, were identified using GATK (The Genome Analysis Toolkit),<sup>13</sup> and Pindel<sup>14</sup> programs. To our knowledge, this is the first time that the whole-genome sequence of an individual from the ethnic Buryat of Mongolian ancestry has been presented.

We selected all rare variants (minor allele frequency (MAF) <5%; 1000 Genomes Project Consortium<sup>15</sup>) located in the specified region on Xp11.21-q24 locus, flanked by the STR markers DXS991 and DXS1001, and identified five rare or novel variants that can alter protein coding exon sequences (Supplementary Table 4). We narrowed down the candidate-gene list by excluding *NHSL2* (Gene ID: 340527), *AMER1* (Gene ID: 139285), *PHKA1* (Gene ID: 5255) and *RGAG1* (Gene ID: 57529) based on evolutionary analysis, gene expression profiles in tissues and protein topology position (Supplementary Figure 1). *NHSL2* gene was excluded because there are no reports on association of rare variants in the *NHSL2* gene, including the rs72630038 (SCV000221324) (MAF=0.045 in 1000 Genomes Project Consortium<sup>15</sup>) found in the Buryat patient, with any disorder; low expression of the gene in the cerebellum at prenatal period;<sup>16,17</sup> and that at least one mammalian species (hedgehog) bears the same rare amino-acid variant in *NHSL2*. Mutations in the *AMER1* gene are known as genetic factors underlying osteopathia striata, cranial sclerosis and intellectual disability syndrome (OMIM: 300647 and 300373). We excluded *AMER1* gene because of the clinical phenotype differences in our patients and location of the variant



**Figure 2** Identification of variants in *ABCB7* and *ATP7A* genes. Representation of genomic region containing mutations using IGV (Integrative Genomics Viewer) tools: (a) exon 16 of *ABCB7* gene (hg19 chrX:g.742734204C>T), (b) 41.4 kb deleted region of the *ATP7A* gene (hg19 chrX:g.77190006\_77231471del), including exon 2; vertical arrows in (a) and (b) indicated the chromosomal loci for *ABCB7* and *ATP7A* genes. (c) Sequencing verification of candidate mutations in exon 16 of *ABCB7* gene. (d) Primers scheme for validation of deletion in *ATP7A* gene. (e) Sequencing validation by Sanger sequencing of deletion in *ATP7A* gene in the patients. (f) PCR products of the *ATP7A* gene deleted region: M, DNA ladder; R, upstream 'right' flanking with deletion region; In, region inner deletion; L, downstream 'left' flanking with deletion region; Del – product is present if there is deletion in DNA sample.

(SCV000221323) in evolutionarily non-conserved region of the protein. Mutations in the *PHKA1* gene lead to X-linked muscle glycogenosis (OMIM: 311870 and OMIM 300559), which is also distant from the observed clinical symptoms in our patients. The amino-acid substitution arginine to glutamine (SCV000221325) in the *PHKA1* protein occurred in non-conserved position. Variant in *RGAG1* (retrotransposon gag domain containing 1 protein) gene (SCV000221326) is also localized in a non-conserved position. Ultimately, we identified the genetic defects in two closely linked genes, both essential for metal transport. We found that the missense mutation in exon 16 of the *ABCB7* (*ABC*-binding cassette transporter B7) gene (SCV000221289), which is in linkage disequilibrium with a large 41.4 kb deletion in the *ATP7A* gene on X-chromosome (SCV000221290) in this family, is the most probable causative factor for the neurological disease of the Buryat patients (Figure 2).

#### *ABCB7* gene

Nonsynonymous missense variant hg19 chrX:g.74273420C>T in exon 16 (AF241887) of *ABCB7* gene (Gene ID: 22) leads to

substitution of glycine to serine in position 682 of ATP-binding cassette member 7 protein (NP\_001258625, UniProt O75027) (Figures 2a and c). As the identified variant is localized near an acceptor splice site within the first coding triplet of exon 16, we examined if the substitution could lead to the formation of a new strong splice site. The bioinformatic tools testing putative splicing sites based on nucleotide frequencies in each site position (from –20 to +3) for constitutive, cassette, inner acceptors and outer acceptors predicted no splicing alterations for the mutant allele<sup>18,19</sup> (Supplementary Table 5).

There are several known transcripts of *ABCB7* gene encoding protein isoforms, and the identified variant affects all predicted protein isoforms. The structure and function of *ABCB7* protein domains are not fully understood. The mutation is located in the nucleotide-binding domain (NBD) of *ABCB7* protein (NP\_001258625, UniProt O75027), and disruption of this region in *ATM1* yeast ortholog leads to the loss of protein function and iron accumulation inside the mitochondrion (Supplementary Figure 3A).<sup>20</sup> We studied structural alterations in the *ABCB7* protein bearing mutant serine variant using the bioinformatics program Phyre2.<sup>21</sup> Comparative analysis of ~1000



and spermatogenesis.<sup>29</sup> Interestingly, although our data indicate that the full sequence of *PGAM4* retrogene was lost, the Buryat patient with cerebellar pathology and carrier of the above-described X-linked *ABCB7-ATP7A-PGAM4* genetic variants was married and fathered three healthy sons. As anticipated, the genotyping of one of the sons available for this study revealed inheritance of X-chromosome wild-type allele from his mother. Given the potential role of the *PGAM4* gene on male fertility, we verified genetic paternity for the patient and his sons. Using homemade and commercial informative STR markers, we confirmed the authentic biological paternity of the patient bearing the X-linked *ABCB7-ATP7A-PGAM4* genetic variants. Thus, these results suggest that the naturally occurring knockout of *PGAM4* does not disrupt the male fertility. However, given that all offsprings of the patient were males, the pathogenic effect of gene loss on generation or maintenance of X-chromosome-bearing spermatozooids cannot be ruled out.

### Genetic heterogeneity

We found the mutation/deletion in *ABCB7* and *ATP7A* genes in all four examined affected male relatives of this Buryat family with the same diagnosis and in four asymptomatic females relatives with the affected sons (Supplementary Table 6). The mutation/deletion was in the hemizygotic state in all examined affected males and in every female carrier it was heterozygous. All four examined healthy men from the same pedigree were hemizygous for wild-type alleles.

We further tested four unrelated families of patients with the very similar clinical manifestation and with diagnosis of congenital nonprogressive ataxia occurring exclusively in males (Supplementary Table 7). The analysis of all exons of the *ABCB7* gene by direct sequencing (Supplementary Table 8) did not exhibit any differences when compared with human reference genome sequences. Similarly, we found no deletion of exon 2 of the *ATP7A* gene and retrogene *PGAM4* in affected males in these families. The genotyping of these families for mutation NM\_001001344.2:c.3321G>A (p.Gly1107Asp) in the *ATP2B3* gene, which was previously described in cases of cerebellum ataxia,<sup>4</sup> also revealed no difference from wild-type reference allele.

### DISCUSSION

The common feature among nonprogressive ataxias is a noticeable delay in early motor development and a disturbance of motor coordination during the life as a result of cerebellar hypoplasia/atrophy. Here, we report a novel mutation in *ABCB7* that is a causative factor of cerebellar hypoplasia/atrophy and nonprogressive ataxia, thus further expanding the list of mutations in this gene linked to cerebellar ataxia. Previously, four different mutations found in *ABCB7* were linked to cerebellar pathology with sideroblastic anemia. In sideroblastic anemia iron is not incorporated into hemoglobin as it is required to produce healthy erythrocytes by bone marrow. However, the remarkable feature of our case is that the patients have nonprogressive ataxia without the classic symptoms of sideroblastic anemia. It is tempting to speculate that this is because of a unique location of the mutation in *ABCB7*. Alternatively, the deletion of N-terminal part of the *ATP7A* may potentially have a role in modifying the genetic factor.

*ABCB7* protein consists of six transmembrane domains and two intramitochondrial regions, forming NBD (Supplementary Figure 3A). It is localized in the mitochondrial inner membrane, as well as on the cytoplasmic membrane and in the cytoplasm.<sup>30</sup> *ABCB7* has an important role in maturation of cytosolic and mitochondrial Fe-S cluster proteins, affecting the function of cytochrome c, NADH

dehydrogenase (nicotinamide adenine dinucleotide, the reduced form) and succinate dehydrogenase. It is involved in DNA repair and nucleotide excision repair, in oxidative DNA damage repair, in ribosome biogenesis and tRNA thiomodification.<sup>31</sup> The mutation of *ABCB7* protein in our patients is located in the domain facing the inner mitochondrial space. All other mutations described to date, causing refractory anemia and ataxia, are localized near or in the transmembrane domains.<sup>20,32–36</sup> As such, this mutation may affect its activity or binding with other co-factors, as *ABCB7* controls mitochondrial iron transport and protoporphyrin binding during hemoglobin synthesis.<sup>20,32,33,36</sup>

The mental and neurological symptoms are varied in patients with described mutations in the *ABCB7* gene. The depression, cognitive declines or intellectual disabilities and even schizophrenia were described for some patients (Supplementary Table 9). Neurological manifestations in the affected members of the Buryat family demonstrate some common features with the *ABCB7* mutation – associated symptoms in other cases, such as cerebellar ataxia, lack of muscle weakness and deficiency of sensation (Supplementary Table 9). As shown by MRI in the Buryat family case, the brain atrophy was mostly localized in the cerebellar hemispheres and the vermis. However, we failed to detect abnormalities in other regions, including pons and medulla shown in other cases.<sup>34</sup>

The *ABCB7* mutation in our patients is coupled with the deletion in *ATPase* copper-transporter gene (*ATP7A*) (Supplementary Figure 7). Surprisingly, the deletion in the evolutionarily conserved N-terminal part of *ATP7A* found in individuals from the Buryat family does not lead to disease manifestations described for carriers of *ATP7A* mutations. The disruption of this gene is linked to several clinically different disorders characterized by copper metabolic disturbance (OMIM: 300011) including MD (OMIM: 309400) and OHS (OMIM: 304150). Depending on the specific mutation, it can lead to early childhood lethality (before 3 years old), which is characterized by infantile-onset cerebral and cerebellar neurodegeneration, heavy mental retardation and arrest of development. A milder form of MD is not lethal and exhibits weaker clinical symptoms.<sup>37</sup> The *ATP7A* gene mutations also lead to OHS, characterized by a connective tissue disorder and skeletal abnormalities often associated with mild mental retardation (OMIM: 304150), or to distal motor neuropathy, characterized by motor nerve degeneration in the anterior horn of the spinal cord and atrophy of lower and upper extremities (OMIM: 300489). Interestingly, the expression of *ATP7A* protein is inversely correlated with the severity of the disease phenotype.<sup>38</sup> Despite the loss of the first of six MBDs of *ATP7A*, the patients from the Buryat family did not show any clinical signs of the *ATP7A*-associated disorders reported to date. Among the mutation spectrum described for *ATP7A* gene, the gross deletions were represented in ~15% of the MD patients.<sup>39</sup> Thus, it is tempting to speculate that in males carrying the *ATP7A* deletion of the first MBD, the remaining five copper-binding domains are efficiently used to retain the function of *ATP7A*.

We cannot rule out that the dysfunction in *ABCB7* and modestly modified activity of *ATP7A* may mutually affect homeostasis in some tissue cells, as *ABCB7* impairment may cause an accumulation of iron ions in the mitochondria, whereas a mutation of *ATP7A* transporter may affect copper ion levels in the cell cytoplasm. The iron deficiency increases intestinal tissue expression of *ATP7A*<sup>40</sup> and brain copper levels.<sup>41</sup> Copper is known to regulate iron utilization in the bone marrow and in hemoglobin synthesis.<sup>42–45</sup> Importantly, both *ABCB7* and *ATP7A* genes are expressed in the bone marrow<sup>26,46</sup> and in various tissues (Illumina Human Body Map 2.0),<sup>26,46,47</sup> (Supplementary Figure 5). Given the mutual regulatory influence of

the copper and iron uptake, the clinical phenotype described in the Buryat patients may depend on presumable interaction of genetic defects in two genes for the MBPs in some tissue cells, an interesting case of syndrome inherited as a X-linked monogenic trait. We, however, argue that the major cause of cerebellar hypoplasia and the clinical phenotype of ataxia in these patients is caused by this novel mutation in the *ABCB7* gene.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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