Molecular findings in symptomatic and pre-symptomatic Alexander disease patients

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Abstract—Background and Objective: Alexander disease is a slowly progressive CNS disorder that most commonly occurs in children. Until recently, the diagnosis could only be established by the histologic finding of Rosenthal fibers in brain specimens. Mutations in the glial fibrillary acidic protein (GFAP) gene have now been shown in a number of biopsy- or autopsy-proven patients with Alexander disease. A prospective study on patients suspected to have Alexander disease was conducted to determine the extent to which clinical and MRI criteria could accurately diagnose affected individuals, using GFAP gene sequencing as the confirmatory assay. Methods: Patients who showed MRI white matter abnormalities consistent with Alexander disease, unremarkable family history, normal karyotype, and normal metabolic screening were included in this study. Genomic DNA from patients was screened for mutations in the entire coding region, including the exon-intron boundaries, of the GFAP gene. Results: Twelve of 13 patients (~90%) were found to have mutations in GFAP. Seven of those 12 patients presented in infancy with seizures and megalencephaly. Five were juvenile-onset patients with more variable symptoms. Two patients in the latter group were asymptomatic or minimally affected at the time of their initial MRI scan. The mutations were distributed throughout the gene, and all involved sporadic single amino acid heterozygous changes that changed the charge of the mutant protein. Four of the nine changes were novel mutations. Conclusions: In symptomatic and asymptomatic patients with a predominantly frontal leukoencephalopathy by MRI, GFAP gene mutation analysis should be included in the initial diagnostic evaluation process for Alexander disease.

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Alexander disease is a fatal disorder of the CNS that occurs primarily in infants and children. In decreasing order of frequency, three forms of Alexander disease are recognized based on the age of onset: infantile, juvenile, and adult. Younger patients typically present with seizures, megalencephaly, developmental delay, and spasticity. In older patients, bulbar or pseudobulbar symptoms predominate (dysphagia and speech difficulties) frequently accompanied by spasticity, particularly of the legs. The disease is progressive, with most patients dying within 10 years of onset.

Imaging studies of the brain (MRI, CT, or ultrasound) have typically shown abnormalities in the cerebral white matter, preferentially affecting the frontal region. Although these findings are suggestive of Alexander disease, other leukodystrophies, especially those with a metabolic basis (e.g., metachromatic leukodystrophy), can have this pattern of brain involvement. When metabolic screening and genetic testing for other disorders reveal no abnormalities in patients suspected of having Alexander disease, it has been necessary to obtain brain biopsy or autopsy material to establish the diagnosis by the histologic finding of astrocyte cytoplasmic inclusions called Rosenthal fibers.

The genetic basis for most cases of Alexander disease recently has been identified. In a study of 11 autopsy- or biopsy-proven patients with Alexander disease, all but 1 patient were confirmed to harbor mutations in the GFAP gene.
heterozygous mutations in the gene for glial fibrillary acidic protein (GFAP) that resulted in single amino acid changes. All of the mutations currently identified have been sporadic and are consistent with a “change-of-function” effect on the GFAP protein, altering its physical and chemical properties.

Recently, the molecular basis for a leukodystrophy that showed clinical overlap with Alexander disease was also identified. A female patient whose clinical presentation resembled Alexander disease was found to harbor a homozygous mutation in NDUFV1, a nuclear gene that encodes a mitochondrial enzyme in complex I.

In this article, we report a mutation screening study of 13 patients with variable ages of onset (8 infantile and 5 juvenile; 11 symptomatic and 2 asymptomatic) who had not undergone histologic characterization but whose metabolic and MRI results were consistent with the possible diagnosis of Alexander disease. The study was conducted to determine the extent to which clinical and MRI criteria could accurately diagnose Alexander disease, using GFAP gene sequencing as the confirmatory assay. We screened the entire coding region of the GFAP gene in all patients. Patients who were GFAP mutation-negative were then subjected to screening for the NDUFV1 gene. The clinical and molecular data on all the patients tested are presented and discussed.

Materials and methods. Patients. Patient samples were sent to our laboratory for GFAP mutation screening after the evaluation by referring physicians suggested the diagnosis of Alexander disease. Patients were included in our study if they fulfilled the following minimum criteria: 1) MRI findings of white matter changes with preferential involvement of the frontal regions of the brain, 2) normal karyotype, 3) normal metabolic screening, and 4) no family history of any leukoencephalopathy. Patients were included in the study only when their parents or legal guardians signed an informed consent form approved by the Ethics Committee of the Children’s National Medical Center (CNMC).

Typical MRI findings included diffuse and symmetrical white matter abnormalities (low signal on T1, high signal on proton density and T2-weighted sequences) that were most pronounced in the frontal regions but sparing the subcortical U-fibers and the posterior areas of the brain. All patients had normal karyotype, normal concentrations of blood lactate and pyruvate, urine organic acids, plasma very long chain fatty acids, thyroid stimulating hormone, urine N-acetylaspartic acid, and normal leukocyte activities of arylsulfatase A and galactosylceramide beta-galactosidase.

Mutation analysis. From each patient, at least 5 mL blood was collected into purple-top tubes, and samples were shipped at room temperature to CNMC for processing. Genomic DNA was then isolated using the protocol provided with the Purigene® DNA Isolation Kit (Genta Systems, Minneapolis, MN). The genomic DNA was used in PCR reactions designed to amplify the entire coding region and exon-intron boundaries of GFAP (GenBank accession no. XM_008388) and NDUFV1 (GenBank accession no. XM_006370). (Primer sequences and PCR conditions are available from the authors by e-mail request.) Both strands of the resulting PCR products were sequenced using an ABI 3100® automated sequencer (Applied Biosystems, Weiterstadt, Germany).

To confirm whether a nucleotide change was the causative mutation in the patient, similarity to previously identified mutations was verified. If no similarity was found, the affected exon was amplified from DNA of the patient, the parents (when available), and 96 ethnically diverse unrelated healthy individuals (192 chromosomes). Then, the PCR products were subjected to denaturing high-performance liquid chromatography (DHPLC) using the WAVE DNA Fragment Analysis System® from Transgenic. The nucleotide change was determined to be the causative mutation when the pattern was not seen in the patient’s parents or in any healthy individuals tested by DHPLC.

Results. Clinical manifestations. Thirteen patients were screened for mutations in the GFAP gene after meeting the minimum clinical and MRI criteria for Alexander disease. From this group, 12 patients (92%) were determined to have mutations in GFAP. Nine of the 12 patients were male (table). Seven of the 12 patients with GFAP mutations showed onset in the infancy period (see the table), with ages ranging from 2 to 18 months. Five of the 12 patients had age of onset between 5 and 9 years, placing their classification under the juvenile type of Alexander disease (see the table). In the infancy-onset group, seizure episodes were the most commonly observed presenting sign (5 of 7 patients), followed by failure to thrive and delayed motor development. The presenting manifestation in the juvenile-onset group was more variable; some patients were completely asymptomatic or minimally affected and were only discovered by chance when an MRI was performed to evaluate other conditions (Patients 10 and 12), whereas others initially presented with severe clinical signs that included linear growth failure, excessive sleepiness, and frequent vomiting (Patients 9 and 11). With advancing age, patients in both groups showed variable progression of megalencephaly, bulbar or pseudobulbar signs, spasticity, cognitive deficits, and developmental delay. However, seizures and spasticity appeared to be less common in those patients whose onset occurred in the juvenile period (see the table).

Imaging studies. All GFAP mutation-positive patients showed strikingly similar findings on imaging studies. All patients showed diffuse and symmetrical white matter abnormality that predominantly affected the frontal regions of the brain but spared the subcortical U-fibers (figure 1). The posterior regions of the brain were relatively spared. Two patients (Patients 9 and 12) showed involvement that extended to the white matter of the cerebellum, medulla, and pons. Curiously, both patients with more widespread MRI changes showed onset in the juvenile period, when the imaging studies were first performed. In addition, Patient 9 showed small lytic lesions in the periventricular regions anteriorly and posteriorly. Patient 11 also showed a small enhancing mass in the pons that progressively extended within 5 months to the cerebellum through the middle cerebellar peduncle.
Using DNA isolated from peripheral blood of each patient, we screened the entire coding region and the exon-intron boundaries of the GFAP gene (see the table). Of those patients with mutations, more than half (7 of 12 patients) showed mutations in exon 1. The remainder of the mutations were distributed among exon 4 (2 of 12 patients), exon 6 (1 of 12 patients), and exon 8 (2 of 12 patients). Eight patients showed mutations that have been previously reported: R79C,6 R79H,6,7 R88C,7 R239C,6,7 R416W,6 and R416W,6. Of the 4 novel mutations (R79G) identified in our patients involved an amino acid change from a positively charged arginine residue to a noncharged residue, as previously found in all 10 patients reported by Brenner et al.6 However, three patients showed other missense changes (M73R, Y242D, and E373K) that did not involve an arginine residue (figure 2).

The parents of three patients showing novel mutations (Patients 1, 6, and 7) were tested for the same nucleotide change as their affected child, and none showed the change confirming the causative mutations in the patients (see the table; figure 3).

The patient who did not show any mutation in the

<table>
<thead>
<tr>
<th>Patient no./sex</th>
<th>Mutation: Exon/Base change/AA change</th>
<th>Initial presentation</th>
<th>Age at onset(\text{a/}) current age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infancy onset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/F</td>
<td>Exon 1 249C→G R79G(\dagger)</td>
<td>Seizures</td>
<td>3 m/3 y</td>
</tr>
<tr>
<td>2/M</td>
<td>Exon 1 249C→T R79C(\dagger)</td>
<td>Seizures</td>
<td>6 m/5 y</td>
</tr>
<tr>
<td>3/M</td>
<td>Exon 1 250G→A R79H(\dagger)</td>
<td>Seizures</td>
<td>18 m/9 y</td>
</tr>
<tr>
<td>4(\dagger)/M</td>
<td>Exon 1 250G→A R79H(\dagger)</td>
<td>Seizures</td>
<td>7 m/2 y</td>
</tr>
<tr>
<td>5/M</td>
<td>Exon 4 729C→T R239C(\dagger)</td>
<td>Delayed motor development</td>
<td>9 m/4 y</td>
</tr>
<tr>
<td>6/M</td>
<td>Exon 4 738T→G Y242D(\dagger)</td>
<td>Failure to thrive; hypotonia</td>
<td>12 m/5 y</td>
</tr>
<tr>
<td>7/F</td>
<td>Exon 6 1131G→A E373K(\dagger)</td>
<td>Seizures</td>
<td>2 m/5 m</td>
</tr>
<tr>
<td>Juvenile onset</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8/M</td>
<td>Exon 1 232T→G M73R</td>
<td>Strabismus</td>
<td>9 y/15 y</td>
</tr>
<tr>
<td>9/M</td>
<td>Exon 1 276C→T R88C(\dagger)</td>
<td>Excessive sleepiness, frequent vomiting</td>
<td>7 y/9 y</td>
</tr>
<tr>
<td>10/M</td>
<td>Exon 1 276C→T R88C(\dagger)</td>
<td>MRI changes observed during evaluation for short stature</td>
<td>—/4 y</td>
</tr>
<tr>
<td>11/F</td>
<td>Exon 8 1260C→T R416W(\dagger)</td>
<td>Intractable vomiting</td>
<td>5 y/7 y</td>
</tr>
<tr>
<td>12(\dagger)/M</td>
<td>Exon 8 1260C→T R416W(\dagger)</td>
<td>MRI changes observed after accidental eye injury</td>
<td>—/11 y</td>
</tr>
<tr>
<td>13**/F</td>
<td></td>
<td>Seizures</td>
<td>2 m/10 y(\dagger)</td>
</tr>
</tbody>
</table>

\(\text{a}\) No age at onset is reported for Patients 10 and 12; evaluation for leukodystrophy was initiated only after incidental findings of white matter changes were discovered by MRI performed at examination for other conditions.

\(\dagger\) Increased head circumference (\(\dagger\) HC) (megalencephaly) is positive (+) when the occipitofrontal circumference is >95\(\text{th}\) percentile for age. Patient 1 had HC trending at 2 percentile although her height and weight were <10\(\text{th}\) percentile as well; no language development is noted at current age (3 years).

\(\ddagger\) Parents’ DNA tested negative for the mutation.

\(\dagger\) These mutations have previously been described: R79C,6 R79H,6,7 R88C,7 R239C,6,7 R416W,6.

\(\ddagger\) Patient 3 was also homozygous for a 879G→A nucleotide change that results in a D295N amino acid change. This nucleotide change has previously been observed in 3% of healthy control subjects.\(\geq\) In addition, this patient was also heterozygous for a silent 872G→A nucleotide change previously found in 9% of control subjects.\(\geq\). Patients 10 and 13 were likewise heterozygous for these 2 nucleotide changes. Both of these nucleotide changes are found in exon 5.

\(\ddagger\) Patient 4 was also heterozygous for a silent 110T→C nucleotide change in exon 1.

\# Patient 12 also carried a 154C→T nucleotide change in exon 1 that is predicted to result in a P47L amino acid change. This nucleotide change has previously been described in another patient with Alexander disease who also had the 729C→T mutation.\(\geq\)

\(\%\) Patient 13 recently died in a drowning accident.

** Mutation studies. Using DNA isolated from peripheral blood of each patient, we screened the entire coding region and the exon-intron boundaries of the GFAP gene (see the table). Of those patients with mutations, more than half (7 of 12 patients) showed mutations in exon 1. The remainder of the mutations were distributed among exon 4 (2 of 12 patients), exon 6 (1 of 12 patients), and exon 8 (2 of 12 patients). Eight patients showed mutations that have been previously reported: R79C,6 R79H,6,7 R88C,7 R239C,6,7 R416W,6 and R416W,6. One of the 4 novel mutations (R79G) identified in our patients involved an amino acid change from a positively charged arginine residue to a noncharged residue, as previously found in all 10 patients reported by Brenner et al.6 However, three patients showed other missense changes (M73R, Y242D, and E373K) that did not involve an arginine residue (figure 2).

The parents of three patients showing novel mutations (Patients 1, 6, and 7) were tested for the same nucleotide change as their affected child, and none showed the change confirming the causative mutations in the patients (see the table; figure 3).

The patient who did not show any mutation in the
GFAP gene was then subjected to mutation screening for the NDUFV1 gene. Patient 13 did not show any nucleotide changes in this second gene. Her clinical summary is presented in the table.

**Discussion.** Alexander disease is often considered as a remote differential diagnosis in infants presenting with megalencephaly, developmental delay, spasticity, and seizures, or in older onset patients with a preponderance of brainstem signs, spasticity, with or without megalencephaly or seizures. The suspicion for this disease is heightened when MRI studies show white matter changes predominantly affecting the frontal cortical white matter. Until recently, the diagnosis could only be established by the detection of Rosenthal fibers on brain specimens, a finding considered to be the histologic hallmark of Alexander disease. In a recent study, 10 of 11 biopsy- or autopsy-proven patients with Alexander disease were shown by sequencing to harbor mutations in the GFAP gene. To determine the accuracy with which the diagnosis could be made without histologic examination, we selected a cohort of patients with suspected Alexander disease whose imaging studies were compatible with previously described MRI findings, with variable age of onset, clinical presentation, and disease progression. Thirteen patients who showed the typical MRI picture, in whom other metabolic causes were excluded, were tested for mutations in the coding regions of the GFAP gene. Of the 13 patients, we found GFAP gene mutations in 12 (~90%), indicating that most, but not all, cases that present with frontal predominance of leukoencephalopathy and normal metabolic results will show mutations in the coding regions of the GFAP gene. Seven of the 12 patients showed the expected clinical presentation and progression of infancy-onset Alexander disease; 5 patients initially presented in the juvenile period. Our study confirms that MRI finding of predominantly frontal region leukoencephalopathy is highly characteristic of Alexander disease. We have tested 11 other patients with overlapping clinical findings (i.e., seizures and megalencephaly) with more variable white matter changes on MRI (non-frontal predominance), and none has shown mutations of GFAP (data not shown). Thus, DNA testing should now abrogate the need for brain biopsy. More
importantly, GFAP gene mutation analysis should be included in the initial diagnostic evaluation of any patient presenting with a predominantly leukoencephalopathy by MRI.

Agreeing with previous reports, our cohort showed that the infantile type (age of onset from 0 to 2 years) was the most common form, followed by the juvenile type (5 of 12 patients). No adult patients were studied. To date, 40 cases (32 infantile and 8 juvenile) have been confirmed by GFAP sequencing, including 2 Japanese juvenile-onset and 14 French infancy-onset cases that were reported during the preparation of this article.

Interestingly, two of the GFAP mutation-positive patients were ascertained based on incidental MRI findings; both patients were asymptomatic or only minimally affected and had MRI studies performed as part of their evaluation for other conditions. Patient 12 underwent head MRI at age 9 years to evaluate an accidental injury to the eye. He was otherwise asymptomatic except for parent-reported nonprogressive clumsiness and uncoordinated movements. He had reportedly been an excellent student prior to the accident and only recently had his math ability declined to the point of requiring home tutoring in this specific area. Patient 10 was being examined for linear growth failure when a head MRI was ordered by the endocrinologist to evaluate the pituitary prior to a planned initiation of growth hormone therapy. Although his head circumference had always been high borderline, there was nothing in his clinical history to suggest any white matter abnormality. These two cases appear to be serendipitous preclinical ascertainment of patients that likely represent late juvenile or adult-onset cases of Alexander disease. Previous reports have described some adult cases of histologically diagnosed Alexander disease that have been incidentally discovered at autopsy after succumbing to other causes, although such patients have not had GFAP gene mutation studies. What clinical symptoms, if any, these two patients will eventually develop are unknown. However, current findings suggest that these patients may experience a slowly progressive course.

Although the MRI image from an Alexander disease patient typically shows frontal preponderance of diffuse and symmetrical white matter abnormality (see figure 1), our review of sequential imaging studies on some patients revealed that the age at which this picture is first appreciated could be variable. For instance, Patient 1 was first evaluated for seizures at 3 months of age, and showed abnormal contrast enhancement confined to the brain parenchyma at the periventricular regions. It was only with subsequent imaging that the classical frontal involvement appeared and the diagnosis of Alexander disease was considered. This is in contrast to Patient 7 who already showed the classical picture at a comparable age of onset (seizures at 2 months, MRI at 3 months).

T232G → M73R
C249G → R79G
T738G → Y242D
G1131A → E373K

Figure 1. Representative MRI of a patient (Patient 9) with glial fibrillary acidic protein mutation-positive Alexander disease. The patient has juvenile-onset Alexander disease and harbors a 276C→T mutation that predicts a R88C amino acid change. Findings included white matter changes that preferentially involved the frontal lobes and anterior temporal areas. The posterior areas of the brain were relatively spared initially.

Figure 2. Novel glial fibrillary acidic protein mutations identified by sequencing. Chromatographic traces of the novel mutations that were identified by direct sequencing of genomic DNA isolated from peripheral blood of patients are shown. The chromatograms illustrated are from the following patients: Patient 8 (M73R), Patient 1 (R79G), Patient 6 (Y242D), and Patient 7 (E373K).
No clear-cut genotype–phenotype correlations were apparent between the mutations identified and the signs and symptoms that were observed. For example, Patients 11 and 12 who both had the R416W mutation showed disparate clinical manifestations (see the table); Patient 11 showed an earlier onset and relatively more severe bulbar symptoms (frequent vomiting and choking). Conversely, Patients 3 and 4 had relatively early onset; both were males who presented with seizures, and both had the same R79H mutation. However, a previously identified female patient (Patient 2 in Brenner et al.6) also had the same R79H mutation but had an older age of onset (10 years; died at age 48 years).

Most of the GFAP mutations we identified were sporadic single amino acid heterozygous missense changes: M73R (1), R79G (1), R79C (1), R79H (2), R88C (2), R239C (1), Y242D (1), E373K (1), R416W (2). The majority of previously described mutations and one of the four novel mutations reported in this article (R79G) change an arginine to a noncharged residue (figure 4), suggesting that a change in the charge of the protein alters its solubility, structural integrity, or both. In this article, we extend the range of mutations beyond the previous reports, including three amino acid changes that alter charge (Y242D, E373K, and M73R; see figure 4). These changes presumably do not affect protein synthesis but result in a defective protein that alters the oligomerization or solubility of the protein synthesized from the normal allele (toxic change-of-function effect). GFAP is an intermediate filament protein, which places Alexander disease in a growing list of autosomal dominant disorders resulting from change-of-function mutations in genes for intermediate filament proteins that includes Emery–Dreifuss muscular dystrophy (lamin A/C),11 epidermolysis bullosa simplex (keratin 14),12 cardiac and skeletal desmin myopathy (desmin),13 and Meesman corneal dystrophy (keratin 3 and keratin 12).14

To determine the molecular basis for the clinical disorder in Patient 13, who did not show mutations in GFAP, the gene for NDUFV1 was screened for possible mutations. NDUFV1, the gene that codes for the nucleus-encoded mitochondrial protein NADH-ubiquinone oxidoreductase flavoprotein 1, has been implicated in one patient whose clinical history and examination was suggestive for Alexander disease.10 Unlike patients with Alexander disease with heterozygous GFAP mutations, the affected girl was homozygous for a NDUFV1 mutation, indicating that this patient had a recessive form of a neurodegenerative disorder. Although Alexander disease was entertained as a diagnosis, no brain biopsy was obtained to determine the presence of Rosenthal fibers. Screening for the entire coding region of NDUFV1 did not reveal mutations in Patient 13. This patient, and Patient 11 in a previous study6 who showed Rosenthal fibers on histologic examination but no
mutations in the candidates for the pharmacologic modulation of the on GFAP expression, these agents conceivably can be precluded from already neurologically compromised patients (obtaining appropriate tissue for study (brain biopsy). To exclude these possibilities, the frequent difficulty in determining the appropriate tissue for study (brain biopsy from already neurologically compromised patients) can preclude their performance.

Regarding potential therapeutics, some agents have been shown to down-regulate GFAP expression, specifically phosphoinositol-3-kinase inhibitors and quercetin. By virtue of their down-regulating effect on GFAP expression, these agents conceivably can be candidates for the pharmacologic modulation of the disease in those patients who are confirmed to have mutations in the GFAP gene.

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References