Archetypal and New Families With Alexander Disease and Novel Mutations in GFAP

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**Objective:** To describe genetic analyses of the 2 most thoroughly studied, historically seminal multigenerational families with Alexander disease described prior to the identification of GFAP as the related gene, as well as 1 newly discovered family.

**Design:** Clinical histories were obtained and DNA was analyzed from blood, cheek epithelial cells, or fixed paraffin-embedded surgical samples.

**Subjects:** Affected and unaffected adult members of 3 families and affected children were included.

**Main Outcome Measures:** Mutations in GFAP and behavior of mutant protein in cellular transfection assays.

**Results:** Family A contains 4 siblings in whom we found a novel p.Ser247Pro mutation that was paternally inherited. The phenotypes of these siblings include 1 unaffected adult, 1 individual with juvenile-onset disease, and 2 individuals with adult-onset disease. Family B spans 4 generations, including the first described patient with adult-onset disease originally reported in 1968. Analysis of members of the later generations revealed a novel p.Asp417Ala mutation. Family C contains 3 generations. We detected a novel p.Gln426Leu mutation that, to our knowledge, is the farthest C-terminal mutation known.

**Conclusions:** These families display clear evidence of variable phenotypes but do not support recessive inheritance. While germline mosaicism cannot be excluded for 1 family (A), we propose that for genetic counseling purposes the risk of germline mosaicism should be described as less than 1%.


Alexander disease is a rare and generally fatal neurodegenerative disorder of the central nervous system that results from mutations in the astrocyte intermediate filament protein, glial fibrillary acidic protein (GFAP). Before the genetic basis of the disease was discovered, diagnosis required autopsy or biopsy identification of Rosenthal fibers, the characteristic cytoplasmic protein aggregates that are found in abundance in subpial, subependymal, and perivascular astrocytes. The most widely recognized form affects young children, whose initial signs occur before the age of 2 years and who have short life spans. In 1968, based on the unique pathology of abundant and disseminated Rosenthal fibers, Seil et al described the first adult-onset form of the disease. Subsequently, other patients with intermediate ages at onset were identified. In 1992, Duckett et al described an unusual family with 2 affected children, 1 with juvenile-onset disease and the other with adult-onset disease, both of whom received biopsy confirmation of their diagnoses.

GFAP was discovered as the gene mutated in Alexander disease by a candidate gene approach. Later studies quickly linked early- and later-onset forms of the disease as having a common origin in mutations in this single gene. All known mutations are heterozygous, acting in an autosomal dominant fashion. Newly all are missense mutations, predicting a change in a single amino acid, although recently several other types of mutations have been described with short in-frame insertions or deletions or frameshifts occurring at the tail end of the protein. Most mutations arise de novo, though in some instances patients live to reproductive age and transmit the mutation to subsequent generations. Germline mosaicism, which might be the explanation for the family described by Duckett et al, has yet to be documented for any families where Alexander disease occurred in multiple siblings. The penetrance of Alexander disease approaches 100%, the few exceptions being patients who may have only been presymptomatic at the time of study.

Despite the rapid advances of the past decade, the genetic basis for many of the origi-
nal patients with Alexander disease described prior to 2001 has not been established. We now report that GFAP mutations, albeit novel ones, account for the patients described by Seil et al² and Duckett et al,³ as well as previously unreported members of their respective families. Analyses of these families also expand the spectrum of phenotypic variability associated with GFAP mutations, even within families, and indicate that some patients not only stabilize but sustain long periods of clinical improvement. We also present a third family, again with a novel mutation, that prompts consideration of additional phenotypes in future studies of Alexander disease.

METHODS

Informed consent for genetic studies was obtained following protocols approved by the institutional review boards at the University of Wisconsin–Madison and University of Alabama–Birmingham. DNA was isolated from samples of peripheral blood using the PureGene kit (Genta Systems, Minneapolis, Minnesota) according to the manufacturer's recommendations. DNA was isolated from fixed tissues in paraffin blocks as previously described. DNA sequencing was performed as previously described,⁴ and the presence of the mutations was confirmed by restriction enzyme digestion.

FUNCTIONAL ANALYSIS OF MUTANT GFAP PROTEINS

Plasmids used were pcDNA3.1 vectors expressing either the human wild-type GFAP coding region (pcDNA3.1-hGF[WT]) or GFAP with the p.Ser247Pro mutation (pcDNA3.1-hGF[S247P]), p.Asp417Ala mutation (pcDNA3.1-hGF[D417A]), or p.Gln426Leu mutation (pcDNA3.1-hGF[Q426L]). Plasmids were constructed and GFAP expression was analyzed by transfection into SW13vim− cells as previously described.⁵

GERMLINE INHERITANCE

The parental origin of the chromosome bearing the p.Ser247Pro mutation was determined as previously described, using IVS4/H11001 and IVS4/H11022 to indicate maternal and paternal wild-type alleles, respectively. Plasmids were constructed and GFAP expression was analyzed by transfection into SW13vim− cells as previously described.⁵

RESULTS

FAMILY A

Family A consists of 8 siblings born to nonconsanguineous parents. Two of these siblings were previously reported by Duckett et al³ to document the existence of “familial” Alexander disease. A pedigree for this family is shown in Figure 1A, with birth order disguised to protect confidentiality. Patient II.c presented at 26 years of age with a 1-year history of headaches and incoordination. Her condition progressed and she died at the age of 50 years. Samples of brain were donated to the Eunice Kennedy Shriver National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland (accession No. 4858). Patient II.d presented at 8 years of age with chronic vomiting and poor weight gain. At the age of 29.5 years, he remained cognitively intact, although he had obstructive sleep apnea, weakness of palatal elevation, and diminished gag reflex on the right, causing slight difficulty swallowing liquids. His head circumference was 59.5 cm (98%). Patient II.f presented at the age of 29 years, 9 months after experiencing an episode of atonia, with an inability to move her arms and legs. She then devel-

Figure 1. Pedigrees of 3 families with Alexander disease. Numbers below each symbol indicate the age at onset (o) and the age at most recent report or death (d). Genotypes, where available, are indicated by a plus or minus sign above a symbol. Affected individuals are indicated by filled symbols. The presence of the mutations was confirmed by restriction enzyme digestion.

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had no significant changes in cerebral white matter. and signal changes in the deep cerebellar white matter. Patients II.d and II.f patient indicated a space-occupying mass in the left cerebellar hemisphere. Previous magnetic resonance images reported by Duckett et al for this patient indicated a space-occupying mass in the left cerebellar hemisphere and signal changes in the deep cerebellar white matter. Patients II.d and II.f had no significant changes in cerebral white matter.

Figure 2. Magnetic resonance images of 2 affected members of family A. A, Sagittal T1-weighted image of patient II.c, at age 46 years, showing a lesion in the pons (dark arrow) and severe atrophy of the medulla oblongata (white arrow). The cerebellar white matter (arrowhead) has a low signal, which is abnormal. B, Sagittal T1-weighted image after contrast of patient II.d, at age 15 years, showing an enhancing lesion in the dorsal brain stem. The cerebellar white matter (arrowhead) has a low signal, which is abnormal. B, Sagittal T1-weighted image after contrast of patient II.d, at age 15 years, showing an enhancing lesion in the dorsal brain stem.

opened episodic headaches and neck pain, with progressive deterioration in balance. In retrospect, she recalled gait abnormalities and dizziness that began during her early 20s. She had intermittent swallowing difficulties. Her head circumference was 58 cm (98%). Magnetic resonance images (MRIs) of patient II.c at age 46 years revealed severe atrophy of the medulla oblongata, a lesion in the pons, and abnormal signal in the cerebellar white matter (Figure 2A) and of patient II.d at age 15 years, an enhancing lesion in the medulla oblongata (Figure 2B). Asymptomatic siblings available for study included II.b and II.e. II.b has no known neurological or cognitive deficits and was healthy at the age of 51 years. II.e had no known neurological or cognitive deficits and was fully employed after receiving a PhD in engineering. He died at the age of 43 years of suicide.

The parents of these siblings are designated I.b (alive and healthy in her late 60s) and I.a (died at the age of 66 years of a myocardial infarction). The father (I.a) had a long-term history of snoring and sleep apnea. His children also reported that he had a long-term history of snoring and sleep apnea.

DNA isolated from blood samples was analyzed for GFAP coding mutations for each of the affected children (II.c, II.d, and II.f) and 2 apparently unaffected children (II.b and II.e). For II.d, all 9 exons and some flanking intronic segments were sequenced as previously described. A heterozygous 739T→C mutation was found in exon 4, which predicts a p.Ser247Pro coding change. Presence of the mutation results in gain of a Haell restriction site, and this alteration was used to confirm the presence of the mutation. The other family members were similarly tested by restriction enzyme digestion, and the 2 other affected children (II.c and II.f) also tested positive for the p.Ser247Pro mutation. Interestingly, while 1 unaffected sibling (II.b) was wild type, the other (II.e) was positive for the p.Ser247Pro mutation. The presence of the heterozygous mutation in II.c and II.f was confirmed by sequencing.

The finding of multiple siblings in the same family with the same mutation suggests either inheritance from one of the parents or germline mosaicism. To attempt to address this question, we initially determined the genotype of the mother, since the father was deceased. However, the mother (I.b) was wild type for both alleles. We then determined which parental allele harbored the mutated GFAP for patient II.d. Eleven of 13 clones tested were consistent with the mutation being on the paternal inherited chromosome, the most frequent pattern for sporadic Alexander disease. We subsequently located paraffin blocks from a testicular biopsy done on the father (I.a) several years before his death and isolated DNA from this tissue for sequencing. I.a was heterozygous for the p.Ser247Pro mutation.

FAMILY B

Family B consists of 4 generations, the earlier members of which were originally described by Seil et al and Schwankhaus et al. A pedigree for this family is shown in Figure 1B, continuing the nomenclature used by Schwankhaus et al to facilitate identification of individuals across publications. The first reported example of adult-onset Alexander disease was patient III.F, who presented at the age of 32 years for rapid-onset paralysis of his left arm. He died at the age of 47 years and received an autopsy diagnosis of Alexander disease. Two of his brothers, patients III.A and III.E, were considered potentially affected. Three daughters of III.F (IV.B, IV.D, and IV.F) were subsequently described by Schwankhaus et al, one of whom received an autopsy diagnosis (IV.B). Our subsequent studies have concentrated on patient IV.B and her descendants.

Patient V.B had onset at the age of 37 years with generalized weakness that worsened over the course of a year, during which time she also had frequent headaches and experienced significant weight gain (18-22.5 kg). She gradually became nonambulatory and died at the age of 42 years. Autopsy confirmed the diagnosis of Alexander disease. Samples of brain were donated to the Eunice Kennedy Shriver National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland (accession No. 5109). Patient VI.A is the nephew of V.B and presented at the age of 7 years for intractable vomiting. His parents were reported as healthy in their mid-30s. Magnetic resonance imaging revealed a lesion in the dorsal medulla and pons, with a mass effect that caused initial consideration of glioma (Figure 3). Subsequent review revealed signal abnormalities and slight swelling of the caudate nucleus and putamen (Figure 3). The brainstem lesion enhanced after contrast (Figure 3). A second MRI obtained shortly thereafter revealed small frontal white matter lesions and enhancement of the subependymal region (data not shown). Re-review of an earlier computed tomographic scan taken at the age of 6.5 years showed a thin rim of increased density around the lateral ventricles, particularly in the frontal region. The radiological findings were considered diagnostic for Alexander disease. At the age of 10 years, a gastric feeding tube was inserted, leading to rapid weight gain. At
the age of 11 years, he was reported as asymptomatic, with above-average intelligence.

DNA isolated from blood samples was analyzed for GFAP coding mutations for selected members of generations V and VI in Figure 1B. For patient VI.A, all 9 exons and flanking intronic segments were sequenced as previously described. A heterozygous 1250A→C mutation in exon 8 was found, which predicts a p.Asp417Ala coding change. Other individuals were tested by BstF51 digestion for the p.Asp417Ala mutation. The clinically affected aunt (V.B) and unaffected father (V.D) of patient VI.A had the same mutation, whereas his mother (V.C) did not. We subsequently obtained autopsy samples of brain from patient IV.B (the mother of both V.B and V.D and reported by Schwankhaus et al) in the form of paraffin blocks and isolated DNA from this tissue. This individual was also heterozygous for the p.Asp417Ala mutation. The presence of the mutations in IV.B, V.B, and V.D were confirmed by sequencing.

**FUNCTIONAL ANALYSIS OF THE MUTANT GFAP PROTEINS**

Given the variability in phenotypes associated with the p.Ser247Pro and p.Asp417Ala mutations, and the unusual site of the p.Gln426Leu mutation near the C-terminal tail, we tested the effects of these mutations on filament assembly using transfection assays in cultured cells. For these experiments, SW13vim− cells, which have no endogenous cytoplasmic intermediate filaments that could complicate the interpretation, were transfected with vectors that expressed either the mutated or wild-type human GFAP protein. Transfection with the wild-type vector often gave rise to normal GFAP filaments (Figure 4A), but normal filaments were never seen in cells transfected with the p.Ser247Pro mutation; instead, the latter often showed ringlike filaments on a background of diffuse staining (Figure 4B). Since the affected family members are all heterozygous for the p.Ser247Pro change, the effect of cotransfection of the mutant and wild-type vectors was also tested. Presence of the wild-type protein failed to rescue the defect in GFAP polymerization, consistent with a dominant effect of the p.Ser247Pro mutation (Figure 4B, insert). Similar re-
Results were obtained for the p.Asp417Ala and p.Gln426Leu mutant proteins, with the additional feature that an aggregate that appeared to be an agglomeration of rings was often seen extending outwards from the nucleus (Figure 4C and D).

Comment

We report genetic findings in 3 families with Alexander disease, 2 of which were previously known from seminal clinicopathologic publications during the pregenetic era. A novel mutation was found for each of the 3 families. Although the clinical presentations now fit within the recognized spectrum of juvenile- and adult-onset Alexander disease, consideration of these families raises a number of important issues, such as the criteria for judging causality when confronted by novel mutations, the possibilities of recessive inheritance, and the sometimes extreme variability in expressivity of the clinical phenotypes.

The GFAP mutation in family C was found to arise de novo in patient II.1, providing strong evidence that it is disease causing. For families A and B, the origin of the mutations could not be determined, requiring other evidence to establish their disease causality. Three critical criteria for being disease causing were met by these mutations: (1) they were present in each affected family member tested; (2) the DNA changes are not simply single-nucleotide polymorphisms, since they have not been reported for more than 100 other individuals whose GFAP coding region has now been sequenced for Alexander disease diagnosis nor are they reported in the National Center for Biotechnology Information single-nucleotide polymorphism database, which includes sequence results from 200 Danish controls; and (3) each mutant GFAP displays grossly aberrant polymerization when expressed in SW13vim− cells.

Each of the new GFAP mutations reported herein, Ser→Pro, Asp→Ala, and Gln→Leu, involves a significant change in amino acid properties; however, GFAP mutations as conservative as Asp→Glu and Leu→Val also cause Alexander disease. Clearly, the context in which an amino acid substitution occurs is critical. Like other cytosolic intermediate filament proteins, the GFAP monomer has been described as having unstructured N- and
C-terminal domains separated by a central rod region consisting of 4 α-helical segments, 1A, 1B, 2A, and 2B, separated by nonhelical linkers L1, L12, and L2. By this scheme, the p.Ser247Pro coding change found in family A occurs at the penultimate amino acid in the 2A helical segment and would be expected to shorten the α-helical segment by just 2 amino acids. However, recent crystallographic evidence suggests a continuity of α-helix from 2A to 2B, providing a more understandable basis for the p.Ser247Pro change having significant consequences. The p.Asp417Ala and p.Gln426Leu mutations occur in the nonhelical tail region and are the farthest C-terminal point mutations reported. Asp417 is part of the RDG sequence that is highly conserved among intermediate filaments. Cell transfection experiments have indicated a role for this sequence in GFAP filament formation, and it has also been suggested to participate in interactions with other proteins. The adjacent Arg416 site is a hot spot for Alexander disease mutations (http://www.waisman.wisc.edu/alexander-disease/ and www.interfil.org). The p.Gln426Leu mutation in family C occurs just 7 amino acids from the end of the protein, making it the farthest C-terminal mutation yet described for Alexander disease. To our knowledge, no evidence for functionality for this region of the protein has previously been reported; in fact, the entire C-terminal region after the RDG sequence can be deleted from GFAP without affecting its polymerization in transfected SW13vim− cells. While the drastic effect of a point mutation within this region might be considered surprising (Figure 4D), it is consistent with a previous study of desmin, also a type III intermediate filament, that found effects on assembly from amino acids 425 to 429. By this scheme, the p.Asp417Ala mutation in family A harbored heterozygous p.Arg276Leu mutations reported by Duckett et al3 were one of the first to raise the possibility of recessive inheritance for Alexander disease. They reported a family with 2 affected siblings, 1 with juvenile-onset disease and the other with adult-onset disease, both of whom received biopsy confirmation of their diagnoses. However, the past decade has shown that all known disease-causing mutations in GFAP are heterozygous. Another possibility to explain multiple siblings with identical mutations is germline mosaicism. While a theoretical possibility, germline mosaicism has not been demonstrated for any family with Alexander disease. Namekawa et al18 reported 2 brothers with onsets in their 30s and 40s who each had the same p.Arg276Leu mutation. While their parents were described as neurologically normal into their 70s, none of their tissues were available for analysis, leaving the question of germline mosaicism unresolved. Our finding that all affected members of family A harbored heterozygous p.Ser247Pro mutations, also present in testicular tissue from their father, shows that inheritance was dominant rather than recessive. Whether germline mosaicism occurred in this family cannot be determined; the father showed clinical signs consistent with mild impairment, but the only tissue available for analysis was testicular, in which the vast majority of DNA is derived from sperm. Estimating risk of germline mosaicism is particularly problematic for rare disorders, as discussed by Edwards,19 and it is likely that a parent with mosaicism of germ cells would possess some degree of somatic mosaicism as well. Given that more than 100 patients/families have now been published who have genetic diagnoses of Alexander disease, with no documented cases of germline mosaicism, we propose that for genetic counseling purposes the risk of germline mosaicism should be described as less than 1%.

While the GFAP mutations causing Alexander disease are typically viewed as 100% penetrant, several examples now exist that warrant reconsideration of this issue. Previous discussions of variable expressivity have been complicated by uncertainty whether genetically positive individuals were being seen at presymptomatic stages and especially by variability in depth of evaluation. One example is a family with p.Leu331Pro mutations that consists of a 16-month-old boy with megalencephaly and MRI changes characteristic of Alexander disease but who is otherwise asymptomatic.20 His sister and mother both had the same mutation, and mild changes on MRI, but no megalencephaly or any other symptoms. Whether this particular mutation is unusually mild, or any of these 3 individuals is truly unaffected, is unclear. A second example is the p.Asp78Glu family reported by Stumpf et al.21 Most of these patients had adult-onset disease, but 1 individual experienced a juvenile onset. In addition, a 30-year-old woman was identified who was positive for the mutation but without any obvious symptoms. However, she was unavailable for a complete neurological examination. The findings in our p.Asp417Ala family clearly support variable expressivity, at least with respect to combined juvenile and adult onsets in the same family. Our findings in the p.Ser247Pro family also provide support for variable expressivity, not only with regard to having both juvenile and adult onsets among siblings, but a father who was possibly affected and lived until 62 years of age with only mild and equivocal signs of disease. Furthermore, this family included 1 mutation-positive brother who had no neurological symptoms at all until his death at age 43 years. Unfortunately, this latter individual’s tissues were not available for postmortem review, and no antemortem imaging had been conducted.

Finally, whether GFAP mutations account for the occasional individuals in whom abundant Rosenthal fibers are found at autopsy without preexisting suspicion of Alexander disease, previously termed Rosenthal fiber encephalopathy,22 is an interesting question. These patients typically had histories of systemic illnesses (such as infection, neoplasia, or hypertension), sometimes combined with substance abuse.23 An alternative possibility is that secondary illness or intoxications can independently lead to the formation of Rosenthal fibers, perhaps by simple upregulation of GFAP expression above a critical threshold (as happens in transgenic mice that overexpress wild-type GFAP24). These questions should be considered in future research on Alexander disease and the interpretive value of Rosenthal fibers.
REFERENCES