Neurodegeneration associated with genetic defects in phospholipase A2

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ABSTRACT

Objective: Mutations in the gene encoding phospholipase A2 group VI (PLA2G6) are associated with two childhood neurologic disorders: infantile neuroaxonal dystrophy (INAD) and idiopathic neurodegeneration with brain iron accumulation (NBIA). INAD is a severe progressive psychomotor disorder in which axonal spheroids are found in brain, spinal cord, and peripheral nerves. High globus pallidus iron is an inconsistent feature of INAD; however, it is a diagnostic criterion of NBIA, which describes a clinically and genetically heterogeneous group of disorders that share this hallmark feature. We sought to delineate the clinical, radiographic, pathologic, and genetic features of disease resulting from defective phospholipase A2.

Methods: We identified 56 patients clinically diagnosed with INAD and 23 with idiopathic NBIA and screened their DNA for PLA2G6 mutations.

Results: Eighty percent of patients with INAD had mutations in PLA2G6, whereas mutations were found in only 20% of those with idiopathic NBIA. All patients with two null mutations had a more severe phenotype. On MRI, nearly all mutation-positive patients had cerebellar atrophy, and half showed brain iron accumulation. We observed Lewy bodies and neurofibrillary tangles in association with PLA2G6 mutations.

Conclusion: Defects in phospholipase A2 lead to a range of phenotypes. PLA2G6 mutations are associated with nearly all cases of classic infantile neuroaxonal dystrophy but a minority of cases of idiopathic neurodegeneration with brain iron accumulation, and genotype correlates with phenotype. Cerebellar atrophy predicts which patients are likely to be mutation-positive. The neuropathologic changes that are caused by defective phospholipase A2 suggest a shared pathogenesis with both Parkinson and Alzheimer diseases.

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GLOSSARY

INAD = infantile neuroaxonal dystrophy; NBIA = idiopathic neurodegeneration with brain iron accumulation; PKAN = pantothenate kinase-associated neurodegeneration.

The neuroaxonal dystrophies are degenerative disorders that share the pathologic feature of axonal spheroids in brain. Spheroids are poorly understood axonal swellings that occur in infantile neuroaxonal dystrophy (INAD), pantothenate kinase-associated neurodegeneration (PKAN, formerly Hallervorden-Spatz syndrome), idiopathic neurodegeneration with brain iron accumulation (NBIA), and Schindler disease. INAD is a severe psychomotor disorder with early onset and rapid progression of hypotonia, hyperreflexia, and tetraparesis. Spheroids are found in both the central and peripheral nervous systems in INAD, and iron accumulates in brain in a subset of these patients. The term “neurodegeneration with brain iron accumulation” is used both as a descriptor for the set of neurologic disorders with high basal ganglia iron and as a specific diagnostic category.
for patients whose molecular basis of disease is unknown.\textsuperscript{4,5} As causative genes are identified, the stratification of patients with idiopathic NBIA will shift to reflect new understanding of etiology.

Recently, we discovered that some cases of idiopathic NBIA are caused by mutations in the same gene that underlies INAD.\textsuperscript{6} This gene, \textit{PLA2G6}, encodes a phospholipase A\textsubscript{2} group VI protein that is suspected to play a key role in cell membrane homeostasis.\textsuperscript{7} Since INAD and some cases of NBIA share a causative gene, we sought to characterize the range of clinical features associated with \textit{PLA2G6} mutations in these two populations. In the current study, we genotyped patients with INAD or idiopathic NBIA and reviewed their medical records to assess whether there was a correlation between clinical, radiographic, or pathologic findings and the presence of a \textit{PLA2G6} mutation.

**METHODS**

**Subjects.** We recruited subjects through a research listing on GeneTests\textsuperscript{8} and on the Oregon Health & Science University Web site. Using data collected from physicians by questionnaire and medical records, we identified 56 patients from 53 families with a clinical diagnosis of INAD and 23 patients from 20 families with a clinical diagnosis of idiopathic NBIA. Most patients underwent neuroimaging, but the specific clinical studies varied widely, and images or interpretation information were sometimes limited or unavailable. Clinical criteria\textsuperscript{1} were used to help classify patients with INAD within the limits of the clinical information available. All the patients classified as INAD had onset before 3 years of age with psychomotor regression and progression of disease. Each patient with INAD had one or more of the following: axonal spheroids on peripheral nerve biopsy, truncal hypotonia, and tetraparesis. Truncal hypotonia was reported in 48 of the patients with INAD (unknown for 8), and 39 of these developed the typical pattern of subsequent tetraparesis. Axonal spheroids were present in most cases (39 of 47, 9 additional patients had not been biopsied). Individuals with iron accumulation in the basal ganglia (most often globus pallidus and substantia nigra), without an eye-of-the-tiger sign on T2-weighted brain MRI, and progressive dystonia and dysarthria were classified as idiopathic NBIA. Patients with idiopathic NBIA were negative for mutations in the \textit{PANK2} gene, confirming that none had PKAN. Although some patients classified with INAD had iron accumulation in the globus pallidus, none had dystonia. Referring physicians provided DNA samples and medical information after participants had given written informed consent according to a protocol approved by the institutional review board at Oregon Health & Science University. Only partial clinical data were available on some patients.

**Analysis of mutations.** We used primer sets to amplify all \textit{PLA2G6} exons and adjacent intronic sequences including splice signals and sequenced DNA in at least one affected individual from each family. Mutations were not routinely confirmed in parental samples of mutation-positive individuals.

**RESULTS**

**Genetic findings.** Of the 79 patients tested, we found \textit{PLA2G6} mutations in 45 individuals from 42 families with INAD (79% of unrelated cases) and in 6 members of 4 families with idiopathic NBIA (20% of unrelated cases). We were unable to identify a \textit{PLA2G6} mutation in 11 patients with clinical evidence of INAD, including 5 in whom spheroids were reported on peripheral nerve biopsy. Analysis for gene dosage alterations is currently underway for these cases. All 28 patients with two null mutations or homozygous for any mutation had early onset, rapidly progressive disease. No mutation accounted for >10% of cases in any population. In two patients, we found only a single mutant allele; one patient had INAD and the other idiopathic NBIA. Individual patients and their corresponding mutations are summarized in table 1.

**Clinical findings.** In mutation-positive patients classified as having INAD, the average age at onset was 1.1 years (range 5 months to 2.5 years, n = 44) presenting with psychomotor regression characterized by early truncal hypotonia progressing to tetraparesis (95%, n = 39). These data are consistent with previous clinical descriptions of the disorder.\textsuperscript{1,11} The weakness was usually spastic (70%) but sometimes areflexic (30%). Nearly half of the patients (46%) presented with ataxia or gait instability. Neuroophthalmologic abnormalities were common: 79% (n = 25) of patients developed optic atrophy at an average age of 3.7 years, with nystagmus and strabismus developing in 73% and 67% of patients, respectively. Most patients had evidence of denervation on electromyogram (78%, n = 36) and fast rhythms on EEG (77%, n = 30). Only one-third had decreased nerve conduction velocity (35%, n = 37) with the same fraction manifesting generalized seizures (29%, n = 38; average onset at 7 years, range 3 to 18 years). The average age at death of the 11 patients who had died was 9.4 years (range 5 to 16 years). Clinical features in the 11 mutation-negative INAD patients did not differ significantly from those of patients with mutations (table 2).
Table 1 PLA2G6 mutations identified in patients

<table>
<thead>
<tr>
<th>Individual</th>
<th>DNA mutation*</th>
<th>Protein change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic INAD</td>
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</tr>
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<tr>
<td>125</td>
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</tr>
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<tr>
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<td>292</td>
<td>PLA2G6IVS13_FLJ22582IVS1del homozygous</td>
<td>Fusion protein</td>
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</table>

—Continued

In contrast to those with INAD, the cohort of patients classified as having idiopathic NBIA had widely varying phenotypes, and mutation-positive patients differed significantly from those lacking mutations (table 2). Patients with mutations had an average age at onset of 4.4 years (range 1.5 to 6.5, n = 6), vs 6.8 years (range 1 to 31, n = 15) for mutation-negative patients. Four patients presented with gait instability or ataxia, three had speech delay, and two were also noted to have diminished social interaction. One maintained a diagnosis of autism until 8 years of age, when his parents noted toe-walking and gait instability. Two-thirds of mutation-positive patients had optic atrophy, but only 25% of those without mutations showed this feature. The frequencies of tetraparesis (spastic or areflexic), nystagmus, and seizures in mutation-positive patients with NBIA did not differ significantly from those in patients with INAD. However, three other common features of INAD were not observed in any mutation-positive patient with NBIA, including truncal hypotonia (p = 0.0001), strabismus (p = 0.008), and fast rhythms on EEG (p = 0.002). Two patients (40%, n = 5) had evidence of denervation on EMG.

The natural history of disease for patients with mutation-positive NBIA included progressive dystonia and dysarthria and neurobehavioral disturbances with impulsivity, distractibility and poor attention span, hyperactivity, and emotional lability.

Radiographic findings. Radiographic data were collected through use of a questionnaire completed by the referring physicians. Whenever possible, the corresponding author also reviewed images. Imaging approaches varied and some studies were lost, leading to incomplete data and varying denominators for the features described.

Most mutation-positive patients had cerebellar atrophy (95% of INAD cases, n = 40; 83% of NBIA cases, n = 6). It is noteworthy that the two patients with INAD without cerebellar atrophy were studied at less than 2 years of age, perhaps before the development of this radiographic feature. Among mutation-negative patients, we saw cerebellar atrophy in 70% (n = 10) of those diagnosed with INAD but in only 12% (n = 17) of those with idiopathic NBIA. Eleven of 18 patients with mutation-positive INAD had cerebellar hyperintensity on proton density, FLAIR, or T2-weighted MRI, which was not found in any patients with NBIA. The entire cohort of patients with mutations had a multitude of additional MRI abnormalities including thin corpus callosum, mixed white and gray matter atrophy, mega cisterna magna, thin optic chiasma, enlarged ventricles, verminal atrophy, and on T2-weighted imaging, hyperintense putamen and cerebral cortex.
Table 1 Continued

<table>
<thead>
<tr>
<th>Individual</th>
<th>DNA mutation*</th>
<th>Protein change†</th>
</tr>
</thead>
<tbody>
<tr>
<td>293-1</td>
<td>1039G&gt;A homozygous</td>
<td>G347R</td>
</tr>
<tr>
<td>293-2</td>
<td>1039G&gt;A homozygous</td>
<td>G347R</td>
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<td>294</td>
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</tr>
<tr>
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<td>R635X</td>
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<td>297</td>
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<tr>
<td>336</td>
<td>2370_2371delTG homozygous</td>
<td>Y790X</td>
</tr>
</tbody>
</table>

Atypical NAD (previously categorized NBIA‡)

16-1 1744G>T V582L
2233C>T R745W
16-2 1744G>T V582L
2233C>T R745W
45 2380G>A A80T
2370_2371delTG Y790X
102 319delC 2nd allele not identified L107fsX110
288-1 1061T>C L354P
2215G>C D739H
288-2 1061T>C L354P
2215G>C D739H

*Changes in the nucleotide sequence (GenBank accession number NM_003551) are shown.
*Changes in the amino acid sequence (GenBank accession number NP_003551) are shown.
†Some patients originally categorized by criteria described in Methods as having NBIA were subsequently categorized as atypical NAD according to nomenclature proposed in Discussion.
‡Some patients originally categorized by criteria described in Methods as having NBIA were subsequently categorized as atypical NAD according to nomenclature proposed in Discussion.

By definition, all of the cases classified as idiopathic NBIA regardless of mutation status had high iron in the globus pallidus. The pattern of iron accumulation was similar to that seen in other forms of NBIA but did not include globus pallidus central hyperintensity on T2-weighted imaging.

Neuropathologic findings. In PLA2G6 mutation-positive INAD, peripheral nerve biopsies (skin, conjunctiva, sural nerve, muscle, areola, and rectal mucosa) demonstrated axonal spheroids in 87% of patients (n = 39). While one child who was negative for spheroids was tested only once at 2 years of age, a second individual had a negative biopsy at 12 years despite the finding of a positive skin biopsy in his affected sibling at 11 years of age.† Five of 8 mutation-negative patients (63%) also had spheroids reported on biopsy. Two patients with NBIA and mutations in PLA2G6 underwent a biopsy for spheroids, only one of which was positive.

Table 2 Frequency of major features in proposed diagnostic categories

<table>
<thead>
<tr>
<th></th>
<th>Spheroids in peripheral nervous system</th>
<th>Optic atrophy</th>
<th>Cerebellar atrophy</th>
<th>Brain iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic INAD,* mutation-positive</td>
<td>87% [34/39]</td>
<td>79% [30/38]</td>
<td>95% [38/40]</td>
<td>48% [14/29]</td>
</tr>
<tr>
<td>Idiopathic NBIA, mutation-negative</td>
<td>Unknown</td>
<td>25% [4/16]</td>
<td>12% [2/17]</td>
<td>100% [16/16]</td>
</tr>
</tbody>
</table>

Forty-eight percent of mutation-positive patients with INAD had abnormal iron accumulation in the globus pallidus (14 of 29 patients), and 4 had increased iron in the substantia nigra as well. Among mutation-negative patients, globus pallidus iron was observed in only 1 of 9 patients (11%) with INAD.

Within a diagnostic category, denominators vary by feature because of missing clinical data points. Data were mainly obtained from medical records, which varied widely based on clinical tests ordered for each subject.

*Classic INAD mutation-positive vs mutation-negative groups did not differ significantly for any of the features shown.
†Atypical NAD cases differed significantly from mutation-negative NBIA cases (p < 0.01).

INAD = infantile neuroaxonal dystrophy; NBIA = neurodegeneration with brain iron accumulation.
appeared as rounded eosinophilic swellings with a diameter of 30–100 \( \mu \text{m} \) that were also stained with Bielschowsky silver stain. We saw brown granular iron deposits in a perivascular morula-like pattern within globus pallidus and substantia nigra (figure 1C). Large spheroids were also present in globus pallidus and stained with neurofilament antibodies.

We also observed pathologic features in this case that define Parkinson disease and dementia with Lewy bodies. Most pigmented neurons in the substantia nigra pars compacta contained classic Lewy bodies (figure 1D) that were strongly labeled with \( \alpha \)-synuclein antibodies (figure 1, E and F). Many cortical regions, including cingulate, prefrontal, midfrontal, and temporal cortex, contained Lewy bodies that were accompanied by synuclein-positive dystrophic neurites and spheroids (figure 1, G–I). Lewy bodies were present in the dorsal raphe nucleus, thalamus, and locus coeruleus, while synuclein-positive spheroids and dystrophic neurites were abundant in locus coeruleus, caudate, putamen, globus pallidus, dorsal raphe nucleus, and hypothalamic nuclei. Many of the spheroids identified on hematoxylin and eosin staining were not stained with synuclein antibodies.

We also observed neurofibrillary tangles in cortical neurons, a pathologic hallmark of Alzheimer disease. Neurofibrillary tangles were visualized by Bielschowsky silver stain (figure 1) and by immuno-
histochemistry with PHF-1 antibody to hyperphosphorylated tau (figure 1, K and L). They were most abundant in entorhinal and temporal cortex but were also present in cingulate, motor, and midfrontal cortex. Tau-positive dystrophic neurites were infrequently observed with PHF-1 staining.

DISCUSSION

In this study, we identified mutations in the phospholipase A2 group VI gene (PLA2G6) in the majority of patients clinically diagnosed with INAD and in a smaller fraction of patients with idiopathic NBIA. This latter condition is thought to encompass several different disorders, accounting for the lower frequency of mutations observed in this group. We found that genotype correlates with phenotype: mutations predicted to lead to absent protein were associated with the INAD profile of early onset and rapid progression, whereas compound heterozygous missense mutations correlated with the less severe phenotype of NBIA, consistent with residual function in the mutant protein (table 1).

The term “infantile neuroaxonal dystrophy” is well established in the literature, as with other disorders historically described based on their clinical and pathologic features. Rather than abandon this terminology in the face of the PLA2G6 gene discovery, we instead propose a functional classification of mutation-positive patients to reflect the shared molecular etiology of their disease. Children presenting with psychomotor regression in the first year of life and loss of ambulation within 5 years should be designated as having “classic INAD.” When disease onset is later and progression slower, the term “atypical neuroaxonal dystrophy” should be used. For patients with brain iron on imaging but without a defined genetic basis for their disease, we propose continuing to use the term “idiopathic NBIA.” As more people are discovered to have mutations in PLA2G6, we anticipate that the phenotypic spectrum of atypical neuroaxonal dystrophy will probably expand but that this classification will remain useful.

Prior to the discovery of the causal gene, a diagnosis of INAD was confirmed by demonstrating axonal spheroids in a peripheral nerve biopsy. In our study, we found that some patients with mutations lacked spheroids and some without mutations had spheroids (table 2), indicating incomplete detection using either pathologic or molecular methods. We were unable to identify a mutation in nearly one-fifth of patients with a strong clinical profile of INAD, including five patients with peripheral spheroids. We presume these patients to have undetected PLA2G6 mutations, indicating an 87% detection rate using current molecular and pathologic methods in combination. With the development of methods to identify large deletions, duplications, and insertions, we predict that the PLA2G6 mutation detection rate will exceed 95% by molecular methods alone, as has been achieved for other rare autosomal recessive disorders. Such assays are being developed for clinical use, and preliminary studies in patients with strong clinical and radiographic features for INAD suggest that up to 25% of alleles negative by sequencing contain large deletions. When INAD is suspected, we recommend that genetic testing precede tissue biopsy, which would be unnecessary when PLA2G6 mutations are identified by DNA analysis. In rare cases, biopsy of peripheral tissue (conjunctiva, rectal mucosa, skin, or sural nerve) may still be useful in the diagnostic evaluation of mutation-negative patients.

The spectrum of clinical features associated with mutations in PLA2G6 is broader than previously described, and genotype correlates with phenotype. We found the clinical profile of classic INAD to be a fairly uniform one of early hypotonia and tetraparesis that progresses rapidly to complete psychomotor retardation. We observed optic atrophy, nystagmus, and strabismus in most patients. We found abnormalities on various electrophysiologic measures, including a decrease in nerve conduction velocity, denervation on EMG, and fast rhythms on EEG. In contrast, we found atypical neuroaxonal dystrophy to be clinically heterogeneous, with patients showing later onset disease and slower progression, with variable ataxia, spasticity, and neurobehavioral abnormalities.

The imaging findings in classic infantile and atypical neuroaxonal dystrophy will guide diagnostic molecular testing. We observed cerebellar atrophy in nearly all PLA2G6 mutation-positive patients, and

(A) High globus pallidus iron (arrow) on T2-weighted imaging in a 9-year-old patient with atypical neuroaxonal dystrophy and PLA2G6 mutations. (B) Cerebellar atrophy (arrow) is evident in a case of classic infantile neuroaxonal dystrophy.
globus pallidus iron accumulation in half, evident as
low signal intensity on T2-weighted MRI (figure 2).2
Since cerebellar atrophy is not typically found in
other disorders associated with high brain iron, this
combination of features is strongly predictive of a
PLA2G6 mutation.12 Thus, if neuroimaging shows
cerebellar atrophy with or without evidence of iron
accumulation in a patient with psychomotor regres-
sion, molecular testing for a PLA2G6 mutation is
indicated.

The pathology associated with mutations in
PLA2G6 suggests a shared pathogenesis with both
Parkinson disease and Alzheimer disease. In this
study, we saw not only the expected peripheral and
central spheroids, but also α-synuclein-positive
Lewy bodies, dystrophic neurites, and neurofibril-
lar tangles in the brain tissue of a 23-year-old
patient with atypical neuroaxonal dystrophy.
Interestingly, although synuclein-positive spheroids
have been observed in classic early onset cases with
death before age 10,13 Lewy bodies and neurofi-
brillary tangles have not been reported in associa-
tion with this phenotype. This suggests that in
order to form these structures either exposure to
the neuropathologic process must be prolonged, or
the brain must reach a certain stage of maturation.
Our patient is among the youngest with any dis-
ease in whom Lewy bodies and neurofibrillary tangles
have been described.

The pathologic and imaging features shared by dis-
orders of distinct molecular etiology may lend further
insights into pathogenesis. The similar appearance of
spheroids and iron in classic and atypical neuroaxonal
dystrophy and in PKAN, another inherited neurode-
gerative disorder with brain iron accumulation, sug-
gests that defects in the respective mutant proteins lead
to a common mechanism of iron dyshomeostasis in
basal ganglia. The PLA2G6 protein is critical for cellu-
lar membrane phospholipid homeostasis and remodel-
ing,7 and defects in this protein may cause
accumulation of membranes, organelles, and protein in
distal axons that represent spheroids. The protein that is
defective in PKAN normally regulates the biosynthesis
of coenzyme A, which is critical to fatty acid metabo-
лизm. Both proteins associate with mitochondria and
play a role in lipid metabolism, which when perturbed
may alter the regulation of iron transport and utiliza-
tion. By identifying genes that underlie neurodegenera-
tive disorders with high brain iron, we have improved
diagnostic testing and gained insights into pathogenesis.
This new knowledge may guide the development of
therapeutic strategies not only for these rare disorders
but also for pathogenically related more common ill-
nesses such as Parkinson disease and Alzheimer disease.

NOTE ADDED IN PROOF
Mutations have now been found in adult-onset parkinsonism-dystonia
PLA2G6 as a locus for dystonia-parkinsonism. Ann Neurol Epub ahead of
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