Patients Homozygous and Heterozygous for SNCA Duplication in a Family With Parkinsonism and Dementia

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Background: Multiplication of the α-synuclein gene (SNCA) (OMIM 163890) has been identified as a causative mutation in hereditary Parkinson disease or dementia with Lewy bodies.

Objective: To determine the genetic, biochemical, and neuropathologic characteristics of patients with autopsy-confirmed autosomal dominant Lewy body disease, with particular reference to the dosage effects of SNCA.

Design: Four-generation family study.

Setting: Academic research.

Patients: We fractionated samples extracted from frozen brain tissues of 4 patients for biochemical characterization, followed by immunoblot analysis.

Main Outcome Measures: We determined the dosages of SNCA and its surrounding genes by quantitative polymerase chain reaction analysis.

Results: Quantitative polymerase chain reaction analysis revealed that 3 patients were heterozygous for SNCA duplication and 1 patient was homozygous for SNCA duplication. The homozygous patient showed earlier age at onset and earlier death, with more severe cognitive impairment than the heterozygous patients. Biochemical analysis revealed that phosphorylated α-synuclein accumulated in the sarkosyl-insoluble urea-extracted fraction of the brains of the patients.

Conclusions: Pathologically confirmed Lewy body disease clinically characterized by progressive parkinsonism and cognitive dysfunction is caused by SNCA duplication. The homozygous patient demonstrated the most severe phenotype, suggesting that SNCA dosage has a considerable effect on disease phenotype even within a family. SNCA duplication results in the hyperaccumulation of phosphorylated α-synuclein in the brains of patients.

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Dementia with Lewy bodies (LBs) is clinically characterized by parkinsonism, recurrent visual hallucinations, and fluctuating cognitive impairment. Cognitive impairment is a common symptom of Parkinson disease, and this condition is referred to as dementia associated with Parkinson disease. Most cases of dementia associated with Parkinson disease or dementia with LBs are sporadic, but familial cases have been reported. In an Iowa family, triplication of the α-synuclein gene (SNCA) was found. The clinical features of patients from the Iowa kindred ranged from Parkinson disease to dementia with LBs, with age at onset ranging from 20 to 48 years. Neuropathologic examinations of the affected members of the Iowa family revealed extensive formation of LBs and α-synuclein-positive glial cytoplasmic inclusions. In addition to parkinsonism, cognitive impairment was observed in the Iowa family and in a Swedish American family, both carrying SNCA triplication. Subsequently, SNCA duplication was documented in familial Parkinson disease, in which late onset and slow progression of the disease were observed. Neither cognitive decline nor dementia was a prominent clinical manifestation in families with SNCA duplication.

A Japanese family presenting with progressive parkinsonism with dementia, transmitted as an autosomal dominant trait, was previously described. Neuropathologic findings in 2 patients showed marked neuronal loss with LBs in pigmented nuclei of the brainstem and numerous cortical LBs. In the present study, we identified an alteration in SNCA dosage in the same family and report the results of our biochemical and neuropathologic analyses.

METHODS

PATIENTS AND SAMPLES

This study was performed under institutional review board–approved protocols. The clinical fea-
tures of the family have been partly described elsewhere. The neuropathologic findings in 2 members of the family (II-4 and III-1) have been reported elsewhere. Another affected member of the family (IV-1) recently underwent autopsy, and the neuropathologic findings are included in this study. This study was approved by the Ethical Committee of Niigata University, Niigata, Japan.

GENETIC ANALYSIS

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures with informed consent. We tested microsatellite markers within or flanking the SNCA locus, which include D4S2458, TC16, Rep1, IVS-4, D4S2304, and D4S2458. Polymerase chain reaction (PCR) was performed in the log linear range, which enables semiquantitative assessment. The ratio of integrated peak heights was standardized using an internal diploid PCR control sample, and the ratio was compared with ratios of patients with diploid to estimate allele copy number. Alleles with a higher peak as determined by semiquantitative PCR analysis using the already mentioned markers were considered disease-associated alleles with gene dosage alteration. We performed realtime PCR analysis to determine SNCA dosage (SYBR Green PCR; Applied Biosystems, Foster City, California). We first targeted exons 2 and 6 of SNCA to detect any alteration in SNCA dosage, followed by real-time PCR analysis of the surrounding genes to determine the duplicated regions in the patients. The albumin gene (OMIM 103600) was amplified as an endogenous reference. SNCA dosage normalized to 2 control samples was determined using the 2−ΔΔCt method. The primer sequences used in this study are available on request from the authors.

NEUROPATHOLOGIC ANALYSIS

Brain tissues were fixed in a phosphate-buffered 20% formalin solution and embedded in paraffin. Histopathologic examination was performed on 4-μm-thick sections using standard stains. Selected sections were immunostained with a rabbit polyclonal antibody against human α-synuclein (α-synuclein; BD Transduction Laboratories, San Jose, California) and mouse monoclonal antibodies were used against phosphorylated α-synuclein (pSyn#64; Wako Pure Chemicals, Tokyo, Japan) and phosphorylated-dependent tau (AT8; Innogenetics, Zwijndrecht, Antwerp, Belgium).

PROTEIN ANALYSIS

Frozen frontal cortical samples from 2 autopsied patients (II-4, III-1, and IV-1) were homogenized on ice. We fractionated the samples by resolubilization in increasingly stringent buffers (Tris-buffered saline, 1% Triton X-100, 1% sarcosyl, and 8M urea) as previously described. Equal amounts of supernatant protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. The mouse monoclonal antibodies Syn-1 (BD Transduction Laboratories, San Jose, California) and LB509 (Zymed Laboratories, South San Francisco, California) were used to detect α-synuclein. The monoclonal antibody pSyn#64 specifically recognizes phosphorylated α-synuclein at serine 129.

RESULTS

GENETIC ANALYSIS FOR SNCA MULTIPLICATION

We performed semiquantitative PCR analysis using microsatellite markers within or flanking SNCA for the family. We found different ratios of integrated peak heights in the affected patients (ratio range, 1.72-2.47); however, the healthy and unaffected individuals showed comparable peak height ratios (ratio range, 1.02-1.14) (Figure 1A). Furthermore, PCR fragments with higher peaks were cosegregated with affected individuals in the family (Figure 1B). Patient IV-2 was homozygous for the microsatellite markers in this region. These results suggest that there is a difference in dosage between 2 alleles in this region among the affected individuals.

Next, we conducted gene dosage analysis using real-time PCR analysis. For exons 2 and 6 of SNCA, the mean gene dosages of 3 patients (II-4, III-1, and IV-1) ranged from 1.41 to 1.58, suggesting that SNCA duplication is present in these patients (Figure 2A). For patient IV-2, the gene dosages of exons 2 and 6 of SNCA were 1.89 and 2.01, respectively, suggesting that this patient carries 4 copies of SNCA (ie, is homozygous for SNCA duplication because...

Figure 1. Analysis of microsatellite markers within or flanking SNCA. A, Electropherograms of amplified polymerase chain reaction products using markers, including TC16, Rep1, and IVS-4. Allele sizes are indicated in base pairs. The integrated peak height normalized to alleles with a lower peak is shown as a ratio (boxed). B, Segregation of alleles with higher peak height ratio amplified using primers. Circle indicates female; square, male; and slash through symbol, deceased individual. The alleles with a higher peak are boxed and cosegregated with the affected individuals.
of the consanguineous marriage of his affected parents). We extended real-time PCR analysis to the surrounding genes and determined the genomic region of duplication in the family (Figure 2B). Gene dosage alteration was detected in approximately the 5-Mb genomic region, which includes the following genes: SNCA (OMIM 603756), MMRN1 (multimerin1) (OMIM 602456), secreted phosphoprotein 1 (SPP1) (OMIM 166490), dentin sialophosphoprotein (DSPP) (OMIM 125485), integrin-binding phosphoprotein (IBSP) (OMIM 147563), poly cystic kidney disease gene 2 (PKD2) (OMIM 173910), glutamate receptor ionotropic δ-2 (GRID2) (OMIM 602368), dentin matrix acidic phosphoprotein 1 (DMP1) (OMIM 600980), adenosine triphosphate–binding cassette subfamily G, and matrix extracellular phosphoglycoprotein with acid serine–aspartate-rich matrix extracellular phosphoglycoprotein (ASARM) motif (MEPE) (OMIM 605912).

**Effects of SNCA Dosage on Clinical and Pathologic Findings**

The clinical and genetic findings among patients in the family are summarized in Table 1. The patient homozygous for SNCA duplication (IV-2) developed gait disturbance and tremor at the age of 28 years (the earliest age at which parkinsonism has developed in the family), followed by the development of severe cognitive impairment at the age of 35 years. He developed orthostatic hypotension and visual hallucination and died at the age of 48 years. For the parents heterozygous for SNCA duplication (II-4 and III-1), onset was late at 71 and 61 years, respectively. Therefore, SNCA dosage has a considerable effect on disease phenotype even within a family.

At autopsy, the fresh brain of patient IV-1 weighed 930 g and showed frontal and temporal lobar atrophy and marked depigmentation of the substantia nigra and locus.
coeruleus. The pathologic findings of patient IV-1 and of his parents are compared in Table 2. The histopathologic features of the brainstem in patient IV-1 were consistent with those of the brainstems of his parents, but the degree of degeneration in each of the affected areas was more severe. The substantia nigra showed an almost complete loss of pigmented neurons and prominent gliosis (Figure 3A), where typical LBs (Figure 3B) distinctly labeled with pSyn#64 (Figure 3C) were observed in the remaining neurons. A striking pathologic finding in the brain of patient IV-1 was the presence of numerous cortical LBs in the amygdala and cerebral cortex. In the amygdala, neuronal loss and gliosis were evident (Figure 3D). Vacuolar changes were observed in the temporal cortex (Figure 3E), where many Lewy neurites were revealed by α-synuclein immunohistochemistry (Figure 3F). In the CA2-3 regions of the hippocampus, severe neuronal loss (Figure 3G) and Lewy neurite accumulation (Figure 3H) were observed. Furthermore, in the cerebral cortex, widespread senile plaques (Figure 3I) and many cortical LBs (Figure 3J) were observed, as revealed by immunohistochemistry using Aβ and α-synuclein antibodies, respectively. Few α-synuclein–positive glial inclusions were observed in this family. More senile plaques and cortical LBs were observed in patient IV-1 than in his parents. The pathologic features of the senile plaques and neurofibrillary tangles were evaluated according to stages (Table 2) as defined by Braak and Braak.14

**BIOCHEMICAL ANALYSIS OF BRAIN TISSUES FROM PATIENTS**

We performed biochemical analysis using samples extracted from frozen brain tissues of 3 affected individuals (II-4, III-1, and IV-1). We fractionated the samples by resolubilization with increasingly stringent buffers containing urea, sarkosyl, Triton X-100, and Tris-buffered saline. In the Tris-buffered saline– and Triton X-100–extracted fractions, monomeric α-synuclein migrating at approximately 15 kDa was equally detected (Figure 4A). In contrast, monomeric α-synuclein visualized using the Syn-1 and LB509 antibodies was detected only in the urea-extracted fraction of the samples from the patients. Moreover, α-synuclein in the urea-extracted fraction was reactive to the anti-pSer129 antibody, indicating that the accumulated α-synuclein in the patients is phosphorylated. Patient IV-1 with the apolipoprotein ε4/ε4 (APOE-ε4/ε4) allele showed larger amounts of α-synuclein–reactive fragments migrating at approximately 30 kDa with higher-molecular-weight smears, which are probably equivalent to the dimer or oligomer fraction of α-synuclein (Figure 4B).

In this study, we detected an alteration in SNCA dosage in an autopsy-confirmed autosomal dominant LB disease. Real-time PCR analysis revealed that 3 patients carry SNCA duplication in the heterozygous state and 1 patient carries SNCA duplication in the homozygous state. The duplicated genomic region in this family encompassed approximately 5 Mb (including SNCA at 4q21), which is the largest to date among the patients with SNCA multiplication previously described.3,9

All 4 patients developed parkinsonism, including tremor, rigidity, bradykinesia, and postural instability, which were initially improved by levodopa treatment. During the late course of the disease, they experienced progressive dementia and visual hallucinations, which are consistent with clinical features reported in LB diseases, including dementia associated with Parkinson disease and dementia with LBs.1

In previous studies,7-10 patients with heterozygous SNCA duplication demonstrated a phenotype similar to that of idiopathic Parkinson disease without apparent cognitive dysfunction except for a few patients who developed mild dementia during the later course of the disease. The prominent cognitive impairment in the present family may be attributed to the presence of at least 1 or 2 alleles of APOE-ε4. Alternatively, the cognitive impairment could be a consequence of the duplication of other genes within the large duplicated genomic region.

In previous studies, the mean age at onset among 15 patients with SNCA tripllication was 35.4 years (age range, 25-48 years),5,6,15,16 and the mean age at onset among 10 patients with SNCA duplication was 50.3 years (age range, 38-71 years).7-10 The mean age at death among 15 patients with SNCA tripllication was 44.5 years (age range, 27-56 years),5,6,15,16 suggesting rapid progression of the disease. For our patient IV-2 with homozygous SNCA duplication (who has the same SNCA copy number as the patients with SNCA tripllication), the ages at onset and death were 28 and 48 years, respectively, which are comparable to those reported for patients with SNCA tripllication. Considering that patient IV-2 had earlier age at onset and earlier death than the patients with heterozygous SNCA duplication in our study, we conclude that age at onset and severity of the phenotype are largely affected by the copy number of SNCA even within the same family sharing the same duplicated genomic region. However, it is possible that additional recessive genes resulting from consanguineous marriage are responsible for the severe phenotype of patient IV-2.

Finally, Western blot analysis revealed that the phosphorylated α-synuclein abundantly accumulated in the sarkosyl-insoluble ura-extractable fraction of the brains of the patients with SNCA duplication. A similar accumulation

### Table 2. Summary of Pathologic Findings

<table>
<thead>
<tr>
<th>Variable</th>
<th>II-4</th>
<th>III-1</th>
<th>IV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain weight, g</td>
<td>1150</td>
<td>1205</td>
<td>930</td>
</tr>
<tr>
<td>Lewy body score</td>
<td>7</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Nigral degeneration</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Vascular change</td>
<td>Mild</td>
<td>Mild</td>
<td>Marked</td>
</tr>
<tr>
<td>Lewy neurites</td>
<td>Several</td>
<td>Several</td>
<td>Many</td>
</tr>
<tr>
<td>Gial inclusion</td>
<td>Slight</td>
<td>Slight</td>
<td>Slight</td>
</tr>
<tr>
<td>Aβ stage&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Neurofibrillary tangles stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>II</td>
<td>II</td>
<td>III</td>
</tr>
</tbody>
</table>

<sup>a</sup>The pathologic changes of amyloid plaque and neurofibrillary tangles were evaluated according to stages as defined by Braak and Braak.14

<sup>b</sup>II indicates transentorhinal; III, limbic.
Figure 3. Neuropathologic findings in patient IV-1. A, Low-power magnification of substantia nigra pars compacta showing severe neuronal loss and fibrillary gliosis. Brainstem type (B) and α-synuclein–positive Lewy body (C) in substantia nigra. D, Marked neuronal loss and gliosis in amygdala. Note the presence of cortical Lewy bodies (arrows) in some remaining neurons. Vacuolar changes (E) and α-synuclein–labeled Lewy neurites (F) in transentorhinal cortex. G, Low-magnification view of hippocampus demonstrating severe neuronal loss in CA2-3 subfields (arrowheads). H, High-magnification view of subfields showing many α-synuclein–labeled Lewy neurites. I, Superior temporal cortex. J, Deeper layer of middle temporal cortex. Demonstration of frequencies of senile plaques (I) and cortical Lewy bodies (J) in patient IV-1 (right panels) compared with those in his mother (III-1, left panels) and father (II-4, center panels). Note that both structures are much more abundant in patient IV-1 than in his parents. Hematoxylin-eosin staining (A, B, D, and E) and Klüver-Barrera staining (G). Immunohistochemistry with anti-Ab42 antibodies (I) and antiphosphorylated α-synuclein antibodies (J). Scale bars indicate 50 µm (A, D, E, F, and H), 10 µm (B and C), 1 mm (G), 300 µm (I), and 200 µm (J).
of phosphorylated α-synuclein in the detergent-resistant fraction was reported in the brains of sporadic patients with synucleinopathy.13 These results suggest that the enhanced level of phosphorylated α-synuclein, possibly induced by increased SNCA dosage, or the modulated clearance or expression of α-synuclein is the key event responsible for the formation of LBs and Lewy neurites, which eventually leads to neuronal cell death.

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