C9ORF72 Repeat Expansion in Amyotrophic Lateral Sclerosis in the Kii Peninsula of Japan

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Background: In the Kii peninsula of Japan, high prevalences of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex have been reported. There are 2 major foci with a high prevalence, which include the southernmost region neighboring the Koza River (Kozagawa and Kushimoto towns in Wakayama prefecture) and the Hohara district (Mie prefecture).

Objective: To delineate the molecular basis of ALS in the Kii peninsula of Japan, we analyzed hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (C9ORF72) gene, which has recently been identified as a frequent cause of ALS and frontotemporal dementia in the white population.

Design: Case series.

Setting: University hospitals.

Patients: Twenty-one patients (1 familial patient and 20 sporadic patients) with ALS from Wakayama prefecture, and 16 patients with ALS and 16 patients with parkinsonism-dementia complex originating from Mie prefecture surveyed in 1994 through 2011 were enrolled in the study. In addition, 40 probands with familial ALS and 217 sporadic patients with ALS recruited from other areas of Japan were also enrolled in this study.

Main Outcome Measures: After screening by repeat-primed polymerase chain reaction, Southern blot hybridization analysis was performed to confirm the expanded alleles.

Results: We identified 3 patients with ALS (20%) with the repeat expansion in 1 of the 2 disease foci. The proportion is significantly higher than those in other regions in Japan. Detailed haplotype analyses revealed an extended shared haplotype in the 3 patients with ALS, suggesting a founder effect.

Conclusions: Our findings indicate that the repeat expansion partly accounts for the high prevalence of ALS in the Kii peninsula.

been identified as the causative mutation in familial and sporadic ALS and frontotemporal dementia (OMIM 105550).12,13 Given the potential clinical overlapping among ALS, frontotemporal dementia, and ALS/PDC, we investigated the GGGGCC hexanucleotide repeat expansion in C9ORF72 in patients with ALS and PDC from the Kii peninsula.

METHODS

SUBJECTS AND DNA EXTRACTION

Sixteen patients with ALS and 16 patients with PDC originating from Mie prefecture and 21 patients (1 familial patient and 20 sporadic patients) with ALS from Wakayama prefecture surveyed in 1994 through 2011 were enrolled in the study. In addition, a total of 40 probands with familial ALS and 217 sporadic patients with ALS recruited from other areas of Japan were also enrolled in this study.14 Genomic DNA was isolated from patients' blood leukocytes, lymphoblastoid cell lines, or autopsied brains using standard procedures. Written informed consent was obtained from all of the participants or the families of the deceased patients. The study was approved by the institutional review boards of the participating institutions.

REPEAT-PRIMED POLYMERASE CHAIN REACTION ANALYSIS

Because the expansion is too large to detect by a standard polymerase chain reaction, screening by repeat-primed polymerase chain reaction was performed, as reported previously.12 Fragment analysis was performed using an ABI PRISM 3130xl sequencer and GeneScan software (Life Technologies).

SOUTHERN BLOT HYBRIDIZATION ANALYSIS

To independently confirm the repeat expansion in C9ORF72, Southern blot hybridization analysis was conducted, as described previously.12

HAPLOTYPE ANALYSIS

To investigate the possibility of a founder effect associated with the expanded alleles in C9ORF72, we genotyped the patients with expanded alleles using Genome-wide Human SNP array 6.0 (Affymetrix). Genotypes were called and extracted using Genotyping Console 4.0 (Affymetrix). In addition, we performed direct nucleotide sequence analysis of 42 single nucleotide polymorphisms to compare the haplotype with the Finnish haplotype.14
Figure 2. Mutational analyses of hexanucleotide repeat expansion in C9ORF72. A, Repeat-primed polymerase chain reaction analysis was performed as previously described. Patients 1-3 show the characteristic sawtooth patterns with a 6-bp periodicity (blue lines). Red lines indicate DNA size markers. B, Southern blot hybridization analysis. Genomic DNA extracted from lymphoblastoid cell lines of patients 1 through 3 were subjected to Southern blot hybridization analysis, as described previously. Patients 1-3 showed expanded alleles. C, Result of haplotype analysis. Physical positions are shown using the reference genome (NCBI36/hg18). An extended haplotype (Kii 9p-haplotype) spanning 3.3-63 Mb was shared by the 3 patients with ALS with the repeat expansions. A 410-kb region (defined by rs9111602 and rs10511810) of the Kii 9p-haplotype was shared with that in another patient with the repeat expansion from another region of Japan. We compared this haplotype with the Finnish haplotype; a 130-kb region (defined by rs10511816 and rs6335835) was shared between the Kii 9p-haplotype and the Finnish haplotype. NC indicates negative control; P, patient.
Patients with hexanucleotide expansion in C9ORF72 were identified in the Kii peninsula of Japan. We screened a total of 37 patients with ALS and 16 patients with PDC identified in the Kii peninsula using repeat-primed polymerase chain reaction analysis. Three of the patients with ALS (patients 1-3) showed the characteristic sawtooth-like electrophoresis pattern (Figure 2A). Southern blot hybridization analysis of the genomic DNA from the 3 patients further confirmed the presence of expanded alleles (Figure 2B).

Interestingly, the 3 patients with ALS with the expansion were from the southernmost Kii peninsula neighboring the Koza River (Kozagawa and Kushimoto towns), which is 1 of the 2 disease foci. When confined to the southernmost Kii peninsula, 3 of the 15 patients with ALS (20%) showed the repeat expansion. In contrast, 30 patients from the Hohara district and its vicinity did not reveal the repeat expansion. Mutational analyses of the repeat expansions in patients with ALS from Kii peninsula were mainly in areas around Tokyo, we found only 1 patient with a family history of ALS, which were included as the summary data in the meta-analysis study.14

The clinical characteristics of the patients are shown in Table 1. Family history of ALS was present only in patient 2, whose sibling was also diagnosed as having ALS. There were no family histories of ALS and related disease in the other 2 patients. They showed both upper and lower motor neuron signs. Two of the patients had lower limb-onset ALS, whereas 1 patient had bulbar-onset ALS. Patient 1 showed moderate cognitive decline, and mild brain atrophy was detected on computed tomographic scans. None of the patients showed parkinsonism. There were no obvious inverse correlations between the age at onset and the size of expanded alleles, as determined by Southern blot hybridization analysis.

Haplotype analysis using a high-density single nucleotide polymorphism array revealed an extended shared haplotype spanning 3.3-6.3 Mb in the 3 patients with ALS, although the kinships among the 3 patients were not evident (Figure 2C). The findings strongly suggest that the expanded alleles in this region originated from a common founder. As just described, we found only 1 patient with the repeat expansion in C9ORF72 in the 40 probands with familial ALS (2.5%) collected in other regions in Japan.14 The haplotype of this patient with ALS shares a 410-kb segment with the Kii 9p-haplotype. When the Kii 9p-haplotype was compared with the Finnish haplotype, a common haplotype of 130 kb was observed.14

We identified the hexanucleotide repeat expansion in C9ORF72 in the 3 patients from the southernmost Kii peninsula neighboring the Koza River. The frequency of patients with expanded alleles was 20% (3 of 15) in this area. In the study of the other cohort of ALS collected mainly in areas around Tokyo, we found only 1 patient with the repeat expansion in C9ORF72 in the 40 probands with familial ALS (2.5%) and none in the 217 sporadic patients with ALS.14 Although the number of patients examined in the southernmost Kii peninsula was small, virtually all the affected patients in this region were enrolled based on a continued epidemiologic study conducted by the authors (T.K. and S.Y.) in this region. Moreover, the difference in the frequency of patients carrying the repeat expansion in C9ORF72 is statistically significant (Table 2). Thus, our findings in this study emphasize that patients with ALS with the repeat expan-

### Table 1. Clinical Characteristics of Kii Patients With ALS With C9ORF72 Repeat Expansions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Age at onset, y</th>
<th>Age at examination, y</th>
<th>Family history</th>
<th>Initial symptom</th>
<th>Cranial UMN signs</th>
<th>Lower limbs UMN signs</th>
<th>LMN signs</th>
<th>Other</th>
<th>Dementia</th>
<th>Neuroimaging</th>
<th>nEMG</th>
<th>Other</th>
</tr>
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<tbody>
<tr>
<td>Patient 1</td>
<td>72</td>
<td>Female</td>
<td>72</td>
<td>72</td>
<td>+</td>
<td>Dysarthria</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Neurogenic changes</td>
<td>−</td>
</tr>
<tr>
<td>Patient 2</td>
<td>71</td>
<td>Female</td>
<td>71</td>
<td>71</td>
<td>+</td>
<td>Leg weakness</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>Neurogenic changes</td>
<td>+</td>
</tr>
<tr>
<td>Patient 3</td>
<td>46</td>
<td>Female</td>
<td>41</td>
<td>46</td>
<td>−</td>
<td>Leg weakness</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Neurogenic changes</td>
<td>+</td>
</tr>
</tbody>
</table>

| Abbreviations: ALS, amyotrophic lateral sclerosis; CT, computed tomography; UMN, upper motor neuron; LMN, lower motor neuron; nEMG, needle electromyography. |

Because all the patients were singletons, we reconstructed the haplotypes using the homozygosity haplotype method.15

### STATISTICAL ANALYSIS

The Fisher exact test was used to compare the frequencies of the repeat expansion in patients with ALS from Kii peninsula and those from other regions in Japan.

### Results

40 probands with familial ALS and the 217 sporadic patients with ALS from other areas of Japan revealed only 1 patient with a family history of ALS, which were included as the summary data in the meta-analysis study.14

The clinical characteristics of the patients are shown in Table 1. Family history of ALS was present only in patient 2, whose sibling was also diagnosed as having ALS. There were no family histories of ALS and related disease in the other 2 patients. They showed both upper and lower motor neuron signs. Two of the patients had lower limb-onset ALS, whereas 1 patient had bulbar-onset ALS. Patient 1 showed moderate cognitive decline, and mild brain atrophy was detected on computed tomographic scans. None of the patients showed parkinsonism. There were no obvious inverse correlations between the age at onset and the size of expanded alleles, as determined by Southern blot hybridization analysis.

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sion in C9ORF72 are concentrated in the southernmost Kii peninsula with a founder effect.

The clinical features of the patients with the repeat expansion are indistinguishable from those with conventional ALS. Moderate cognitive decline was present in 1 patient, whereas none of them showed parkinsonism (Table 1). Because autopsy findings of patients with the repeat expansion are unavailable, further investigations will be certainly needed to address the relationship between the ALS with the repeat expansion in C9ORF72 identified in the southernmost Kii peninsula and ALS/ PDC identified in the Kii peninsula.

However, it should also be noted that the repeat expansion did not account for all the ALS cases, even in the southernmost Kii peninsula. It is also of interest that patients with the repeat expansion were not identified in the Hohara district or other areas of Wakayama and Mie prefectures. Taken together, our study demonstrates that the patients with the repeat expansion are concentrated in the southernmost Kii peninsula, but simultaneously raises the possibility of genetic heterogeneities even in these 2 regions in the Kii peninsula where ALS is prevalent.

In summary, we identified that the C9ORF72 repeat expansion is concentrated in the patients with ALS in the Kii peninsula. Our finding suggests that the repeat expansion partly accounted for the high prevalence of ALS in the Kii peninsula of Japan.

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