Novel Mutations in the Guanosine Triphosphate Cyclohydrolase 1 Gene Associated With DYT5 Dystonia

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Objectives: To better understand the relationship between mutation of the guanosine triphosphate cyclohydrolase I (GCH1) gene and the etiology of DYT5 dystonia and to accumulate data on the mutation in the Japanese population for genetic diagnosis of the disease.

Setting: Japanese population.

Patients: Eight Japanese patients with suspected DYT5 dystonia were analyzed.

Intervention: Direct genomic sequencing of 6 exons of GCH1 was performed.

Main Outcome Measures: For patients who did not exhibit any abnormality in the sequence analysis, the possibility of exon deletions was examined. In cases for which cerebrospinal fluid was available, the concentrations of neopterin and biopterin were measured as an index of GCH1 enzyme activity.

Results: In 2 patients, we found a new T106I mutation in exon 1 of GCH1, a position involved in the helix-turn-helix structure of the enzyme. In the third patient, we found a new mutation (a 15-base pair nucleotide deletion) in exon 5 that may cause a frameshift involving the active site. In the fourth patient, we detected a known nucleotide G>A substitution in the splice site of intron 5, which has been reported to produce exon 5–skipped messenger RNA. The concentrations of both neopterin and biopterin in the cerebrospinal fluid of the third and fourth patients were markedly lower than the normal range, indicating that the GCH1 enzyme was functionally abnormal in these mutations. Gene dosage analysis showed that the fifth patient had a deletion of both exon 3 and exon 4, whereas the sixth patient had a deletion of exon 3.

Conclusions: We found several novel, as well as known, GCH1 mutations in Japanese patients with DYT5 dystonia. In some of them, the GCH1 enzyme activity was proved to be impaired.

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YT5 DYSTONIA (SEGAWA disease) is an autosomal dominant hereditary progressive dystonia with marked diurnal fluctuation, characterized by bilateral foot dystonia that becomes apparent in childhood or adolescence.1 Molecular genetic studies have revealed that DYT5 dystonia is caused by mutations of the guanosine triphosphate cyclohydrolase 1 (GCH1) gene located in 14q22.1-22.2.2-4 This enzyme catalyzes the rate-limiting step of tetrahydrobiopterin biosynthesis.5 Tetrahydrobiopterin is a cofactor for tyrosine hydroxylase, which is involved in the production of dopamine.6 It remains to be clarified, however, how a low dopamine level results in the onset of this disease. Tetrahydrobiopterin is also a cofactor for phenylalanine hydroxylase, and deficient activity of this enzyme causes hyperphenylalaninemia, which is clinically distinct from DYT5 dystonia.7,8 The human GCH1 gene is composed of 6 exons spanning approximately 30 kilobases.8 To date, various types of mutation have been found throughout the 6 exons, as well as introns, of the GCH1 gene, including missense and nonsense mutations, large and small deletions and insertions, and splice site mutations.4,8-10 (Figure 1). The reason why such a variety of mutations occur in the GCH1 gene remains unknown. Patients with DYT5 dystonia in each pedigree, but not in different pedigrees, have identical mutations. It is not known how the single GCH1 gene is associated with 2 distinct diseases, DYT5 dystonia and hyperphenylalaninemia. In our previous short report, we described a novel T106I mutation found in exon 1 of the GCH1 gene in patients with DYT5 dystonia.17 In the present article, we provide further details of this mutation. In addition, analysis of other patients revealed a novel small deletion and a new type of exon deletion, as well as the known splice site mutation.
Genomic DNA was isolated from peripheral blood leukocytes of 7 female patients (P1, P2, P3, P4, P5, P7, and P8) and 1 male patient (P6) with clinically suspected DYT5 dystonia (Table 1) and 100 healthy control individuals, with the informed consent of the donors. The 6 exons of the \textit{GCH1} gene were amplified by polymerase chain reaction (PCR) using the primers reported by Ichinose et al\textsuperscript{3} and subjected to sequence analysis.

**HAPLOTYPE ANALYSIS**

Five microsatellite loci (D14S288, D14S978, D14S991, D14S1057, and D14S980) around the \textit{GCH1} gene of patients P1 and P2 were amplified by PCR using fluorescence-labeled primers for each locus. Allele frequencies in the Japanese population at each locus were obtained by genotyping 54 Japanese volunteers.

**ANALYSIS OF 15–BASE PAIR DELETION IN THE NORMAL POPULATION**

The exon 5 genomic sequence around a 15–base pair (bp) deletion in 100 normal individuals was amplified using fluorescence-labeled primers for each exon. The mutations found in this study are indicated by arrows. Mutations associated with hyperphenylalaninemia are underlined. fs indicates frameshift; del, deletion; and x, stop codon.

**Table 1. Clinical Characteristics and \textit{GCH1} Mutations of 8 Patients Analyzed in This Study**

<table>
<thead>
<tr>
<th>Patient/ Sex/Age, y</th>
<th>\textit{GCH1} Mutation</th>
<th>Site of Onset</th>
<th>Clinical Signs</th>
<th>Family History of Dystonia</th>
<th>Levodopa/DCI Effective Dose, mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1/F/8</td>
<td>T106I</td>
<td>Leg</td>
<td>Foot dystonia</td>
<td>Not known</td>
<td>100</td>
</tr>
<tr>
<td>P2/F/30</td>
<td>T106I</td>
<td>Arm</td>
<td>Writer’s cramp; foot dystonia</td>
<td>Yes</td>
<td>200</td>
</tr>
<tr>
<td>P3/F/7</td>
<td>V206fs</td>
<td>Arm, leg, neck</td>
<td>Cervix rotation</td>
<td>Yes</td>
<td>Data unavailable</td>
</tr>
<tr>
<td>P4/F/4</td>
<td>IVS5+1G&gt;A</td>
<td>Unknown</td>
<td>Foot dystonia</td>
<td>Not known</td>
<td>400-600</td>
</tr>
<tr>
<td>P5/F/8</td>
<td>Exons 3-4 deletion</td>
<td>Arm</td>
<td>Dystonia of lower and upper limbs</td>
<td>Yes</td>
<td>300</td>
</tr>
<tr>
<td>P6/M/8</td>
<td>Not detected</td>
<td>Leg</td>
<td>Foot dystonia</td>
<td>Not known</td>
<td>Data unavailable</td>
</tr>
<tr>
<td>P7/F/20</td>
<td>Not detected</td>
<td>Trunk</td>
<td>Anteversion posture</td>
<td>Not known</td>
<td>Data unavailable</td>
</tr>
<tr>
<td>P8/F/18</td>
<td>Not detected</td>
<td>Leg</td>
<td>Tremor</td>
<td>Not known</td>
<td>500</td>
</tr>
</tbody>
</table>

**METHODS**

**POLYMERASE CHAIN REACTION DIRECT SEQUENCING**

Genomic DNA was isolated from peripheral blood leukocytes of 7 female patients (P1, P2, P3, P4, P5, P7, and P8) and 1 male patient (P6) with clinically suspected DYT5 dystonia (Table 1) and 100 healthy control individuals, with the informed consent of the donors. The 6 exons of the \textit{GCH1} gene were amplified by polymerase chain reaction (PCR) using the primers reported by Ichinose et al\textsuperscript{3} and subjected to sequence analysis.
cience-labeled primers, and the PCR products were analyzed by GeneScan.

MEASUREMENT OF NEOPTERIN AND BIOPTERIN CONCENTRATIONS

Concentrations of neopterin and biopterin in cerebrospinal fluid (CSF) were measured by high-performance liquid chromatography (HPLC) as described previously. In brief, the CSF was oxidized in iodine solution. The oxidized samples were analyzed by HPLC using an organized delivery system reverse-phase column (GL Sciences).

GENE DOSAGE ANALYSIS

Six exons of the GCH1 gene were subjected to real-time PCR analysis using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif) and a PRISM 7700 Sequence Detection System (Applied Biosystems). The Ct value for each exon was normalized using those for the beta-globin gene. A ratio between 0.8 and 1.2 was considered normal, and a ratio between 0.4 and 0.6 was considered to represent a heterozygous deletion.

RESULTS

ANALYSIS OF PATIENTS WITH A MISSENSE MUTATION

Eight patients (P1, P2, P3, P4, P5, P6, P7, and P8) who had been suspected to have DYT5 dystonia from their levodopa responsiveness and clinical characteristics (Table 1) were analyzed. The 6 exons of the GCH1 gene of these patients were amplified by PCR and subjected to direct sequence analysis. In 2 patients (P1 and P2), we found a novel and identical heterozygous mutation. These patients had a C317>T nucleotide mutation in exon 1, leading to an amino acid substitution of T106I (Figure 1 and Figure 2). To our knowledge, this mutation has not been reported previously in Japanese or other populations. The deletion was not represented in 100 normal controls, indicating that it does not represent a normal polymorphism. Since the 2 patients (P1 and P2) were not aware of any blood relationship with each other but lived close together, we performed haplotype analysis using 5 microsatellite markers mapped around the GCH1 gene. We discovered that the 2 patients shared 1 of the 2 alleles of each locus (Table 2). The probability of sharing all the 5 alleles is calculated to be $2.1 \times 10^{-4}$ (1 in about 5000) according to the frequencies in the Japanese population, suggesting that they originated from a common founder.

ANALYSIS OF A PATIENT WITH A SMALL DELETION

In 1 patient (P3), we found a novel heterozygous 15-bp deletion that, to our knowledge, has not been reported previously in Japanese or other populations. The deletion, starting from T618 in exon 5, results in amino acid–sequence framsheffs starting from V206 (Figure 1 and Figure 3). All of the possible 3 framsheffs give rise to downstream termination codons and would disrupt the active site of the GCH1 enzyme. This deletion was not detected in the 100 normal controls when analyzed by GeneScan, indicating that the mutation does not represent a normal polymorphism.

A CSF sample from this patient was available, and the concentrations of neopterin and biopterin, the metabolic byproducts of tetrahydrobiopterin biosynthesis, were measured by HPLC to assess the enzyme activity of GCH1. The concentrations of neopterin (8.2 pmol/mL) and biopterin (8.5 pmol/mL) were found to be markedly lower than the normal range, indicating that this mutation leads to functional impairment of the GCH1 enzyme (Table 3).

ANALYSIS OF A PATIENT WITH A SPLICE SITE MUTATION

In 1 patient (P4), we detected a G>A nucleotide substitution in the splice site of intron 5 (Figure 1). This nu-
tation had been reported to produce messenger RNA without exon 5 and cause impaired activity of recombinant enzymes, although the enzyme activity in the affected patient had not been analyzed.11 In the present study, we found that the concentrations of neopterin and biopterin in the CSF of the patient were 3.9 pmol/mL and 1.3 pmol/mL, respectively (Table 3). These concentrations were strikingly lower than the normal range, indicating that this mutation markedly impairs the function of the GCH1 enzyme.

ANALYSIS OF PATIENTS WITH EXON DELETIONS

In 4 patients (P5, P6, P7, and P8), we did not detect any mutation by sequence analysis of the 6 exons. Therefore, gene dosage analysis was performed by real-time PCR. It was found that in 1 patient (P5) the gene dosage values for exons 3 and 4 were 0.42 and 0.60, respectively, whereas in another patient (P6), the value for exon 3 was 0.41 (Figure 4). The gene dosage values for other exons of these patients were within the normal range (0.8-1.2). These concentrations were strikingly lower than the normal range, indicating that this mutation markedly impairs the function of the GCH1 enzyme.

A CSF sample from patient P5 was available and was found to contain concentrations of neopterin (8.6 pmol/mL) and biopterin (15.7 pmol/mL) that were both lower than the normal range, although to a lesser extent in the latter case (Table 3).

In our previous short report, we described a novel T106I mutation in the GCH1 gene in 2 patients with DYT5 dystonia.17 In the present article, we have provided more detailed results and discussion related to this mutation. T106 is conserved in human, mouse, rat, chicken, and Xenopus and is located at the turning position of the helix-turn-helix structure of the guanosine triphosphate cyclohydrolase I molecule.9,19,20 Because the G90V mutation located at the same helix-turn-helix structure has been reported to cause a dominant negative effect,11 it is possible that the T106I mutation impairs the enzyme activity by a similar mechanism. The 2 patients with this mutation were suspected by haplotype analysis to originate from a common founder, although family studies would be necessary to prove this, as well as to infer the generation in which the founder appeared. A notably high rate of childhood dystonia or dystonia in general is not evident in the geographic region where the patients live, suggesting...
that the occurrence of the mutation was not such a distant event as to cause a founder effect in this region.

We also found 2 additional novel GCH1 mutations. In 1 patient, we identified a V206fs mutation that would terminate the translation of active-site sequences of the GCH1 enzyme. The markedly low neopterin and biopterin levels in the patient's CSF proved the pathogenicity of this mutation. In the other patient, we detected a deletion of both exons 3 and 4, which is a new type of genomic exon deletion, although a patient of an English-Canadian family with a genomic exon 3 deletion has been reported to express messenger RNA lacking both exons 3 and 4. The pathogenesis of this mutation was proved by the low levels of neopterin and biopterin in the patient's CSF.

In 2 of the 8 patients, none of the known mutations was detected in any of the 6 exons or the proximal splice sites of the GCH1 gene. Thus, the frequency of detectable GCH1 mutation was 6 in 8. It is possible that these patients may have a mutation in the promoter region or the untranslated region of the gene. They did not harbor a known mutation in other genes related to dystonia, such as tyrosine hydroxylase, epsilon-sarcoglycan, dopamine receptor D2, and pantothenate kinase 2. Both patients were levodopa responsive and showed no particular clinical characteristics except for a relatively high age at onset (20 years and 18 years). According to a cohort study on nervous and mental disorders in the Japanese population, DYT5 dystonia is about 1.9 times more frequent than DYT1 dystonia (74 and 40 patients among 259 patients with hereditary dystonia, respectively) (K.H. et al, unpublished data). Thus, mutation analysis of the GCH1 gene is particularly important in Japan for conclusive diagnosis.

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### Table 3. Concentrations of Neopterin and Biopterin in Cerebrospinal Fluid of Patients P3, P4, and P5

<table>
<thead>
<tr>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>Normal Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
<td>V206fs</td>
<td>Possible</td>
<td>Exons 3-4</td>
</tr>
<tr>
<td></td>
<td>site change</td>
<td>deletion</td>
<td></td>
</tr>
<tr>
<td>Age at onset, y</td>
<td>7</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Neopterin level, pmol/mL</td>
<td>8.5</td>
<td>3.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Biopterin level, pmol/mL</td>
<td>8.2</td>
<td>1.3</td>
<td>15.7</td>
</tr>
</tbody>
</table>

*Normal values have been cited from Fujishiro et al.† Mean ± SD.
Author Contributions: Study concept and design: Ohta and Obata. Acquisition of data: Ohta, Toyoshima, Matsuo, Yukihiko, Yoshino, Yokoyama, Shimazu, and Maeda. Analysis and interpretation of data: Ohta, Funayama, Ichinose, Toyoshima, Urano, Tomoko, Hasegawa, and Obata. Drafting of the manuscript: Ohta, Tomoko, Yukihiko, Yoshino, and Obata. Critical revision of the manuscript for important intellectual content: Funayama, Ichinose, Toyoshima, Urano, Matsuo, Yokoyama, Shimazu, Maeda, Hasegawa, and Obata. Obtained funding: Shimazu and Maeda. Administrative, technical, and material support: Ohta, Funayama, Ichinose, Urano, Yokoyama, and Obata. Study supervision: Hasegawa and Obata.

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