Molecular heterogeneity of Krabbe disease

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Summary: Krabbe disease (globoid cell leukodystrophy) is an autosomal recessive neurodegenerative disorder that affects both the central and peripheral nervous system due to an enzymatic defect of galactocerebrosidase (GALC). Following its cloning, many mutations in the galactocerebrosidase gene have been reported, but the correlation between phenotype and genotype was not clear in many cases. In this study we further investigated the molecular defects in another 10 patients (6 Japanese and 4 non-Japanese), using cultured skin fibroblasts, and found 10 mutations, of which 8 were novel, including a nonsense mutation (W647X) and 7 missense mutations (G43R, S52F, T262I, Y319C, W410G, R515H, T652R) in the coding region. Some phenotype-specific mutations were found but the other mutations were private. Mutations reported so far have been distributed over the whole GALC gene and it is difficult to speculate on functional domains of the GALC protein and phenotypically specific regions.

Globoid cell leukodystrophy (GLD; Krabbe disease; McKusick 245200) is an autosomal recessive inherited neurodegenerative disorder caused by the deficiency of galactocerebrosidase (GALC, EC 3.2.1.46), the lysosomal enzyme responsible for the degradation of galactocerebroside (Suzuki and Suzuki 1970). Neuropathologically, the disease is characterized by numerous globoid cells, severe loss of myelin, and marked gliosis of the white matter (Austin 1968). These changes result from accumulation of the toxic metabolite of psychosine (galactosylsphingosine) (Svennerholm et al 1980). Clinically, most patients with the disease show hypertonicity, irritability and mental retardation within the first year of life. However, there have been several reports of later onset in childhood and even in adulthood (Kolodny et al 1991; Suzuki et al 1995). Recently, the enzyme was purified from human urine and lymphocytes (Chen and Wenger 1993; Sakai et al 1994a), and cloning of the cDNA was reported by two groups (Chen et al 1993; Sakai et al 1994b). The 3780 bp cDNA contains an open reading frame of 2007 bp, which encodes 669 amino acids, including 26 residues of a signal peptide. The 80–90 kDa precursor protein is processed to
50 kDa and 30 kDa. To clarify the clinical heterogeneity and substrate specificity of the enzyme, it is essential to clarify the molecular defects of the gene in patients.

Following the cloning of the gene, many mutations in the galactocerebrosidase gene have been reported. In the infantile type, a large deletion of the gene (about 30 kb), which begins in the middle of intron 10 and results in the loss of the sequences encoded by exons 11–17 associated with the 502T to C polymorphism, appears to be very common in patients of northern European origin (Luzi et al 1995; Raffi et al 1995), and 12-base-deletion and 3-base-insertion mutation (12Del-3Ins) was reported in several Japanese infantile patients (Tatsumi et al 1995). In late-onset forms, half of the patients were heterozygous for the large deletion and the other mutations differ among patients (de Gasperi et al 1996). In the adult patients, only a few mutations have been reported (Bernardini et al 1997; Furuya et al 1997; Luzi et al 1996). Recently, Wenger and colleagues (1997) reviewed over 40 mutations in Krabbe disease, including 26 new mutations, some of which have yet to be confirmed by expression study. In this study we further investigated the molecular defects in another 10 patients (6 Japanese and 4 non-Japanese), using cultured skin fibroblasts, and found 10 mutations, of which 8 mutations were novel, including a nonsense mutation and 7 missense mutations in the coding region. The low enzymatic activities expressed from the cDNAs with mutations were confirmed and the genetic status of the mutations in genomic DNAs were also confirmed in some patients.

**MATERIALS AND METHODS**

**Patients:** Patients were diagnosed with GLD in our laboratory through their low GALC activity in the presence of other characteristic clinical and laboratory findings. Patient 10 (onset at the age of 3 years) was of a late-onset form and the others were of the infantile form.

**Mutation analysis of cDNA by RT-PCR and direct sequencing:** Total RNA was isolated from cultured skin fibroblasts from 7 patients. This was subjected to reverse-transcribed PCR (RT-PCR) amplification using two primer sets (Table 1) to cover the protein coding region of GALC. Amplification using primer set I was done under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C in the presence of 3% DMSO. For primer set II, the amplification conditions were initial denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C without DMSO. PCR products were directly sequenced using ABI 373 and the ABI primer dye termination cycle sequencing kit.

**Genomic DNA analysis by PCR and restriction enzyme digestion:** Genomic DNA was extracted from cultured skin fibroblasts and PCR was performed using a series of primer pairs described previously (Tatsumi et al 1995) and mismatch primers designed for each mutation, under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for