Identification and Expression of a Missense Mutation (Y446C) in the Acid Sphingomyelinase Gene from a Japanese Patient with Type A Niemann-Pick Disease

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Department of Pediatrics, Akita University School of Medicine, Akita 010, *Department of Pediatrics, Nagoya City University Medical School, Nagoya 467, †National Center of Neurology and Psychiatry, Kodaira 187, and ‡Department of Human Genetics, Mount Sinai School of Medicine, New York, NY, USA

Takahashi, T., Suchi, M., Sato, W., Ten, S.B., Sakuragawa, N., Desnick, R.J., Schuchman, E.H. and Takada, G. Identification and Expression of a Missense Mutation (Y446C) in the Acid Sphingomyelinase Gene from a Japanese Patient with Type A Niemann-Pick Disease. Tohoku J. Exp. Med., 1995, 177 (2), 117-123 — Types A and B Niemann-Pick disease (NPD), an autosomal recessive lysosomal storage disorder, are caused by deficiency of acid sphingomyelinase (ASM). The recent identification of mutations in ASM gene causing types A and B NPD has led to the investigation of the phenotypic heterogeneity and the ethnic distribution of this disease, especially in Ashkenazi Jewish population. To characterize the mutations causing NPD in Japanese population, we analyzed the genomic sequence of ASM from a Japanese patient with type A NPD by PCR amplification and sequencing. A new mutation, Y446C, was identified. The authenticity of this lesion was demonstrated by the expression of the Y446C allele in COS-1 cells. No residual ASM activity was detected from the expression of the Y446C.

Types A and B Niemann-Pick disease (NPD) result from the deficient activity of the lysosomal hydrolase, acid sphingomyelinase (ASM). Type A disease is a fatal neurodegenerative disorder of infancy, whereas a type B disease patient has no neurologic manifestations and survives into adulthood (Spence and Callahan 1989). The isolation and characterization of cDNA and genomic sequences encoding ASM has led to the identification of mutations in ASM gene...
and the molecular characterization of this disease (Schuchman et al. 1991, 1992). To date, twelve mutations have been described (Ferlinz et al. 1991; Levran et al. 1991a, b, 1992a, b, 1993; Takahashi et al. 1992a, b). In the Ashkenazi Jewish population, in which the incidence of NPD is higher than in the general population, common mutations have been identified and provided the basis for molecular-based carrier detection. In the Japanese population, we identified a mutation, S436R, in a patient with type B Niemann-Pick disease (Takahashi et al. 1992a). Here, we described the molecular lesion in a Japanese patient with type A Niemann-Pick disease.

**Materials and Methods**

*Genomic amplification and single-stranded solid-phase direct sequencing*

To identify the ASM mutation in a Japanese type A NPD patient, a 6-month-old boy with hepatosplenomegaly, failure to thrive, and neurologic deterioration, PCR amplification was performed on genomic DNA isolated from cultured skin fibroblasts. The complete ASM coding region, including the intron/exon boundaries, was amplified in four fragments using the PCR primers described below. The four sense oligonucleotide primers (P1, P3, P5, P7) were biotinylated for single-stranded solid-phase direct sequencing. PCR product 1 (469 bp) containing exon 1 was amplified using sense and antisense primers P1 (5'-CTCGAGATCGGGACAGACGAACCA-3') and P2 (5'-GAATTTCATCAGGGA TGCAATTC-3'), respectively. The sense and antisense primers for PCR product 2 (391 bp) containing the 5'-portion of exon 2 were P3 (5'-GAATTCTGATTTCTCACCATGC-3') and P4 (5'-CTCGAGGCCCTCCAAGGTAGTCATG-3'), respectively. PCR product 3 including the 3'-portion of exon 2 (561 bp) was amplified using sense and antisense primers P5 (5'-TCATCCTCGAGCACTGACCTGCCTGGG-3') and P6 (5'-CTCGAGCTTTTTTCTTTTCTTTCTCGTCTG-3'), respectively, and the sense and antisense primers for PCR product 4 (1708BP) including exons 3-6 were P7 (5'-GAATTCTGGGCGAGACTGAGTCATG-3') and P8 (5'-GAATTCTAAACCAGCCTCCAGGA-3'), respectively. PCR amplifications were performed for 35 cycles with Taq polymerase (Promega, Madison, WI, USA). Each amplification cycle consisted of denaturation for 1 min at 94°C, annealing for 1 to 2 min at temperatures varying from 60°C to 72°C, and extension for 1 to 2 min at 72°C. For PCR product 2, which included 391bp of exon 2, a PCR “boost” procedure was required and a slightly modified PCR buffer was used. Double-stranded PCR products were denatured by standard techniques, and the biotinylated single strands were isolated by affinity capture using streptavidin-coated magnetic beads (Dynabeads M-280; DYNAL, New York, NY, USA) (Hultman et al. 1989). The biotinylated single-stranded PCR products were subjected to dideoxy chain termination sequencing using ASM-specific sequencing primers at a final concentration of 0.5 pmol.
Genomic amplification of exon 5 including the Y446C mutation, subcloning, and double-stranded sequencing

PCR amplification of the exon including the Y446C mutation was performed as the procedure shown above using the primers, which included EcoRI and BamHI restriction sites for directional subcloning. The purified fragment was double-digested with the appropriate restriction enzymes and subcloned into pUC18 (Takara Shuzo, Otsu). Minipreps of multiple ampicillin-resistant colonies were sequenced by the dideoxy chain termination method using ASM gene sequencing primers. In order to rule out the possibility of a mutation resulting from amplification errors, several independent clones were sequenced to confirm the putative mutation.

Site-directed mutagenesis

To evaluate the effect of the Y446C mutation on ASM catalytic activity, the mutation was introduced into the full-length ASM cDNA, pASM-3b, using the PCR-overlap mutagenesis technique (Ho et al. 1989). Each round of PCR amplification was performed for 20 cycles and the primers containing the mutated sequences were constructed to be 25 nucleotides long and completely overlapping. For the Y446C mutation, sense primer P9 (5'-ATCATCGGTAACCATGAAAGCACACCTGTCAA-3'), which contained a BstEII site at the 5'-end, was used with antisense primer P10 (5'-AGGGTGTTCTCACACCTGGCTACAA-3') to amplify a 407-bp fragment from pASM-3b containing the Y446C mutation. Sense primer P11 (5'-TTGTAGCCAGGTGAGAACACCCT-3') (complementary to P2) was then used with antisense primer P12 (5'-ATCATCGGTAACCAGGATTAGG-3') to amplify a 168-bp fragment containing a BstEII site at the 3'-end. These two fragments were then combined and used as templates for a second amplification with primers P1 and P4. Following DNA sequencing, the fragment was inserted into pASM-3b by cassette exchange with the wild type BstEII fragment.

Transient expression of the Y446C mutation in COS-1 cells

To evaluate the effect of the Y446C mutation on ASM catalytic activity, the mutated full-length ASM cDNA was inserted into the eukaryotic expression vector p91023 (B). DNA (15 to 20 μg) from the construct, as well as the wild-type, full-length pASM-3b, was introduced into COS-1 cells by the method of Chen and Okayama (1987). The transfected cells were harvested after 72 hr and ASM activities were determined using the radioactive substrate, 14C-sphingomyelin as previously described (Sloan et al. 1969).
**RESULTS**

*Identification of the Y446C mutation*

To identify the molecular lesion in a Japanese patient with type A NPD, the complete ASM coding region from the patient was PCR amplified from genomic DNA and sequenced by the dideoxy method. Fig. 1 shows the DNA sequence obtained from a region of exon 5; note that there is an A-to-G transition at nucleotide 1337, which causes a tyrosine (TAT) to cysteine (TUT) change at codon 446. The presence of the A residue suggests that this mutation is one of two heteroallelic mutations. In the other regions of the gene, no mutation was found with this method. To eliminate the possibility of a sequencing artifact, the region containing exon 5 was PCR amplified and subcloned and several independent clones were sequenced by the dideoxy chain termination method. Two types of clones were found. One contained the G residue at nucleotide 1337 and the other contained the A residue identical to wild type (Fig. 2). The existence of these two different clones was consistent with the authenticity of the Y446C mutation.

*Transient expression of the Y446C mutation*

To confirm the authenticity of the Y446C mutation, the base substitution was introduced into the full-length ASM cDNA, pASM-3b, by the PCR-overlap method and transiently expressed in COS-1 cells. Table 1 shows the ASM activities expressed in COS-1 cells using the radioactive natural substrate, $^{14}$C-sphingomyelin. Untransfected COS-1 cells had a mean activity of 4.0 nmol/hr/mg. In comparison, COS-1 cells transfected with the full-length ASM cDNA,
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pASM-3b, had a mean activity of 30.9 nmol/hr/mg. Notably, the mutant construct did not express catalytically active ASM, consistent with the fact that type A NPD patients have less than 1% residual ASM activity in cultured cells.

**DISCUSSION**

In general, type A patients have less than 5% of normal ASM activity in cultured fibroblasts and lymphoblasts upon in vitro or in situ determination, whereas type B patients have from 3 to 10% of normal levels (Levade et al. 1986). However, at present it is not possible to reliably predict the subtype or clinical severity of newly diagnosed NPD patients by the level of residual ASM activity. Since the isolation and characterization of cDNA and genomic sequences encoding ASM, three mutations (R496L, L302P, and fsP330) causing type A NPD and one mutation (ΔR608) causing type B NPD were identified from Ashkenazi Jewish

![Fig. 2. Sequences identified from PCR amplified genomic DNAs of a Japanese patient with type A Niemann-Pick disease. Two types of sequence were identified from several identical subclones of the PCR products. Normal, a TAT codon (Try); Mutant, a TGT codon (Cys).](image-url)

**TABLE 1. Transient expression of a ASM mutation in COS-1 cells**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Intracellular ASM activity (nmol/hr/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS-1 cells</td>
<td>4.0</td>
</tr>
<tr>
<td>ASM constructs</td>
<td></td>
</tr>
<tr>
<td>pASM-3b (sense)</td>
<td>30.9</td>
</tr>
<tr>
<td>Y446C</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Values represent the mean of four independent transfections.

pASM-3b, had a mean activity of 30.9 nmol/hr/mg. Notably, the mutant construct did not express catalytically active ASM, consistent with the fact that type A NPD patients have less than 1% residual ASM activity in cultured cells.
patients. The three type A mutations are responsible for more than 90% of the alleles causing type A NPD in the Ashkenazi Jewish population. The existence of common mutations should facilitate carrier testing for this debilitating disease in this population.

On the other hand, five mutations (g2610c, G577S, L261X, fsL178, and M382I) causing type A NPD and three mutations (S436R, G242R, and N383S) in type B NPD were isolated from non-Jewish population. Although the frequency of this disease in the non-Jewish population is low, the 4R608 mutation, which was identified from Jewish NPD patients as a type B NPD genotype, is prevalent in the non-Jewish NPD patients from Northern Africa and Europe.

In the Japanese population, we reported a S436R mutation from Japanese siblings with type B NPD (Takahashi et al. 1992a). These patients had typical hepatosplenomegaly, bilateral cherry-red spot, diffuse pulmonary infiltration, and no neurological deterioration. We found the existence of short stature with partial growth hormone deficiency as a part of the phenotype in the S436R type B NPD patients. The Y446C mutation reported here is the first type A NPD mutation identified in the Japanese population. In order to know the frequency of these mutations in the Japanese population, it will be necessary to screen sufficient number of unrelated individuals from various regions in Japan.

References

9) Levran, O., Desnick, R.J. & Schuchman, E.H. (1992b) Identification of a 3' acceptor splice site mutation (g2610c) in the acid sphingomyelinase gene of patients with
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