Identification and characterisation of mutations underlying Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB)

G-J Lee-Chen, S-P Lin, S-Z Lin, C-K Chuang, K-T Hsiao, C-F Huang, W-C Lien

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Sanfilippo syndrome type B or mucopolysaccharidosis type IIIB (MPS IIIB) is an autosomal recessive disorder caused by deficiency of the lysosomal enzyme α-N-acetylgalactosaminidase (NAG, EC 3.2.1.50). NAG catalyses the removal of terminal α-N-acetylgalactosamine residues from heparan sulphate. In the absence of NAG, partially degraded heparan sulphate accumulates in tissues and is excreted in the urine. Affected subjects show developmental delay, mental retardation, and symptoms and enzyme deficiency diagnosed during early childhood. Patients 773 and 155 died at 8 and 17 years, respectively. The four living patients are 4 to 9 years old, all with moderate mental retardation.

MATERIALS AND METHODS

DNA preparation and PCR amplification

The six patients with MPS IIIB had a typical, severe disease course with symptoms and enzyme deficiency diagnosed during early childhood. Patients 773 and 155 died at 8 and 17 years, respectively. The four living patients are 4 to 9 years old, all with moderate mental retardation. Genomic DNA was extracted from whole blood according to standard procedures. Polymerase chain reaction (PCR) of NAG exons including adjacent intronic regions was done with primers and conditions listed in table 1. Genomic DNA (100 ng) was used for a 25 µl PCR reaction containing 10 mmol/l Tris, pH 8.3, 50 mmol/l KCl, 0.1% triton X-100, 0.2 mmol/l dNTPs, 0.4 µmol/l of each primer, and 0.5 U Taq polymerase (Promega). After initial denaturation for six minutes at 94°C, 35 cycles of 30 seconds at 94°C, 45 seconds at annealing temperature (table 1), and 45 seconds of extension at 72°C followed, with a final extension step for 10 minutes at 72°C. Exon 6 was amplified in four overlapping fragments.

SSCP/heteroduplex analyses and DNA sequencing

PCR products were mixed with an equal volume of 95% formamide buffer and electrophoresed on a low cross-linking polyacrylamide gel. PCR products were restricted to generate exon containing subfragments of approximately 100-300 bp for SSCP analysis.13 Fragments showing mobility shifts of single strands and/or heteroduplex formation were sequenced directly using the ABI Prism Dye Terminator Kit (PE Applied Biosystems) and an ABI Prism 377 DNA Sequencer (PE Applied Biosystems). In patients 1362 and 1377, all exon containing fragments were sequenced to identify mutations.

Mutation and haplotype analyses

All mutations were verified by either restriction analysis or by allele specific oligonucleotide (ASO) hybridisation of PCR amplified products (table 2). One hundred control chromosomes were analysed for missense mutations using restriction analysis or ASO hybridisation. ASO analysis was performed as described previously with washing at 42°C. ASO sequences are available on request. The NAG gene IVS2+50G>C

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
<th>Annealing temp (°C)</th>
<th>MgCl₂ (mmol/l)/ DMSO (%)</th>
<th>Product size (bp)</th>
<th>Restriction enzyme before SSCP</th>
<th>Fragment sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: AGAACCTCCCAGGAGAATAT R: AATTGGCTGACCGCGCTCC</td>
<td>57</td>
<td>1.5/10</td>
<td>608</td>
<td>BsalI (315, 235, 58)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F: CCCGCTCTGATGCACTA R: GCACCTGGAACGCACCTTA</td>
<td>55</td>
<td>1.5/10</td>
<td>596</td>
<td>Avall (308, 203, 85)</td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>F: AGCGCggcAGCAAGAGGAAG R: AAAACTCCCTCTGCTAGAAT</td>
<td>57</td>
<td>1.0</td>
<td>561</td>
<td>PsI (315, 246)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F: AACACCAGGAGCTTAGAGAAGGTT R: CTGGGCTACCTGCTGACT</td>
<td>54</td>
<td>1.5</td>
<td>434</td>
<td>BsalI (259, 175)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F: GGCCTCTCTCATCTCATCCTAG R: AAATCTGGCACTGGGTCCTT</td>
<td>57</td>
<td>1.0</td>
<td>444</td>
<td>Spal (251, 193)</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>F: GCACGCGCGCTGGGCTCC</td>
<td>52</td>
<td>1.5</td>
<td>436</td>
<td>PsI (236, 190)</td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>F: CAACGCTCTGCTGCTCTGTTAG R: CCACGCTCTTCTGCTGAGTAG</td>
<td>55</td>
<td>1.5</td>
<td>387</td>
<td>Hhal (234, 153)</td>
<td></td>
</tr>
<tr>
<td>6.3</td>
<td>F: GCCGAGCGCGAGTTTCTAC R: GCGATCTATCACCAAGGCG</td>
<td>57</td>
<td>1.0</td>
<td>345</td>
<td>SmaI (180, 165)</td>
<td></td>
</tr>
</tbody>
</table>
polymorphism was detected by Msel restriction on PCR amplified exon 2 containing DNA. The microsatellite markers D17S946 and S17S800 were examined using primers as described by Gyapay et al.

Construction of wild type and mutated NAG cDNA plasmids

Polyadenylated RNA was isolated from human cultured fibroblasts using the Dynabeads mRNA DIRECT kit (Dynal AS). Reverse transcription and subsequent PCR amplification of cDNA were carried out with 200 ng mRNA using the Thermoscript RT-PCR system (GIBCO) according to the manufacturer's instructions. Sense and antisense primers used for amplification of specific cDNA were 5'-TTAGCCTTCGG GTCCACGTG-3' and 5'-CCACACTTTGGTGGTGTT-3', respectively. After the amplified full length 2.5 kb NAG cDNA were cloned into pGEM-T Easy vector (Promega) and sequenced, the mutated sequence constructs were confirmed by DNA sequencing.

Expression and analysis of NAG cDNA mutants

pcDNA3, pcDNA3-NAG, or the mutated cDNA plasmids were transfected into COS-7 cells as previously described. Forty-eight hours after transfection, cells were harvested and extracts prepared by freeze-thawing. The assay for NAG activity was carried out as previously described. The PCR segment containing the desired mutation was digested with PstI and HinfI restriction enzymes and cloned into the corresponding restriction sites of pcDNA3-NAG. The mutated sequence constructs were confirmed by DNA sequencing.

RESULTS AND DISCUSSION

All NAG exons from six patients with the severe form of MPS IIIB were amplified for SSCP and heteroduplex analyses. As shown in table 2, 660delC, Y309C, G412E, and R565W mutations were detectable by heteroduplex formation, whereas only R626X produced clear, single strand mobility shifts. Since no disease alleles could be identified by SSCP and heteroduplex screening in patients 1362 and 1377, all of the amplified PCR products were directly sequenced. Using these techniques, seven different mutations (table 2, 100% disease alleles) were found, five of which have not been previously reported. The seven mutations were confirmed by restriction enzyme digestion or ASO hybridization. Five (R130C, I154R, Y309C, G412E, and R565W) of the mutations identified here predict the replacement of one evolutionarily conserved amino acid (table 2). In each case, 100 control chromosomes were screened for the presence or absence of mutations and none was found. Family analyses verified that the two mutations found in patients 773 and 155 are located on separate alleles.

The effect of the missense mutations on enzyme function was investigated by transient expression in COS-7 cells. The retention of only 0.0-3.9% NAG activity (table 3) indicates the deleterious nature of the mutations. None of the mutations caused an apparent reduction in NAG mRNA level when compared to that of wild type (fig 1A, lanes 2-4, 6-8). Proteins of 80-83 and 70 kDa were detected in both wild type and mutant transfected cells (fig 1B, lanes 2-4, 6-8). The 80-83 kDa band could include the precursor and mature forms reported previously on NAG processing in culture cells. The smaller 70 kDa form might be a degraded form of NAG protein.

**Table 2** Mutations identified in Chinese patients with MPS IIIB

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide alteration</th>
<th>Protein alteration</th>
<th>Patient</th>
<th>Allele</th>
<th>SSCP shift/ heteroduplex formation</th>
<th>Restriction enzyme (fragment sizes (bp))/ASO test</th>
</tr>
</thead>
<tbody>
<tr>
<td>R130C</td>
<td>CGC-&gt;TGC</td>
<td>Arg-&gt;Cys</td>
<td>1362</td>
<td>Hom</td>
<td>–</td>
<td>–KpnI [419]</td>
</tr>
<tr>
<td>I154R</td>
<td>ATA-&gt;AAG</td>
<td>Ile-&gt;Arg</td>
<td>1377</td>
<td>Hom</td>
<td>+</td>
<td>+HinfI [379, 21]</td>
</tr>
<tr>
<td>660delC*</td>
<td>1 bp del</td>
<td>17 altered aa, term</td>
<td>773</td>
<td>Het</td>
<td>+ (heteroduplex)</td>
<td>ASO</td>
</tr>
<tr>
<td>Y309C</td>
<td>TAT-&gt;TGC</td>
<td>Tyr-&gt;Cys</td>
<td>155</td>
<td>Het</td>
<td>+ (heteroduplex)</td>
<td>ASO</td>
</tr>
<tr>
<td>G412E</td>
<td>GGA-&gt;GAA</td>
<td>Gly-&gt;Glu</td>
<td>155</td>
<td>Het</td>
<td>+ (heteroduplex)</td>
<td>ASO</td>
</tr>
<tr>
<td>R565W†</td>
<td>CGG-&gt;TGG</td>
<td>Arg-&gt;Trp</td>
<td>2092</td>
<td>Hom</td>
<td>+</td>
<td>ASO</td>
</tr>
<tr>
<td>R626X‡</td>
<td>CGA-&gt;TGA</td>
<td>Arg-&gt;Stop</td>
<td>1146</td>
<td>Hom</td>
<td>+ (SSCP)</td>
<td>+Ddel [125, 103]</td>
</tr>
</tbody>
</table>

*Number of nucleotides according to Zhao et al.†Found previously.‡The HinfI site generated by amplification created restriction site PCR using exon 2 forward primer and mismatch reverse primer (CCATTACGCGCATCCAGAC).§The microsatellite markers D17S946 and S17S800 were examined using primers as described by Gyapay et al.

**Table 3** Expression of NAG in transfected COS-7 cells

<table>
<thead>
<tr>
<th>Transfected vector</th>
<th>α-N-acetylglucosaminidase activity (mnmol/h/mg protein)</th>
<th>Total (% of wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3</td>
<td>3.80</td>
<td>0.0</td>
</tr>
<tr>
<td>pcDNA3-NAG</td>
<td>54.25</td>
<td>100.0</td>
</tr>
<tr>
<td>pcDNA3-NAG/R130C</td>
<td>3.80</td>
<td>0.0</td>
</tr>
<tr>
<td>pcDNA3-NAG/I154R</td>
<td>4.00</td>
<td>0.4</td>
</tr>
<tr>
<td>pcDNA3-NAG/660delC</td>
<td>3.80</td>
<td>0.0</td>
</tr>
<tr>
<td>pcDNA3-NAG/Y309C</td>
<td>5.41</td>
<td>3.2</td>
</tr>
<tr>
<td>pcDNA3-NAG/G412E</td>
<td>3.85</td>
<td>0.1</td>
</tr>
<tr>
<td>pcDNA3-NAG/R565W</td>
<td>5.77</td>
<td>3.9</td>
</tr>
<tr>
<td>pcDNA3-NAG/R626X</td>
<td>3.80</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The NAG activities in COS-7 cells are the mean of two independent experiments, with a range of less than 5%. The endogenous NAG activity in COS-7 cells was subtracted before the percentage of wild type was calculated.
1B, lane 1), no NAG protein was seen with 660delC (fig 1B, lane 5); the largely truncated protein was apparently unstable. Conversely, a truncated 70 kDa NAG protein and a smaller 55 kDa form were seen with R626X (fig 1B, lane 9), but it had no enzyme function (table 3).

Of the six point mutations reported here, three (R130C, R565W, R626X) affect highly mutable CpG dinucleotides (table 2). The latter two (R565W and R626X) have been found in other patient series.

The methylation mediated deamination of 5-methylcytosine in the hypermutable CpG dinucleotide is frequently associated with point mutations of various genes.22 Three polymorphic markers, NAG intron 2 IVS2+50G>C and microsatellites D17S946 and D17S800,15 were used for haplotype analysis on the R565W alleles in patients 773 and 2092. The different haplotypes observed (not shown) suggest that the alterations are probably of independent origin.

Six out of these seven mutations were present in only one patient. Four of the six patients were homozygous for their mutations, while the remaining two were compound heterozygotes (table 2). In three of four homozygous patients (1362, 1377, and 2092), the parents were consanguineous, but in one (1146) they were not. By Ddel restriction analysis, the R626X mutation was detected in the father of 1146 (fig 2A, lane 1), but not in the mother (fig 2A, lane 3). The sample identity was verified by examining the IDUA gene intron 2 VNTR polymorphism7 and the apoB-100 gene 3' HVR polymorphism7 (not shown). The family were homozygous for IVS2+50G>C but heterozygous for D17S946 and D17S800 (fig 2B). Although a de novo mutation or hemizygosity owing to a deletion of the NAG gene in the patient cannot be completely excluded, a double stranded break gene conversion event may have resulted in homozygosity for R626X. A high incidence of homozygosity in MPS IIIB has previously been reported.7,15

In conclusion, we have identified seven mutations (R130C, I154R, 660delC, Y309C, G412E, R565W, and R626X) in the NAG gene in six unrelated Chinese MPS IIIB patients, five of which were novel. Transfection of COS-7 cells by cDNA with the mutations did not yield active enzyme, indicating the deleterious nature of the mutations. These results add to the growing body of knowledge documenting molecular heterogeneity and racial differences in mutations in MPS IIIB. The fact that the missense mutations described here were associated with a severe clinical picture may help to pinpoint amino acids essential for enzyme function.

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