ELECTRONIC LETTER

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

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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-acetylglucosaminidase (NAG, EC 3.2.1.50). NAG catalyses the removal of terminal α -N-acetylglucosamine 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, attention deficit disorder, uncontrollable hyperactivity, and aggressive behaviour, followed by progressive dementia with death usually in the late teens.¹

Cloning of the NAG gene and cDNA has enabled studies of the molecular basis of this syndrome.^{2,3} At present, over 50 different mutations underlying MPS IIIB have been identified, including deletions, insertions, point mutations, and splicing site mutations.³⁻¹⁰ The extensive allelic heterogeneity reflects the wide spectrum of clinical phenotypes reported in MPS IIIB patients.¹¹ In this study, the molecular lesions of six unrelated Chinese patients with MPS IIIB were investigated. Five novel and two previously reported mutations were found. We used transient expression studies to examine the effects of the mutations on NAG catalytic activity, mRNA stability, and protein stability.

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.¹² 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 μ l 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 μ mol/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.¹³ 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 previously¹⁴ with washing at 42°C. ASO sequences are available on request. The NAG gene IVS2+50G>C

Table 1 Primers and conditions for PCR amplification of NAG exons

Exon	Primer sequence	Annealing temp (°C)	MgCl ₂ , (mmol/l)/ DMSO (%)	Product size (bp)	Restriction enzyme before SSCP (fragment sizes (bp))
1	F: AGACGCCCAAGGGAGTAT R: ATTTGGGTGGCAGCGGCTCC	57	1.5/10	608	<i>Bss</i> HI (315, 235, 58)
2	F: CCCTGCCCATCTGTAGACT R: GCACGTGAAAGCACTTCTA	55	1.5/10	596	<i>Av</i> all (308, 203, 85)
3-4	F: AGCGCCCGAGCAGAAAGAAG R: AAATTCCTCTCTGAGCTAA	57	1.0	561	<i>Pst</i> I (315, 246)
5	F: AAACCAGGAGCTGTAGAGAAGT R: CTGCCTACCCCTACTGACATCT	54	1.5	434	<i>Bst</i> XI (259, 175)
6.1	F: GGCCCTCTGTTCATCACTC R: AAATCTGGCACTGGGTCTCT	57	1.0	444	<i>Sph</i> I (251, 193)
6.2	F: GCATCAGCCAGAACAAGTG R: CCAGCTCCTTGCTCAGGTAG	52	1.5	436	<i>Pst</i> I (236, 190)
6.3	F: CAACCGATCTGATGTGTTTG R: TTGGCATAGTCCAGGATGTT	55	1.5	387	<i>Hha</i> I (234, 153)
6.4	F: GCCGAGGCCGATTCTAC R: GCGAATCTATACCAAGAGC	57	1.0	345	<i>Sfa</i> NI (180, 165)

Table 2 Mutations identified in Chinese patients with MPS IIIB

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

*Number of nucleotides according to Zhao *et al.*³†‡Found previously.^{3,5,7}§The *HinfI* site generated by amplification created restriction site PCR using exon 2 forward primer and mismatch reverse primer (CCATTCAGCGCCATCCAGAC).

polymorphism⁷ was detected by *MaeI* restriction on PCR amplified exon 2 containing DNA. The microsatellite markers D17S946 and S17S800 were examined using primers as described by Gyapay *et al.*¹⁵

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'-CCCACACTTGGGTGGTGGT-3', respectively. After the amplified full length 2.5 kb *NAG* cDNA were cloned into pGEM-T Easy vector (Promega) and sequenced, the *NAG* cDNA was then excised with *EcoRI* and subcloned into pcDNA3 (Invitrogen) to produce plasmid pcDNA3-NAG. The mutation in the *NAG* cDNA was generated by two step PCR¹⁶ (sequences of primers are available on request). The PCR segment containing the desired mutation was digested with *PmaCI* and *HinfI* restriction enzymes and cloned into the corresponding restriction sites of pcDNA3-NAG. 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.¹⁴ 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.⁶ The 1.3 kb *EagI-StuI* fragment containing *NAG* codon 85-507 was cloned into pET-22b(+) *HindIII* and *EagI* sites and introduced into BL21(DE3)pLysS (Novagen). After IPTG induction, the 50 kDa antigen was purified by His-Tag resin and used to raise polyclonal antiserum in rabbits. Total RNA and proteins from transfected cells were used in northern and western blot analyses as previously described.¹⁷

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

Table 3 Expression of NAG in transfected COS-7 cells

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

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.

reported. The seven mutations were confirmed by restriction enzyme digestion or ASO hybridisation.

Five (R130C, I154R, Y309C, G412E, and R565W) of the mutations identified here predict the replacement of one evolutionarily conserved^{2,3,18} 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.^{10,19,20} The smaller 70 kDa form might be a degraded form of *NAG* protein.

The frameshift mutation 660delC predicts the addition of 17 unrelated amino acids at codon 221 followed by termination. The nonsense mutation R626X predicts lack of 118 amino acids at the carboxyl terminus of the protein. The two mutations can be assumed to cause disease. In transfected COS-7 cells, both mutations showed no appreciable NAG activity (0.0% of normal activity) (table 3), although they did not cause an apparent reduction in *NAG* mRNA level (fig 1A, lanes 5, 9). Frameshift and nonsense mutations are likely to lead to instability of the mRNA.^{21,22} With the exception of the non-specific protein also seen in pcDNA3 transfected cells (fig

Figure 1 Northern and western analyses of NAG cDNA mutant. (A) Total RNA (10 µg) was separated on formaldehyde-agarose gel, transferred onto nylon membrane, and hybridised to NAG probe. (B) Total protein (10 µg) was separated on 8% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with NAG polyclonal antibody. Molecular mass is indicated as determined from a plot of distances migrated of standard proteins against the logarithms of molecular masses. Lane 1, pcDNA3; lane 2, pcDNA3-NAG; lane 3, pcDNA3-NAG/R130C; lane 4, pcDNA3-NAG/I154R; lane 5, pcDNA3-NAG/660delC; lane 6, pcDNA3-NAG/Y309C; lane 7, pcDNA3-NAG/G412E; lane 8, pcDNA3-NAG/R565W; lane 9, pcDNA3-NAG/R626X.

Figure 2 (A) DNA PCR and *DdeI* restriction analysis of R626X mutation. The amplified 387 bp DNA fragments from the father of 1146 (lane 1), 1146 (lane 2), the mother of 1146 (lane 3), and a normal control (lane 4) were digested with *DdeI* and fractionated on a 2% agarose gel. The normal control was digested into two fragments of 228 and 159 bp. The mutation R626X created a new *DdeI* restriction site on the PCR products so that, on digestion, 125 and 103 bp fragments appeared instead of the wild type 228 bp fragment. Lane M (*HindIII* digest of pGEM4 DNA) is used as a size marker. (B) Pedigree and haplotype analysis of the family of 1146. Haplotypes for polymorphic markers IVS2+50G>C, D17S946, and D17S800 are shown under each person. Haplotypes cosegregating with the disease phenotype are indicated by bold numbers.

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.^{3 5 7} The methylation mediated deamination of 5-methylcytosine in the hypermutable CpG dinucleotide is frequently associated with point mutations of various genes.²³ Three polymorphic markers, NAG intron 2 IVS2+50G>C⁷ 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 *DdeI* 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 polymorphism²⁴ and the apoB-100 gene 3' HVR polymorphism²⁵ (not shown). The family were homozygous for IVS2+50G>C but heterozygous for D17S946 and D17S800 (fig 2B). Although a de novo mutation or hemizyosity owing to a deletion of the NAG gene in the patient cannot be completely excluded, a double stranded break²⁶ gene conversion event may have resulted in homozygosity for R626X. A high incidence of homozygosity in MPS IIIB has previously been reported.^{3 5 6 8 10}

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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