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Mutational analysis of Sanfilippo syndrome type A (MPS IIIA): identification of 13 novel mutations

EDITOR—Sanfilippo syndrome or mucopolysaccharidosis type III (MPS III) encompasses a group of four lysosomal storage disorders resulting from a failure to break down the glycosaminoglycan heparan sulphate. Each of the four subtypes, A, B, C, and D, is caused by the deficiency of a different enzyme in the degradative pathway of heparan sulphate: heparan-N-sulphatase (EC 3.10.1.1), α-N-acetylglucosaminidase (EC 3.2.1.50), acetyl-CoA N-acetyl transferase (EC 2.3.1.3), and N-acetylglucosamine-6-sulphatase (EC 3.1.6.14), respectively.[1] Clinical symptoms usually occur after two years of apparently normal development and include hyperactivity, aggressive behaviour, delayed development (particularly in speech), sleep disturbances, coarse hair, hirsutism, and diarrhoea. There are only relatively mild somatic manifestations. There then follows a period of progressive mental retardation with death usually between the second and third decade of life. In a small number of patients with Sanfilippo syndrome type B, there is a more slowly progressive form of the disease with later onset known as the attenuated phenotype.[2–4] A late onset phenotype has also been described for Sanfilippo syndrome type A.[5]

Sanfilippo syndrome type A (MPS IIIA) is caused by a deficiency in the enzyme heparan-N-sulphatase (sulphamidase). The disease is autosomal recessive and the gene encoding the enzyme is situated on chromosome 17q25.3, contains eight exons, and encodes a protein of 502 amino acids.[6 7] To date, 46 different mutations have been identified in Sanfilippo A patients,[6 8–13] several of which have been found at high frequencies in particular populations. The R245H, R74C, 1091delC, and S66W were the most frequent mutations in the Dutch (56.7%),[11] Polish (56%),[8] Spanish (45.5%),[13] and Italian (33%)[12] populations, respectively. Several polymorphisms have been identified in the sulphamidase gene including R456H, which has a high frequency of 55% in the normal Australian population.[9] In this study, mutational analysis has been carried out on the sulphamidase gene from 23 patients with Sanfilippo syndrome type A in the UK. Twenty three different mutations have been found, 13 of which have not been reported previously. The novel mutations comprise one insertion (1156ins6), two nonsense mutations (R233X, E369X), and 10 missense mutations (D32G, H84Y, R150W, D235N, D273N, I322S, E355K, Y374H, R433W, V486F).

All except one of the 23 Sanfilippo A patients under investigation had the classical Sanfilippo phenotype. The one milder patient had slight developmental delay but no hyperactivity or sleep problem. Sulphamidase enzyme activity in leucocytes from the patients ranged from 0 to 5 pmol/h/mg protein (reference range 52-458 pmol/h/mg protein).[14] The average age at diagnosis was 5 years 6 months. A modified version of the ammonium acetate salting out method was used to extract genomic DNA from either venous blood or fibroblast cell lines of the patients.[15 16]

Each of the eight exons and intron/exon boundaries of the sulphamidase gene was amplified by PCR using intronic primers (table 1). Exon 8 was amplified as two overlapping fragments (exons 8a and 8b). An M13(-21) forward primer sequence (5'-TGTAAAACGACGGCC AGT-3') and an M13 reverse primer sequence (5'-CAGGAAACAGCTATGACC-3') were tagged at the 5' end of the sense and antisense primers, respectively. These sites were used as universal primer binding sites in the fluorescent DNA sequencing procedure.

A typical PCR reaction using 100 ng of genomic DNA contained 25 pmol of each primer, 1 × NH_4 reaction buffer (Bioline), 4% (v/v) DMSO (dimethylsulphoxide), 0.2 mmol/l dNTPs, and 0.5 µl (2.5 units) BioPro™ DNA polymerase (Bioline) (added after a hot start). Details of annealing temperatures and $MgCl_2$ concentrations for each particular amplification reaction are provided in table 1. Cycling conditions were typically 96°C for 10 minutes, followed by 35 cycles of one minute at 96°C, one minute at 60-64°C, one minute at 72°C, and a final extension at 72°C for 10 minutes.

Following amplification, the PCR products were subjected to SSCP (single strand conformation polymorphism) analysis using MDE™ gel (Mutation Detection Enhancement) (FMC Bioproducts). The nine PCR products were digested with a restriction enzyme before SSCP analysis (table 1). To 5 µl of digestion mix, 2 µl of loading dye (95% (v/v) formamide, 10 mmol/l NaOH, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added. Samples were denatured at 94°C for four minutes before loading on a 0.5 × MDE™ gel. Electrophoresis was carried out in 0.5 × TBE at 15 W overnight at 4°C or at 45 W for four hours at room temperature. Bands were detected by silver staining.[17]

Fragments of interest were concentrated and separated from excess primers and dNTPs by ultrafiltration through Microcon™-100 columns (Millipore) before sequencing. Products were sequenced in both the forward and reverse direction using the appropriate M13 dye labelled primer kits (Perkin Elmer Applied Biosystems). Reactions were performed as instructed and samples were analysed on an ABI Prism™ 377 DNA Sequencer (Perkin Elmer Applied Biosystems).

Sequence changes were confirmed either by digestion with a restriction enzyme or by ACRS (amplification created restriction site) PCR.[18] Primer sequences, annealing

Table 1 Primers and PCR conditions used for the amplification of the sulphamidase gene

Fragment	Primer	Nucleotide position	Sequence	Product size (bp)	$MgCl_2$ conc (mmol/l)	Annealing temp (°C)	Restriction enzyme before SSCP (fragment sizes (bp))
1	SFA1(+)	3-19*	M13 (-21)- GCGGGAGACCAGAGAGC				
	SFA1(−)	267-251	M13 rev-CAGCGGGGGATACAAGG	301	1.5	64	*Bst*NI (118+183)
2	SFA2(+)	11-30†	M13 (-21)- ACAGTCCCAGCCTCCCTACT				
	SFA2(−)	336-319	M13 rev-ATGGGAGACGTGGCAGAG	362	1.5	60	*Hae*II (142+220)
3	SFA3(+)	79-95‡	M13 (-21)- GGGCCATGGGAGAACAG				
	SFA3(−)	348-330	M13 rev- CGTGCCTTGGTACAAGGTG	306	1.5	62	*Apa*LI (129+177)
4	SFA4(+)	426-442‡	M13 (-21)- CGAGAACCCACAGTGCG				
	SFA4(−)	727-711	M13 rev- GTGCTCTGGTACCGGCC	338	1.5	64	*Bsa*AI (138+200)
5	SFA5(+)	27-43§	M13 (-21)- CCTTCCTGTGCCACGTG				
	SFA5(−)	344-323	M13 rev- TCTTTCTTCATCATCTAGGGCC	354	1.0	62	*Rsa*I (139+215)
6	SFA6(+)	493-511§	M13 (-21)- TGTTCTAAGCCTGGCTCCC				
	SFA6(−)	718-701	M13 rev- CTGCCACACTGGACCCTC	262	1.0	62	*Sty*I (130+132)
7	SFA7(+)	40-56¶	M13 (-21)- GGATGCAGCAGCAGGTG				
	SFA7(−)	425-406	M13 rev- AGGTAATGGGTGTGGAGCAG	422	1.5	64	*Rsa*I (219+203)
8a	SFA8a(+)	1286-1304¶	M13 (-21)- TTGGATTGGAGAAGGGAGC				
	SFA8a(−)	1742-1721	M13 rev- CCGGTAGTAGTAATGACGGAGG	493	1.5	66	*Hae*II (230+263)
8b	SFA8b(+)	1632-1651¶	M13 (-21)- CCCATCGACCAGGACTTCTA				
	SFA8b(−)	2002-1984	M13 rev- GGATGTGTCTGGGACATGC	407	1.0	62	*Sac*I (143+264)

*†‡§¶Positions of primers are numbered according to Genbank database entry u60107, u60108, u60109, u60110, and u60111, respectively.[7]

temperatures, and $MgCl_2$ concentrations for the ACRS PCR reactions are provided in table 2. All other PCR parameters were as described previously.

The eight exons of the sulphamidase gene from 23 patients with Sanfilippo syndrome type A were amplified as nine fragments by PCR using intronic primers. Exon 8 is 559 bp in length and was amplified as two overlapping fragments of 493 bp and 407 bp. The PCR products were digested with a restriction enzyme before SSCP analysis at 4°C. Fragments showing a shift were purified and sequenced directly in both the forward and reverse direction using fluorescent DNA sequencing technology (Perkin Elmer Applied Biosystems). In six patient samples where no or only one heterozygous shift was observed, restricted PCR products were also subjected to SSCP analysis at room temperature (RT). Two of the mutations identified only showed a shift at RT. In four patients where no SSCP shifts were observed at either 4°C or RT, the mutations were identified by directly sequencing all nine PCR products. In this study, 23 different mutations were found, 13 of which have not been reported previously (table 3). Of the 23 mutations found, 82.6% could be detected by SSCP under the conditions used, comparable to the expected detection rate of SSCP. The previously known mutations found in this study include three deletions (1307del9, 1091delC, and 1284del11), one insertion (1039insC), and six missense mutations (S66W, R74C, T79P, G122R, R245H, and S298P). Three of the known mutations, R74C, R245H, and 1091delC, accounted for 8.7%, 13.1%, and 15.2% of the mutant alleles, respectively, and together accounted for 37%. The 13 novel mutations comprised one insertion (1156ins6), two nonsense mutations (R233X, E369X), and 10 missense mutations (D32G, H84Y, R150W, D235N, D273N, I322S, E355K, Y374H, R433W, and V486F). A second PCR fragment was generated to confirm the presence of the mutation in the patient sample. Restriction digests were performed for 18 of the mutations (table 3) and for the remaining five sequence changes region specific primers were designed that would lead to the creation or loss of restriction sites at those known mutation sites, a technique known as ACRS PCR (table 3). Table 2 details the amplification conditions for the reactions and subsequent restriction enzyme analysis.

Both putative mutations were found in all 23 Sanfilippo A patients (table 4). Nine of the patients were homozygous for the sequence change and the remaining 14 were compound heterozygotes. The patients were also screened for the R456H polymorphism[9] by *Bst*UI enzyme digestion and the frequency of the "A" allele (CAC encoding histidine) was 41.3%. Six of the 23 mutations, 1091delC, R74C, R245H, V486F, S66W, and 1156ins6, were found in more than one unrelated family. For patients 1, 3, 5, 8, and 23 (table 4), parent samples were available and all were shown to be carriers of one of the mutant alleles found in their affected child.

In this study, 10 novel missense mutations were found but their pathogenic effect on enzyme function has yet to be investigated. However, 100 control chromosomes were screened for all of these changes. SSCP analysis was used to screen for four of the changes (H84Y, R150W, D273N, and I322S). Digestion with the restriction enzymes *Bsp*1286I, *Bsr*I, and *Bsa*HI was used to detect E355K, R433W, and V486F, respectively. ACRS PCR and *Hin*cII, *Taq*I, or *Sau*96I digestion were used to screen for D32G, D235N, and Y374H, respectively. None of the missense mutations was found in any of the 100 control chromosomes. A rare polymorphism which has not been previously reported was found in heterozygous form in exon 4 of one control, A137A (GCG→GCA: 423G>A).

Mutational analysis on 23 patients from the UK with Sanfilippo syndrome type A has resulted in the identification of both putative mutations in all of the patients. Twenty three different mutations, including 13 which have not been reported previously, were detected.

One of the novel mutations is an insertion of 6 bp (AGCGCC) in exon 8. The insertion is a duplication of six nucleotides upstream of the insertion site and the presence of direct repeat elements (GCAC) in the vicinity of the mutation suggests that it has arisen because of slippage

Table 2 Primers and PCR conditions used for the ACRS PCR reactions

Mutation	Sense primer (nucleotide position)	Antisense primer (table 1)	Product size (bp)	Annealing temp (°C)	$MgCl_2$ conc (mmol/l)	Restriction enzyme (fragment sizes (bp))
D32G	5'ACTCTCTGTCTCCCACCTCACGCAGCGGTT3' (67-96*)	SFA2(−)	288	64	1.5	−*Hin*cII (288)
G122R	5'GCCAGGCCTCTCTTCCCGCCCAGGCATCCT3' (503-532†)	SFA4(−)	243	62	1.0	+*Dde*I (29+133+81)
D235N	5'TTCGTCCCCAACACCCCGGCAGCCCGAGTC3' (536-565‡)	SFA6(−)	201	64	1.5	−*Taq*I (201)
D273N	5'CCTGAACGACACACTGGTGATCTTCACGAC3' (170-199§)	SFA7(−)	274	64	1.5	−*Bsi*EI (274)
Y374H	5'CAGCCAGAGCCACCACGAGGTCACCATGGC3' (1532-1561§)	SFA8a(−)	229	64	1.5	+*Sau*96I (28+111+48+42)

*†‡§Positions of primers are numbered according to Genbank database entry u60108, u60109, u60110, and u60111, respectively.[7]

Table 3 Mutations found in the sulphamidase gene from patients with MPS IIIA in this study

Exon (fragment)	*Mutation**	*Nucleotide alteration**	*Protein alteration*	*SSCP shift*	*RE test*	*ACRS test (table 2)*
2 (2)	D32G†	GAC→GGC 107A>G	Asp→Gly	−		−*Hin*cII
2 (2)	S66W	TCG→TGG 209C>G	Ser→Trp	−	+*Bst*NI	
2 (2)	R74C	CGC→TGC 232C>T	Arg→Cys	+ 4°C	−*Bst*UI	
2 (2)	T79P	ACT→CCT 247A>C	Thr→Pro	+ 4°C	+*Bst*NI	
3 (3)	H84Y†	CAT→TAT 262C>T	His→Tyr	+ 4°C	−*Fnu*4HI	
4 (4)	G122R	GGG→AGG 376G>A	Gly→Arg	+ 4°C		+*Dde*I
4 (4)	R150W†	CGG→TGG 460C>T	Arg→Trp	+ 4°C	−*Aci*I	
6 (6)	R233X†	CGA→TGA 709C>T	Arg→Stop	+ 4°C	−*Ava*I	
6 (6)	D235N†	GAC→AAC 715G>A	Asp→Asn	+ RT		−*Taq*I
6 (6)	R245H	CGC→CAC 746G>A	Arg→His	+ 4°C	−*Eag*I	
7 (7)	D273N†	GAC→AAC 829G>A	Asp→Asn	+ 4°C		−*Bsi*EI
7 (7)	S298P	TCC→CCC 904T>C	Ser→Pro	+ 4°C	+*Mae*III	
8 (8a)	I322S†	ATC→AGC 977T>G	Ile→Ser	+ 4°C	+*Alu*I	
8 (8a)	1039insC	1 bp ins	158 altered aa, term	+ 4°C	−*Bst*XI	
8 (8a)	E355K†	GAG→AAG 1075G>A	Glu→Lys	+ 4°C	−*Bsp*1286I	
8 (8a)	1091delC	1 bp del	51 altered aa, term	+ 4°C	+*Bst*XI	
8 (8a)	E369X†	GAG→TAG 1117G>T	Glu→Stop	+ 4°C	+*Bfa*I	
8 (8a)	Y374H†	TAC→CAC 1132T>C	Tyr→His	−		+*Sau*96I
8 (8a)	1156ins6†	6 bp ins AGCGCC	ins Gln Arg	+ 4°C	+*Hae*II	
8 (8b)	1284del11	11 bp del	1 altered aa, term	+ 4°C	−*Rsa*I	
8 (8b)	1307del9	9 bp del	del 3 aa	+ 4°C	−*Bst*UI	
8 (8b)	R433W†	CGG→TGG 1309C>T	Arg→Trp	+ RT	+*Bsr*I	
8 (8b)	V486F†	GTC→TTC 1468G>T	Val→Phe	−	−*Bsa*HI	

*Number of codons and nucleotides according to ref 6.
†Novel mutations.

during DNA replication.[19 20] However, the 6 bp insertion is not an identical copy of the previous six nucleotides because the fourth base has been changed from A to G (normal = AGC**A**CC, mutant = AGC**G**CC). The insertion does not lead to a frameshift but results in the introduction of two additional amino acids into the protein chain (glutamine and arginine). This is likely to have a detrimental effect on the functioning of the protein and the absence of the insertion in 100 control chromosomes supports the likelihood that it is disease causing. Three unrelated British patients were found to be heterozygous for the insertion and since it has not been reported previously, it may have a founder effect in the UK population.

The two novel nonsense mutations that were found, R233X and E369X, will both be disease causing because of the production of a truncated product. All of the 10 novel missense mutations result in non-conservative amino acid substitutions and their absence in 100 control chromosomes further supports their suspected pathogenicity. Comparison of sulphamidase to O- and N-sulphatases has highlighted two highly conserved regions, residues 70-80 containing the CTPSR active site motif and residues 115-124.[6] None of the 10 novel missense mutations in this study is situated within these regions, although H84Y is four amino acids downstream of the conserved CTPSR region and may have an effect on the active site environment. Five glycosylation sites have been proposed in the sulphamidase enzyme[6] and although none of the missense mutations directly affects the NXS/T motif, R150W is immediately upstream of the third proposed glycosylation sequence and the basic to hydrophobic amino acid change may have a detrimental effect on the local conformation. For many of the novel mutations there is a significant change in the charge of the amino acid side chain and this is likely to affect the conformation, stability, or catalytic function of the sulphamidase enzyme.

Table 4 Genotype and national origin of the MPS IIIA patients in this study

Patient	*Allele 1*	*Allele 2*	*R456H poly CGC→CAC*	*National origin*
1	1091delC	E355K	G/A	UK
2	R74C	S298P	G/A	UK
3	R433W	R245H	G/A	UK
4	H84Y	H84Y	G/G	Pakistan
5	G122R	G122R	G/G	Pakistan
6	R74C	R74C	G/G	UK
7	V486F	V486F	A/A	Czech Rep
8	I322S	I322S	A/A	Pakistan
9	R245H	S66W	G/A	UK
10	E369X	T79P	G/G	UK
11	R245H	R245H	A/A	UK
12	R233X	1156ins6	G/A	UK
13	1091delC	D235N	G/A	Spain
14	1307del9	D32G	G/G	UK
15	1091delC	1091delC	G/G	Malta
16	S66W	1091delC	G/G	UK
17	V486F	V486F	A/A	Greece
18	D273N	Y374H	G/G	Turkey/UK
19	R245H	1156ins6	A/A	UK
20	R150W	1039insC	G/A	UK
21	1284del11	1156ins6	G/A	UK
22	R74C	R245H	G/A	UK
23	1091delC	1091delC	G/G	UK

Interestingly, three of the novel mutations in this study have occurred at amino acid sites in the sulphamidase protein where other mutations have been reported. In our study, R150W, D235N, and E369X occur at the same codons as the previously reported mutations R150Q, D235V, and E369K, respectively. Mutations at different nucleotides within the specific codon are responsible for the variation in the mutant amino acid but these results suggest that certain codons in the sulphamidase gene may be mutational hotspots. CpG dinucleotides are known to be mutational hotspots within many genes[21] and six of the 13 novel mutations in our study, R150W, R233X, D235N, D273N, E355K, and R433W, are at such sites.

All except one of the Sanfilippo patients in this study showed the classical Sanfilippo phenotype and had very low or no detectable sulphamidase enzyme activity in their leucocytes. The one patient who did have a milder phenotype was homozygous for I322S. The amino acid change from a neutral hydrophobic to a neutral polar residue may allow the production of some residual enzyme but when sulphamidase enzyme activity was measured in leucocytes from this patient using the natural substrate [N-*sulphonate*-^{35}S] heparin,[14] no activity was detectable. In contrast, two patients who were homozygous for the V486F novel mutation had very similar residual enzyme activities of 3 and 3.8 pmol/h/mg protein (reference range 52-458 pmol/h/mg protein) but they both had the classical Sanfilippo phenotype associated with no detectable enzyme activity. Consequently, it is difficult to provide evidence of a genotype/phenotype correlation in Sanfilippo syndrome type A. Further expression work on the I322S, V486F, and the other novel missense mutations is required to confirm their pathogenicity and to investigate their effect on enzyme function. In MPS I where there are three phenotypes,

Hurler (severe), Hurler-Scheie (intermediate), and Scheie (mild), polymorphisms in the α-L-iduronidase gene are thought to modify expression and affect enzyme function.[22] In Sanfilippo syndrome type A, despite the high frequency of the R456H polymorphism (41.3% in our study), there is no evidence yet that it modifies the sulphamidase enzyme. However, expression of this polymorphism in isolation and in combination with known pathogenic mutations is necessary to investigate the possibility of such an effect.

Six mutations identified in this study, S66W, R74C, R245H, 1091delC, 1156ins6, and V486F, were found in more than one unrelated family. The 6 bp insertion has not been reported previously and appears to be unique to the British Sanfilippo A population. The novel V486F mutation was found in homozygous form in a Greek and a Czech patient and although these patients were unrelated, haplotype analysis for three common polymorphisms (R456H, IVS5+17, and IVS2-26) showed that the mutant alleles were identical, suggestive of a common ancestor. The remaining four mutations, R74C, R245H, S66W, and 1091delC, are known to be prevalent in Polish, Dutch, Italian, and Spanish populations, respectively.[8 11–13] In our study, although the majority of patients with these four mutations were British, the haplotype of the mutant alleles corresponds to that associated with the mutations and suggests that they are all ancient mutations. The most common mutation in the 15 British patients was R245H with a frequency of 20% (6/30 alleles). Two patients heterozygous and homozygous for the 1091delC mutation originated from Spain and Malta, respectively, confirming the prevalence of the mutation in this population. Altogether, the six mutations accounted for 56.5% of the mutant alleles in this study and this information in combination with knowledge of the ethnic background of patients will be important for future mutational analysis on newly diagnosed Sanfilippo A patients in the UK. However, 17 of the mutations found in this study were unique to a particular family, further highlighting the extensive heterogeneity of Sanfilippo syndrome type A at the genetic level.

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Genotype-phenotype relationship of Niemann-Pick disease type C: a possible correlation between clinical onset and levels of NPC1 protein in isolated skin fibroblasts

EDITOR—Niemann-Pick disease type C (NP-C, MIM 257220) is a fatal autosomal recessive disorder characterised by progressive neurological deterioration and hepatosplenomegaly. NP-C patients can be classified into four major groups according to the onset of neurological symptoms, that is, early infantile, late infantile, juvenile, and adult forms, and the earlier the clinical onset the more quickly progressive are the symptoms and the shorter is the life span.[1–4] Complementation analysis using cultured skin fibroblasts indicated the presence of at least two subgroups of NP-C, NPC1 (the major subgroup that comprises >90% of NP-C patients) and NPC2 (the minor subgroup).[2–4] In 1997, the NPC1 gene (*NPC1*) (accession No AF002020) that is responsible for the NPC1 subgroup was identified by positional cloning.[5 6] The number of *NPC1* mutations known to date is not far off 100,[7–11] taking into account the accumulated data from seven groups presented in a recent international workshop (International Workshop, The Niemann-Pick C Lesion and the Role of Intracellular Lipid Sorting in Human Disease, Bethesda, USA, October 1999).

Because the genomic structure of *NPC1* was unknown, initial mutation screening was performed on RT-PCR