Molecular basis of iduronate-2-sulphatase gene mutations in patients with mucopolysaccharidosis type II (Hunter syndrome)

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Abstract

Mucopolysaccharidosis type II (Hunter syndrome) is an X linked lysosomal storage disorder resulting from heterogeneous mutations in the iduronate-2-sulphatase (IDS) gene. To detect IDS gene mutations, direct sequencing of IDS cDNA fragments coupled with assays on IDS genomic amplicons was applied to 18 unrelated patients with MPS II. Seventeen mutations were detected from the 18 patients including seven missense mutations (S71R, A82E, A85T, R88C, R468W, R468Q, and E521V), five deletions (ΔR95, 383delAT, 596delAACA, 1148delC, and 1216delCT), two insertions (208insC and 1063insA), two splicing mutations (1006+5g→c in intron 7, 1122C→T in exon 8), and an intragenic deletion of IDS exons 4, 5, 6, and 7. Nine of the small mutations were novel mutations. Mutation 596delAACA was detected in two unrelated patients. The mutation in intron 7 was found to cause aberrant splicing and resulted in a 22 bp insertion into its mRNA transcript. The intragenic deleted IDS gene expressed two aberrant mRNA transcripts consisting of exons 1-2-8-9 and 3-8-9. Analysis of mutations A85T, R88C, R468Q, R468W, and 438C/T found no polymorphism for the four missense mutations but about 36% heterozygosity for the 438C/T silent mutation. These results provide further evidence of mutational heterogeneity for MPS II. Also, underlying sequence directed mutagenesis mechanisms for some recurrent mutations in the IDS gene were proposed.

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Keywords: mucopolysaccharidosis type II; Hunter syndrome; iduronate-2-sulphatase gene; mutation detection

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Mucopolysaccharidosis type II (MPS II, Hunter syndrome, MIM 309900) is an X linked recessive disorder caused by various lesions in the iduronate-2-sulphatase (IDS) gene.[1–3] The predominant clinical features of MPS II include coarse facial features, stiff joints, hepatosplenomegaly, cardiovascular and respiratory disorders, developmental delay, and mental retardation. Two clinical extremes of MPS II, mild and severe, have been recognised. In general, the severe form has early onset at 2-4 years, global retardation, seriously disturbed behaviour, and death before adulthood. In contrast, mildly affected subjects preserve normal intelligence and survive into late adulthood.[4 5]

The enzymatic defect in MPS II patients was found to be the deficiency of a lysosomal enzyme, sulphoiduronate sulphatase (now termed iduronate-2-sulphatase, IDS).[6] The characterisation of IDS made possible the definitive diagnosis of MPS II by enzymatic assay.[7 8] However, the in vitro measurement of deficient IDS enzyme activity, ranging from no detectable activity to residual activity, has not been correlated with clinical severity. In 1990, the IDS cDNA sequence, encoding an IDS protein of 550 amino acids, was characterised.[9] Subsequent studies deciphered the organisation of the IDS gene that spans approximately 24 kb and contains nine exons and eight introns.[10 11] Recently, an alternative IDS transcript containing exons 1 to 7b was discovered[12] and a unique IDS-2 locus or IDSψ sequence located approximately 20 kb distal to the active IDS gene was delineated.[13] However, the functional implication of the IDS alternative transcript and the unique IDS-2 locus remains to be determined.

Molecular studies using Southern blot analysis and cDNA sequencing have shown a wide spectrum of genetic alterations in the human IDS gene.[1–3] It has been concluded that all MPS II patients resulting from full deletions and gross rearrangements have severe clinical presentation.[1] However, for MPS II patients caused by small mutations in the IDS gene, the clinical effects of mutations are evaluated on an individual basis. Therefore, further accumulation of small mutation data and their clinical information could contribute to a better understanding of their disease causing mechanisms.

As a first step towards a systematic analysis of molecular defects of the IDS gene, we used single strand conformation polymorphism (SSCP) analysis to screen for mutations in the IDS exon specific amplicons.[14] Subsequently, a reverse transcription polymerase chain reaction (RT-PCR) approach coupled with direct cycle sequencing was developed to detect mutations in the IDS cDNA fragments.[15 16] The present study exploits the PCR based techniques for 18 unrelated patients and identifies 17 IDS gene mutations, including 16 small mutations (seven missense mutations, five small deletions, two insertions, and two splicing mutations) and an intragenic deletion.

*Table 1 Oligonucleotides for amplification of IDS and IDS-2 sequences**

Fragment	*Primer sequence (5' to 3')*	*Amplicon (bp)*
	E1L: TGTTGCGCAGTCTTCATGGGTT	
Exon 1	E1R: GGAGAAGAGATGGCAGGGAG	255
	E2L: CAGTGTCAGTGCAGGTTACC	
Exon 2	E2R: TCAGTGCACGAAGCAGCACA	233
	E3L: CCTAAGAGATGGCAGACATG	
Exon 3	E3R: GAGAACCCAGACTCTGGACATG	272
	E4L: CAAGGGATATCTTCTAACCA	
Exon 4	E4R: GGTTCCTCTTCAGAAATGTC	129
	E5L: CTGAGTGACTAACACGTGAA	
Exon 5	E5R: TCACAGCTGTGCTGGATCAG	356
	E6L: CAGTGATAGAGCCACAAGCT	
Exon 6	E6R: ACCTACGACACTATGTCATC	299
	E7L: TGTATGCCTTGGCAATTTAA	
Exon 7	E7R: CATGTTTCACAGGAAAGTTC	245
	E8L: CAAGCTGTGGTATGATGATT	
Exon 8	E8R: CTAAAGGTGATCTTACTGTC	280
	E9L: GCAGGCTTTTATAATGTAAC	
Exon 9	E9R: CGACCAGCTCTAACTCCTCC	595
IDS-2	2F: GCCTCGAACTCCTGATCTCA	
IVS 7	7S: TGGCACAGGGTCTGACACAT	

*Primers designed based on IDS exon/intron boundary sequences[11] and IDS-2 sequence.[13]

Materials and methods

Eighteen MPS II patients from different regions of the United States were referred for molecular analysis. All cases were diagnosed with deficient IDS activity in serum, leucocytes, or fibroblasts. Whenever possible, clinical information and family data were obtained to evaluate phenotypes and construct pedigrees. Clinical phenotype was evaluated according to the criteria described by Young *et al*,[4 5] which differentiated the severe type from mild by early onset age, developmental delay, and neurological involvement.

Fibroblasts, peripheral blood leucocytes, or developed lymphoblastoid cells were obtained from patients and normal controls. Total RNA was extracted from each specimen using the Purescript RNA Isolation Kit (Gentra System). Genomic DNA was isolated from each specimen using the Puregene DNA Isolation Kit (Gentra System). The extracted total RNA was used as a template for RT-PCR amplification of IDS cDNA fragments using a previously described method.[15] The extracted DNA was used as a template for PCR synthesis of IDS and IDS-2 specific amplicons. A previously described exon by exon IDS PCR scheme[14] was routinely performed to generate IDS exon specific amplicons using nine primer pairs each flanking one of the nine exons of the IDS gene. Oligonucleotide primers designed and used for the amplification of IDS and IDS-2 sequences are shown in table 1. Modification of this scheme included the expanded amplification of large fragments of IDS exons 1-3 (primers E1L/E3R), exons 3-8 (primers E3L/E8R), and the IDS-2 locus (primers 2F/7S). The expanded PCR was performed at similar conditions as routine PCR except for the addition of *Taq* extender (Stratagene) in equal units as *Taq* polymerase and the elongation of extension time. The thermal cycling conditions for the expanded PCR were 94°C for one minute, 60°C for one minute, and 72°C for three minutes.

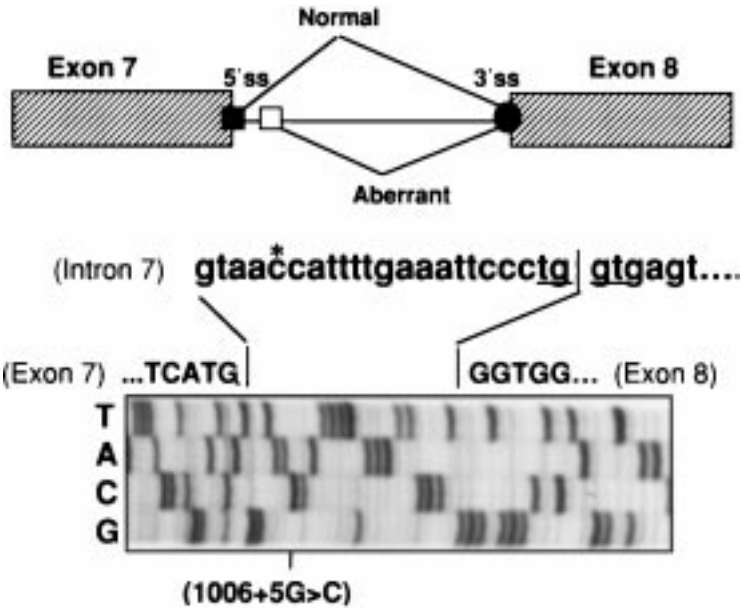

Figure 1 RT-PCR sequencing showing an aberrant splicing. A 1006+5g→c substitution in intron 7 (indicated by asterisk) resulted in the use of a cryptic 5' splicing site (denoted by underline) and the insertion of 22 nucleotides from intron 7 sequence.

Direct cycle sequencing of amplified IDS cDNA fragments was performed as the primary procedure for mutation identification. Mutation confirmation and heterozygote detection were subsequently performed by sequencing IDS genomic amplicons containing the identified mutation. RT-PCR cDNA fragments and genomic amplicons prepared for cycle sequencing were purified using Geneclean procedure (Bio 101). Purified products were sequenced using Δ*Taq* Cycle Sequencing Kit (United States Biochemical) according to the protocol provided by the manufacturer. The designation of mutations followed the suggested nomenclature of Beaudet and Tsui.[18]

Four recurrent base substitutions at presumed CpG hot spots (A85T, R88C, R468W/Q) and a previously described silent mutation (438C/T)[15] were subjected to polymorphic analysis. Control DNA samples were extracted from 34 unrelated white males and 35 unrelated white females. Mutation A85T (TGCGCC to TGCACC, underline denotes mutant codon) eliminated a *Hha*I site (5'GCGC). Mutations R468Q and R468W (CCCCGG to CCCTGG and CCCCAG, respectively) eliminated a *Hpa*II site (5'CCGG). Mutation R88C (AGCCGC to AGCTGC) introduced a new *Alu*I site (5'AGCT). These mutations were studied by selected restriction enzyme digestion of corresponding PCR amplicons. Owing to the absence of restriction enzyme recognition sites, the silent mutation 438C/T was analysed by a limited primer extension (LPE) method.[17] A primer, 5'CTGGGATATCTTCTAACCAT3', was designed with its 3' end immediately adjacent to the 5' side of ACC (codon 146). The LPE reaction was performed in a 20 µl mixture containing 1 pmol/l of designed primer, 10 ng of exon IV amplicon, 10 µmol/l of dCTP, 0.1 µl of α-[^{35}S]dATP (10 µCi/µl, Amersham), and 0.5 unit of *Taq* polymerase (Boehringer Mannheim). The reaction mixtures underwent 30 thermal cycles at 95°C for 15 seconds and 60°C for 30 seconds. The reactions were stopped by adding 10 µl of sequencing gel loading buffer. The final mixture (4 µl) was

*Table 2 IDS gene mutations detected in 29 patients with MPS II**

Case No	*Mutation*	*Exon*	*Codon*	*Nucleotide change*	*Effect on coding*	*Phenotype*	*References*
	Missense						
1	S71R	2	71	AGC→AG**A** at 213	Ser→Arg	Severe	This report
2	A82E	3	82	GCA→G**A**A at 245	Ala→Glu	NA	This report
3	A85T (438T)	3	85	GCC→A**C**C at 253	Ala→Thr	Severe	This report
4	R88C (438T)	3	88	CGC→T**G**C at 262	Arg→Cys	NA	This report
5	S333L	7	333	TCG→T**T**G at 998	Ser→Leu	Severe	14
6	D334G	7	334	GAT→G**G**T at 1001	Asp→Gly	Severe	14
7	1006G→A	7	336	G/gt→**A**/gt	Splicing error (?)	Severe	14
8	A346V (438T)	8	346	GCC→G**T**C at 1037	Ala→Val	Severe	15
9	R468W	9	468	CGG→T**G**G at 1402	Arg→Trp	NA	This report
10/11	R468Q/(438T)	9	468	CGG→C**A**G at 1403	Arg→Gln	Severe	15/this report
12	E521V	9	521	GAA→G**T**A at 1562	Glu→Val	NA	This report
	Nonsense						
13	Y234X	5	234	TAC→TA**A** at 702	Tyr→term	Severe	14
14	L279X	6	279	TTA→T**G**A at 836	Leu→term	Severe	16
15	Q465X	9	465	CAG→T**A**G at 1393	Gln→term	Severe	14
16	W502X	9	502	TGG→T**A**G at 1505	Trp→term	NA	17
	Deletions						
17	ΔR95 (438T)	3	95	†GGC**AGG**AGA at 283	Lost 1 aa	Severe	This report
18	383delAT	3	128	†GGCT**AT**GTG at 383	FS/PT	Severe	This report
19/20	596delAACA	5	199	†GACA**AACA**G at 596	FS/PT	Severe	This report
21	1148delC	8	383	†GACC**C**TTTT at 1148	FS/PT	Severe	This report
22	1216delCT	9	406	†TCT**CT**TTTT at 1216	FS/PT	NA	This report
23	1220delTT	9	407	†CTTT**TT**CCC at 1220	FS/PT	Severe	17
	Insertion						
24	208insC (438T)	2	70	†TCC**C**CACAGC after 208	FS/PT	NA	This report
25	1063insA	8	355	†GCT**A**ACCCAT after 1063	FS/PT	Severe	This report
26	1269insCC (438T)	9	423	†TGCCC**C**CCGTT after 1269	FS/PT	Severe	15
	Splicing						
27	1006+5g→c	(IVS-7)		g→c at 1006+5	Splicing in 22 nuct	NA	This report
28	1122C→T	8	374	AGGC→AG/gt	Lost 20 aa	NA	This report
	Large deletion						
29	Intragenic del			del exons 4-7	Aberrant transcripts	Severe	This report
	Polymorphism						
	438C/T	4	146	ACC→ACT	Silent mutation		15

*Nucleotide numbered from translational initiation site.[9]
Underline denotes mutant nucleotide or nucleotide immediately before insertion.
†Denotes the wild type codon before deletion or insertion.
Small capital letters denote deleted or inserted nucleotides.
aa = amino acid, del = deletion, ins = insertion, IVS = intervening sequence (intron), FS = frameshifting, PT = premature termination, term = stop codon, NA = clinical data not available for subtype classification.

subjected to electrophoresis in an 8% Sequagel (National Diagnostics). The gel was dried and exposed to Biomax film (Eastman Kodak) for 8-12 hours. The LPE reaction was designed to generate a 23 bp fragment from the normal allele and a 22 bp fragment from the mutant allele. Heterozygosity of a base substitution in the polymorphic analysis was defined as the number of mutant alleles divided by total number of alleles studied.

Results

Direct RT-PCR sequencing showed 16 small mutations. Seven missense mutations, S71R (AGC to AGA), A82E (GCA to GAA), A85T (GCC to ACC), R88C (CGC to TGC), R468W (CGG to TGG), R468Q (CGG to CAG), and E521V (GAA to GTA), were detected. Five small nucleotide deletions, ΔR95, 383delAT, 596delAACA, 1148delC, and 1216delCT, were found. It is noteworthy that two unrelated cases were determined to have the same deletion, 596delAACA. Two insertions, 208insC and 1063insA, were detected. Two splicing mutations, 1006+5g→c and 1122C→T were identified. RT-PCR sequencing showed that the 1006+5g→c caused an insertion of 22 bp of intron 7 sequence into the cDNA (fig 1). Mutations A85T, R88C, ΔR95, and 208insC were found coexisting with a 438T silent mutation (ACC to ACT in codon 146). Of the 16 small mutations, seven mutations (A85T, R88C, R468W, R468Q, E521V, 596delAACA, and 1122C→T) have previously been published.[1–3] The remaining nine small mutations were novel mutations. Table 2 summarises the detected mutations.

All 16 small mutations detected from RT-PCR sequencing were further confirmed in IDS genomic sequence. Four mutations (A85T, R88C, R468W/Q) introducing changes in restriction enzyme recognition sites were confirmed using direct restriction enzyme digestion of corresponding exon specific amplicons. The other 12 mutations not involved with changes in any common restriction enzyme recognition sites were confirmed by direct PCR sequencing of the correspondent exon specific amplicons.

Polymorphic analysis was performed for four missense mutations and a silent mutation. None of the four missense mutations was found in the study of 104 chromosomes from normal controls, providing indirect evidence for a causative role of the four mutations. However, the 438T mutation was found in 31 of the 87 chromosomes studied, estimating it to be present in about one in three alleles of the normal population. The silent mutation occurs at almost equal frequency in control males (10/29, 34%) and females (21/58, 36%). These results provide further evidence that the 438C/T is an IDS intragenic polymorphic site. Table 3 summarises the polymorphic analysis results.

RT-PCR sequencing performed on case 29 found two aberrant IDS transcripts, one containing exons 1, 2, 8, and 9 and another containing exons 3, 8, and 9. Routine exon by exon PCR for genomic DNA suggested a dele-

Table 3 Polymorphic analysis of five IDS mutations in normal controls

Mutation	Assays	Male		Female			Freq*
		N*	M*	N/N	N/M	M/M	M/(N+M)
A85T	Exon 3/*Hha*I	34	0	35	0	0	0/104
R88C	Exon 3/*Alu*I	34	0	35	0	0	0/104
R468W/Q	Exon 9/*Hpa*II	33	0	35	0	0	0/103
438C/T	Exon 4/LPE	19	10	12	13	4	31/87

*Freq, frequency; N, normal allele; M, mutant allele.

tion of exons 4, 5, 6, and 7 in the IDS gene. Expanded PCR for IDS exons 1 to 3 using primers E1L and E3R amplified an expected 1976 bp product, which showed the presence of exons 1, 2, and 3 of the IDS gene. PCR for IDS-2 locus using primers 2F and 7S amplified an expected 1950 bp product, which indicated the presence of an intact IDS-2 locus. In the normal IDS gene, primers E3L and E8R are located more than 16 kb apart, so no amplicon was generated using the primer pair in normal controls. For case 29, PCR using primer pair E3L-E8R amplified an approximately 2900 bp fragment, which suggested that the IDS exon 3 was linked to exon 8 by an intronic junction. Direct PCR cycle sequencing of the E3L-E8R PCR fragment showed normal IDS sequence except for a 400 bp segment that was identical to the intron 3-intron 7 junction of the IDS-2 locus. Therefore, the IDS gene defect in case 29 was a deletion of exons 4 to 7 of the IDS gene and a junction of intron 3 to intron 7 through an IDS-2 specific segment. Fig 2 shows the characterisation of the intragenic deletion and the chiasmatic sequence of the junction segment.

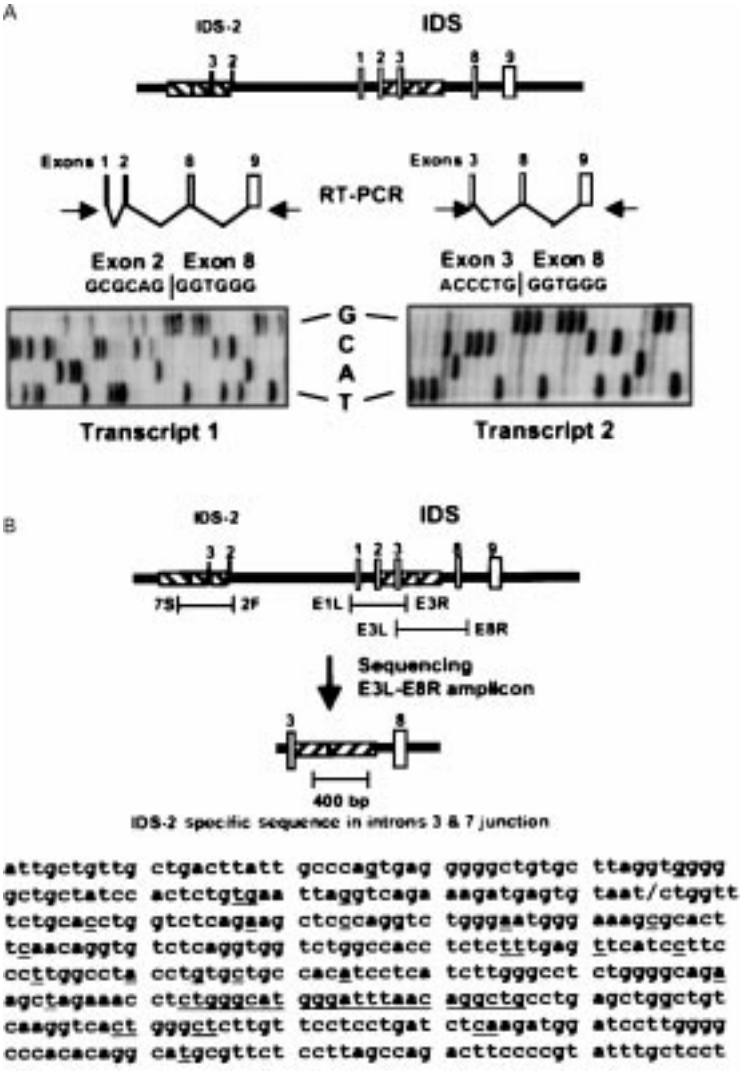

Figure 2 Characterisation of an intragenic deletion of IDS exons 4, 5, 6, and 7. (A) RT-PCR sequencing showed two IDS mRNA transcripts: one transcript contained exons 1, 2, 8, and 9, and another transcript contained exons 3, 8, and 9. The cross hatches indicate homologous sequences between IDS and IDS-2. (B) PCR amplification of IDS and IDS-2 showed the presence of the intact IDS-2 locus (7S-2F) and a fragment of IDS exon 3 linked to exon 8 (E3L-E8R). Sequencing of the exon 3/exon 8 fragment (E3L-E8R) showed a 400 bp junction segment identical to that in the IDS-2 locus. Nucleotide sequence of this 400 bp segment is shown. Underlines denote nucleotides specific for IDS-2 region and slash (/) denotes the junction point of intron 3 and intron 7 in the defective IDS gene.

Discussion

Hunter syndrome, MPS II, is an inborn error of lysosomal catabolism for which the spectrum of clinical severity is thought to result from mutational heterogeneity of the IDS gene.[1–3] It has been suggested that approximately 19-25% of MPS II patients result from a whole or partial IDS gene deletion and major IDS gene rearrangements.[1 19 20] Therefore, point mutations and small lesions (deletions, insertions, and duplications of <20 bp) cause approximately 75-80% of MPS II cases. Analysis of a compiled 135 small mutations in the IDS gene showed a non-random distribution of point mutations over the exons and a relatively frequent occurrence of point mutations in exons III, VIII, and IX.[3]

We previously identified 11 small mutations from 11 patients (Y234X, L279X, S333L, D334G, A346V, Q465X, R468Q, W502X, 1006G→A, 1220delTT, and 1269insCC).[14–17] In this report, we present 17 mutations detected from 18 MPS II patients. Nine of the 17 mutations were novel mutations including S71R, A82E, ΔR95, 383delAT, 1148delC, 1216delCT, 208insC, 1063insA, and 1006+5g→c. Combining the previous data[14–17] with data from the present results gives a total of 27 different mutations and a polymorphic site (table 2). A search of IDS mutations in the human gene mutation database (HGMD)[21] found a compilation of 105 small mutations including 65 missense/nonsense mutations, 19 small deletions, eight small insertions, 12 intronic splicing mutations, and one dinucleotide transversion. From table 2, 13 mutations, including the present nine novel mutations and four previously reported mutations (L279X, W502X, 1220delTT, and 1269insCC[16 17]), could be added to the mutation database. Mutations A85T, R88C, S333L, R468W, R468Q, 596delAACA, and 1122C→T in table 2 have been previously reported[1–3] from most probably unrelated patients. These mutations are considered as recurrent mutations in the IDS gene.

Generally, recurrent mutations that involve C→T or G→A transition in CpG dinucleotides are suggested to be caused by an in situ methylation-deamination process. The involved CpG dinucleotides are defined as mutation "hotspots".[22] Review of published reports and IDS mutation databases indicated that recurrent mutations A85T, R88C/H, S333L,

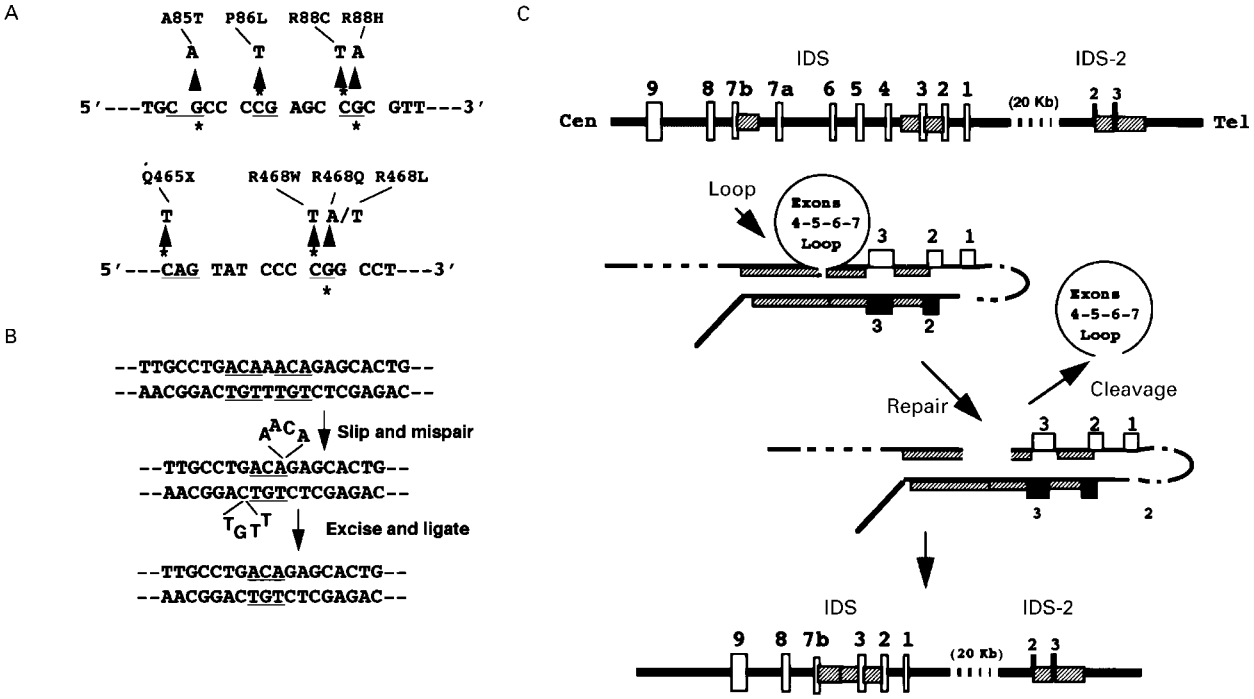

Figure 3 Proposed sequence directed mutagenesis mechanisms in the IDS gene. (A) CpG hotspots and CpNpG locus around R88 and R468. Underline denotes possible CpG hotspots and postulated CpNpG sites, asterisk indicates possible methylated nucleotides (sense strand or antisense strand), and arrow points to mutant nucleotides. (B) Direct repeats around mutation 596delAACA. The ACA/TGT (indicated by underline) "slips" and mispairs, forming a single strand loop. Repairing the loop by excise and ligation would generate the 4 bp deletion. (C) "Loop-cleavage-repair" model of IDS intragenic deletion. The pairing of IDS-2 and IDS homologous sequences induces an intrachromosomal configuration that loops out IDS exons 4 to 7. A cleavage of the loop and repair of the junction using IDS-2 sequence as template resulted in the intragenic deletion and chiasmatic junction.

1122C→T, R443X, and R468Q/W were all C→T or G→A transitions in CpG dinucleotides and therefore considered as IDS "hotspot" mutations. We identified five of these "hotspot" mutations in six patients, which accounted for about 21% (six out of 28) of cases with small mutations. In addition, methylation at the CpNpG site, especially at CAG and CTG, has been observed in plasmid DNA stably transfected into mammalian cells.[23] The observation suggested a possibility of CpNpG methylation of endogenous genes of mammalian cells. Of the 65 IDS point mutation entries in the HGMD, six CAG to TAG mutations (Q80X, Q298X, Q389X, Q465X, Q474X, Q531X) were found. However, none of the six reported CAG to TAG mutations in the IDS gene was observed in two or more patients. The postulated role of CpNpG methylation in mutagenesis needs to be addressed by further analysis of corresponding mutation patterns and methylation patterns in human genes. Fig 3A shows the suggested CpG or CpNpG methylation sites around codons 88 and 468 and related "hotspot" mutations.

The recurrence of mutation 596delAACA in two unrelated cases and reported in a most probably unrelated patient[24] indicated that the deletion may be a common mutation resulting from a sequence directed mutagenesis mechanism. One hypothesis is that direct repeats (2 bp or more) flanking or overlapping the locus, or both, may cause slipped mispairing and eventually loop out a small segment of nucleotides.[22] Fig 3B presents the putative slipped mispairing mechanism using sequences flanking the identified 4 bp deletions. Since the slipped mispairing may occur during replication when the DNA becomes single stranded, the mechanism might be an important cause of germinal small deletions. Small deletions 1148delC, 1216delCT, and 1220delTT, might also be readily explicable by the slipped mispairing model.

Among various types of large IDS gene deletions and rearrangements,[1 19 20] specific types of rearrangements were suggested as IDS-2 related.[25 26] An inversion of the IDS gene resulting from homologous intrachromosomal recombination between IDS and IDS-2 loci was estimated to occur in about 13% of patients with MPS II.[25] An intragenic deletion of IDS exons 4-7 caused by IDS-2 induced exchange accounted for about 1.6% of patients with MPS II.[26] In the present communication, we characterised an intragenic deletion of IDS exons 4-7 (case 29) with similar features as described above[26] except that the junction segment was different. Based on the presence of a 400 bp IDS-2 specific sequence joining intron 3 and 7 of the IDS gene (fig 2), we suggest that the putative breakpoints lie outside the 400 bp region. To explain the mechanism of the deletion in this patiant, we propose a "loop-cleavage-repair" model as shown in fig 3C. The model assumes that an intrachromosomal mispairing between the IDS and IDS-2 might be expected to occur. In order to juxtapose the IDS sequences necessary for pairing with homologous sequences at the IDS-2 locus, a loop of genomic IDS DNA containing part of intron 3, exons 4, 5, 6, and 7, and introns 4, 5, 6, and a portion of intron 7 could be created. If this loop were recognised and excised by DNA

repair nucleases, the resulting gap could be repaired using the IDS-2 sequences as a template. The model explains the two possible rearrangements (inversion and deletion) based on a unique intrachromosomal configuration. Another important finding from the case was the variant IDS mRNA transcripts. Although the intervening IDS-2 sequence in our case is different from the case reported previously,[26] both cases present with similar aberrant transcripts. However, the mechanism causing the aberrant transcription requires further investigation.

The silent mutation 438C/T (T146T, ACC→ACT) has been reported previously as a polymorphic site in coexistence with other disease causing mutations.[1 15 24 27 28] In this report, the 438T was found along with four disease causing mutations, A85T, R88C, ΔR95, and 208insC (table 2). Combined with three cases reported previously,[15] the 438T mutant allele existed in 32% of our MPS II cases (seven out of 22 cases studied by the RT-PCR approach). The frequency was close to that of normal controls (table 3). The presence of the IDS intragenic polymorphic site may be used as a marker in haplotyping and linkage analysis.

In the present investigation, approximately 41% (12/29) of our MPS II cases were the result of missense mutations. An important question arises as to whether or not a single base pair change is indeed the pathological lesion causing disease. Cooper and Krawczak[22] have suggested several sources for evidence of the causality. Basically, the evidence of causality for missense mutations should involve three aspects. The first aspect involves clinical observation, such as the documentation of independent occurrence of the mutation in unrelated patients, or novel appearance and subsequent cosegregation of the gene lesion and disease phenotype through a family pedigree. The second aspect takes advantage of sequence analysis to define the occurrence of the mutation in a region of known structure or function and in a region of known evolutionarily conserved residues. The third aspect requires well designed in vitro or in vivo experiments. For example, a polymorphic analysis could suggest the absence of a given mutation in a large sample of normal controls, in vitro expression of the mutant gene could indicate the enzymatic defect, or in vivo replacement of the mutant gene or protein with its wild type counterpart could show the reversal of the mutant effect. To date, sequence analysis is the most commonly used approach to determine causality for detected IDS missense mutations.[1–3] Documentation of mutations in unrelated patients was also presented but limited mostly to recurrent mutations.[3] Probably because of technical difficulty, experimental approaches were rarely applied. Crotty *et al*[29] conducted an in vitro mutagenesis and expression to show that the defective enzyme activity resulted from R468W. We present here the polymorphic analysis to indicate the absence of mutations A85T, R88C, and R468Q/W in normal controls.

In summary, our results provide further evidence of mutational heterogeneity of the IDS gene observed in patients with MPS II. We propose that certain sequence directed mechanisms, such as methylation-deamination at CpG hot spots, slipped mispairing at short direct repeats, and the intrachromosomal recombination induced by unique IDS-2 loci, could contribute to the occurrence of recurrent mutations in the IDS gene.

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