CHARTER IV
RESULTS

1. *IDUA* gene analysis

1.1 Mutation analysis of mRNA

For mutation screening, RT-PCR was performed. RNA was prepared from leukocytes of two unrelated probands, and reverse-transcribed. PCR-amplification of the cDNA using the forward primer, IDUA-F1 and the reverse primer, IDUA-R2 produced a 1,271- bp product (Figure 10). The cDNAs were then sequenced.

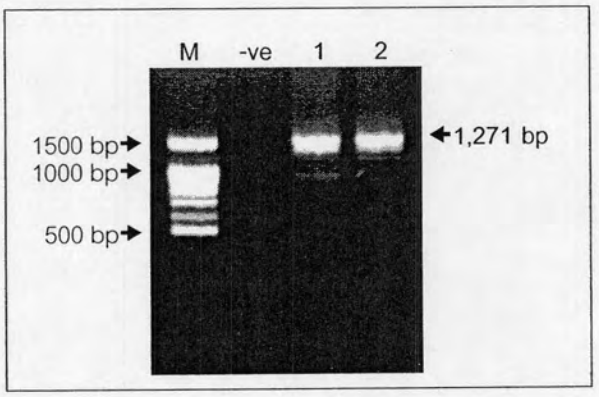


Figure 10 RT-PCR amplification of the *IDUA* gene. M = 100 bp DNA marker; -ve = no template; Lane 1 = patient 1; Lane 2 = patient 2

1.2 Mutation analysis of gDNA

Mutation analysis by PCR-sequencing of the entire coding region of the *IDUA* gene revealed two different potential pathogenic mutations, one for each patient. The mutant alleles identified in the gDNA of the MPS I patients studied are summarized in Table 13.

Table 13 Clinical and molecular features of patients with MPS I.

	Patient 1	Patient 2
Age (year)	2	29
Sex	Male	Female
Province	Chonburi	Nakhon Si Thammarat
Phenotype	Hurler syndrome (Fig. 11)	Scheie syndrome (Fig. 12)
Mutation (homozygous)	c.252insC	c.826G>A (p.E276K)
Frequency in 100 control alleles	-	0

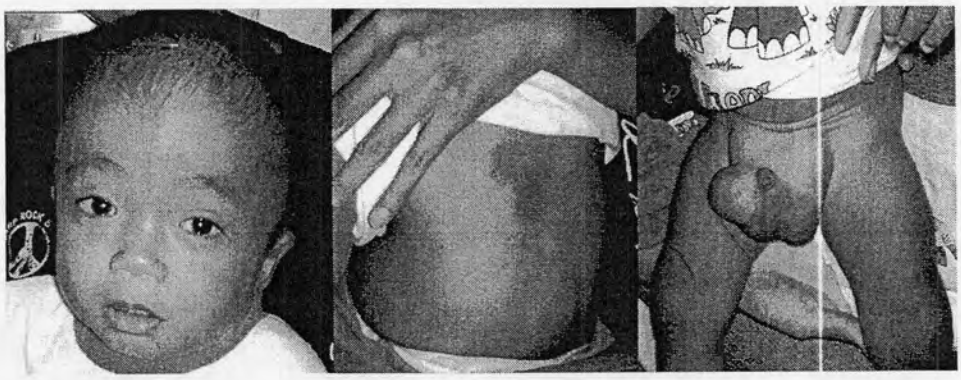


Figure 11 Clinical features of patient 1 with Hurler syndrome.

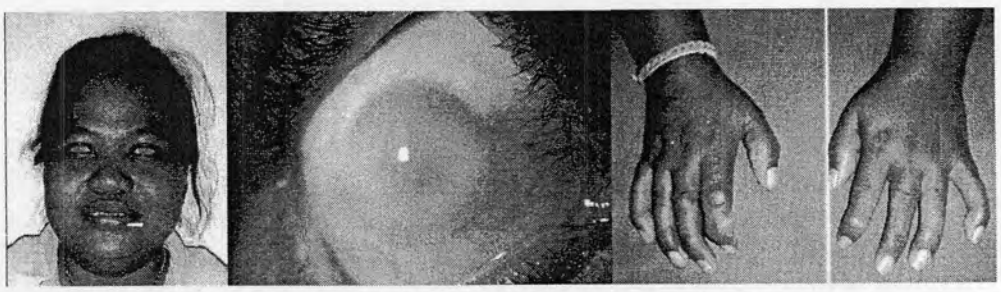


Figure 12 Clinical features of patient 2 with Scheie syndrome.

Patient 1 was found to be homozygous for the known c.252insC mutation (Figure 13). This mutation has been previously described[51] and found to be associated with a severe phenotype.

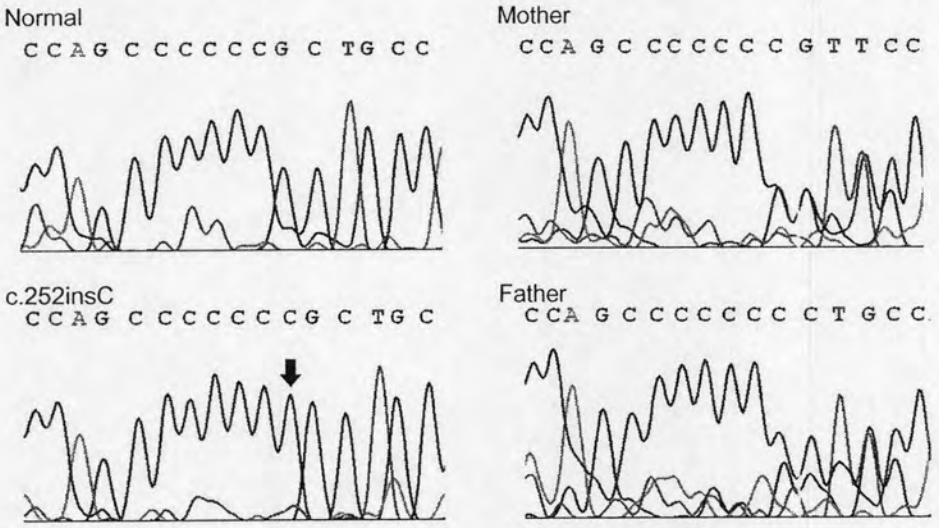


Figure 13 Mutation analysis. An electropherogram of the patient with Hurler syndrome showing a homozygous c.252insC (an arrow, left lower panel). Both parents were found to be heterozygous for the mutation (right panel). The left upper panel was a sense sequence of the Thai unaffected control.

Patient 2 was found to be homozygous for the missense mutation located in exon 7. A G to A transition was identified at nucleotide position 826 (c.826G>A). This was expected to result in a glutamic acid to lysine substitution at codon 276 (p.E276K) (Figure 14). This mutation has never been previously described and was not detected in 100 ethnic-matched unaffected control chromosomes.

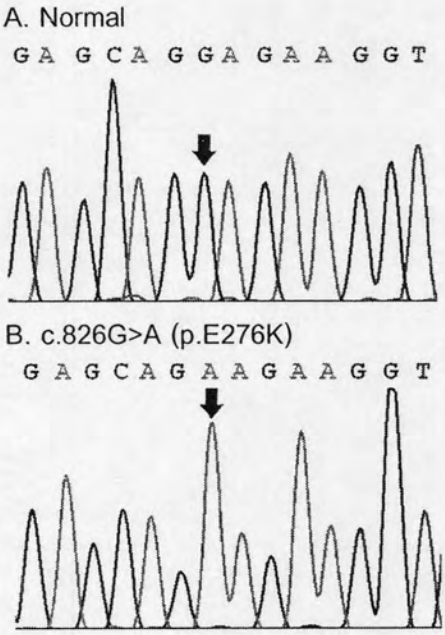
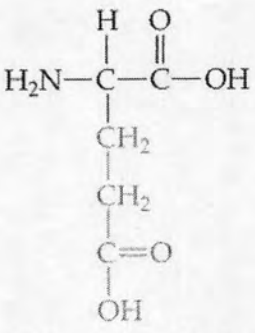


Figure 14 Mutation analysis. An electropherograms of the patient with Scheie syndrome showing (B) a missense mutation, c.826G>A (an arrow) resulting in a glutamic acid (GAG) to lysine (AAG) substitution at codon 276 (p.E276K) and the unaffected Thai control (A) showing normal genotype.

2. Confirmation of the novel mutant allele

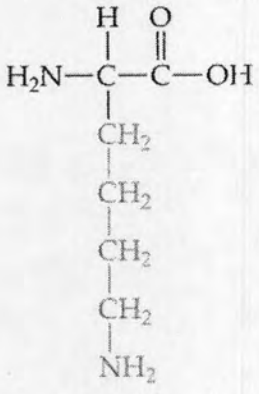
2.1 Amino acid change

The c.826G>A mutation causing a glutamic acid to lysine substitution, resulting in acidic or negatively charged groups to basic or positively charged groups.



Glutamic acid (Glu)

acidic or negatively charged groups



Lysine (Lys)

basic or positively charged groups

Figure 15 Structure of the glutamic acid (Glu) and lysine (Lys)

2.2 Multiple protein sequence alignment of IDUA

The amino acid E276 is conserved among cow, dog, chicken, frog, mouse, and zebrafish but not in fruit fly.

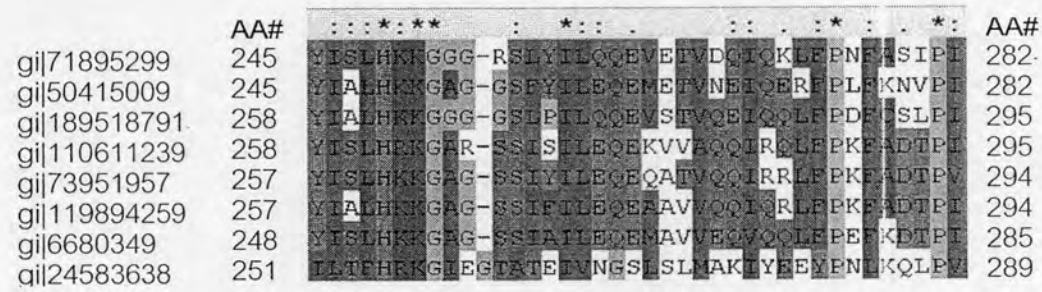


Figure 16 The IDUA protein sequence comparison. AA# = amino acid

gi|71895299 = *Gallus gallus*
gi|50415009 = *Xenopus laevis*
gi|189518791 = *Danio rerio*
gi|110611239 = *Homo sapiens*
gi|73951957 = *Canis familiaris*
gi|119894259 = *Bos taurus*
gi|6680349 = *Mus musculus*
gi|24583638 = *Drosophila melanogaster*

2.3 Restriction enzyme digestion with *Mbo*II

Restriction enzyme digestion of the PCR products was carried out to confirm the presence of the identified mutation. To confirm the novel mutation, c.826G>A (p.E276K), the primers and the restriction enzyme used are shown in Table 14.

Table 14 The primers and restriction enzyme

Mutation	Primer Name	Expected size before digestion(bp)	Restriction enzyme	Expected sizes after digestion (bp)		
				Normal	Homozygous mutant alleles	Heterozygous mutant alleles
c.826G>A	IDUA-Ex7F	448	*Mbo*II	247	247	247
(p.E276K)	IDUA-Ex7R			201	169	201
					32	169
						32

The PCR products were digested with *Mbo*II and analyzed on 3.0 % agarose gel stained with ethidium bromide. *Mbo*II digested the wild type allele of a control into 247 and 201-bp products. The c.826G>A mutation creates another *Mbo*II site, generating 247, 169 and 32-bp products (the 32-bp band was invisualized). The digestion patterns are shown in Figures 17.

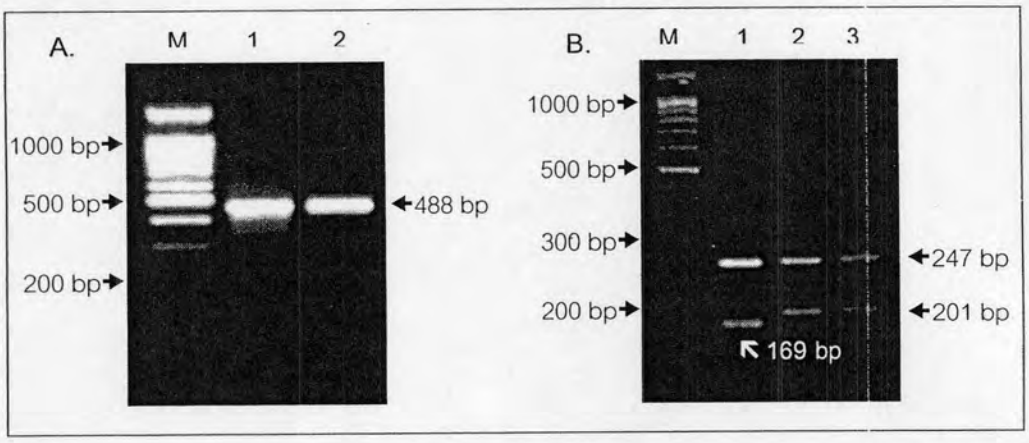


Figure 17 Restriction enzyme digestion analysis. M = 100-bp marker. In the left panel, lanes 1 and 2 = the PCR products without restriction enzyme. in the right panel, lane 1 = patient (p.E276K); lanes 2 and 3 = unaffected controls.

3. Alpha-L-iduronidase activity assay in leukocytes

Diagnosis of MPS I can be made by measuring levels of alpha-L-iduronidase activity in leukocytes using 4-MU-alpha-L-iduronide as a substrate. Alpha-L-iduronidase activity in a patient and eight unaffected adults was shown in Table 15. The mean value of alpha-L-iduronidase activity in unaffected controls with S.D. was 23.10±8.80 nmol/h/mg. The patient with Scheie syndrome had an enzyme activity level of 0.61± 0.06 nmol/h/mg. Biochemical studies revealed a significant reduction of alpha-L-iduronidase activity in the patient's leukocytes when compared with that in Thai unaffected controls.

Table 15 Alpha-L-iduronidase activity in leukocytes of an MPS I patient.

Samples	Alpha-L-iduronidase activity (nmol/h/mg)
Unaffected controls (Mean ± SD) (Range) (n= 8)	23.10±8.80 (14.3-31.9)
Patient 2 (Scheie syndrome)	0.60±0.06 (0.66-0.54)

4. Functional analysis of mutant *IDUA*

The objective of this experiment is to determine the effect of the identified mutation on the protein function using the fluorometric assay. In transient transfection studies, the wild-type or mutant IDUA constructs were generated. The constructs were transiently transfected in COS-7 cells. All experiments were performed in triplicate and repeated two times. The mutant IDUA constructs included the c.826G>A (p.E276K), and the c.314G>A (p.W402X). The previously described p.W402X in Hurler patients was also used[50]. The pEFNeo was used as a vector control. Transfection of the wild-type IDUA in COS-7 cells resulted in an increase of alpha-L-iduronidase activity (435.04±56.23 nmol/h/mg) compared to the baseline activity of COS-7 cells. As shown in Table 16, the p.E276K mutant had reduced activity (31.88±6.05 nmol/h/mg). The p.W402X mutant expressed an enzyme activity of 21.10 ± 12.57 nmol/h/mg.

Table 16 Alpha-L-iduronidase activity in COS-7 cells transiently transfected with either wide-type (WT) or mutant cDNAs. Values are the mean of two experiments.

Constructs	Alpha-L-iduronidase activity (nmol/h/mg) mean ± SD	Phenotype
None	27.17±4.89	-
pEFNeo	32.52±10.58	-
pEFNeo/WT-IDUA	435.04±56.23	Normal
pEFNeo/p.W402X	21.10±12.57	Hurler
pEFNeo/p.E276K	31.88±6.05	Scheie

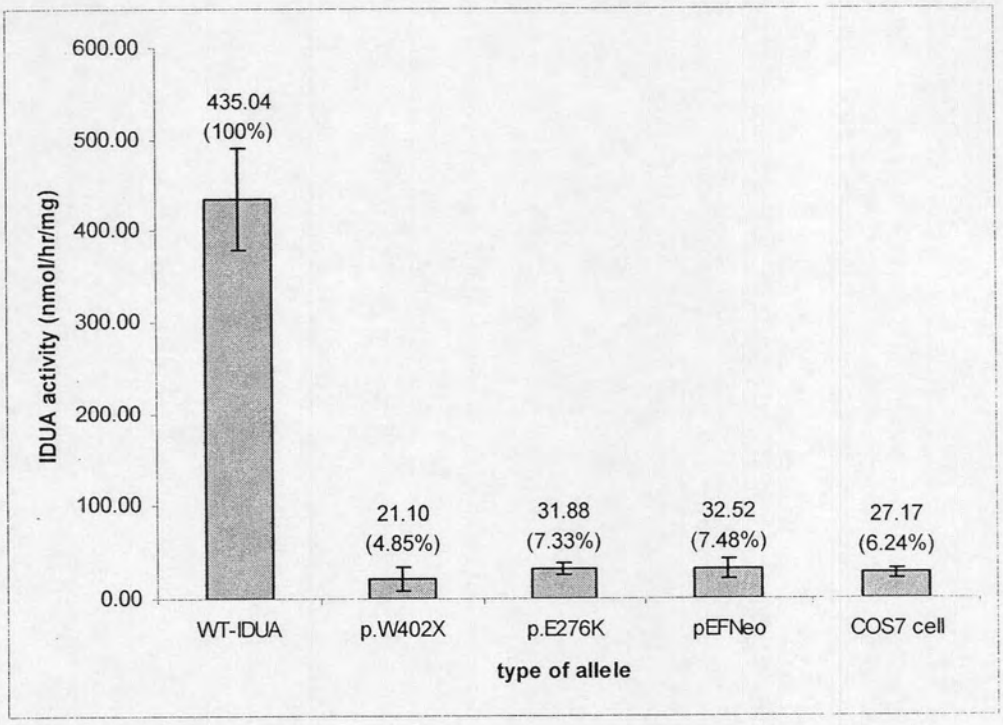


Figure 18 IDUA activity in COS-7 cells transiently transfected with different constructs. The p.W402X and p.E276K mutants showed a significant reduction of the IDUA activity compared with that of the WT-IDUA (values in parentheses are expressed as percentages of the enzyme activity in the WT-IDUA).