ภาคผนวก

Two novel mutations including a large deletion of the *SLC4A11* gene causing autosomal recessive hereditary endothelial dystrophy

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Abstract

Aims To characterize the clinical and molecular features of three affected individuals from a non-consanguineous Thai family with autosomal recessive congenital hereditary endothelial dystrophy (CHED2).

Methods Physical and slit-lamp examinations were performed in all three affected siblings and their parents. Genomic DNA was extracted from peripheral blood leukocytes. The entire coding and putative promoter regions of the solute carrier family 4 (sodium borate cotransporter) member 11 (*SLC4A11*) gene were subjected to PCR and direct sequencing. Array CGH on chromosome 20 of the patient's DNA was also performed.

Results All three patients were found to harbor two novel pathogenic mutations. The c.778A>G mutation inherited from the mother was expected to result in a lysine to glutamic acid substitution (p.K260E) at codon 260. It was not identified in 100 ethnic-matched unaffected control chromosomes. The lysine residue at codon 260 was evolutionarily highly conserved. The 68-kb deletion encompassing the whole *SLC4A11* gene inherited from the father was the largest deletion described to date identified in patients with CHED2.

Conclusions This study reported two newly identified mutations in a Thai family with CHED2, extending the mutational spectrum of *SLC4A11*. As there are previously reported CHED2 patients with undetected *SLC4A11* mutations using conventional PCR-sequencing techniques, further analysis to identify chromosomal deletions in these patients is warranted.

INTRODUCTION

Congenital hereditary endothelial dystrophy (CHED) is an inherited disorder of the corneal endothelium, characterized by bilateral non-inflammatory corneal clouding ranging from a diffuse haze to a ground-glass, milky appearance. CHED can be inherited either as an autosomal dominant (CHED1; MIM 121700) or recessive (CHED2; MIM 217700) manner. CHED2 is more common and severe, usually presenting at birth or early infancy. Bilateral corneal clouding can lead to visual impairment often accompanied by nystagmus in CHED2 patients requiring corneal transplantation.[1 2]

Mutations in the solute carrier family 4 (sodium borate cotransporter) member 11 (*SLC4A11*) gene have been identified in most of the patients with CHED2.[3 4] The *SLC4A11* gene contains 18 coding exons and encodes the bicarbonate transporter-related protein 1 (BTR1) of 891 amino acids. Its protein product is also called NaBC1, known to function as a membrane bound electrogenic sodium-borate cotransporter, involved in cell growth and proliferation by increasing intracellular borate and activating the mitogen-activated protein kinase pathway.[5] Interestingly, *SLC4A11* gene mutations have also been reported to be causative for two different inherited corneal disorders; corneal dystrophy and perceptive deafness (CDPD, MIM 217400) or Harboyan syndrome and dominant late-onset Fuchs endothelial corneal dystrophy (FECD4, MIM 613268).[6 7]

At least 80 different disease-causing mutations scattered throughout the *SLC4A11* gene have been described with the most common being missense mutations (Human Gene Mutation Database, http://www.hgmd.cf.ac.uk, accessed May, 2014). The nonsense, splice-junction alterations, nucleotide deletions or insertions and exon deletions have also been reported. The

SLC4A11 has been the only gene found to be responsible for CHED2. With PCR-sequencing of the entire coding and putative promoter regions of the *SLC4A11* gene, there were, however, some clinically-confirmed CHED2 patients with undetected mutations in the *SLC4A11* gene. [4 8-11]

Here, we reported three affected siblings from a Thai family with CHED2. Two novel alterations in the *SLC4A11* gene including a missense mutation and a 68-kb deletion encompassing the whole *SLC4A11* gene and its putative promoter were identified. This is the first study to describe the deletion covering the large genomic region associated with CHED2.

METHODS

Patients and controls

Three affected individuals with CHED2 from a non-consanguineous Thai family were reported. The pedigree is shown in figure 1A. A diagnosis of CHED was made by clinical course and features and histopathological findings. The study was approved by the institutional review board of the Faculty of Medicine of Chulalongkorn University and written informed consent was obtained from all the patients and parents. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

All three siblings were referred to King Chulalongkorn Memorial Hospital at the age of 7, 17 and 20 years, respectively. All were reported to have corneal haze since birth. Nystagmus was present in the 20-year-old brother and the 7-year-old sister. None had sensorineural hearing loss. Corneal pachymetry revealed increased corneal thickness in all three individuals (> 1000 μm). All had normal intraocular pressure (below 21 mmHg). Both parents had clear corneas

and denied a family history of corneal disorders. The clinical features of three patients were summarized in Table 1

The youngest sibling (7-year-old) had a visual acuity of counting fingers at 2 and 1.5 feet in the right and left eye, respectively. She had horizontal nystagmus with esotropia of 30 prism diopters. There were bilateral corneal haziness and thickening with microcystic edema of the epithelium (figure 1B-C). Her vertical and horizontal corneal diameters were 10 and 11 millimeters, respectively. She received a penetrating keratoplasty of the right eye two years later (figure 1D). Histopathological examination of the excised corneal buttons revealed widening of the stroma and marked thickening of the Descemet's membrane. The corneal tissues had completely absent endothelium (figure 1E). The electron microscopic findings were consistent with the diagnosis of CHED2 (figure 1F).

An *in vivo* confocal microscopy of the left cornea of the elder brother revealed multiple hypo-reflective bullae in the epithelial layer, and loss of sub-basal epithelial nerve plexus (figure 1G). There was a reduction in the number of stromal keratocytes and loss of normal stromal structures which were replaced by hyper-reflective interwoven bands resembling a "honey-comb" appearance (figure 1H). The endothelial cells could not be demonstrated.

Mutation analysis of *SLC4A11* using PCR-direct sequencing

After informed consent, peripheral blood samples were collected from three affected siblings and their parents. Genomic DNA was extracted from peripheral blood leukocytes using the ArchivePure DNA Blood Kit according to manufacturer's instructions (5 Prime Inc., Gaithersburg, MD). Direct sequencing of polymerase chain reaction (PCR) products representing the entire coding region of *SLC4A11* was performed. We used the primers from the

previous study[4] except for the primers for exon 1 which were designed using Primer3 (v. 0.4.0) software (Table 2). The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH) and directly sequenced at the Macrogen, Inc. (Seoul, Korea). The identified mutation was confirmed by direct sequencing in the opposite direction. PCR-RFLP analysis with the restriction enzyme *Avr*II was used to confirm the presence of the novel missense mutation in the patients and to screen in 100 ethnic-matched unaffected control chromosomes. Specific primers were used to amplify two regions that were predicted to be the *SLC4A11* promoter from the previous study.[9] The PCR products were sent for sequencing at the Macrogen, Inc. (Seoul, Korea).

Array comparative genomic hybridization (CGH)

Genomic DNA of the patient (II-2) was sent to Macrogen Inc. (Seoul, Korea) for array CGH analysis on chromosome 20 using NimbleGen 385K, according to the company's instruction. Briefly, genomic DNA was labeled with fluorescent dyes, co-hybridized to a NimbleGen Human CGH 385K Chromosome 20 Tiling array, and scanned using a 2-μm scanner. Log2-ratio values of the probe signal intensities (Cy3/Cy5) were calculated and plotted versus genomic position using Roche NimbleGen NimbleScan 2.5 software. Data were displayed in Roche NimbleGen SignalMap software. The deletion breakpoints were further analyzed by PCR using three newly designed primers (Table 2). A primer pair, SLC4A11_Deletion-F and SLC4A11_No Del-R, was designed to amplify the non-deleted allele giving the PCR product of 679 bp and another primer pair, SLC4A11_Deletion-F and SLC4A11_Del-R, was used to detect the deleted allele giving the PCR product of 523 bp.

RESULTS

Direct sequencing of PCR products covering the entire coding region of the *SLC4A11* gene revealed a novel c.778A>G mutation in exon 8. This mutation was expected to result in a lysine to glutamic acid substitution (p.K260E) at codon 260 (figure 2A). No other variants were observed. PCR-RFLP analysis with the restriction enzyme *Avr*II of the patients and parents' genomic DNA showed that the mutation was present in all three affected siblings and the mother but was absent in the father (figure 2B). This mutation was not identified in 100 ethnic-matched unaffected control chromosomes (data not shown). It is located at a highly conserved residue (figure 2C) within the anion exchange domain which was predicted with Simple Modular Architecture Research Tool (SMART) software. As only one mutant allele transmitted from the mother was detected, PCR-sequencing of the putative promoter region was performed and revealed no pathogenic variants.

The fact that direct sequencing of the entire coding region and the putative promoter of the *SLC4A11* gene demonstrated only the c.778A>G (p.K260E) mutation which was inherited from the mother suggested that the patients were hemizygous for the mutant allele. In addition, there were no other heterozygous variants identified. These results prompted us to investigate the possibility of a whole gene deletion occurring in the other allele. Further experiments using array CGH covering chromosome 20 revealed that the patient (II-2) had a chromosomal deletion from position 3107501-3174468 which included the region where the *SLC4A11* gene was located (3156063-3166373_NCBI36/hg18) (figure 3A). This loss of chromosomal region is in the structure variant (variation_5121, deletion type, 3076070-3238538_NCBI36/hg18) which has been reported in Database of Genomic Variants (DGV, http://dgv.tcag.ca/dgv/app/home).

The presence of the heterozygous deletion was verified in all of the patients by PCR analysis across the predicted deletion points. The size of the PCR products was 523 bp and 679 bp for the deleted and non-deleted alleles, respectively (figure 3B-C). The deletion was present in the father but absent in the mother (figure 3C). We also attempted to identify the precise breakpoints of the deletion by direct sequencing (figure 3D) and confirmed the deletion size of 67,733 base pairs.

DISCUSSION

We described three affected siblings from a Thai non-consanguineous family with CHED2. All were compound heterozygous for a single base pair transition (c.778A>G; p.K260E) and a 68-kb deletion encompassing the *SLC4A11* gene. Both mutations have never been previously described.

The newly identified missense variant was inherited from the patients' mother. Several lines of evidence support the variant as the disease-causing mutation. It was not identified in 100 ethnic-matched unaffected control chromosomes. ClustalX showed the lysine residue at codon 260 which was conserved from *Homo sapiens* to *Caenorhabditis elegans* (figure 2C). The mutation changes polarity of the amino acid residue from a positive charge to a negative charge. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicted the variant (c.778A>G; p.K260E) to be probably damaging with a score of 1.000 and SIFT (http://sift.jcvi.org/) predicted it to be deleterious with a score of 0.91.

Previous studies have failed to identify pathogenic mutations in some patients with clinically-confirmed CHED2 using conventional PCR-sequencing of the entire coding and the putative promoter regions of *SLC4A11*.[4 8-11] The *SLC4A11* has been the only gene found to be

responsible for CHED2. The evidence derived from studies in this CHED2 family suggested us to investigate the possibility of a whole gene deletion occurring in the other allele. Using array CGH, we were able to identify a novel 68-kb deletion encompassing the *SLC4A11* gene as another disease-causing allele inherited from the patients' father. PCR-directing sequencing was able to detect the precise deletion points which gave the deletion size of 67,733 base pairs. This deletion resides in the structural variant reported to be a deletion polymorphism in DGV (variation_5121). In addition to the variation_5121, there are four deletion variants with different sizes encompassing the *SLC4A11* gene. The deletion identified in our patient overlaps with these deletions. Copy number variations (CNVs) including microdeletions and microduplications have been demonstrated as a significant cause of structural variation in the human genome.[12 13] Several studies have suggested deletion polymorphisms may play a significant role in the genetic basis of complex traits as well as in genome evolution.[14-17] In addition, several genes including those responsible for autosomal recessive diseases such as *NEB* in nemaline myopathy and *GCNT2* in congenital cataracts and i blood group, have been found to be affected by such deletions.[18-21] It has been demonstrated that CNVs preferentially occur near or within the duplicated sequences. CNVs including deletions can be caused by a recombination between flanking direct repeats. This newly identified 68-kb deletion is flanked by repeat sequences which can lead to an aberrant recombination event resulting in loss of the intervening unique sequence in our case (figure 3D). The microdeletions at this genomic region could be the disease-causing alleles responsible for CHED2 in the previously reported cases with unidentified mutations in the coding region of the *SLC4A11* gene. Considering the existence of deletion polymorphisms covering the *SLC4A11* gene, there is a high possibility of finding such deletions in CHED2 patients with unidentified *SLC4A11* mutations on conventional mutation detection

techniques. It would be interesting to investigate what proportions of the chromosomal deletions contribute to CHED2. This information will have significant implications for developing an algorithm for genetic testing in patients with CHED2 leading to more accurate genetic diagnosis and effective genetic counseling.

In summary, we successfully identified two novel mutations in *SLC4A11* in a Thai family and expanded the mutational spectrum of *SLC4A11* causing CHED2. This is the first to characterize the largest deletion identified in CHED2 patients. Microdeletions could be the disease-causing alleles responsible for some of the previously reported cases with undetected mutations in the coding and the putative promoter regions of *SLC4A11*. Our findings support the use of additional mutation detection techniques to identify the deletions if no causative mutations are identified using the conventional PCR-sequencing techniques. It would help increase the detection rate of mutations in patients with CHED2.

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Contributors VP collected the data, helped with interpretation of histological data and drafted the manuscript. PY performed molecular studies, analyzed the data and drafted the manuscript.

CC collected the data and helped with analysis of the images. K.S and V.S. designed the study, undertook data analysis and interpretation and wrote the manuscript.

Competing interests None

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Table 1 Clinical features of three affected siblings with CHED2

Case no.	Age/Sex	Nystagmus	BCVA RE	BCVA LE	CCT RE/LE (microns)	Surgical course
1	20/M	Present	CF 1/2 ft	20/200	1087/1089	Underwent PKP RE at age 7, graft rejection
2	17/F	Absent	20/70-1	20/200	1115/1116	Not done
3	7/F	Present	CF 2 ft	CF 1.5 ft	1115/1119	Underwent PKP RE at age 9

M, male; F, female; BCVA, best corrected visual acuity; CCT, central corneal thickness; CF, counting fingers; ft, feet; PKP, penetrating keratoplasty; RE, right eye.

Table 2 Newly designed primers and PCR conditions for *SLC4A11* mutation analysis.

Primer name	Sequence 5'- 3'
SLC4A11-Ex1-F	GAGTTTGGGGTCCAGAAGC
SLC4A11-Ex1-R	GCAGAGCCCTAATGAAACCA
SLC4A11_Deletion-F	GGGACCTTACCCACAGAACA
SLC4A11_No Del-R	ATTGCATTGGATGCCTTGTA
SLC4A11_Del-R	CACAACCCAGGGATACCAGT

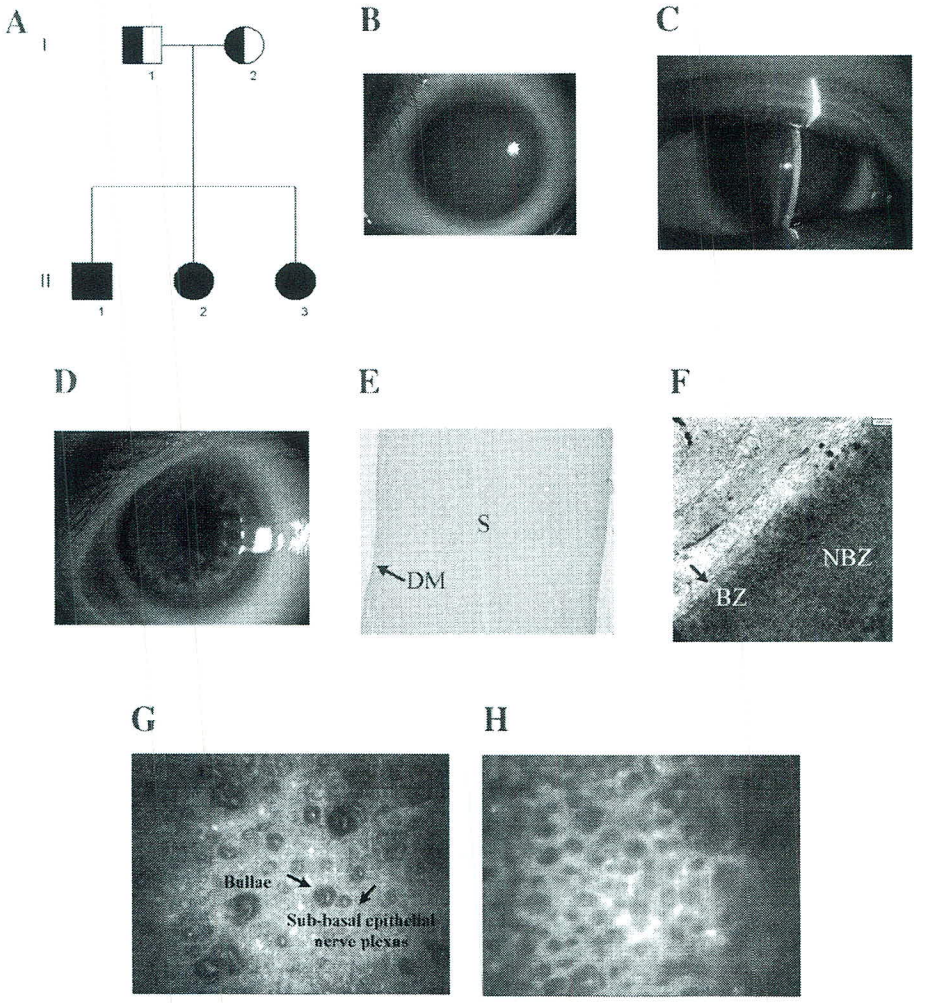
Figure legends

Figure 1. Pedigree of a Thai family with CHED2 and clinical features. (A) Pedigree. (B-C) Slit-lamp photograph of the patient (II-3)'s cornea showing corneal edema and haziness. (D) The patient (II-3)'s cornea after penetrating keratoplasty. (E) Histopathology of corneal button of the patient (II-3) with hematoxylin-eosin staining showing marked stromal and Descemet's membrane thickening. The endothelial cells were absent. S, stroma; DM, Descemet's membrane. (F) Electron microscopy (EM) revealed increased thickness of the Descemet's membrane, due to an increase thickness of the non-banded zone (NBZ). Fragmentation and disorganization of collagen fibers was seen. (G) Confocal image showing multiple hypo-reflective bullae in the epithelial layer. (H) Loss of normal stromal structures including keratocytes, replaced by hyper-reflective interwoven bands giving a "honey-comb" appearance.

Figure 2. Mutation analysis of the missense mutation. (A) Electropherograms of the patient and unaffected control. The c.778A>G mutation is indicated by an arrow. (B) PCR-RFLP analysis for the c.778A>G mutation. *Avr*II digested the mutant allele into 490 and 149-bp products (an arrow head). The wild-type allele does not harbor the recognition site, leaving the 639-bp PCR product intact. Note that the 149-bp band is not visualized. Lane 1, 100-bp marker; lane 2, father; lane 3, mother; lanes 4-6, affected family members; lane 7, uncut. The 500-bp band is indicated by an arrow head. (C) Sequence alignment of SLC4A11 centering around residue 260 from various species. The site of the p.K260E mutation is indicated by an arrow.

Figure 3. Mutation analysis of the large deletion. (A) Array-CGH analysis showed that the patient (II-2) had a heterozygous deletion from position 3107501 to 3174468 (according to chromosome 20 of hg18) encompassing the *SLC4A11* gene. (B) Schematic representation of the deleted region identified in the patients showing the location of the primers and the expected

size. (C) PCR analysis showing the deletion was present in all patients and their father but not present in their mother. The size of the PCR products was 523 bp and 679 bp for the deleted and non-deleted alleles, respectively (an arrow head). (D) Electropherograms of the patient and unaffected control showing the precise breakpoints of the 67733-bp deletion and the flanking short direct repeats confirming the results from the array-CGH studies.



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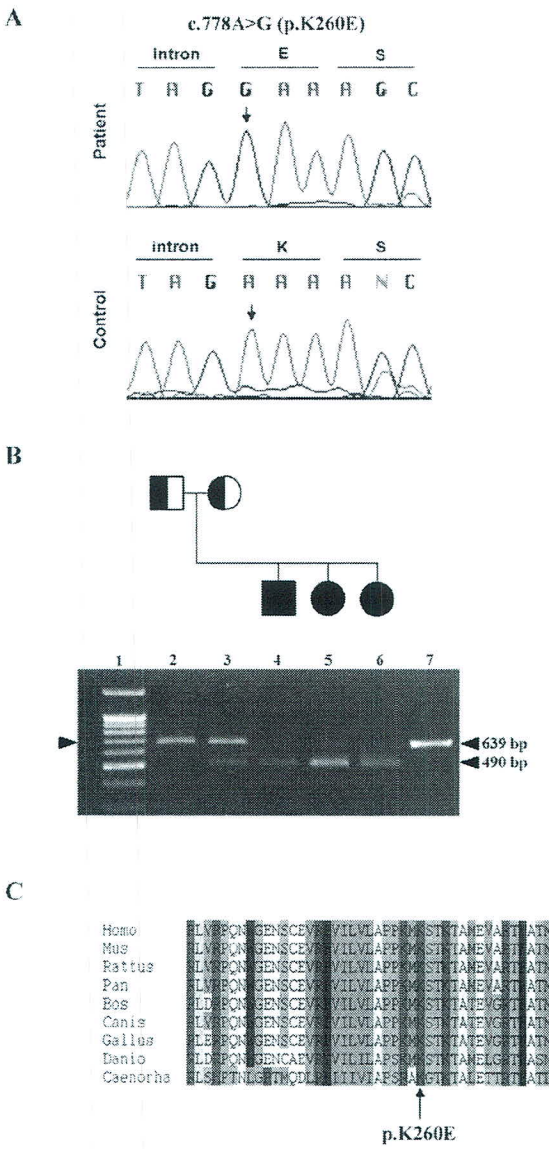

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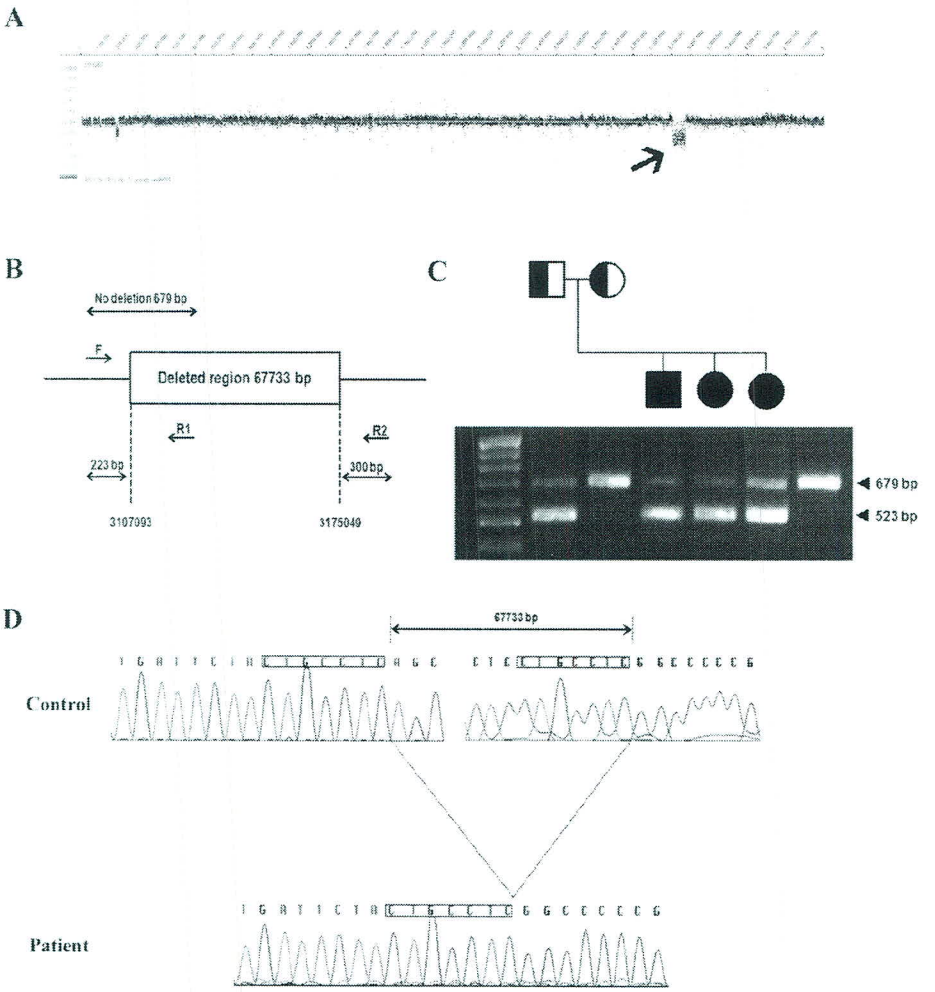

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Short communication

Two novel *CTNS* mutations in cystinosis patients in Thailand

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ABSTRACT

Cystinosis is an autosomal recessive disorder characterized by defective transport of cystine across the lysosomal membrane and resulting in renal, ophthalmic, and other organ abnormalities. Mutations in the *CTNS* gene cause a deficiency of the transport protein, cystinosin. We performed mutation analysis of *CTNS* in six cystinosis patients from four families in Thailand. Using PCR sequencing of the entire coding regions, we identified all eight mutant alleles, including two mutations, p.G309D and p.Q284X, that have not been previously reported. This study expands the mutational and population spectrum of nephropathic cystinosis.

1. Introduction

Cystinosis is an autosomal recessive lysosomal storage disorder caused by impaired transport of free cystine out of lysosomes; it affects many organs and tissues, but early manifestations involve the kidney (Gahl et al., 2002). The different severities of the disease include classical nephropathic cystinosis, with renal tubular Fanconi syndrome in the first year of life, glomerular failure by 10 years of age, and later involvement of other organ systems. Intermediate cystinosis is characterized by all the clinical manifestations of nephropathic cystinosis, but with later onset. Non-nephropathic, or ocular cystinosis manifests with only corneal crystals and photophobia (Gahl et al., 2001; Nesterova and Gahl, 2008).

In 1995, the cystinosis gene was mapped to chromosome 17p13 (McDowell et al., 1995), and in 1998 Town et al. found that mutations in the *CTNS* gene caused cystinosis (Town et al., 1998). *CTNS* consists of 12 exons with coding regions of 1104 bp. The *CTNS* gene product, cystinosin, contains 367 amino acids and 7 transmembrane domains and serves as an integral lysosomal membrane protein. At least 85 different mutations in *CTNS* have been identified in the Human Gene Mutation Database (http://www.hgmd.org/).

We identified six affected individuals with cystinosis from four families in Thailand, and performed mutation analysis of the *CTNS* gene. Two novel mutations were found in this, the first evaluation of the molecular biology of cystinosis in Thailand.

2. Materials and methods

2.1. Patients

Six patients from four different families were studied. Patients 1, 2 and 3 were single cases. Patients 4, 5 and 6 were siblings originally from Cambodia and had intermediate cystinosis; their clinical characteristics were previously reported (Kitnarong et al., 2005). These siblings had short stature and leg deformity. They did not have renal dysfunction or ocular symptoms. Further investigations demonstrated Fanconi syndrome and rickets. In all cases, the diagnosis was based upon finding typical corneal crystals and elevated leukocyte cystine levels. Clinical details and leukocyte cystine levels are in Table 1. Parental DNA was available only for the fourth family.

2.2. CTNS mutation analysis

Genomic DNA and total RNA were extracted from peripheral leukocytes according to standard protocols. Direct sequencing of PCR-amplified DNA representing the entire coding region of *CTNS* was performed as previously described (Shotelersuk et al., 1998). The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, Ohio) and directly sequenced. A novel missense mutation,

Abbreviations: CTNS, cystinosin; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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Table 1
Clinical and molecular characteristics of Thai patients with cystinosis.

Patient/Ethnic group	Age of onset	Type	Inbred	WBC cystine[a]	DNA change	Amino acid change	Novel
1/Thai	9 mo	Nephropathic	No	4.5	c.926G>A/c.969C>G	p.G309D/p.N323K	Yes No
2/Thai	7 mo	Nephropathic	Yes	NA	c.850C>T/c.850C>T	p.Q284X/p.Q284X	Yes Yes
3/Thai	15 mo	Nephropathic	No	NA	c.18-21del/c.971-12G>A	T7fsX13	No No
4/Cambodian	13 y	Intermediate	Yes	0.5	c.969C>G/c.969C>G	p.N323K/p.N323K	No
5/Cambodian	10 y	Intermediate	Yes	0.5	c.969C>G/c.969C>G	p.N323K/p.N323K	No
6/Cambodian	18 mo	Intermediate	Yes	0.6	c.969C>G/c.969C>G	p.N323K/p.N323K	No

[a] Nanomoles of half-cystine/mg of WBC protein. Normal values are <0.2 and heterozygous values are less than 1.0. See text for details.

c.926G>A, was analyzed by PCR-RFLP, using *Mwo*I restriction enzyme.

3. Results

Patient 1 was compound heterozygous for c.926G>A (p.G309D) and c.969C>G (p.N323K) (Fig. 1). The former mutation has never been described, and was not detected in 50 Thai controls (100 chromosomes), using PCR-RFLP with *Mwo*I. Patient 2 had a history of parental consanguinity and was homozygous for a nonsense mutation, c.850C>T (p.Q284X) (Fig. 1), not previously described. Patient 3 was compound heterozygous for a 4 bp deletion (c.18-21del) and a G>A substitution at the −12 position of exon 11 (c.971-12G>A). Both mutations were previously reported (Town et al., 1998).

Patients 4, 5, and 6 were homozygous for a previously described missense mutation at nucleotide position c.969C>G (p.N323K) (Thoene et al., 1999). Both parents were carriers for the mutation. The affected siblings had leukocyte cystine levels approximately 5 times the normal level (Table 1), which is always <0.2 nmol half-cystine/mg protein but averages ~0.1 nmol half-cystine/mg protein.

4. Discussion

We report the *CTNS* mutations in 6 patients from 4 families in Thailand; three had classical nephropathic cystinosis and three had intermediate cystinosis. Patients 1 and 3, who were products of non-consanguineous parents, had compound heterozygous mutations, while patient 2 and the 3 siblings from family 4, from consanguineous matings, were homozygous. Five different mutations were identified, including a 4 bp deletion, 2 missense mutations, 1 nonsense and a single base pair substitution in intron 11. One missense (c.926G>A, p.G309D) and the nonsense (c.850C>T, p.Q284X) mutations were not previously reported.

The newly identified missense mutation in patient 1 (c.926G>A; p.G309D) was predicted by PolyPhen (http://coot.embl.de/PolyPhen/) to be probably damaging with a score of 0.999. ClustalX showed the nucleotide was conserved from *Homo sapiens* to *Oryza sativa* (Fig. 2). Patient 2 was homozygous for a single base pair transition (c.850C>T), expected to result in changing a glutamine at amino acid position 284 into a stop codon (p.Q284X).

Several studies supported the correlation of genotype with phenotype in cystinosis. Among our patients, p.N323K was present in the homozygous state in patients 4, 5, and 6, who had intermediate cystinosis. This is consistent with a previous study reporting patients with intermediate cystinosis, who were also homozygous for p.N323K (Thoene et al., 1999). Both patients had fibroblast cystine values of 1.1 and 1.2 nmol half-cystine/mg protein; our patients' values, 0.5 and 0.6 nmol half-cystine/mg protein, are within the range for individuals who are heterozygous for severe *CTNS* mutations (i.e., <1.0 nmol half-cystine/mg protein). This discrepancy might be related to the difference in cell types assayed, but in any event, it suggests that p.N323K allows for considerable residual cysteine-transporting

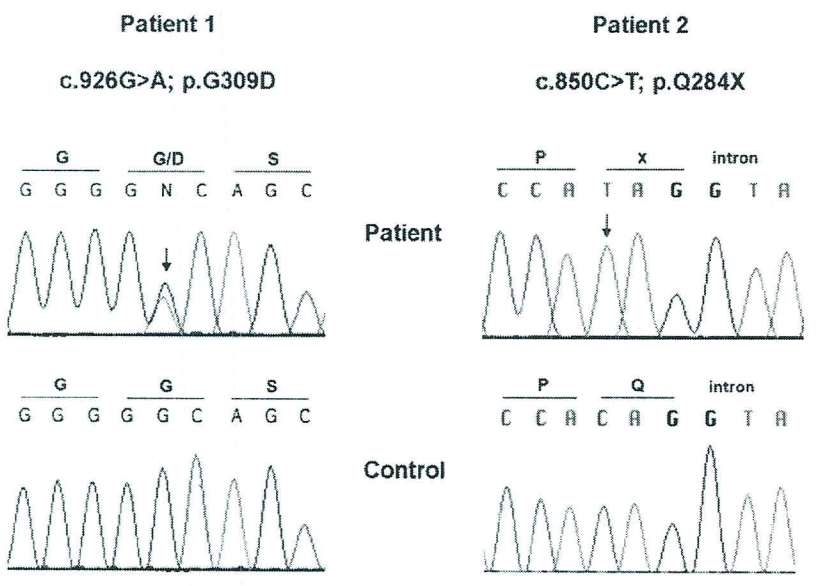

Fig. 1. *CTNS* mutation analysis for patients (upper panels) compared with controls (lower panels). Left panel: Electropherogram of patient 1 using complementary DNA as template shows a heterozygous novel missense mutation c.926G>A (p.G309D) (arrow). Right panel: Electropherogram of patient 2 using genomic DNA as template reveals a homozygous novel nonsense mutation c.850C>T (p.Q284X) (arrow).

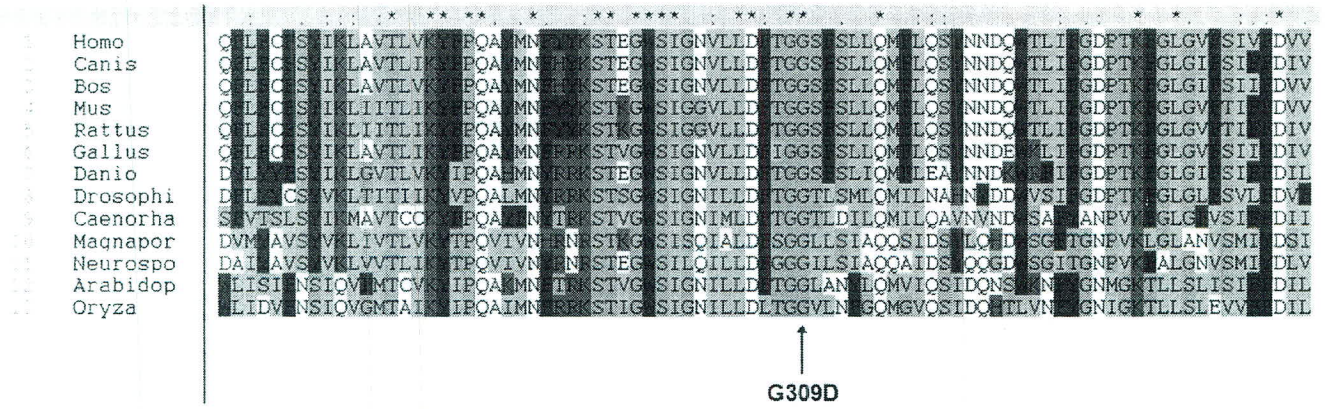

Fig. 2. ClustalX. Multiple sequence alignment of CTNS in different species. The position of amino acid (G309D) is 100% conserved across all sequences (arrow).

activity, perhaps as much as 50%. The p.N323K was also found in the heterozygous state in our patient 1 with nephropathic cystinosis. The other two previously reported mutations in our cases, c.18-21del and c.971-12G>A, have been reported in patients with classical nephropathic cystinosis (Attard et al., 1999; Town et al., 1998). Our findings support a correlation between *CTNS* mutations and clinical severity in cystinosis, and identify *CTNS* mutations that are, to date, novel to Thai families.

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A novel p.E276K *IDUA* mutation decreasing α-L-iduronidase activity causes mucopolysaccharidosis type I

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Purpose: To characterize the pathogenic mutations causing mucopolysaccharidosis type I (MPS I) in two Thai patients: one with Hurler syndrome (MPS IH), the most severe form, and the other with Scheie syndrome (MPS IS), the mildest. Both presented with distinctive phenotype including corneal clouding.

Methods: The entire coding regions of the α-L-iduronidase (*IDUA*) gene were amplified by PCR and sequenced. Functional characterization of the mutant *IDUA* was determined by transient transfection of the construct into COS-7 cells.

Results: Mutation analyses revealed that the MPS IH patient was homozygous for a previously reported mutation, c. 252insC, while the MPS IS patient was found to harbor a novel c.826G>A (p.E276K) mutation. The novel p.E276K mutation was not detected in 100 unaffected ethnic-matched control chromosomes. In addition, the glutamic acid residue at codon 276 was located at a well conserved residue. Transient transfection of the p.E276K construct revealed a significant reduction of IDUA activity compared to that of the wild-type IDUA suggesting it as a disease-causing mutation.

Conclusions: This study reports a novel mutation, expanding the mutational spectrum for MPS I.

Mucopolysaccharidoses (MPs) are a group of inherited lysosomal storage disorders resulting from a deficiency of enzymes that catalyze the degradation of glycosaminoglycans (GAGs). MPS I is considered as the prototypic lysosomal storage disease of the MPS group which is caused by a deficiency of lysosomal α-L-iduronidase (IDUA, EC 3.2.1.76). As a result of defects inside the lysosomes, partially degraded GAGs, heparan, and dermatan sulfate accumulate in these organelles leading to progressive cellular dysfunction and characteristic features of the disorder. MPS I has been classified into three clinical phenotypes, with different levels of severity: a severe form (Hurler syndrome; MPS IH; OMIM 607014), an intermediate form (Hurler-Scheie syndrome; MPS IH/S; OMIM 607015), and a mild form (Scheie syndrome; MPS IS; OMIM 607016) [1,2].

MPS I can be diagnosed biochemically by the presence of urinary dermatan sulfate and heparan sulfate and the significant reduction or absence of IDUA activity in patients' leukocytes or skin fibroblasts [1]. The *IDUA* gene contains 14 exons encoding a 653-amino acid precursor protein [3,4]. At least 110 different disease-causing mutations in *IDUA* have been described with the majority being missense/nonsense mutations. The splice-junction alterations and nucleotide insertions/deletions have also been reported (Human Gene Mutation Database, accessed September, 2010). The p.W402X and p.Q70X mutations are most commonly found in Caucasians and are accountable for as much as 70% of the disease alleles in some European countries [5-7].

There has been only one report on Thai patients with molecularly confirmed Hurler syndrome [8]. Here, we described two unrelated Thai patients with MPS I and identified one recurrent and one novel mutations in *IDUA*. The functional consequence of the novel missense mutation was also further elucidated.

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METHODS

Subjects: Two unrelated Thai patients with clinically diagnosed MPS I were reported. Patient 1, a product of a consanguineous marriage, with clinical features consistent with MPS IH was diagnosed at one year of age. The clinical findings included coarse facial features, corneal clouding, hepatosplenomegaly, and skeletal deformities. He was noted to have delayed development and passed away at age two from severe respiratory infection. Patient 2 was adopted and was diagnosed with MPS IS at the age of 29 years. The clinical features included coarse facial features, corneal clouding and claw hand deformity. The α-L-iduronidase activity in leukocytes from patient 2 was measured and revealed a significant reduction in enzyme activity (0.61 nmol/h/mg protein). The mean of α-L-iduronidase activity in leukocytes obtained from eight unaffected adult Thai controls was

Table 1. Primers and PCR conditions for *IDUA* mutation analysis.

Exon	Primer name	Primer sequences for PCR (5′ to 3′)	Product size (bp)	Melting temperature (°C)
1	IDUA-Ex1F	F-ACCCAACCCCTCCCAC	398	58
	IDUA-Ex1R	R-AGCTTCAGAGACCGGAG		
2	IDUA-Ex2F	F-GAACGTGTGTGTCAGCCG	304	62
	IDUA-Ex2R	R-GCTCGGAAGACCCCTTGT		
3–4	IDUA-Ex3/4F	F-TTCCAGCCTGGAGCATGGAG	516	62
	IDUA-Ex3/4R	R-GTTGCACCCCTATGACGCAG		
5–6	IDUA-Ex5/6F	F-TCACCTTGCACCCTCCCTCC	576	62
	IDUA-Ex5/6R	R-GCTGACCCTGGTGGTGCTGA		
7	IDUA-Ex7F	F-TGCGGCTGGACTACATCTC	448	62
	IDUA-Ex7R	R-GCAGCATCAGAACCTGCTACT		
8	IDUA-Ex8F	F-CCACCTTCCTCCCGAGAC	386	62
	IDUA-Ex8R	R-GGAGCGCACTTCCTCCAG		
9–10	IDUA-Ex9F	F-TCCTTCACCAAGGGGAGG	701	58
	IDUA-Ex10R	R-TCCTCAGGGTTCTCCAGG		
11–12	IDUA-Ex11/12F	F-GTGTGGGTGGGAGGTGGA	466	62
	IDUA-Ex11/12R	R-CTTCACCCATGCGGTCAC		
13–14	IDUA-Ex13/14F	F-CTGCCTGCTCCCACCTTTGHA	530	62
	IDUA-Ex13/14R	R-CCCATGCTGCCCTCCCATCA		

23.10±8.80 nmol/h/mg protein. Unfortunately, leukocytes from patient 1 were unavailable for analysis.

Mutation analysis of the IDUA gene: After informed consent, genomic DNA was extracted from peripheral blood leukocytes from patients and available parents according to standard protocols. The entire coding regions of *IDUA* were assessed by polymerase chain reaction (PCR) and direct sequencing. Most of the oligonucleotide primers were used as previously described [9] and presented in Table 1. Exons 3–6, 13–14, and their intron-exon boundaries were amplified using newly designed primers (Table 1). The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH), according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen, Inc. (Seoul, Korea). The sequences were analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI).

For a novel missense mutation, c.826G>A (p.E276K), PCR-RFLP analysis with the MboII restriction enzyme was used to confirm its presence in the patient and to screen in 100 ethnic-matched unaffected control chromosomes. The forward and reverse primers for amplification of *IDUA* exon 7 were used to generate a 448-bp PCR product. The mutant PCR product creates another MboII site, which allowed detection of the mutation by agarose gel electrophoresis. As the proband was adopted, the parental DNA was not available for analysis.

Protein sequence comparison: IDUA orthologs were first identified through a BLAST search of the non-redundant database using *Homo sapiens* IDUA, accession NP_000194.2 as the reference sequence. All known and complete IDUA sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by ClustalX program.version 2.0.12. The human IDUA was aligned with rat (*Rattus norvegicus*; NP_001165555.1), mouse (*Mus musculus*; NP_032351.1), cow (*Bos taurus*; XP_877410.2), chicken (*Gallus gallus*; NP_001026604.1), frog (*Xenopus laevis*; NP_001087031.1), and zebrafish (*Danio rerio*; XP_001923689.1). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

Construction of plasmids and site-directed mutagenesis: The expression vector wild-type pEFNeo-IDUA was kindly provided by Hopwood's laboratory. The mutant *IDUA* constructs of c.1206G>A (p.W402X) and c.826G>A (p.E276K) were generated by in vitro site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Stratagene, La Jolla, CA) on the pEFNeo-IDUA using oligonucleotide primers. The p.W402X was a previously described mutation in MPS IH and was used as a mutant control. All mutant *IDUA* constructs were verified by direct sequencing.

Transient transfection and enzyme assay: COS-7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. COS-7 cells were transfected with the wild-type or mutant constructs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cells were harvested after 48 h and assayed for IDUA activity.

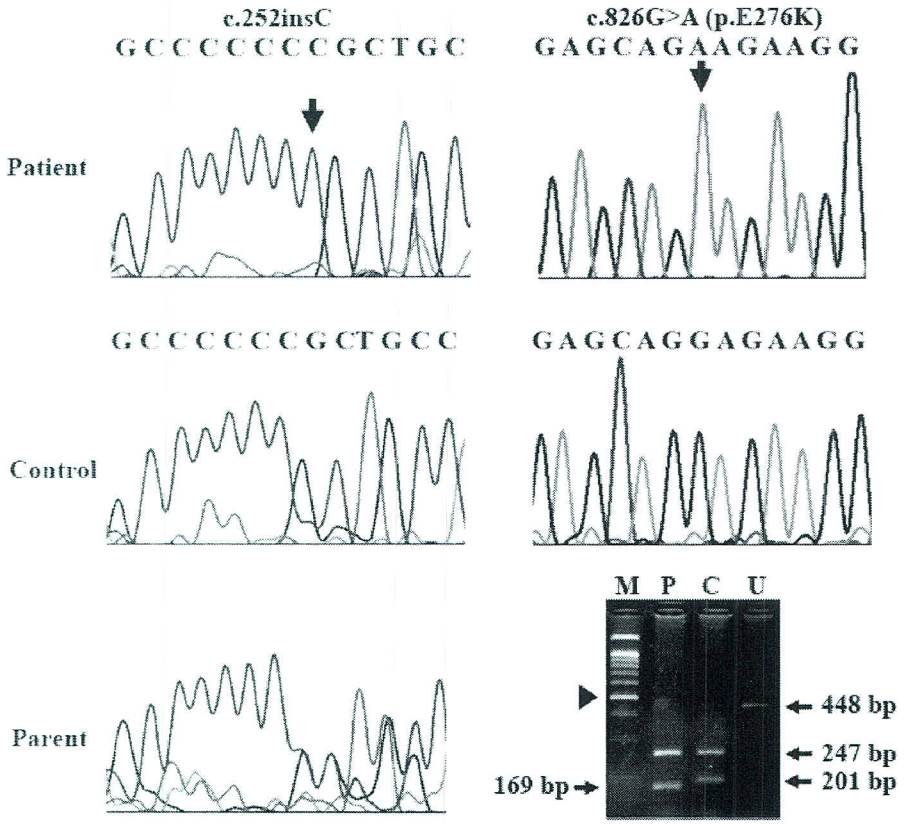

Figure 1. Mutation analysis. The left and right panels relate to c.252insC and c. 826G>A (p.E276K) mutations, respectively. Upper, middle and left lower panels are electropherograms of patients, unaffected controls, and one of the parents, respectively. Each identified mutation is indicated by an arrow. Right lower panel showing RFLP analysis for the c.826G>A mutation in patient 2. MboII digested the wild-type allele of a control into 247 and 201-bp products. The c.826G>A creates another cleavage site for MboII resulting in 169 and 32-bp bands. Note that the 32-bp band is not visualized. (M=100-bp marker, P=patient, C=control, U=uncut amplified product). The 500-bp band is indicated by an arrowhead.

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Hs HCHDGTNFFTGEAGVRLDYISLHRKGARSSISILEQEKVVAQQIRQLFPKFADTPIYNDEADPLVGWSLPQPWRA
Bt ..NN.S.....V.......A..K...G...F....EAA.V...QR.............................
Rn ..AN.....V.........K...G...A....EVA.VE..Q......K.........................
Mm ..AN.....V.........K...G...A....EMA.VE.VQ....E.K.........................
Gg ..YN.....T.........K..GGR.LY..Q.EVETVD..QK...N..SI.........M....V..L....
Xl ..YN...Y....KR.....A..K...GG.FY....EMETVNE.QER..L.KNV.............T..S..G
Dr ..YN...Y....S.......A..K..GGG.LP..Q.EVSTV.E.Q....D.CSL.............NK.LT...
```

Figure 2. Protein sequence alignment of IDUA from different species. The site of the amino acid variant found in this study is indicated in bold red in all conserved species. Sites that are 100% conserved across all sequences are indicated by dots (.). Hs, *Homo sapiens*; Bt, *Bos taurus*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Gg, *Gallus gallus*; X1, *Xenopus laevis*; Dr, *Danio rerio*.

Experiments were performed twice with triplicate per experiment.

An assay for IDUA activity was performed using the fluorogenic substrate 4-methylumbelliferyl-α-L-iduronide (Glycosynth, Cheshire, UK) as previously described [10]. The protein concentration of the cell lysates was determined by the Bradford assay.

RESULTS

PCR-sequencing revealed a homozygous c.252insC mutation in patient 1 (Figure 1, left upper panel). Sequence analysis of the parental genomic DNA confirmed that both parents were heterozygous for the c.252insC. This mutation has been previously described to be associated with a severe phenotype in different populations including Thai [6,8].

A novel homozygous c.826G>A mutation in exon 7 was identified in patient 2. This was expected to result in a glutamic acid to lysine substitution (p.E276K) at codon 276 (Figure 1, right upper panel). No other variants were observed.

Restriction enzyme digestion of the PCR products with MboII was used to confirm the presence of the c.826G>A mutation in the patient 2 and its absence in 100 ethnic-matched unaffected control chromosomes. Alignment of the IDUA protein sequences revealed that the glutamic acid residue at codon 276 was located at a well conserved residue (Figure 2).

The functional effect of the novel p.E276K and the previously identified p.W402X mutations on IDUA activity were analyzed by transient transfection of each IDUA construct into COS-7 cells. Comparing to the enzyme activity

TABLE 2. α-L-IDURONIDASE ACTIVITY IN TRANSIENTLY TRANSFECTED COS-7 CELLS WITH EITHER WILD-TYPE OR MUTANT IDUA CONSTRUCTS.

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

of the wild-type IDUA (435.04±56.23 nmol/h/mg protein), the p.E276K and the p.W402X had reduced activity to background levels (31.88±6.05 nmol/h/mg protein and 21.10±12.57 nmol/h/mg protein, respectively, Table 2).

DISCUSSION

We identified two unrelated Thai patients with MPS I. Patient 1 was found to be homozygous for the c.252insC mutation. It has been previously described to be responsible for a severe phenotype. The novel missense mutation, c.826G>A (p.E276K), was identified in patient 2 with MPS IS and caused a significant reduction of IDUA activity.

There was only one report describing two patients with MPS IH in the Thai population. Four different mutations including the c.252insC were detected. Combined this previous report with our findings, the c.252insC mutation is responsible for 40% (2 out of 5 alleles), making it a possible common mutant allele in Thai patients with MPS IH. Continued studies for mutations in patients with MPS I will be required for a definite conclusion. If this is proved to be the case, it will benefit a molecular diagnosis for this population.

Our patient with MPS IS was found to carry the novel c.826G>A (p.E276K) mutation. Due to the unavailability of her parent's DNA, a possibility that the other allele is deleted making her hemizygous for the c.826G>A remains. As no other variants were observed, the patient could be either homozygous or hemizygous for this particular mutation.

Several lines of evidence support the pathogenicity of this novel mutation. First, it is not found in 100 ethnic-matched control chromosomes. Second, the glutamic acid at codon 276 is located at a well conserved residue (Figure 2). And most importantly, when transiently transfected COS-7 cells with the p.E276K construct, IDUA enzyme activity was reduced to background levels. We also tested for the effect of the p.W402X, one of the most common mutations found in Caucasian individuals with MPS I, and revealed a significant reduction of IDUA activity similar to previous reports [9]. Our studies therefore suggested the p.E276K as a disease-causing mutation.

Prediction of a patient's clinical phenotype through genetic analysis of *IDUA* has been complicated by the high number of disease-causing mutations and polymorphic variants present in the *IDUA* gene. It has been hypothesized that a combination of mutations, polymorphisms, genetic background, and environmental factors contribute to the clinical phenotypic spectrum [9,11,12]. Even though studies of genotype-phenotype correlations for MPS I patients are sometimes inconclusive, the nonsense mutations if present on both *IDUA* alleles have been shown to cause a severe form of MPS I. The c.252insC causing frameshift identified in our patient with MPS IH has been previously reported in two patients, one from the Netherlands and the other from Thailand [6,8]. Both were compound heterozygotes for the c.252insC and other nonsense mutations. Our finding of another MPS IH patient associated with the homozygous c.252insC mutation further supports the deleterious effect of this particular mutation on the enzyme activity.

In summary, we reported two unrelated Thai patients with MPS I with different clinical severity who were found to carry different mutations. Combined with a previous study in Thai patients, the c.252insC mutation identified in our patient with MPS IH, could be a common mutation causing Hurler syndrome in the Thai population. We also detected a novel missense mutation of the *IDUA* gene, c.826G>A (p.E276K) causing Scheie syndrome.

Transient transfection studies and assay for IDUA activity confirmed its pathogenic role. This study expands the mutational spectrum of MPS I.

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