

## Deletion of Exon 4 in the N-Acetylgalactosamine-4-Sulfatase Gene in a Taiwanese Patient with Mucopolysaccharidosis Type VI

Wei-De Lin,<sup>1,2,\*</sup> Yu-Yuan Ke,<sup>3,\*</sup> I-Ching Chou,<sup>4,5</sup> Chung-Hsing Wang<sup>4,6</sup> and Fuu-Jen Tsai<sup>4,7,8,9</sup>

<sup>1</sup>Department of Medical Research, China Medical University Hospital, Taichung, Taiwan

<sup>2</sup>School of Post Baccalaureate Chinese Medicine, China Medical University, Taichung, Taiwan

<sup>3</sup>Department of Pediatrics, Taichung Veterans General Hospital, Taichung, Taiwan

<sup>4</sup>Department of Pediatrics, China Medical University Hospital, Taichung, Taiwan

<sup>5</sup>Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan

<sup>6</sup>School of Medicine, China Medical University, Taichung, Taiwan

<sup>7</sup>Department of Medical Genetics, China Medical University Hospital, Taichung, Taiwan

<sup>8</sup>School of Chinese Medicine, China Medical University, Taichung, Taiwan

<sup>9</sup>Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan

Mucopolysaccharidosis type VI (MPS VI) is a rare autosomal recessive disorder caused by a deficiency of N-acetylgalactosamine-4-sulfatase (ARSB), one of the enzymes required for the degradation of dermatan sulfate (DS). Accumulation of DS in connective tissue causes growth failure, resulting in short stature. Here, we observed a 5-year-old girl who was the only one affected member of her family and who presented with an exaggerated, convex curvature of the back at the age of one year. Abnormal excretion of DS in the urine and extremely low leukocyte ARSB activity were noted. The patient was suspected to have MPS VI. Direct DNA sequencing indicated that there was no mutation in the coding region of *ARSB*. However, RT-PCR analysis of RNA prepared from blood samples indicated the deletion of the entire exon 4. Further analysis of the genomic DNA by quantitative PCR confirmed a homozygous deletion of exon 4, an unusual intragenic deletion in *ARSB*. The deletion led to a truncated protein that lacks most of the catalytic domain. The patient received recombinant human ARSB as enzyme replacement therapy (ERT) at an early stage (2 years), and responded positively in terms of skeletal development and other developmental milestones. The early identification of type VI MPS patients and subsequent treatment with ERT may be beneficial for the clinical outcome of MPS VI patients. In addition, detailed gene analysis may enhance the ability to provide genetic counseling to families of patients affected by MPS VI.

**Keywords:** deletion mutation; dermatan sulfate accumulation; enzyme replacement therapy; mucopolysaccharidosis type VI; N-acetylgalactosamine-4-sulfatase

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### Introduction

Mucopolysaccharidosis type VI (MPS VI; Maroteaux-Lamy disease) is a rare autosomal recessive disorder caused by a deficiency in the lysosomal enzyme N-acetylgalactosamine-4-sulfatase (ARSB, arylsulfatase B; EC 3.1.6.1). This enzyme deficiency leads to the incomplete degradation of the glycosaminoglycan (GAG) - dermatan sulfate (DS), and the widespread accumulation of this partially degraded GAG in the lysosomes of connective tissue. This accumulation results in the distortion of tissue structure, induction

of inflammatory responses, and increased secretion of DS into the urine (Neufeld and Muenzer 2001).

MPS VI is heterogeneous, with a wide variety of clinical phenotypes, including growth retardation, hepatosplenomegaly, corneal opacity, joint/skeletal malformations, and cardiac abnormalities. However, the mental development of MPS VI patients is typically normal. The majority of patients with the severe form of MPS VI die in childhood or adolescence because of cardiopulmonary complications. In contrast, patients with the mild form of MPS VI have a later onset of the disease and exhibit clinical manifestations

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\*These two authors contributed equally to this work.

Correspondence: Chung-Hsing Wang, Department of Pediatrics, China Medical University Hospital, No. 2, Yude Road, 404, Taichung, Taiwan.

e-mail: d5894@mail.cmuh.org.tw

Fuu-Jen Tsai, Department of Medical Genetics, China Medical University Hospital, No. 2, Yude Road, 404, Taichung, Taiwan.

e-mail: d0704@mail.cmuh.org.tw

that are less severe. These patients often survive for several decades (Quigley and Kenyon 1974; Wald and Schimdek 1984). The incidence of MPS VI varies among different populations, ranging from 1 in 248,000 to 1 in 1,300,000 live births (Poorthuis et al. 1999; Neufeld and Muenzer 2001; Pinto et al. 2004; Brooks et al. 2005; Lin et al. 2008; Valayannopoulos et al. 2010). Over the past decade, enzyme replacement therapy (ERT), using recombinant human ARSB (rhASB), has significantly improved the efficacy of treating early stages of MPS VI (Harmatz et al. 2004, 2006, 2010) and similar improvements have been reported for treated patients living in Asia (Lin et al. 2010; Furujo et al. 2011).

The human gene encoding ARSB has been identified and localized to chromosome 5q11-q13 (Fidziańska et al. 1990; Peters et al. 1990; Schuchman et al. 1990). This gene spans 440 kb, contains 8 exons, and encodes a 533 amino acids polypeptide that contains a 37 amino acids signal peptide (Modaressi et al. 1993; Bond et al. 1997). According to the records provided by the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>) and other recent publications, over 140 mutations in *ARSB* have been identified to date (Lin et al. 2008; Villani et al. 2010; Costa-Motta et al. 2011; Jurecka et al. 2012). Most of these mutations are single base, missense or nonsense variations, while large fragment deletions or insertions occur rarely. In this study, a phenotypically and biochemically diagnosed MPS VI patient from a consanguineous family from central Taiwan was analyzed using a detailed genetic protocol.

## Materials and Methods

### Patient information

The patient was a 5-year old girl, the third child of consanguineous, healthy Taiwanese parents (Fig. 1). She initially presented to the orthopedics outpatient department at the age of 1 year and 3 months with kyphosis, which had been evident for roughly 6 months. Thoraco-lumbar spine (anterior-posterior and lateral) radiologic studies were performed, and were interpreted as consistent with muco-

polysaccharidosis. She initially was referred to psychiatrists for physical therapy, and then to the Division of Pediatric Genetics and Metabolism for MPS evaluation and management. After quantitative analysis of her urine, it was noted that the patient excreted substantial amount of GAGs. Subsequent analysis of leukocyte and plasma lysosomal enzyme indicated low arylsulfatase B activity ( $5.54 \text{ nmol h}^{-1} \text{ mg-protein}^{-1}$ ; normal range:  $> 121 \text{ nmol h}^{-1} \text{ mg-protein}^{-1}$ ). Based on these clinical and biochemical findings, the patient was diagnosed with MPS VI. ERT protocols were carefully designed for this patient and performed in accordance with previously published literature and guidelines (Lin et al. 2010).

To determine the genetic profile of this patient, molecular genetic analyses were performed after the ethics committee of China Medical University Hospital approved the study and informed consent was obtained from the parents.

### DNA preparation and sequencing

Genomic DNA was extracted from the peripheral blood leukocytes of both the patient and the family members using a MagNA Pure LC DNA Isolation Kit (Roche, Mannheim, Germany). The complete *ARSB* coding region, including the intron/exon boundaries, was amplified by PCR as previously reported (Isbrandt et al. 1994). The binding locations for the primer sets used for amplification are shown in Table 1 and Fig. 2. PCR products were purified and then used for direct sequencing to identify gene mutations (Lin et al. 2008). The DNA sequences were compared to GenBank entry NG\_007089 (genomic DNA) and NM\_000046 (mRNA).

### Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed with total RNA isolated from the blood samples of the patient and the family members using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). Total RNA was isolated using the QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany). Amplification of the fragment spanning exons 3-5 of the *ARSB* cDNA was achieved using exonic primers, including RT-F1: 5'-CCTACTTTGGATATCTCCTG (forward, located at the exon 2-3 junction) and RT-R1: 5'-CTCCATTTTCTTCCTCGAAG (reverse, located in exon 5). The RT-PCR parameters recommended by the manufacture were used. RT-PCR products were then used in the direct sequencing procedure described above.

### Deletion detection using real-time quantitative-PCR (qPCR)

To characterize the deletion mutation at the genomic DNA level, qPCR with fluorescently labeled probes was performed. Primers and probes were designed using ProbeFinder v2.45 (Roche, Mannheim, Germany, <http://www.roche-applied-science.com>) and purchased from Universal Probe Library (UPL, Roche, Mannheim, Germany). Sequences of the primers and probes are listed in Table 1, and their binding locations are shown in Fig. 2. The targeted exons of *ARSB* included exons 3, 4 and 5. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference gene. All reactions were performed in triplicate: each 96-well plate included 3 control samples and 4 genes (1 reference and 3 targets), as well as a no-template control for each gene. The plate was analyzed with a LightCycler 480 Real-Time PCR system (Roche, Mannheim, Germany), and raw data were acquired using LightCycler 480 software (Version 1.2). The formula used to calculate copy number was:  $\text{copy number} = 2 \times 2^{-(\Delta C_{\text{TP}} - \Delta C_{\text{Tn}})}$  where  $C_{\text{t}}$  is the threshold cycle, defined as the mean cycle at which the fluorescence curve reaches an arbitrary

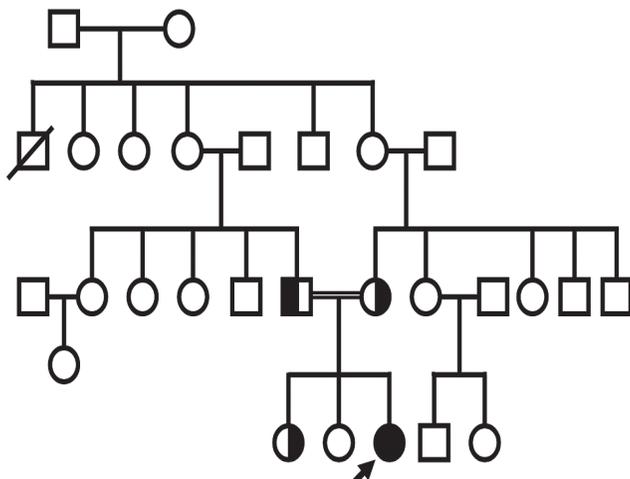
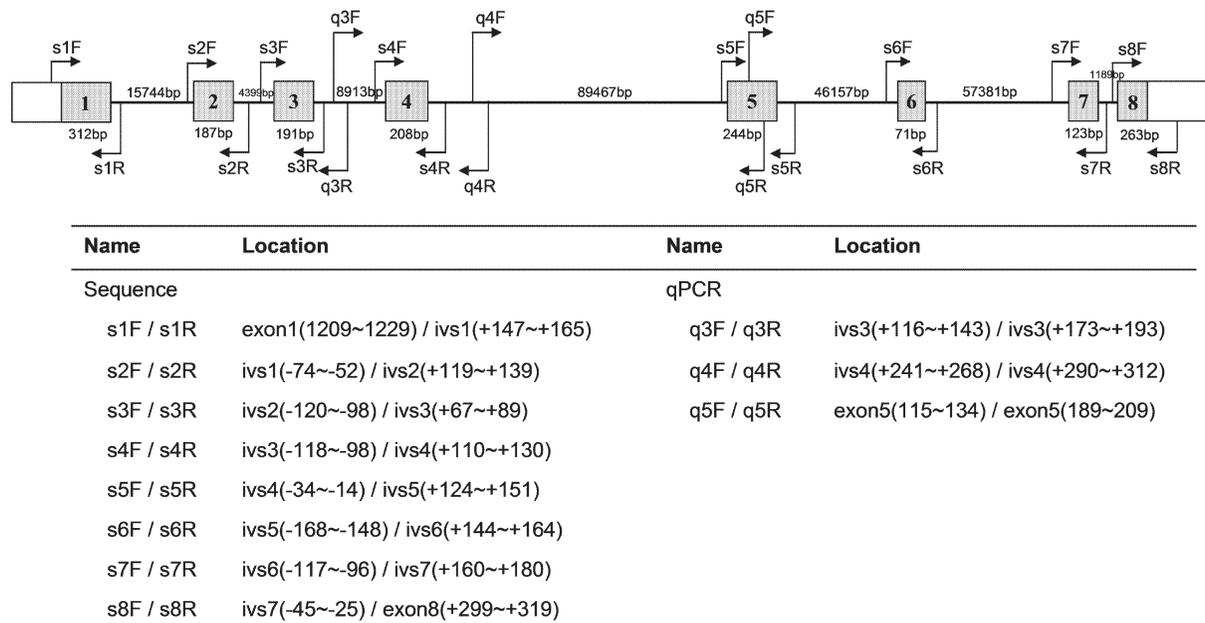


Fig. 1. Pedigree of the MPS VI case in this study. The proband is indicated with an arrow.

Table 1. Sequences of primers and probes used in the qPCR.

Target	Primer (5'→3')	Probe (5'→3')
<i>ARSB</i> exon 3	Forward: cctttcaaattatacagtatcccaga Reverse: tagaggtgggctccaggaat	cagcctgg
<i>ARSB</i> exon 4	Forward: aactgagtaagtatgagtggacatcac Reverse: acaagccactctaggattcac	ggctgctg
<i>ARSB</i> exon 5	Forward: gcagaaggcgtgaagaac Reverse: ctttgccattgggtgtc	ctggctgc
<i>GAPDH</i>	Forward: gcatcctgggctactactga Reverse: ccagcgtcaaagggtggag	ctcctctg

Fig. 2. Schematic representation of the *ARSB* gene structure.

The *ARSB* gene structure is shown with the locations of primer sets used for PCR-directed sequencing and real-time quantitative PCR (qPCR). The lengths of exons and introns are according to GenBank accession number NG\_007089.

threshold;  $\Delta C_t$  is the difference between the  $C_t$  of the target gene and that of *GAPDH*;  $\Delta C_{tp}$  is the  $\Delta C_t$  of the affected individuals; and  $\Delta C_{tn}$  is the  $\Delta C_t$  of normal individuals.

## Results and Discussion

The clinical phenotypes and biochemical profiles of the patient, including elevated urinary GAG content and low leukocyte *ARSB* activity, were consistent with those observed previously in MPS VI patients (Lin et al. 2008; Lin et al. 2010). For this reason, we performed a molecular analysis of *ARSB*. It was hypothesized that the patient would carry homozygous mutant alleles of *ARSB* because her parents were consanguineous. Exons 1-3 and 5-8 from the affected patient were strongly amplified, and no mutations were detected in the coding regions after they were sequenced. Exon 4 consistently failed to amplify when the patient's DNA was used as the template, which led to the hypothesis that the patient carried a homozygous deletion of exon 4 region of the *ARSB* gene. When the genomic DNAs from parents were used as the template, exon 4 was

successfully amplified, and sequencing indicated that there were no genetic variations. This result would be expected in individuals who are heterozygous for the deletion of the presence of the one wild type allele.

To identify the exon 4 deletion within the putative mutated gene, total RNA was isolated from peripheral blood samples taken from the patient and her immediate family members, after which we performed RT-PCR on each sample to amplify specific exon regions. The normal size of the full-length of PCR product is 469 base pairs (bp). When exon 4 is deleted, the size of the PCR product is reduced to 261 bp. As shown in Fig. 3A, when the RT-PCR product was amplified using RNA from the patient, only a single 261-base pair band was detected by agarose gel electrophoresis. This fragment was sequenced directly, and the results confirmed that exon 4 was not included in the *ARSB* mRNA from the patient (Fig. 3B).

According to the crystal structure, the *ARSB* protein has 2 domains. The large domain, extending from residue 46 to residue 382, has an  $\alpha/\beta$  topology and contains the

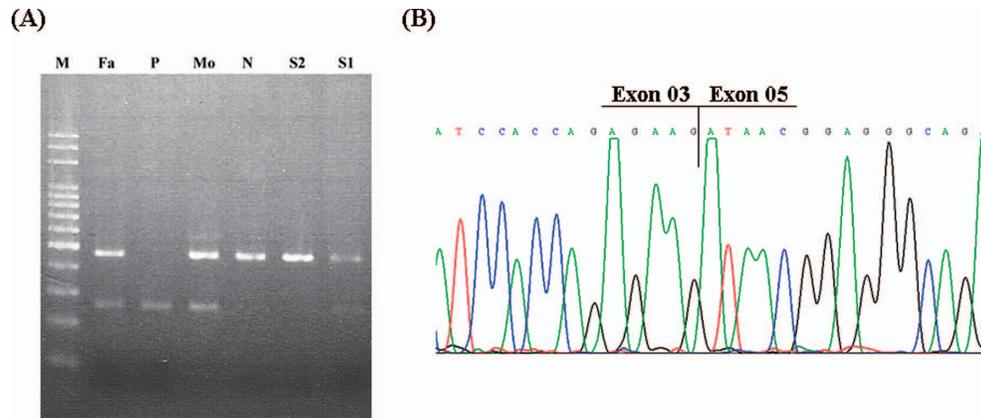
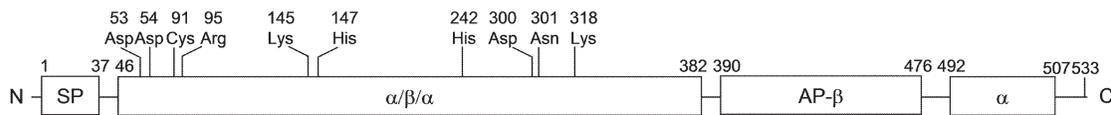


Fig. 3. RT-PCR detection of a partial mRNA region of *ARSB* from the MPS VI family.

(A) Agarose gel depicting of RT-PCR detection of exons 2 to 5 of the *ARSB* mRNA. The size of the full-length product is 469 bp. Loss of exon 4 results in a 261 bp product. The patient was homozygous for deletion allele, while the father (Fa), mother (Mo) and 1 sister (S1) were heterozygous. The other sister (S2) was homozygous for the full-length allele. M: 100-bp ladder DNA marker; N: normal control. (B) The shorter cDNA fragment amplified from the patient's *ARSB* was applied to direct sequencing and lacked exon 4.

(A) Wild type



(B) Mutant type- truncated protein

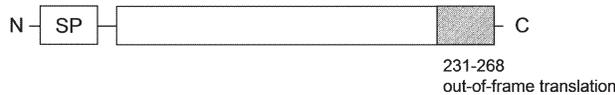


Fig. 4. Schematic diagram of *ARSB* protein structure.

(A) The full length of wild type. (B) Exon 4 deletion mutant identified in this study results in a truncated protein. The amino acids labeled above the wild type make up the active-site pocket. N: N-terminus; C: C-terminus; SP: signal peptide.  $\alpha/\beta/\alpha$ : large domain,  $\alpha/\beta$  topology with a 10-stranded mixed  $\beta$  sheet flanked by 2  $\alpha$  helices. AP- $\beta$  +  $\alpha$ : small domain, 4-stranded antiparallel  $\beta$  sheet with  $\alpha$  helix. Residue numbers are designated according to GenBank accession number NM\_000046.

enzyme's active site; it included Asp53, Asp54, Cys91, Arg95, Lys145, His147, His242, Asp300, Asn301, and Lys318. The small domain, extending from residue 390 to residue 507 consists of a 4-stranded, antiparallel  $\beta$  sheet with an orthogonal  $\alpha$  helix (Fig. 4A) (Bond et al. 1997). The resulting protein, in this case, was shifted out-of-frame at codon 231 and terminated early at codon 268. Relative to the wild-type protein, which has 533 residues, the truncated *ARSB* transcript would produce a protein that was only half the length and that would lose the functional domain (Fig. 4B), likely leading to the rapid degradation of the *ARSB* protein. This deletion mutation was also consistent with the severity of the patient's phenotype, which included the early onset of clinical manifestations and the extremely low blood levels of *ARSB* enzyme activity. The same RT-PCR analysis of samples taken from the patient's family members revealed that both her parents and 1 sister (S1), whose electrophoresis patterns were heterozygous

(469 and 261 bp), carried 1 deleted allele. The other sister (S2), whose pattern was a single 469-bp band, was presumed to have 2 wild type alleles (Fig. 3A).

To confirm that the deletion in the *ARSB* mRNA could also be detected at the genomic DNA level, we performed qPCR using the DNA collected from the patient, her parents, her sisters, and healthy controls. Consistently, no signal was detected when amplifying the *ARSB* exon 4 using genomic DNA from the proband (Fig. 5), while 1 copy was detected using genomic DNA from the parents and sister S1, and 2 copies were detected using genomic DNA from sister S2. We also checked for the ability to amplify exon 3 and exon 5 of the *ARSB* gene, which are located in the nearby upstream and downstream regions, respectively, of the deleted exon, and we found that copy number remained the same in every sample (Fig. 5).

When the patient's diagnosis was confirmed as MPS VI, she received ERT with rhASB (1.0 mg kg<sup>-1</sup> week<sup>-1</sup> intra-

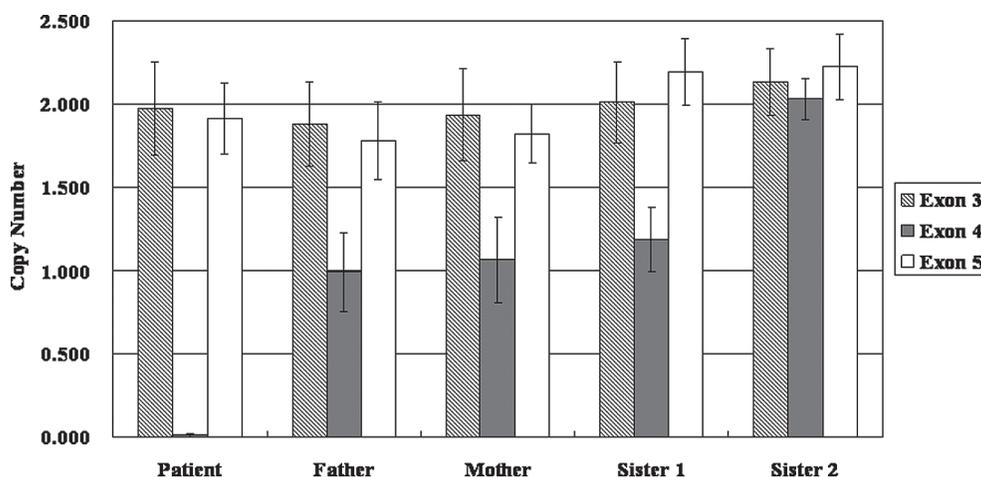


Fig. 5. Analysis of *ARSB* copy number in genomic DNA from all members of the MPS VI family. Copy number of *ARSB* was determined for exons 3-5 using qPCR. Exon 4 was undetectable in the patient; only 1 copy was detected in each parents and 1 sister (S1), and 2 copies were detected in the other sister (S2). A copy number change was not detected in either exon 3 or exon 5 in any sample, implying that these 2 exons were normal. Each bar represents the mean  $\pm$  s.d. of 3 independent experiments.

venously). After 3 years of treatment and a follow-up visit, her urine GAG levels had reduced from 593.51 mg-GAG g-creatinine<sup>-1</sup> to 34.01 mg-GAG g-creatinine<sup>-1</sup>. Her weight and height were 11.5 kg (< 3 percentile weight curve) and 87.5 cm (< 3 percentile growth curve of the Taiwanese population), respectively, at the age of 4 years and 7 months. She underwent acetabuloplasties by osteotomy for bilateral hip dysplasia and dislocation at the ages of 2 and 2.5 years. Measures of function also improved, with her disability index score falling from 2.5 to 0.75. She was able to walk for more than 30 min, jump, and pick up 10 coins easily. Her range of shoulder motion was roughly between 90 and 135 degrees. These improvements all suggest that she responded to ERT.

At present, more than 140 mutations in the *ARSB* gene have been reported. However, only 2 cases of large deletion have been reported, and both involved deletions of exon 5 (Arlt et al. 1994; Villani et al. 2010). To our knowledge, the exon 4 deletion described here has not been reported previously. The PCR primers that we used to screen for the exon 4 deletion amplified 639 bp flanking the *ARSB* exon 4 region (from IVS3 -118 to IVS4 +312). Neither PCR nor qPCR produced a signal for exon 4 when using genomic DNA from the patient studied; alternative splicing was not detected at the RNA level. We expect that the break/junction point for this deletion is located within the intron regions that flank exon 4. However, because exons 3 and 5 are nearly 78 kb apart (Fig. 2), a more elaborate experiment or sequencing procedure would have to be performed in order to detect the correct break/junction point.

In the *ARSB* gene, several polymorphisms have already been described (Karageorgos et al. 2007; Litjens and Hopwood 2007), and 8 polymorphisms located in the coding region, including c.246G/A (p.L82L), c.342C/T

(p.I114I), c.370C/T (L124L), c.972A/G (G324G), c.1072G/A (p.V358M), c.1126G/A (p.V376M), c.1191A/G (p.P397P), and c.1515C/T (p.Y505Y), were analyzed in this study. In our previous study, only c.1072G/A (p.V358M) and c.1191A/G (p.P397P) polymorphisms were detected in Taiwanese MPS VI families (Lin et al. 2008). In this family, all 8 polymorphisms were present as homozygous alleles in the patient and her family members, and the indicated haplotype was G-T-T-G-G-G-G-C. The homozygosity for these 8 polymorphisms is likely the result of their consanguineous relationship and the reduced heterozygosity in the *ARSB* gene region within the Taiwanese population.

PCR-directed DNA sequencing is an effective method for mutation analysis. However, if the mutations are large deletions or insertions in 1 allele, they easily can be missed during this analytic procedure. Southern hybridization and fluorescence *in situ* hybridization are alternative methods for detecting large deletion/insertion mutations, but these techniques usually are time consuming. Currently, qPCR is the standard method used in most laboratories. Combined with fluorescent probes, qPCR allows fast and highly-sensitive detection of copy number variations (Shi et al. 2002; Ceulemans et al. 2012). As we observed in this study, if mutations are not detected when using a standard PCR-directed DNA sequencing method, qPCR and RT-PCR should be performed to determine whether any large deletion or insertion is present within the target gene.

Between 2000 and 2011, 2,626,292 babies were live-born in Taiwan, and a total of 3 MPS VI patients were identified (the first 2 patients have previously been reported (Lin et al. 2008)). The live birth incidence of MPS VI in Taiwan over that period, therefore, was approximately 1 in 875,000. ERT has been proven effective at reducing MPS VI symptoms (Lin et al. 2010; Furujo et al. 2011), and the total cost of ERT is now covered by the Taiwanese government. For

this reason, it is crucial to diagnose this now-treatable metabolic disease early and to begin effective therapy before harmful and irreversible complications occur. Urine GAG 2D-electrophoresis and a blood ARSB enzyme assay can help to identify MPS VI patients quickly. Meanwhile, understanding the mutation profile of MPS VI patients improves carrier detection, and aids in genetic counseling and prenatal diagnosis, all of which may help to minimize the impact of this disease on families and society.

### Conclusions

This study extended the spectrum of *ARSB* mutations in MPS VI patients, uncovering an unusual deletion mutation within the *ARSB* gene. DNA and RNA analyses are necessary for the identification of such mutation. Our patient was administered ERT at an early stage and responded well to treatment in terms of both skeletal development and milestone progression. In addition, *ARSB* gene profiling may be useful for genetic counseling of families affected by MPS VI.

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### Conflict of Interest

The authors declare no conflict of interest.

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