Taiwan National Newborn Screening Program by Tandem Mass Spectrometry for Mucopolysaccharidoses Types I, II, and VI

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Objective To evaluate the initial cutoff values, rates of screen positives, and genotypes for the large-scale newborn screening program for multiple mucopolysaccharidoses (MPS) in Taiwan.

Study design More than 100 000 dried blood spots were collected consecutively as part of the national Taiwan newborn screening programs. Enzyme activities were measured by tandem mass spectrometry from dried blood spot punches. Genotypes were obtained when a second newborn screening specimen again had a decreased enzyme activity. Additional clinical evaluation was then initiated based on enzyme activity and/or genotype.

Results Molecular genetic analysis for cases with low enzyme activity revealed 5 newborns with pathogenic alpha-L-iduronidase mutations, 3 newborns with pathogenic iduronate-2-sulfatase mutations, and 1 newborn was a carrier of an arylsulfatase B mutation. Several variants of unknown pathogenic significance were also identified, most likely causing pseudodeficiency.

Conclusions The highly robust tandem mass spectrometry-based enzyme assays for MPS-I, MPS-II, and MPS-VI allow for high-throughput newborn screening for these lysosomal storage disorders. Optimized cutoff values combined with second tier testing could largely eliminate false-positive results. Accordingly, newborn screening for these lysosomal storage disorders is possible. *(J Pediatr 2019;205:176-82).*

Mucopolysaccharidoses (MPSs) are a group of rare, inherited metabolic disorders that result from deficiency of specific enzymes responsible for the degradation of glycosaminoglycans (GAG) present in lysosomes.[1] The accumulation of GAGs causes progressive damage, which affects patient appearance, physical abilities, organ function, and mental development.[2] MPS types I, II, and VI are associated with deficiencies in alpha-L-iduronidase (IDUA), iduronate-2-sulfatase (IDS), and *N*-acetylgalactosamine-4-sulfatase (or arylsulfatase B), respectively.[3,4] Early initiation of enzyme replacement therapy (ERT) has shown clinical benefit; thus, the US Food and Drug Administration has approved ERT products for MPS-I, MPS-II, MPS-IVA, MPS-VI, and MPS-VII diseases.[5] ERT can stabilize the condition and prevent disease progression.[6] Other therapeutic modalities such as hematopoietic stem cell transplantation and gene therapy have been reported to be beneficial.[7] Clinical trials and recent reports have emphasized that early intervention may prevent irreversible pathology, avoid or significantly minimize disease manifestations, and improve long-term outcomes.[8,9]

MPSs have been incorporated into newborn screening panels. In 2016, MPS-I was added to the Recommended Uniform Screening Panel for newborn screening in the US.[10] In addition to the current pilot newborn screening for lysosomal storage diseases, a retrospective epidemiologic survey in Taiwan revealed that MPS-II had the highest calculated birth incidence (52% of all MPS cases diagnosed), followed by MPS-III (19%), MPS-IV (16%), and MPS-VI (7%).[11] In other Asian countries, there is also a relatively higher incidence of MPS-II compared with other types of MPSs.[12]

Different methods have been considered for newborn screening of MPSs including measurement of lysosomal enzymatic activity,[13,14] lysosomal enzyme abundance, and MPS biomarker quantification.[15,16] Tandem mass spectrometry (MS/MS) and fluorometry techniques for direct assay of lysosomal enzymatic activity in dried blood spots (DBSs) have emerged as the most studied approaches.[17] The fluorometry method using DBS for enzyme assays has been successfully developed to diagnose MPSs by using a fluorescent artificial substrate.[18-20] Using the MS/MS technology, several lysosomal enzymatic activities can be determined when multiple substrates and internal standards are combined

DBS	Dried blood spot
ERT	Enzyme replacement therapy
GAG	Glycosaminoglycans
IDS	Iduronate-2-sulfatase
IDUA	Alpha-L-iduronidase
MPS	Mucopolysaccharidose
MS/MS	Tandem mass spectrometry
VOUS	Variants of uncertain significance

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into a single buffer.[21] First-generation multiplexed assays were developed for 6 lysosomal storage disease lysosomal storage disorders, including Pompe, Fabry, Gaucher, Niemann–Pick type A/B, Krabbe disease, and MPS-I.[22] Furthermore, the second-generation multiplexed assays for MPS-II, MPS-IIIB, MPS-IV, MPS-VI, MPS-VII, and type 2 neuronal ceroid lipofuscinosis have been developed recently.[23] Our group have used MS/MS multiplex technology for large scale screening for lysosomal storage disorders since 2010.[24,25] Herein we report the population-based screening by the MS/MS method for MPS-I, MPS-II, and MPS-VI diseases.

Methods

For the large-scale newborn screening program in Taiwan, informed consent was obtained from subjects' parents whose children were enrolled in the MPS study. Live screening of MPS-I, MPS-II, and MPS-VI was started on August, 2015. Until August, 2017, 130 237 newborns were enrolled in the MPS-I study. For MPS-II and MPS-VI, the reporting timeframe was divided into 2 periods due to different cutoffs being used. From August to December 2015, 28 799 newborns were enrolled, and 101 376 were enrolled from January 2016 to August, 2017.

Enzyme Activity Test

Newborn DBS were collected and shipped within 3 days of birth at ambient temperature. DBS were submitted to MPS analysis on the day of arrival in the newborn screening laboratory. MS/MS assay for MPS-I (IDUA) activity was carried out by using the previously reported flow injection MS/MS method, which was combined with Fabry, Pompe, and Gaucher screening.[26] The enzyme activities of MPS-II (IDS) and MPS-VI (arylsulfatase B) in DBS were carried out by a liquid chromatography MS/MS method as described.[23] We used the blank (filter paper without blood), low (enzyme activity below cutoff value), medium, and high (enzyme activity above cutoff value) controls from the Centers for Disease Control and Prevention for each run for validation.

Establishment of Reference Ranges

For MPS-I, the cutoff value was adopted form our previous work, which was 3 μmol/L/h (approximately 25% of the mean activity).[24] Six clinical confirmed patients with MPS-I enzyme activity fell under the cutoff value; 5 of them (0.17-0.73 μmol/L/h) could be clearly distinguished, and the remaining one was a patient with mild form and had an enzyme activity around 20% of mean activity (2.7 μmol/L/h, the outlier dot in the **Figure**, A).

For MPS-II, enzyme activities from DBS of >3000 anonymous newborns and 14 clinical confirmed patients with MPS-II were measured. Thirteen patients with MPS-II have enzymatic activities ranging from 0.02 to 0.38 μmol/L/h (<2% of mean activity; mean activity, 21.75 μmol/L/h). One patient, who was under ERT treatment, had an enzyme activity of around 20% of the mean activity (4.1 μmol/L/h, the outlier dot in the **Figure**, B). Because the contribution of ERT to total enzyme activity is not known, we used a conservative cutoff value (30% of mean activity; 6.5 μmol/L/h) for both the first and second DBS in the beginning of our study. Later, we adjusted the cutoff value from 30% to 10% of mean activity for the second DBS (**Table I**).

For MPS-VI, enzyme activities from DBS of >3000 anonymous newborns and 4 clinical confirmed patients with MPS-VI were measured. The enzyme activities of 3 patients with MPS-VI (0.01-4.30 μmol/L/h) could be clearly distinguished from the normal newborns (average, 61 μmol/L/h); however, 1 patient with MPS-VI receiving ERT had an enzymatic activity around 25% of mean activity (15.9 μmol/L/h; the outlier dot in the **Figure**, C). For the same reason as in the case of MPS-II, a conservative cutoff value of 21 μmol/L/h (30% of mean activity) was then used for the first and the second DBS in the beginning of the study and was also adjusted for the second DBS later, from 30% to 20%.

Screening Algorithm

After screening, DBS with enzyme activity under the preset cutoff value were retested using 2 additional punches from the same DBS. Newborns with an average enzyme activity lower than

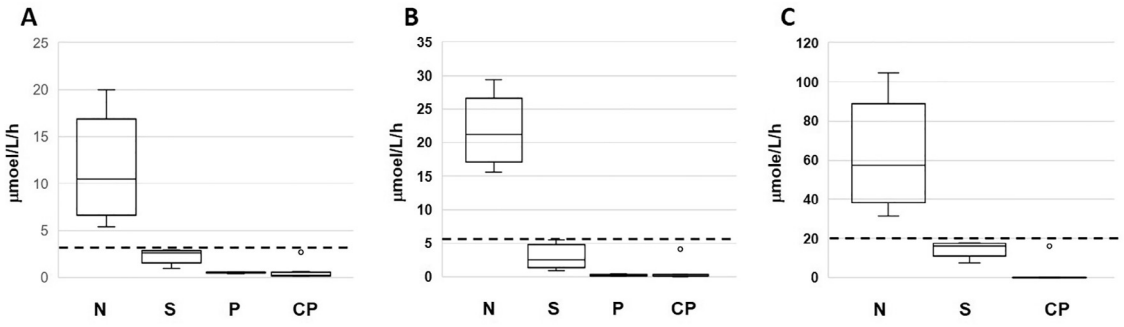

Figure. Overview of the enzyme activities from the first DBS in MPS-I, -II, and -VI screening. **A,** MPS-I (IDUA). **B,** MPS-II (IDS), **C,** MPS-VI (ARSB). *CP*, Patients found from clinics with or without ERT served as positive controls; *N*, normal population; *P*, patients found from newborn screening and confirmed with extremely low enzyme activity and/or elevated GAG disaccharide; *S*, range of enzymatic activities for all cases with a below cutoff value for the first DBS. Error bars are 5th and 95th percentiles. The open circles are for confirmed affected patients with or without ERT. Top and bottom of the box represent the 75th and 25th percentiles. The line through the box is the median. The dotted lines are the screening cutoff values.

the screening cutoff value were recalled, and the second DBS was generated. For newborns with results consistently below the cutoff values, genotyping was performed. DNA was isolated from DBS using the alkaline analysis method.[27] Genetic mutation assays were performed by direct exon and exon/intron boundary sequencing. Each amplicon was generated by using appropriate primers for the polymerase chain reactions.[28-30] Suspicious cases with genetic variants or with consistently low enzyme activities were referred to Mackay Memorial Hospital. The percentage of referred cases is defined as the referred rate. Leukocyte enzyme activity,[31,32] urine GAG dimethylmethylene blue (DMB) examination, 2-dimensional electrophoresis,[33,34] urine GAG disaccharide MS/MS analysis,[35] physical examination,[36,37] and abdominal ultrasonography were performed.

Results

MPS-I

In the MPS-I (IDUA) live screening, 130 237 newborns joined this program, and 120 newborns (0.09%) had enzyme activities lower than the cutoff value and were recalled for a second DBS (**Table I**). Most of the cases were ruled out based on the normal enzyme activities from their second DBS. This left only 5 newborns (0.004%) with decreased enzyme activity from both DBSs; DNA analysis was then carried out for these newborns. All 5 cases were found to have compound heterozygous mutations. They were then advised by certified genetic counselors and referred to the hospital. Although the parents of case 4 refused further analysis, the other 4 suspected newborns (cases 2 and 3 are twins and cases 1 and 5 are siblings) underwent further examinations.

In the *IDUA* mutation analysis, 6 variants were detected, including 1 intronic splicing mutation c.300-3C>G, 4 missense variants (c.1037T>G, c.1079T>G, c.1091C>T, and c.1874A>C), and 1 deletion (c.1359_1384del) (**Table II**). Mutations c.300-3C>G, c.1037T>G, and c.1091C>T were reported as pathogenic mutations from previous databases.[38,39] Variants c.1079T>G, c.1359_1384del, and c.1874A>C have not been reported previously. Based on the very low enzyme activities (<5% of the mean activity), mutation analysis and/or the significantly high GAG disaccharide level (**Table II**), we believed all of these 5 cases are affected patients with MPS-I; however, on follow-up none of them have developed any obvious clinical symptoms at ≤2 years of age. Thus, these attenuated MPS-I cases will require long-term follow-up.

MPS-II

From August to December 2015, 56 newborns were recalled out of 28 799 participants, and genetic sequencing was performed on 53 newborns with low enzyme activities from their recalled DBS. Among these newborns, 16 cases carry the c.301C>T variant, 18 cases carry the c.1499C>T variant, 16 cases have the linked variants of intronic variant c.103 + 34_56 dup and missense variant c.851C>T, and 1 case has c.890G>A. The range of DBS enzyme activities from newborns with c.301 C > T was 11%-25% of mean activity. Because variant c.301C>T was previously reported as a benign polymorphism,[40] we did not refer these cases. Variants of c.1499C>T, c.103 + 34_56 dup, and c.851C>T have unknown pathogenic significance. A total of 34 patients with these variants were referred to the hospital for further examinations, including leukocyte enzyme activity, urine GAG disaccharide analysis, and physical examination. For c.1499C>T, leukocyte enzyme activity and GAG disaccharides from all suspicious cases were in the normal range; thus, this variant is suggested to be nonpathogenic (**Table II**), and newborns harboring this variant will not be referred hereafter. For all of the cases with the linked variants (c.103 + 34_56dup and c.851C>T), urinary GAG disaccharides were in the normal range, but the leukocyte enzyme activities were lower than the reference range (3.2 nmol/h/mg protein; **Table II**). (Variant c.890G>A is discussed elsewhere in this article.)

From January 2016 to August 2017, of the 101 376 screened newborns, 184 were recalled for a second DBS; genotyping was performed on 96 of them with enzyme activities of <10% of the mean. Among these, 50 cases were found to have the c.1499C>T variant and 38 cases have the linked variants (c.103 + 34_56 dup and c.851C>T). All 38 cases with the linked variants were referred to hospitals for follow-up. Although leukocyte enzyme activity was below the cutoff, the urinary GAG and physical examinations were normal. These results suggest that the identified variants cause pseudodeficient IDS activity (see Discussion).

Six additional variants of uncertain significance (VOUS) were detected from 9 cases and first reported in our study. They are c.311A>T, c.589 C > T, c.817C>T, c.890G>A, c.1025A>G, and c.1478G>A (**Table II**). One case with c.890G>A and 4 cases with

Table I. Cutoff values for MPS-I, II, and VI and the number of screen positive and confirmed cases

	MPS-I	MPS-II		MPS-VI	
Period	August 2015-August 2017	August 2015-December 2015	January 2016-August 2017	August 2015-December 2015	January 2016-August 2017
No. of newborns	130 237	28 799	101 376	28 799	101 376
Cutoff for first DBS (% of mean)*	25	30	30	30	30
Cutoff for second DBS (% of mean)†	25	30	10	30	20
No. of recalled cases (%)*	120 (0.09)	56 (0.19)	184 (0.18)	35 (0.12)	141 (0.14)
No. of referred cases (%)†	4 (0.03)	35 (1.22)	46 (0.45)	1 (0.03)	1 (0.02)
No. of confirmed cases (%)	4 (0.03)	0	3 (0.03)	0	0

*All the cases with enzyme activity below the cutoff value from the first DBS would be recalled for the second DBS.
†All the cases with enzyme activity below the cutoff value from the second DBS and/or positive results with genetic analysis were referred to the hospital for further confirmation.

Table II. Referred and/or confirmed MPS-I, -II, and -VI cases

	Variants	Sex	n	DBS enzyme activity*		WBC enzyme activity	Urine DMB GAG ratio	Urine disaccharide level			Clinical decision	Ref. of variants
				μmol/L/h	% of mean	nmol/h/mg protein	mg/mmol creatinine	DS (μg/mL)	HS (μg/mL)	KS (μg/mL)		
MPS-I screening												
MPS-I_case 01	c.300-3C>G c.1874A>C, p.Y625S	Male	1	0.42	3.50	0.4	107	10.9	2.67	7.75	Confirmed	Pathogenic mutation Novel variant
MPS-I_case 02	c.1037T>G, p.L346R; c.1091C>T, p.T364M	Female	1	0.59	4.92	0.2	204.7	99.60	13.31	0.83	Confirmed	Pathogenic mutation Pathogenic mutation
MPS-I_case 03	c.1037T>G, p.L346R; c.1091C>T, p.T364M	Female	1	0.51	4.25	0.2	239.3	41.04	2.93	0.00	Confirmed	Pathogenic mutation Pathogenic mutation
MPS-I_case 04	c.1079T>G, p.F360C c.1359_1384del, p.S453Rfs*47	Male	1	0.54	4.50	Refused follow-up	—	—	—	—	—	Novel variant Novel variant
MPS-I_case 05	c.300-3C>G c.1874A>C, p.Y625S	Female	1	0.56	4.50	0.1	65.7	4.76	3.53	0.17	Confirmed	Pathogenic mutation Novel variant
Cutoff value				>4	>25	>1.7	<68.3	<0.80	<0.41	<17.8		
MPS-II screening												
Non–MPS-II cases 01-54	c.103 + 34_56dup c.851C>T, p.P284L	53M1F	54	0.58-1.94	2.61-8.92	0.3-2.0	21.4-74.1	0-0.64	0-0.54	0-7.51	Normal, keep follow-up	Likely benign polymorphism Likely benign polymorphism
Non–MPS-II cases 55-72	c.1499C>T, p.T500I	Male	18	1.53-2.92	7.03-13.90	3.3-8.6	39.4-62.4	0-0.38	0-0.04	0-5.80	Normal	Benign polymorphism
Non–MPS-II cases 73-76	c.1478G>A, p.R493H	Male	4	0.85-1.52	3.91-6.99	3.2-9.9	22.5-68.4	0-0.38	0-0.1	0-2.7	Normal, keep follow-up	Novel variant
Non–MPS-II case 77	c.890G>A, p.R297H	Male	1	1.14	5.24	2.3	69.7	0.08	0.04	3.47	Normal, keep follow-up	Novel variant
Non–MPS-II case 78	c.589C>T, p.P197S	Male	1	2.05	9.43	2.0	63.8	0.38	1.46	6.35	Normal, keep follow-up	Novel variant
MPS-II_case 01	c.817C>T, p.R273W	Male	1	0.11	0.51	0.1	77.3	7.40	1.83	6.13	Confirmed	Novel variant
MPS-II_case 02	c.1025A>G, p.H342R	Male	1	0.44	2.02	0.1	70.9	21.20	12.10	6.47	Confirmed	Novel variant
MPS-II_case 03	c.311A>T, p.D104V	Male	1	0.20	0.92	0.1	114.0	15.60	103.40	1.40	Confirmed	Novel variant
Cutoff value				>6.5	>30	>3.2	<68.3	<0.8	<0.41	<17.8		
MPS-VI screening												
Non–MPS-VI case 01	Not found	Male	1	17.79	28.81	4.1	44.4	0.20	0.10	2.50	Normal	—
Non–MPS-VI case 02	c.716A>G, p.Q293R	Female	1	18.49	29.90	4.9	35.8	0.09	0.02	3.50	Carrier, normal	Novel variant
Cutoff value				>21	>30	>3.5	<68.3	<0.80	<0.41	<17.8		

DMB, dimethylmethylene blue; *DS*, dermatan sulfate; *HS*, heparan sulfate; *KS*, keratan sulfate; *WBC*, white blood cells.
*Enzyme activities from the first DBS.

c.1478G>A had borderline leukocyte IDS enzyme activities in the range from 2.3 to 9.9 nmol/h/mg protein, and they all had urinary GAG disaccharides in the normal range. One case with c.589 C > T had a decreased leukocyte enzyme activity of 2.0 nmol/h/mg protein, and the level of heparan sulfate was slightly elevated (1.46 μg/mL) compared with the cutoff value (<0.41 μg/mL). All 6 of these newborns were diagnosed as normal at the moment. However, harboring VOUS, they will continue to be followed every 3-6 months for the next several years.

Newborns with c.311A>T, c.817C>T, or c.1025A>G had extremely low enzymatic activities of <3% of mean in both DBS and leukocytes. Urinary heparan sulfate (7.4-21.2 μg/mL) and dermatan sulfate (1.8-103.4 μg/mL) were significantly elevated in these cases compared with reference cutoff (<0.8 μg/mL for heparan sulfate and <0.41 μg/mL for dermatan sulfate). These 3 patients have received a diagnosis of MPS-II, but are so far asymptomatic.

MPS-VI

A total of 131 075 newborns joined this program, and 176 (0.13%) were recalled for a second DBS. From August to December 2015, 1 case was detected with an enzyme activity of <30% of the mean activity from the first and the second DBSs (17.8 and 11.8 μmol/L/h, respectively). Genotype analysis revealed no variations. During the second period, another newborn was identified with low enzyme activity for both the first and second DBS (18.5 and 12.0 μmol/L/h, respectively). Genotyping revealed 1 novel variant c.716A>G. These 2 cases were referred to the hospital; leukocyte enzyme activity and urinary GAG disaccharides were all in the normal range. Thus, no confirmed patients with MPS-VI were identified in our study.

Discussion

In our initial pilot studies described herein, we collected several confirmed MPS cases with or without ERT as our positive controls. Because the collection time was unavailable, the effect of residual enzyme from ERT on the DBS enzyme activity test could not be estimated. We used relatively conservative cutoffs to minimize any possibility of false-negative results.

In the case of MPS-II, the vast majority of newborns with enzymatic activities below the cutoff were false-positive results; thus, we plan to decrease our current cutoff values to lower ones in the future. This step is the typical order of events in newborn screening pilot studies. **Table III** summarizes the recall rates and the number of the second DBSs, which would have been requested as a function of the cutoff values for the first DBSs.

For MPS-I (IDUA), the referred rate was found to be 5 of 130 237 (3.8 per 100 000 live births), including a pair of twins and a pair of siblings. All 5 newborns have mutations and/or urinary GAG levels consistent with the strong possibility to develop MPS-I disease; however, the patients are asymptomatic so far. Newborn screening laboratories in the US are reporting much higher screen positive rates for MPS-I. This markedly higher screening positive rate is in part due to the prevalence of a relatively high pseudodeficiency in the US population and false-positive findings (decreased enzyme activity in DBS but normal leukocyte enzyme activity). The Illinois NBS laboratory later modified their method and lowered their cutoff value. In our study, the screening cutoff value could be optimized from 25% of the mean to 10%. The recall rate would then be lowered from 0.92% to 0.05% (**Table III**).

Table III. Optimum cutoff values for first DBS and the corresponding recall rate (%)

Cut offs (% of mean activity)	Recall number	Recall rate (%‰)
MPS-I screening number: 130 237		
25	120	0.92
15	10	0.08
10	7	0.05
MPS-II screening number: 130 175		
30	240	1.84
20	203	1.56
10	102	0.78
10 + hot spot variants sequence	9	0.07
MPS-VI screening number: 130 175		
30	176	1.35
20	36	0.28
10	3	0.02

For MPS-II (IDS) screening, we started our study with a very conservative cutoff value of 30% of the mean activity. Later, we adjusted the cutoff to 10% of the mean activity for the second DBS because all confirmed patients with MPS-II had activity below this value except the one who was under ERT. With an optimum cutoff of 10% of the mean activity for the first and second DBSs, the recall rate was at most 102 per 100 000 (**Table III**), and the positive rate (number need to be referred) was 49 per 100 000. However, it is noticeable that, even with the optimum cutoff value, this is still a relatively large number compared with the results recently obtained in the MPS-II pilot NBS study in the Washington NBS laboratory, showing a screening positive rate of 7.5 per 100 000.[41] Because full exon sequencing of the IDS gene was carried out on all DBS that were below the cutoff, we realized the relatively high rate of the screening positives for MPS-II was mainly due to common VOUS in the Taiwanese population.

Variants c.301 C > T, c.1499C>T, and the linked variants were widely found in *IDS* mutation analysis in our population. The range of DBS enzyme activity of the c.301 C > T variant is from 11.1% to 25.3% of the mean. According to a previous report, in vitro functional assays showed that the c.301 C > T containing construct expressed high activity (97% ± 9% of the wild-type activity). Thus, the c.301 C > T variant is likely to be a rare polymorphism.[40] The range of DBS enzyme activities of the c.1499C>T variants is from 7.0% to 13.4% of mean. The allele count of the c.1499C>T from the East Asia population is 8 in 6638 (0.12%) (data obtained from the ExAC browser). All cases carrying c.301 C > T or c.1499C>T variant have normal leukocyte enzyme activities, urine GAGs, and urine disaccharide levels. Hence, these 2 variants are now defined as nonpathogenic variants.

Fifty-four cases expressing both low DBS and leukocyte IDS enzyme activities were found with the linked variants. The

linked variants include one intronic variant c.103 + 34_56dup and 1 missense variant c.851C>T. All of these 54 cases also carry one silent variant c.684A>G and 1 polymorphism variant c.1180 + 184T>C. The minor allele frequencies of c.103 + 34_56dup, c.684A>G, c.851C>T and c.1180 + 184T>C from the East Asia population are 5 in 1008 (0.5%), 10 in 6638 (0.15%), 10 in 6637 (0.12%), and 207 in 1008 (28%), respectively (from the ExAC browser and the Single Nucleotide Polymorphism database, [dbSNP]). Because variants c.103 + 34_56 dup and c.1180 + 184T>C are located in introns, and c.684A>G is a silent variant, we believe that the low enzyme activity results from the variant c.851C>T.

Variant c.851C>T has also been reported in Japan.[41] The structural modeling of IDS with p.P284L (c.851C>T variant) suggests a minor structural change occurring on the molecular surface without a predicted structural modification in the active site of IDS. In the Japanese study it was suggested that the mutated enzyme is deficient, low but finite residual activity of this enzyme may be sufficient to prevent GAG buildup *in vivo*. The latter suggestion is supported by the fact that all of the newborns with these linked variants had normal physical examination results, normal urinary-GAGs and disaccharide levels after more than two years of following-up. Furthermore, pedigree studies from the newborns and their relatives were performed. One of the probands' great-grandfather (in his nineties) with the linked variants was found; and he had normal physical examination results, normal urinary-GAGs and disaccharide levels. Together, the high incidence (54 per 130 174 or one per 2400) and family study imply that the linked variants are non-pathogenic. However, further comprehensive family studies are needed to fill the gap of knowledge.

For MPS-VI (ARSB) screening, we also adopted a very conservative cutoff of 30% of mean activity based on the level of activity seen in patients with confirmed MPS-VI disease. All of the patients had <10% mean activity, except the patient already on ERT. In the future, we plan to lower the cutoff to 10% of mean activity as we did for MPS-II, and this would give a recall rate of 2 per 100 000 (**Table III**). The data is comparable with the screening positive rate of 6 per 100 000 from the pilot study for MPS-VI in the Washington state NBS laboratory.[42]

In addition to specific enzyme analyses, biomarker assay has also been regarded as one of the methods for newborn screening and diagnosis.[17,43] GAG and GAG-disaccharides are putative markers for MPS diseases progression and can be measured in DBS and urine samples.[44] The finding of low MPS enzyme activity combined with significantly elevated disaccharides suggests early onset type of MPS diseases and helps evaluation for treatment. Assays using MS/MS have also been recently established to measure GAG-disaccharides in DBS of patients with MPS and mucolipidoses.[45] Disaccharide determination as a second tier test performed on samples with low enzyme activity could be accomplished in a newborn screening laboratory using existing equipment.[46] Presumably, when enzymatic activity is low in the first-tier MS/MS test in DBS, an elevated GAG-disaccharides result as a second-tier test in DBS would mark an affected patient rather than a false positive. The feasibility of measuring DBS disaccharides as a first-tier newborn screening method is still controversial due to the higher false positive rate (0.03-0.9%) found in a recent pilot study for MPS-I, -II, and -III by measuring GAG-disaccharides by LC-MS/MS.[16]

The studies reported herein show that newborn screening for MPS-I, -II, and -VI in Taiwan by MS/MS is feasible. The rate of false positives is low for MPS-I and MPS-VI, but higher for MPS-II. In an Asian population, the high frequency of psuedodeficient-like alleles in MPS-II (IDS) gene gives rise to a high-false positive rate, which highlights a need for a second-tier test in the screening system. In this study, if GAG disaccharides was used as the second tier test, the false-positive rate would decrease remarkably, because those with the linked variants would not be referred. However, cases with VOUS (c.1478G>A. c. 890G>A, and c. 589C>T; **Table II**) would, therefore, not be picked. In contrast, if genotype was in use, all the false-positive cases would be identified and not be referred, yet cases with VOUS would be kept. The optimum cutoff value coupled with specific genotype analysis for pseudodeficiency-like variant can be used to identify real MPS cases and rule out the false positive cases owing to the low enzyme activity-causing variants. ■

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