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

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Abstract

Objective: The first live and large-scale newborn screening program of multiple mucopolysaccharidoses (MPS) was developed in Taiwan. The initial cutoff values, rates of screen positives, and genotypes were evaluated.

Study Design: More than 100,000 dried blood spots (DBSs) were collected consecutively as part of the national Taiwan newborn screening programs. Enzyme activities were measured by tandem mass spectrometry (MS/MS) from DBS punches. Genotypes were obtained when a second newborn screening specimen again had a reduced 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 IDUA mutations, 3 newborns with pathogenic IDS mutations, and one newborn was a carrier of an ARSB mutation. Several variants of unknown pathogenic significance were also identified, most likely causing pseudodeficiency.

Conclusions: The highly robust MS/MS-based enzyme assays for MPS I, MPS II and MPS VI allow for high throughput newborn screening for these lysosomal storage disorders (LSDs). Optimized cutoff values combined with second tier testing could largely eliminate false positive results. Accordingly, newborn screening for these LSDs is possible.

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Keywords

Mucopolysaccharidosis; lysosomal storage disease; newborn screening; tandem mass spectrometry; inborn errors of metabolism

Introduction

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). Accumulation of GAGs causes progressive damage, which affects patient's appearance, physical abilities, organ function and mental development (2). Seven different clinical types and numerous subtypes of the MPSs have been identified. 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, ARSB), respectively (3, 4). Early initiation of enzyme replacement therapy (ERT) has shown clinical benefit in patients with these MPSs. The US Food and Drug Administration approved ERT products are available for several MPS including 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 outcome (8, 9). For all of these reasons, 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 USA (10). In addition to the current pilot newborn screening for lysosomal storage diseases, a retrospective epidemiological 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 to 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 (DBS) 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 into a single buffer (21). First generation multiplexed assays were developed for six LSDs, 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 (NCL2) have been recently developed (23). Our group have used MS/MS multiplex technology for large scale screening for LSDs since 2010 (24, 25). Here we report the population-based screening by the MS/MS method for MPS I, MPS II, and MPS VI diseases.

Methods

Study population

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, 130237 newborns were enrolled in the MPS-I study. For MPS-II and MPS-VI, the reporting timeframe was divided into two periods due to different cutoffs being used. From August to December, 2015, 28799 newborns were enrolled, while 101376 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 (ARSB) in DBS were carried out by LC-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 CDC for each run for validation (30, 31).

Establishment of reference ranges

For MPS-I, the cutoff value was adopted from our previous work, which was $3\mu\text{mol/L/h}$ ($\sim 25\%$ of the mean activity) (20). Six clinical confirmed MPS-I patients' enzyme activity fell under the cutoff value; five of them ($0.17\text{--}0.73\mu\text{mol/L/h}$) could be clearly distinguished, while the remaining one was a mild-form patient and had an enzyme activity around 20% of mean activity ($2.7\mu\text{mol/L/h}$, the outlier dot in figure 1A).

For MPS-II, enzyme activities from DBS of more than 3000 anonymous newborns and 14 clinical confirmed MPS-II patients were measured. Thirteen MPS-II patients have enzymatic activities ranging from 0.02 to $0.38\mu\text{mol/L/h}$ ($<2\%$ of mean activity; mean activity: $21.75\mu\text{mol/L/h}$). One patient, who was under ERT treatment, had an enzyme activity around 20% of the mean activity ($4.1\mu\text{mol/L/h}$, the outlier dot in figure 1B). Since the contribution of ERT to total enzyme activity is not known, we employed a conservative cutoff value (30% of mean activity, $6.5\mu\text{mol/L/h}$) 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 1).

For MPS-VI, enzyme activities from DBS of more than 3000 anonymous newborns and 4 clinical confirmed MPS-VI patients were measured. The enzyme activities of 3 MPS VI patients ($0.01\text{--}4.3\mu\text{mol/L/h}$) could be clearly distinguished from the normal newborns (average $61\mu\text{mol/L/h}$); however, one MPS VI patient receiving ERT had an enzymatic activity around 25% of mean activity ($15.9\mu\text{mol/L/h}$, the outlier dot in figure 1C). For the same reason as in the case of MPS-II, a conservative cutoff value of $21\mu\text{mol/L/h}$ (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 pre-set cutoff value were retested using two additional punches from the same DBS. Newborns with an average enzyme activity lower than the screening cutoff value were recalled, and the second DBS was generated. For newborns with consistent below-cutoff results, 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 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 (0.09%) newborns had enzyme activities lower than the cutoff value and were recalled for a second DBS (Table 1). Most of the cases were ruled out based on the normal enzyme activities from their second DBS. This left only 5 (0.004%) newborns with decreased enzyme activity from both DBSs, DNA analysis was then carried out for these newborns. All five cases were found to have compound heterozygous mutations. They were then advised by certified genetic counselors and referred to the hospital. While the parents of case 4 refused further analysis, the other four suspected newborns (case 2 and 3 are twins, and case 1 and 5 are siblings) underwent further examinations.

In the *IDUA* mutation analysis, 6 variants were detected, including one intronic splicing mutation c.300–3C>G, four missense variants (c.1037T>G, c.1079T>G, c.1091C>T, c.1874A>C), and one deletion (c.1359_1384del) (Table 2). Mutations c.300–3C>G, c.1037T>G, 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 previously reported. Based on the very low enzyme activities (<5% of mean activity), mutation analysis, and/or the significantly high GAG-disaccharide level (Table 2), we believed all of these five cases are affected MPS-I patients; however, none of them being followed up have had any obvious clinical symptoms to date (up to two years of age). Thus, these attenuated MPS I cases will require long-term follow up.

MPS-II

In the period from August to December 2015, fifty-six 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 one case is with c.890G>A. The range of DBS enzyme activities from newborns with c.301 C>T was 11–25% of mean activity. Since 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 non-pathogenic (Table 2), and newborns harbor 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/Hr/mg protein, Table 2). (For variant c.890G>A see below)

From January 2016 to August 2017, 184 newborns, out of 101,376, were recalled for a second DBS; and genotyping was performed on 96 of them with enzyme activities lower than 10% of 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 these 38 cases with the linked variants were referred to hospitals for follow-up. Although leukocyte enzyme activity were below the cutoff, the urinary-GAG and physical examination 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 nine 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 2). One case with c.890G>A and four cases with c.1478G>A had borderline leukocyte IDS enzyme activities in the range from 2.3 to 9.9 nmol/Hr/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/Hr/mg protein, and the level of heparan sulfate was slightly elevated (1.46 ug/ml) compared to the cutoff value (<0.41 ug/ml). All six of these newborns were diagnosed as normal at the moment. However, harboring variants of unknown significance (VOUS), they will continue to be followed-up every three to six 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 ug/ml) and dermatan sulfate (1.8–103.4 ug/ml) were significantly elevated in these cases compared to reference cutoff (<0.8 ug/ml for heparan sulfate and <0.41 ug/ml for dermatan sulfate). These three 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, one case was detected with an enzyme activity lower than 30% of mean activity from the first and the second DBSs (17.8 and 11.8 μmol/L/h). 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 $\mu\text{mol/L/h}$). Genotyping revealed one novel variant c.716A>G. These two cases were referred to the hospital; leukocyte enzyme activity and urinary GAG-disaccharides were all in the normal range. Thus, no confirmed MPS-VI patient were identified in our study.

Discussion

In our initial pilot studies described here, we collected several confirmed MPS cases with or without ERT as our positive controls. Since 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 negatives.

In the case of MPS-II, the vast majority of newborns with enzymatic activities below the cutoff were false positives, and thus we plan to decrease our current cutoff values to lower ones in the future. This is the typical order of events in newborn screening pilot studies. In Table 3, we summarize 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 out of 130,237 (3.8 per 100,000 live birth), 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 labs in the USA 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). 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 mean to 10%. The recall rate would then be lowered from 0.92‰ to 0.05‰ (Table 3).

For MPS-II (IDS) screening we started our study with a very conservative cutoff value of 30% of mean activity. Later, we adjusted the cutoff to 10% of mean activity for the second DBS as all confirmed MPS-II patients had activity below this value except the one who was under ERT. With an optimum cutoff of 10% of mean activity for the first and second DBS, the recall rate was at most 102 per 100,000 (Table 3), 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 to 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). As full exon sequencing of the IDS gene was carried out on all below-cutoff DBS, we realized the relatively large rate of the screening positives for MPS-II was mainly due to common variants of uncertain significance 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 mean. According to a previous report, *in vitro* functional assay showed that the c.301 C>T containing construct expressed high activity ($97 \pm 9\%$ 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 8/6638 (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 two variants are now defined as non-pathogenic 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 one missense variant c.851C>T. All of these 54 cases also carry one silent variant c.684A>G and one 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 5/1008 (0.5%), 10/6638 (0.15%), 10/6637 (0.12%), and 207/1008 (28%), respectively (from the ExAC browser and dbSNP). Since 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 (44). 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 2,400) 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 3). 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 (41).

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, 45). 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 mucopolidoses (46). 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 (47). 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 here show that newborn screening for MPS-I, -II, and -VI in Taiwan by MS/MS is feasible. The rate of false positives is very low for MPS-I and MPS-VI, but much higher for MPS-II. In Asian population, the high frequency of pseudodeficient-like alleles in MPS-II (IDS) gene gives rise to a high false positive rate, which highlights a need of second tier test in the screening system. In this study, if GAG-disaccharides was used as the second tier test, false positive rate would remarkably decrease, as those with the linked variants would not be referred. However, cases with variants of uncertain significance (c.1478G>A, c. 890G>A, and c. 589C>T, Table 2) would therefore not be picked. In the contrary, if genotype was in use, all the false positive case 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 due to the low-enzyme-activity-causing variants.

Abbreviations:

MPS	Mucopolysaccharidosis
GAG	glycosaminoglycans
DBS	dried blood spot
IDUA	alpha-L-iduronidase
IDS	iduronate-2-sulfatase
ARSB	arylsulfatase B
MS/MS	tandem mass spectrometry

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figure 1.

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.

Table 1:

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

Period	MPS I		MPS II		MPS VI	
	2015.08–2017.08	2015.08–2015.12	2016.01–2017.08	2015.08–2015.12	2016.01–2017.08	
Number of newborns	130,237	28,799	101,376	28,799	101,376	
cutoff for first DBS (% of mean) ¹	25	30	30	30	30	
cutoff for second DBS (% of mean) ²	25	30	10	30	20	
Number of recalled cases (%) ¹	120 (0.09)	56 (0.19)	184 (0.18)	35 (0.12)	141 (0.14)	
Number of referred cases (%) ²	4 (0.03)	35 (1.22)	46 (0.45)	1 (0.03)	1 (0.02)	
Number of confirmed cases (%)	4 (0.03)	0	3 (0.03)	0	0	

¹ All the cases with enzyme activity below the cutoff value from the first DBS would be recalled for the second DBS

² 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 2.

Referred and/or confirmed MPS I, II, and VI cases

	Variants	Gender	Number	DBS enzyme activity ¹		WBC enzyme activity	Urine DMB GAG ratio	Urine disaccharide level ²			Clinical decision	Ref. of variants
				(umole/L/Hr)	(% of mean)			DS (ug/ml)	HS (ug/ml)	KS (ug/ml)		
MPS I screening												
MPS I_Case 01	c.300-3C>G c.1874A>C, p.Y625S	M	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	F	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	F	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	M	1	0.54	4.50	Refused F/U	--	--	--	--	--	Novel variant Novel variant
MPS I_Case 05	c.300-3C>G c.1874A>C, p.Y625S	F	1	0.56	4.50	0.1	65.7	4.76	3.53	0.17	confirmed	Pathogenic mutation Novel variant
Cut-off 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 F/U	likely benign polymorphism likely benign polymorphism
Non-MPS II cases 55~72	c.1499C>T, p.T500I	M	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	M	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 F/U	Novel variant
Non-MPS II case 77	c.890G>A, p.R297H	M	1	1.14	5.24	2.3	69.7	0.08	0.04	3.47	Normal, keep F/U	Novel variant
Non-MPS II case 78	c.589C>T, p.P197S	M	1	2.05	9.43	2.0	63.8	0.38	1.46	6.35	Normal, keep F/U	Novel variant
MPS II_Case 01	c.817C>T, p.R273W	M	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	M	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	M	1	0.20	0.92	0.1	114.0	15.60	103.40	1.40	confirmed	Novel variant

Table 3.

Optimum cut-off value for first DBS and the corresponding recall rate (%)

MPS I screening number : 130,237			
Cut off (% of mean activity)	recall number	recall rate (%)	
25	120	0.92	
15	10	0.08	
10	7	0.05	
MPS II screening number : 130,175			
Cut off (% of mean activity)	recall number	recall rate (%)	
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			
Cut off (% of mean activity)	recall number	recall rate (%)	
30	176	1.35	
20	36	0.28	
10	3	0.02	