TABLE I. Summary of Patients with Paternal Uniparental Disomy for Chromosome 14

	This study			Reported patients	
	Patient 1	Patient 2	Patient 3	Full disomy	Seg. disomy
Age at description	5 6/12 years	Deceased (at 2 hr of age)	6 7/12 years	Fetus–9 years (n = 14)[c]	Not described (n = 1)[d]
Sex	Female	Male	Female	M:F = 4:9[e]	Male
Disomy region	Full	Full	14q23.3–qter	Full	14q12–qter
Disomy pattern	Isodisomy	Isodisomy	Isodisomy	I:H:M = 7:4:2[f]	Isodisomy
Polyhydramnios	+	+	+	12/12	+
Placental abnormality	+ (abruption)	+ (forelying)	–	1/1 (large)	+ (abruption)
Delivery	Caesarean	Caesarean	Vaginal	V:C = 4:3[g]	Caesarean
Premature delivery	+ (32 weeks)	+ (34 weeks)	+ (36 weeks)	8/9	+ (29 weeks)
APS at 1 and 5 min	1 and 6	1 and 6	6 and 7	4/6[h]	N.D.
Birth length	41.0 cm	45.7 cm (+0.6 SD)[a]	46.0 cm (–0.3 SD)[a]	...	25 centile
Birth weight	1.85 kg	3.17 kg (+2.5 SD)[a]	2.94 kg (+1.2 SD)[a]	...	95 centile
Birth HC	30.6 cm	35.5 cm (+2.5 SD)[a]	29.4 cm (–2.3 SD)[a]	...	80 centile
IUGR	...	–	–	1/7	–
Present height	102.1 cm (–1.7 SD)[b]	...	121.3 cm (+1.0 SD)[b]	...	N.D.
Present weight	15.1 kg (–1.4 SD)[b]	...	25.8 kg (+1.4 SD)[b]	...	N.D.
Present HC	51.1 cm (+0.4 SD)[b]	...	52.0 cm (+0.6 SD)[b]	...	N.D.
Growth failure	–	...	–	3/3	N.D.
Developmental retardation	+ (DQ = 50)	...	+ (DQ = 52)	6/6	+
Seizure	–	...	–	2/3	–
Frontal bossing	+	–	–	1/1	+
Hairy forehead	+	+	+	3/4	+
Blepharophimosis	+	–	+	9/9	+
Depressed nasal bridge	+	+	+	7/7	+
Anteverted nares	+	+	–	2/4	–
Small ears	±	+	–	6/6	+
Feeding difficulty	+	...	+	5/5	+
Protruding philtrum	+	+	+	8/8	+
Puckered lips	–	–	–	2/4	–
Micrognathia/retrognathia	+	+	+	5/6	+
Short/webbed neck	+	+	+	7/7	+
Laryngomalacia	–	...	+	1/1	N.D.
Small thorax	+	+	+	10/10	+
Mechanical ventilation	+	+	–	9/10	+
Lethal respiratory failure	–	+	–	4/13	–
Congenital heart disease	–	+ (ASD)	–	3/4[i]	ASD, VSD, PS
Abdominal wall defect	+ (diastasis recti)	+ (omphalocele)	+ (diastasis recti)	10/10[j]	+ (omphalocele)
Inguinal hernia	–	–	–	1/1	+
Kyphoscoliosis	+	–	+	1/1	+
Coxa valga	+	...	–	1/1	+
Short limbs	–	–	–	6/8	+
Long fingers	–	–	–	3/3	–
Joint contractures	+	–	+	6/7	–
Paternal age at birth	36	N.E.	24	...	34
Maternal age at birth	36	42	27	...	33
Paternal height	178 cm (+1.6 SD)[b]	N.E.	171 cm (+0.1 SD)[b]	...	...
Maternal height	153 cm (–1.0 SD)[b]	N.E.	155 cm (–0.6 SD)[b]	...	...

APS, Apgar score; HC, head circumference; IUGR, intrauterine growth retardation; SD, standard deviation; DQ, developmental quotient; ASD, atrial septal defect; VSD, ventricular septal defect; PS, pulmonary stenosis; N.E., not examined; and N.D., not described.

[a]Assessed by the gestational age- and sex-matched Japanese reference data from the Ministry of Health, Labor, and Welfare (http://wwwdbtk.mhlw.go.jp/toukei/); no data are available for neonates born at 32 weeks of gestational age.

[b]Assessed by the age- and sex-matched Japanese reference data [Suwa et al., 1992].

[c]Wang et al. [1991]; Papenhausen et al. [1995]; Walter et al. [1996]; Cotter et al. [1997]; Klein et al. [1999]; Berend et al. [2000]; Yano et al. [2001]; Kurosawa et al. [2002]; McGowan et al. [2002]; Offiah et al. [2003]; Chu et al. [2004]; and Stevenson et al. [2004].

[d]Coveler et al. [2002].

[e]Male: Female = 4:9; the sex is not described in one of the two patients reported by Berend et al. [2000].

[f]Isodisomy:Heterodisomy:Mixture of isodisomy and heterodisomy = 7:4:2; the type of disomy is unreported in the patient described by Offiah et al. [2003].

[g]Vaginal:Caesarean = 4:3; the delivery type is unreported in the remaining patients.

[h]APS at 1 min $\leq$ 3.

[i]ASD, VSD, and aortic anomaly are manifested by three different patients, respectively.

[j]All the 10 patients have diastasis recti, and omphalocele is absent in the 10 patients.

In the column summarizing the clinical features in 14 patients with full disomy reported in the literature, the denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature; thus, the differences between the denominators and the numerators denote the number of patients evaluated to be negative for that feature.

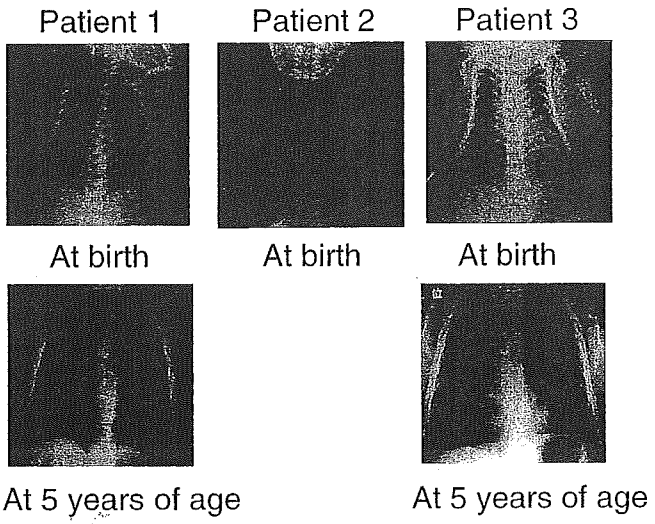
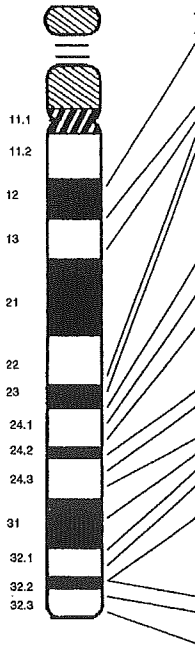


Fig. 1. Roentgenograms of Patients 1–3. The unique bell-shaped thorax with an arched appearance of the ribs is obvious in Patients 1–3 at birth, and the thoracic deformity has ameliorated in Patients 1 and 3 at 5 years of age.

chromosome 14 from a monosomic zygote (monosomy rescue), although loss of maternally derived chromosome 14 from a trisomic zygote (trisomy rescue) or fertilization between a disomic gamete and a nullisomic gamete (gamete complementation) with no preceding recombination at meiosis I might also be possible [Robinson, 2000]. This mechanism could also be applied to Patient 2. However, since karyotype was not examined in Patient 2, it is also possible that Patient 2 had a paternally derived chromosomal abnormality such as i(14q) often associated with upd(14)pat [Kotzot, 2001]. The segmental paternal isodisomy in Patient 3 with a normal karyotype would be explained by assuming mitotic recombination in the first zygotic division or in a very early postzygotic division, followed by loss of the normal cell lineages and reciprocal maternal disomic cell lineage (post-fertilization errors) [Robinson, 2000], although a more complex mechanism assuming errors in both meiosis I and mitosis (another form of trisomy rescues) might also be possible [Kotzot, 2001]. Furthermore, the results of methylation specific PCR assay are consistent with upd(14)pat for the examined DMR of *GTL2* in Patients 1–3.

Genotype–phenotype correlations in Patients 1–3, in conjunction with those in the previously described patients, are informative for the localization of the major locus or loci relevant to the development of the upd(14)pat phenotype (Table I). Patient 3 with segmental paternal isodisomy had a constellation of clinical features characteristic of upd(14)pat at birth, as did Patients 1 and 2 with full paternal isodisomy for chromosome 14. In addition, the clinical phenotype was comparable between Patients 1 and 3 in childhood. Furthermore, although Patient 2 had lethal respiratory failure, atrial septal defect, and omphalocele, such severe features were absent in Patient 1 with full paternal isodisomy as well as in Patient 3 with segmental paternal isodisomy. Similarly, although the previously described patient with segmental isodisomy for 14q12–qter [Coveler et al., 2002] had cardiovascular lesion and omphalocele that were absent in Patient 3 with segmental isodisomy for 14q23.3–qter, cardiovascular lesion is not a consistent feature even in patients with full upd(14)pat, and omphalocele is absent in patients with full upd(14)pat

A



	Patient 1				Patient 2				Patient 3			
Locus	Mother	Patient	Father	Assesment	Mother	Patient	Father	Assesment	Mother	Patient	Father	Assesment
D14S80	105/109	105	105/105	N.I.	95/105	97	97	upd(14)pat	97/105	97	97/105	N.I.
D14S1021	263	265	263/265	upd(14)pat	263/271	263	263	N.I.	263	261/263	261/263	(Biparental)*
D14S608	196/208	196	196/208	N.I	212/216	204	204	upd(14)pat	216/220	196/220	196/204	Biparental
D14S121	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	141/151	141/151	147/151	(Biparental)†
D14S75	188/194	184	180/184	upd(14)pat	184	186	184/186	upd(14)pat	190/194	184/194	184/192	Biparental
D14S980	163/167	167	165/167	N.I.	168/182	168	168	N.I.	155/167	167/183	163/183	Biparental
D14S1038	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	221/225	219/221	219/225	Biparental
D14S63	206/212	214	206/214	upd(14)pat	202/206	204	202/204	upd(14)pat	202/214	206/214	206	Biparental
D14S1026	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	134/142	124/134	124/132	Biparental
D14S271	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	227/229	227/229	227	(Biparental)†
D14S1046	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	214/216	212/214	212/218	Biparental
D14S981	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	193/197	195/197	195/197	(Biparental)*
D14S1069	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	206/212	212	206/212	N.I.
D14S1065	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	256	256	256/258	N.I.
D14S1011	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	184/186*	184	184	N.I.
D14S588	118/126	114	114	upd(14)pat	114/126	114	114/122	N.I.	114/126	114	114/122	N.I.
D14S1029	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	176	176	176	N.I.
D14S258	171/175	169	169/175	upd(14)pat	171/175	169	169/175	upd(14)pat	169	169	169/175	N.I.
D14S1028	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	231/241	231	231	N.I.
D14S1025	145/149	143	143	upd(14)pat	143/145	143	143	N.I.	143	143	143/149	N.I.
D14S1036	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	198/206	206	206/210	N.I.
D14S1000	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	124/126	128	122/128	upd(14)pat
D14S81	172/180	172	172/180	N.I.	175/181	181	179/181	N.I.	172/180	180	180	N.I.
D14S617	165	161	161/165	upd(14)pat	137/165	169	143/169	upd(14)pat	137/165	137	137	N.I.
D14S979	199/201	191	191/195	upd(14)pat	196/208	202	202	upd(14)pat	197/203	197	197	N.I.
D14S267	215/217	189	189/197	upd(14)pat	201/211	209	201/209	upd(14)pat	201	209	201/209	upd(14)pat
D14S250	158	160	160/166	upd(14)pat	154/162	160	160/170	upd(14)pat	158/160	158	158	N.I.
D14S1006	126/138	126	126/138	N.I.	126/130	140	126/140	upd(14)pat	136	136	136/140	N.I.
D14S985	132/136	136	128/136	N.I.	131/137	133	133	upd(14)pat	128/132	130	130	upd(14)pat
D14S1010	142/148	144	144/146	upd(14)pat	142	134	134/144	upd(14)pat	134/152	142	142	upd(14)pat
D14S292	106/110	112	108/112	upd(14)pat	106/108	110	110/112	upd(14)pat	110	104	104	upd(14)pat
D14S1007	111/123	111	111	N.I.	111/121	121	121/123	N.I.	111/121	121	121/123	N.I.

B

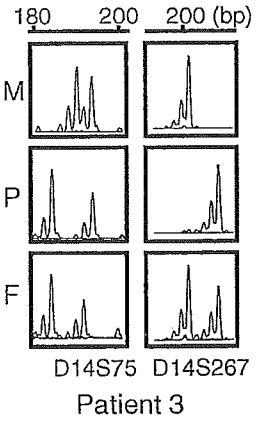


Fig. 2. Microsatellite analysis. **A**: Summary of the results. The ideogram of chromosome 14 is shown with the cytogenetic location of the microsatellite loci examined. The locus order is based on Ensembl Genome Browser (http://www.ensembl.org). The Arabic numbers indicate the PCR product sizes in bp. * Paternal uniparental heterodisomy could theoretically be possible. † Maternal uniparental heterodisomy could theoretically be possible. upd(14)pat, paternal uniparental disomy for chromosome 14; N.E., not examined; and N.I., not informative. **B**: Representative microsatellite results (M, mother; P, patient; and F, father). D14S75: Patient 3 is heterozygous with the paternally and maternally derived peaks, indicating biparental origin of this locus. D14S267: One of the two paternal peaks only is transmitted to Patient 3, indicating paternal uniparental isodisomy for this locus.

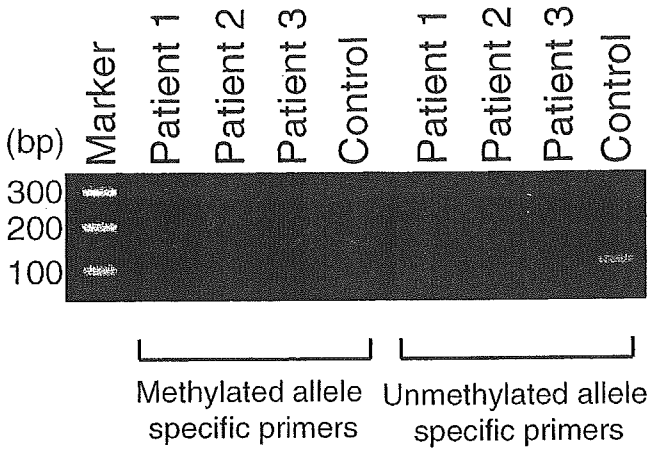


Fig. 3. Methylation specific PCR assay. In patients 1–3, PCR products have been obtained with methylated allele specific primers, but not with unmethylated allele specific primers.

except for Patient 2 in this study. These findings suggest that clinical phenotype in upd(14)pat can be associated with variable expressivity and penetrance; thus, for example, omphalocele and diastasis recti could be regarded as clinical features of different expressivity in a single phenotypic spectrum. It is inferred, therefore, that the isodisomic 14q23.3–14qter segment distal to *D14S981* in Patient 3 is the critical region for the development of upd(14)pat phenotype, and that an imprinted gene with a significant phenotypic effect is absent from the region proximal to 14q23.3. The critical region defined in this study is smaller than that defined previously [Coveler et al., 2002] and spans ~40 Mb in physical length (Ensembl Genome Browser Database, http://www.ensembl.org/).

The present location would be supported by the previous data. First, the 14q32 segment within the critical region is known to harbor both paternally expressed genes such as *DLK1* and *PEG11*, and maternally expressed genes such as *GTL2* and *MEG8* [Charlier et al., 2001; Cavaille et al., 2002]. Second, phenotypic comparisons of paternal or maternal disomy for chromosome 14 with 14q deletions of known parental origin suggest the presence of imprinted genes on a 14q23–q32 region [Sutton and Shaffer, 2000]. Third, the 14q23.3–14qter critical region shares homology with mouse chromosome 12 that contains a cluster of imprinted genes including *Dlk*1 and *Gtl*2 (Jackson Laboratory, http://www.informatics.jax.org/), and murine upd(12)pat leads to skeletal features reminiscent of those observed in human upd(14)pat [Sutton et al., 2003]. These findings would argue for the relevance of an imprinted gene(s) on the critical region to the development of upd(14)pat phenotype.

The present study extends much of the characteristic phenotype through early development. However, the unique bell-shaped thorax with coat-hanger appearance of the ribs, though it was obvious at birth, became non-recognizable by mid-childhood in Patients 1 and 3. Furthermore, the improvement of radiographic findings was apparently associated with that of respiratory function. These findings suggest that thoracic deformity and resultant respiratory distress may ameliorate with age. Thus, the life-prognosis can be expected to be fairly good for the patients who survived the infantile period.

To date, there has been only a single report documenting a patient with upd(14)pat after infancy. Wang et al. [1991] described a 9-year-old girl with characteristic somatic features and mental retardation who had a 13;14 Robertsonian translocation and molecularly confirmed paternal disomy. Although long-term clinical course and radiological findings have not been described, the patient is apparently free from a life-threatening respiratory dysfunction at the time of description. The findings would also support the fairly good life prognosis in survivors.

In summary, the results imply that an imprinted gene(s) for the development of upd(14)pat phenotype is localized to the ~40 Mb segment distal to *D14S981* at 14q23.3, and that the characteristic thoracic deformity may ameliorate with age. Further studies are needed to identify possible additional imprinted loci contributing to the upd(14)pat phenotype and to clarify the natural clinical course of the syndrome.

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Minireview

DHPLC in clinical molecular diagnostic services

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Abstract

A high-capacity low-cost mutation scanning method based on denaturing high-performance liquid chromatography (DHPLC) has been recently introduced. We have implemented an automated and cost-effective strategy using DHPLC. To facilitate the semi-automated analysis of multiple exons, two steps were taken. The first step was the development of a PCR protocol for the amplification of multiple exons under the same conditions. Primer sets, which amplify each exon in the entire gene, were aliquoted to and air-dried on a 96-well format PCR plate. In this way, all the exons in a gene can be simultaneously amplified on a single PCR machine. The second step was the serial DHPLC analysis of multiple amplicons under conditions optimal for each amplicon. We named the 96-well plate containing the primer pairs and the corresponding computer file used to analyze each amplicon under the pre-determined optimal conditions as the "Condition-Oriented-PCR primer-Embedded-Reactor plate," or the COPPER plate. We have developed COPPER plate systems for more than 20 congenital disorders including classic congenital syndromes like Marfan syndrome (*FBN1*: 65 amplicons), CHARGE syndrome (*CHD7*: 39 amplicons), de Lange syndrome (*NIPBL*: 46 amplicons), Sotos syndrome (*NSD1*: 30 amplicons), and Rubinstein–Taybi syndrome (*CREBBP*: 41 amplicons). Using the COPPER plate system, we are functioning as a reference laboratory for the clinical molecular diagnosis of congenital malformation syndromes and are presently analyzing more than 200 samples annually from all over Japan.

Keywords: Mutation analysis; Multiple malformation syndrome; DHPLC; Heteroduplex analysis; Molecular diagnosis

Introduction

As catalogued in pediatric genetics textbooks like "*Metabolic and Molecular Basis of Inherited Diseases*" [1] and "*Inborn Errors of Development*" [2], several hundreds of genes have been shown to cause human congenital disorders. The identification of these causative genes has offered us a wonderful opportunity to delineate the molecular basis of these disorders. Molecular diagnosis offers valuable information to the patients and their families in terms of prognosis, preventing complications, and providing accurate genetic counseling. Thanks to the completion of the human genome project, information on the human genome sequence is now readily accessible to everybody and, theoretically, any gene can be tested by the direct sequencing of PCR products amplified from the patient's genomic DNA. However, identifying pathogenic mutations is difficult when the causative gene has a large number of exons. In such cases, direct sequencing is expensive, technically demanding, and time consuming. Recently, a high-capacity low-cost mutation scanning method based on denaturing high-performance liquid chromatography (DHPLC) has been introduced [3–5]. Over the past six years, we have developed an automated and cost-effective strategy using DHPLC [6–12]. In this review, we would like to share our strategies with the readers of this journal.

DHPLC compares two chromosomes as a mixture of denatured and reannealed PCR amplicons [13]. When the PCR amplicon does not contain a mutation, the sense strand and the anti-sense strand of the PCR amplicons are completely complementary to each other. In this case, the

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molecule is referred to as a homoduplex. When a patient is heterozygous for a mutation, the mutant sense and anti-sense strands will not only form a homoduplex, but also heteroduplices with their corresponding wild type sense and anti-sense strands upon re-naturation of the denatured PCR product. The physicochemical difference between a homoduplex and heteroduplices can be detected by HPLC using a reversed-phase chromatography column with an affinity to double-stranded DNA. The differential retention of homo- and heteroduplex DNA on the column after partial denaturation indicates the presence of a mutation. The temperature of the column determines sensitivity, and the optimal temperature is predicted by a mathematical formula based on the amplicon sequence; the output of this formula can be calculated using a computer program (WAVE Maker, Transgenomic, Omaha, Nebraska, USA). Single-nucleotide substitutions, deletions, and insertions have been successfully detected within 5–6 min using on-line UV or fluorescence monitoring. Numerous articles have been published describing the successful use of this method to detect mutations in various diseases; some of these articles have appeared in recent issues of *Molecular Genetics and Metabolism* [14–18].

COPPER plate technology

In research settings where samples from multiple patients are available for analysis, all the patient samples are simultaneously screened one exon at a time; in other words, exon 1 is screened in all the samples, then exon 2 and so forth [12]. This strategy can be regarded as an "exon-by-exon" approach to mutation screening. In clinical settings, on the other hand, patient samples are usually studied one at a time because most genetic disorders are so rare. This strategy can be regarded as a "patient-by-patient" approach [8,11]. When utilizing a patient-by-patient approach, the key to success lies in the semi-automated serial analysis of multiple exons.

To facilitate the semi-automated analysis of multiple exons, two steps should be taken. First, a PCR protocol for the amplification of multiple exons under the same conditions is needed [11]. Primer sets, which amplify each exon of the entire gene, are aliquoted and air-dried on a 96-well format PCR plate (Fig. 1). No PCR multiplexes were generated. Rather, each exon was amplified individually in different wells of a 96-well plate. All of the PCR primers must be designed so that they have the same annealing temperature. In this way, all the exons can be amplified simultaneously by a single PCR machine. For illustrative purposes, agarose gel electropherograms of the PCR products are shown in Fig. 2. More than one negative control with different combinations of primers was run to exclude PCR contamination. At the same time, several positive controls, for mutations previously detected by DHPLC on the same 96-well plate, were run to ensure optimal and reproducible DHPLC performance from one patient to the next.

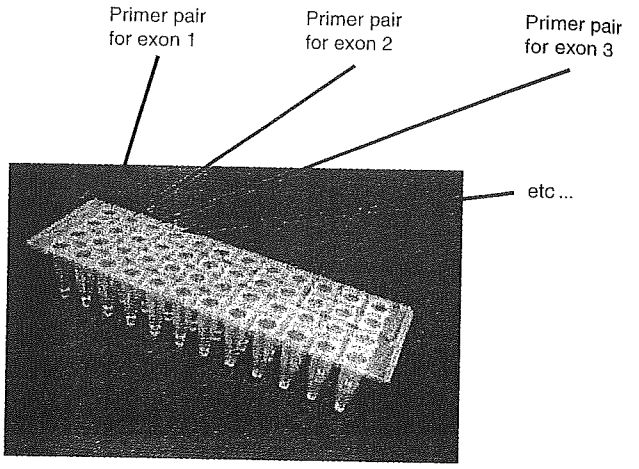

Fig. 1. Simultaneous amplification of all the exons in a gene under a single condition on a 96-well format PCR plate. Primer sets, which amplify each exon of the entire gene, are aliquoted to and air-dried in each well.

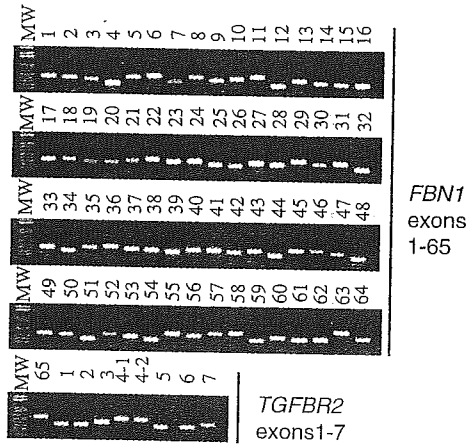

Fig. 2. PCR amplification of all coding exons of the *FBN1* and *TGFBR2* genes under a single condition. The leftmost lane (MW) represents the 1-kb ladder marker showing 100-, 200-, 300-, 400-, 500-, 650-, 850-, and 1000-bp fragments; the other lanes were loaded with 3 μl of PCR products. Please note that the figure was presented for illustrative purposes. We do not usually subject each plate to agarose gel electrophoresis prior to DHPLC analysis.

The second step involves the serial DHPLC analysis of multiple amplicons under optimal conditions for each amplicon. The DHPLC system (WAVE, Transgenomic) is equipped with an auto-sampler and is capable of running each PCR amplicon at a pre-determined optimal chromatographic condition. When a particular exon had more than one melting domain, the exon was amplified in multiple amplicons. The typical injection volume was 5 μl. Hence, there was enough for three injections from each 20 μl of PCR product. Exons from a particular gene were analyzed in order of increasing melting temperature. The typical delay between runs, when the temperature had to be changed, was 1.3 min. The typical total run times and number of runs necessary for each gene are presented in Table 1.

Table 1
List of syndromes for which COPPER plate system is available

Syndrome	Gene	Number of exons	Number of amplicons	Number of runs	Total run times (h)
Marfan syndrome	*FBN1, TGFBR2*	65, 7	65, 8	136, 17	22, 3
Beals syndrome	*FBN2*	65	65	157	25
CHARGE association	*CHD7*	38	39	81	12.5
de Lange syndrome	*NIPBL*	47	46	84	13
Rubinstein–Taybi syndrome	*CREBBP*	32	41	77	12
Sotos syndrome	*NSD1*	23	30	58	9.5
Noonan syndrome	*PTPN11*	15	15	27	4
Blepharophimosis syndrome	*FOXL2*	1	4	12	2
Alagille syndrome	*JAG1*	26	30	51	8
EEC syndrome	*P63*	16	10	19	3
Rieger syndrome	*PITX2*	5	6	13	2
Townes–Brocks syndrome	*SALL1*	3	13	29	5
Holt–Oram syndrome	*TBX5*	9	9	19	3
Treacher–Collins syndrome	*TCOF1*	26	26	46	7.5
Opitz syndrome	*MID1*	10	11	18	3
Cleidocranial dysplasia	*RUNX2*	7	8	17	3
Waardenburg syndrome	*PAX3 and MITF*	10, 9	11, 10	19, 19	3, 3
Craniosynostosis syndromes	*FGFR2*	18	20	33	5.5
Saethre–Chotzen syndrome	*TWIST*	1	2	3	0.5
Holoprosencephaly	*ZIC2, TGIF, SHH, SIX3*	3, 3, 3, 2	5, 4, 4, 3	9, 7, 10, 7	1.5, 1, 1.5, 1
Cleft lip and palate	*IRF6, MSX1, TBX22*	9, 2, 8	7, 4, 9	15, 11, 18	2.5, 2, 3

We named the 96-well plate containing the primer pairs and the corresponding computer file used to analyze each amplicon under the pre-determined optimal conditions as the "Condition-Oriented-PCR primer-Embedded-Reactor," or COPPER plate.

The plate-based system greatly simplifies the labor involved in mutation screening (Fig. 3). When a patient's sample arrives, the laboratory technician removes a dried array containing the appropriate PCR primer pairs from storage. Then, the laboratory technician adds the patient's genomic DNA to the PCR premix and places an aliquot of the mixture in each well of the plate using a multi-channel pipette. The technician then places the plate in a PCR machine and starts the amplification. When the PCR is completed, the technician transfers the plate to a DHPLC apparatus and initiates the computer routine optimized for the gene of interest. We have developed COPPER plate system for more than 20 congenital disorders (Table 1). Specific PCR conditions and DHPLC conditions are listed on our website at http://www.dhplc.jp. Included within the list are causative genes with large exons: *FBN1* (Marfan syndrome, FBN1; 65 exons, 65 amplicons), *CHD7* (CHARGE association; 38 exons, 39 amplicons), *CREBBP* (Rubinstein–Taybi syndrome; 32 exons, 41 amplicons), *NSD1* (Sotos syndrome; 23 exons, 30 amplicons), and *NIPBL* (de Lange syndrome; 47 exons, 46 amplicons). For the analysis of autosomal recessive and X-linked recessive disorders, genomic DNA from normal individuals is added to each well when analyzing autosomal recessive disorders or male patients with X-linked recessive disorders so that a heteroduplex will be formed whether the patient is homozygous or hemizygous for the mutant allele. Using the COPPER plate system, we are functioning as a reference laboratory for the clinical molecular diagnosis of congenital malformation syndromes and are presently analyzing more than 200 samples annually from all over Japan.

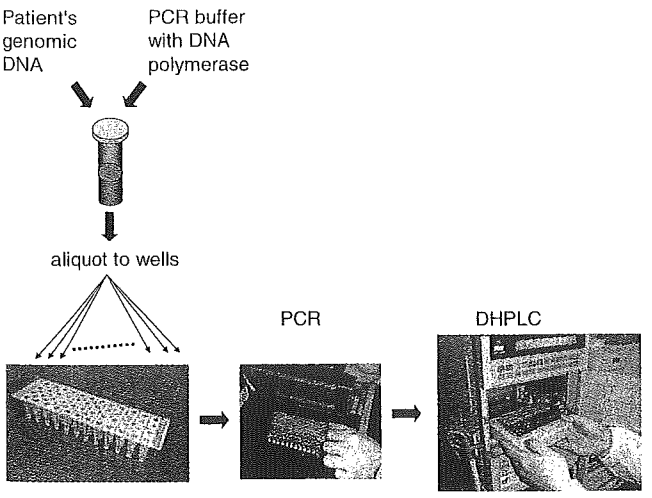

Fig. 3. COPPER plate-based system. This plate-based system greatly simplifies the labor involved in mutation screening. When a patient's sample arrives, the laboratory technician removes a dried array containing the appropriate PCR primer pairs from storage. Then, technician adds the patient's genomic DNA to the PCR premix and places an aliquot of the mixture in each well of the plate using a multi-channel pipette. The subsequent PCR and DHPLC analysis can be performed using the same 96-well plate without any need to transfer solutions.

If a laboratory adopts state-of-the-art Applied Biosystems DNA Sequencers, such as the ABI Prism 3100 and the 3730, >200 chromatograms can be generated in several hours. However, generation of 200 chromatograms is one thing while complete analysis of 192 chromatograms is another. When 200 chromatograms are generated in a day, it is not an easy task for a single technician to visually

inspect all the chromatograms. Moreover, it is unlikely that all of the 200 chromatogram traces will be completely clean. In other words, some of the sequence reactions would need to be repeated. By adopting the COPPER plate system, we could handle molecular diagnosis of various relatively rare diseases. Typically, only one gene was analyzed per patient. Thus, a new sample arrived every other day on average. We analyzed the samples without waiting for other samples from individuals with the same syndrome because this would be the typical situation when analyzing rare diseases in actual clinical settings.

Illustrative examples

For illustrative purposes, we here describe the mutation analysis system for Noonan syndrome, which is characterized by multiple malformations including short stature, characteristic facies, webbed neck, and pulmonary stenosis. Noonan syndrome is caused by mutations in the protein-tyrosine phosphatase, nonreceptor-type gene (*PTPN11*) [8]. We compared the mutation detection rate of direct sequencing and DHPLC by analyzing 34 patients with Noonan syndrome in parallel. One technician perfor the direct sequencing and another technician performed DHPLC screening and samples with abnormal chroma graphic patterns were directly sequenced. Both meth detected the same 12 patients with *PTPN11* mutatic Hence, the concordance between the two methods 100%. The abnormal chromatograms from the patie with Noonan syndrome are depicted in Fig. 4. In e panel, the differences between the normal and the abnor chromatograms were self-evident.

In addition to screening for congenital disorders, COPPER plate system can also be used to screen for ph macogenetic traits [9,11]. We have developed a mutat analysis system for the enzyme thiopurine methyltransf ase (TPMT), which inactivates 6-mercaptopurine (used the treatment of acute leukemia). Patients w homozygous mutations in *TPMT* suffer life-threaten myelosuppression when treated with conventional doses 6-mercaptopurine. We developed a DHPLC-based pro col to analyze the entire coding region of *TPMT* and va dated a protocol to detect all 16 previously descrit variant alleles. We further analyzed the entire coding regi

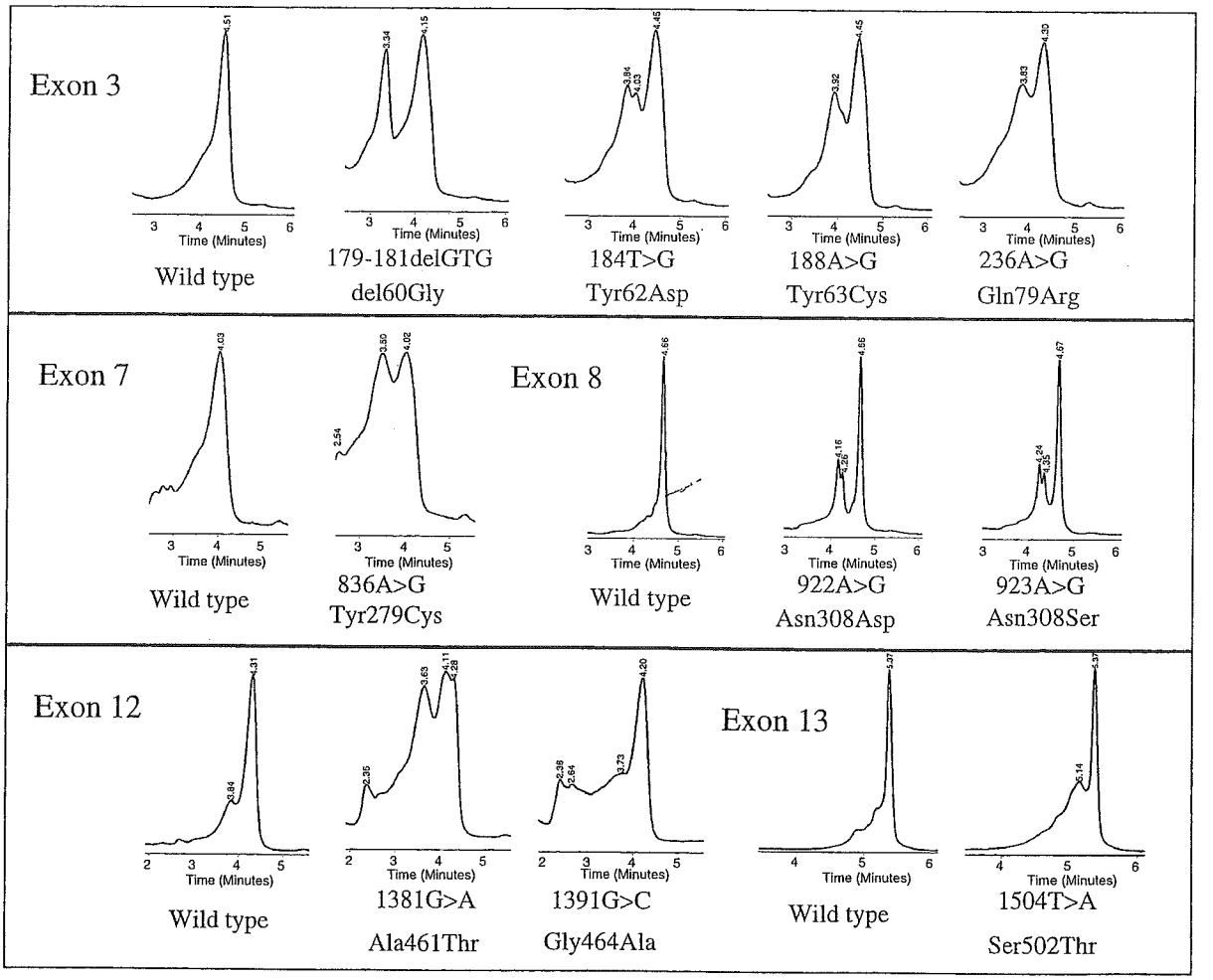

Fig. 4. DHPLC chromatograms of 12 *PTPN11* mutations identified in patients with Noonan syndrome. In each panel, the top and bottom chromat grams were derived from a normal control and a patient, respectively. *x*-axis, retention time in minutes; *y*-axis, fluorescence intensity. The Gln79A mutation was detected in 3 patients.

of *TPMT* in 288 control samples collected worldwide and identified two novel amino acid substitutions, Arg163Cys (487C > T) and Arg226Gln (677G > A) within exons 7 and 10, respectively. The clinical application of this comprehensive screening system to examine the entire *TPMT* gene would help to identify patients at risk of bone marrow failure prior to 6-mercaptopurine therapy [11].

Tips for effective use

DHPLC is a very sensitive analytic method. Therefore, false-positive results can occur if appropriate measures are not taken. Here, we discuss two issues. First, impure oligonucleotide primers for PCR amplification can lead to false-positive results. Sometimes, the PCR primers ordered from custom oligonucleotide synthesis services are contaminated with incompletely synthesized primers that are a base-length shorter than expected. In such cases, the DHPLC chromatogram pattern may show a split and might be falsely interpreted as mutation-positive. The purity of the primer can be tested by the DHPLC machine (Fig. 5). Second, the quality of the DNA polymerase can result in false-positive results. Some DNA polymerase adds an extra base at the 3′ end of the PCR amplicon. The addition of the extra base can modify the chromatographic pattern, possibly leading to misinterpretation. As with other assays based on HPLC, the maintenance of the chromatography column is essential to ensure a high level of sensitivity. Standard operating procedures for the instrument's operation and maintenance and for mutation detection by DHPLC should be strictly followed [19].

The cost-effectiveness of DHPLC in comparison to state-of-the-art sequencing will depend to a certain extent

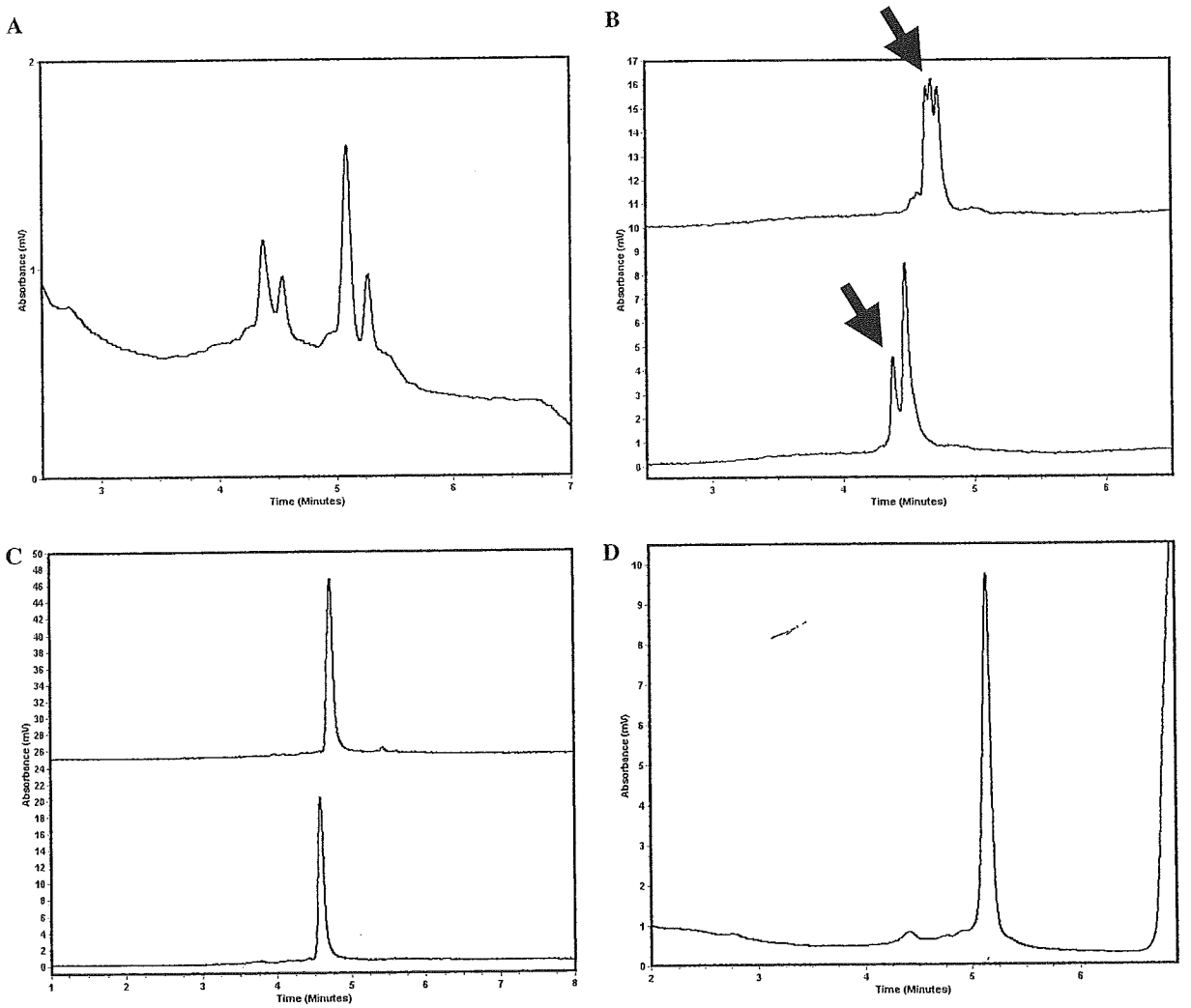

Fig. 5. Detrimental effect of impure primers. *x*-axis, retention time in minutes; *y*-axis, fluorescence intensity. (A) PCR product of exon 16 of the *FGFR2* gene amplified from a known homozygote using an impure primer. Because the PCR product was derived from a known homozygote, the chromatographic pattern was expected to have a single peak. However, the pattern was characterized by multiple peaks, indicating the presence of a heteroduplex. (B) Primer impurity revealed by DHPLC analysis. We evaluated the oligonucleotide primer (not the PCR product). Top, forward primer; bottom, reverse primer. The arrows indicate the oligonucleotide whose primer length was shorter than expected. (C) DHPLC analysis of the re-synthesized primer. We asked the primer manufacturer to re-synthesize the primers. Note that both primers had the expected length. (D) PCR product amplified from a known homozygote using a re-synthesized primer. The chromatographic pattern was characterized by a single peak, as expected.

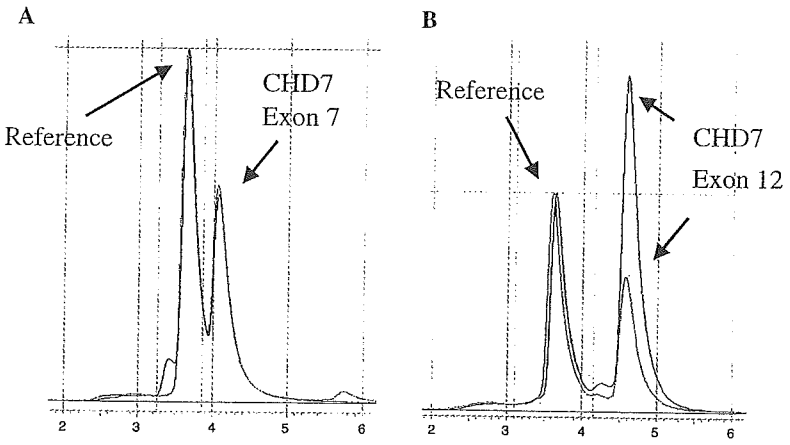

Fig. 6. Detection of whole exon deletion. The deletion of exons 8–12 of *CHD7* was identified using a DHPLC-based semi-quantitative assay. Together with the *CHD7*, another reference gene on a different chromosome was co-amplified by multiplex PCR. *x*-axis, retention time in minutes; *y*-axis, fluorescence intensity. The profiles were superimposed and then normalized using the reference amplicon. The chromatograms in black and red represent the results for a normal control subject and a patient, respectively. The peak corresponding to exon 7 of *CHD7* was comparable in the patient's sample and in the control's sample (A) whereas, the peak corresponding to exon 12 of *CHD7* was lower in the patient's sample than in the control's sample (B). Similarly, the peaks corresponding to exons 8, 9, 10, and 11 were decreased (data not shown). We concluded that exons 8–13 were deleted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

on the frequency, with which more or less common polymorphisms are observed in the genes of interest. We recommend sequencing of all aberrant chromatographic profiles. When relatively common polymorphisms were identified in the introns, we redesigned the PCR primers so as to exclude the polymorphic site from the amplicon.

Future directions

So far, we have been focusing on small aberrations (e.g., single-nucleotide substitutions, deletions, and insertions) of the genome sequences of patients with congenital disorders. However, larger scale abnormalities, including deletions spanning several exons or whole gene deletions, can also be pathogenic. Recently, a French group developed a DHPLC-based semi-quantitative assay for assessing the copy numbers of individual exons of the tumor suppressor retinoblastoma 1 gene (*RB1*) [20]. Multiple exons are amplified using unlabeled primers, then separated by ion-pair reversed-phase high-performance liquid chromatography and quantified using fluorescent detection with a post-column intercalation dye (SYBR Green). The relative peak intensities for each target directly reflect the exon copy number [20]. When no small aberrations are identified, a whole exon deletion can be sought using this DHPLC-based semi-quantitative assay (Fig. 6).

Optimized analytic conditions should be shared to prevent the unnecessary duplication of research. To facilitate such collaborations, we have created a website containing optimized analytic conditions, including the primer sequences, PCR conditions, and DHPLC conditions (http://www.dhplc.jp). All the information required for mutation analysis can be downloaded from the website. In addition, PCR primers could also be physically shared. If an international "primer bank" were to be established, PCR primers could be distributed to collaborating institutions performing DHPLC analysis. At the centralized primer bank, replica plates could be made from the master plate. Once the replica plate has dried, the plate could be delivered to other collaborating institutions at room temperature. We are currently running a program supported by the Japanese government to distribute COPPER plates nationally.

In summary, when complemented with the COPPER plate system, DHPLC technology offers an ideal platform for the clinical molecular diagnosis of congenital disorders. We hope this review will facilitate the formation of an international collaborative network of DHPLC users sharing standardized analytic conditions and PCR primer resources.

Acknowledgments

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Keratan sulphate levels in mucopolysaccharidoses and mucolipidoses

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Summary: The mucopolysaccharidoses (MPS) is characterized by accumulation of glycosaminoglycans (GAGs), and mucolipidosis (ML) by accumulation of GAGs and sphingolipids. Each type of MPS accumulates specific GAGs. The lysosomal enzymes *N*-acetylgalactosamine-6-sulphate sulphatase and β-galactosidase involve the stepwise degradation of keratan sulphate (KS). Deficiency of these enzymes results in elevation of KS levels in the body fluids and in tissues, leading to MPS IV disease. In this study, we evaluated blood and urine KS levels in types of MPS and ML other than MPS IV. Eighty-five plasma samples came from MPS I ($n=18$), MPS II ($n=28$), MPS III ($n=20$), MPS VI ($n=3$), MPS VII ($n=5$) and ML ($n=11$) patients while 127 urine samples came from MPS I

($n = 34$), MPS II ($n = 34$), MPS III ($n = 32$), MPS VI ($n = 7$), MPS VII ($n = 9$) and ML ($n = 11$) patients. KS levels were determined using the ELISA method. Plasma KS levels varied with age in both control and patient populations. In all age groups, the mean values of plasma KS in MPS and ML patients were significantly higher than those in the age-matched controls. Plasma KS values in four newborn patients were above the mean + 2SD of the age-matched controls (mean, 41 ng/ml). Overall, 85.9% of individual values in non-type IV MPS and ML patients were above the mean + 2SD of the age-matched controls. For urine KS levels, 24.4% of individual values in patients were above the mean + 2SD of the age-matched controls. In conclusion, KS in blood is elevated in each type of non-type IV MPS examined, in contrast to the conventional understanding. This finding suggests that measurement of KS level provides a new diagnostic biomarker in a wide variety of mucopolysaccharidoses and mucolipidoses in addition to MPS IV.

The mucopolysaccharidoses (MPSs) are a group of lysosomal storage diseases (LSDs) caused by deficiency of the lysosomal enzymes needed to degrade glycosaminoglycans (GAGs) such as dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), chondroitin sulphate (CS) and hyaluronan (Neufeld and Muenzer 2001). In the MPSs, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the bloodstream and excreted in urine. Mucolipidosis II or III (ML) is a disorder of the lysosomal enzyme phosphorylation and localization caused by deficiency of *N*-acetylglucosaminyl-1-phosphotransferase, leading to accumulation of GAGs and sphingolipids in cells (Kornfeld and Sly 2001). In ML, a number of biochemical defects have been observed including multiple lysosomal enzyme deficiencies in cultured fibroblasts and their presence in the cell culture medium at abnormally high levels (Tondeur et al 1971). ML II (so-called I-cell disease) has characteristics of both MPS and sphingolipidosis, storing GAGs and sphingolipids in the cells; however, no mucopolysaccharidurie (excessive urinary excretion of GAGs) is observed (Leroy et al 1967). In spite of the fact that both MPS and ML involve abnormal metabolism of GAGs, neither total GAG nor a specific GAG level in blood has been measured owing to lack of an appropriate method.

The incidence of MPS and ML is estimated as 1 in 20 000–50 000 live births (Meikle et al 1999). In general, MPS and ML are asymptomatic in newborns, with subsequent onset of clinical signs that include abnormal development of bones, short stature and coarse hair during infancy or childhood. In some patients, mental retardation progresses gradually over years. MPSs are theoretically amenable to exogenously supplied enzymes. Clinical trials of enzyme replacement therapy on MPS I, II and VI are in progress (Kakkis et al 2001; Wraith et al 2004). Successful treatment of these disorders depends on early diagnosis and a protocol for enzyme replacement therapy that quantifies its clinical effectiveness. Identification of disease markers is of paramount importance in diagnosis and treatment.

There are established procedures for measuring total urinary GAGs using dye-spectrometric methods such as dimethylmethylene blue (de Jong et al 1992; Whitley

et al 2002) and alcian blue (Karlsson et al 2000). Thin-layer chromatography has been utilized for identification of each specific GAG. These methods are not applicable to blood or tissue extracts without prior protease, nuclease or hyaluronidase digestion. HPLC is a sensitive and accurate method to measure each specific GAG (Kinoshita and Sugahara 1999; Toyoda et al 1998; Yamada et al 2000). Tandem mass spectrometry is sensitive and accurate but its application is costly (Chai et al 1998; Oguma et al 2001a,b). The lysosome-associated membrane proteins were also measured as potential markers for the screening of LSD patients using the ELISA method (Chang et al 2000; Hua et al 1998; Meikle et al 1997). The newly developed ELISA assay of KS in blood and urine has been evaluated and it clearly separated MPS IVA patients from the controls, indicating a better solution compared with the dimethylene blue method (Tomatsu et al 2004). The KS concentrations correlated with clinical severity, especially with the progression of bone dysplasia (Tomatsu et al 2004).

It is generally accepted that each type of MPS is characterized by accumulation of specific GAGs (Neufeld and Muenzer 2001). MPS IV patients have deficiency of lysosomal enzymes involving the stepwise degradation of keratan sulphate (KS), leading to accumulation of KS in the body fluid. However, accumulation of KS in other types of MPS has not been considered or found, since the enzyme deficient in those types of MPS does not involve KS catabolism and no sensitive method has been available to detect KS in blood.

In this study we have evaluated KS levels in blood and urine for other types of MPS and ML patients in addition to MPS IV patients. We report, surprisingly, that all mucopolysaccharidoses and mucolipidoses are associated with elevation of KS levels.

MATERIALS AND METHODS

Materials

Eighty-five blood (plasma) samples came from MPS and ML patients except MPS IV ranging between 0 and 55 years of age (MPS I, $n=18$; MPS II, $n=28$; MPS III, $n=20$; MPS VI, $n=3$; MPS VII, $n=5$; ML, $n=11$). Sixty-two blood samples from MPS IV patients including 49 type A and 3 type B (2–66 years old) and 59 samples from other LSD patients (0–55 years old) were collected for comparison. Forty-five MPS IVA patients have been described previously (Tomatsu et al 2004). Four hundred and fifty control samples were collected (0–80 years old).

One hundred and twenty-seven urine samples came from MPS and ML patients except MPS IV ranging between 0 and 40 years of age (MPS I, $n=34$; MPS II, $n=34$; MPS III, $n=32$; MPS VI, $n=7$; MPS VII, $n=9$; ML, $n=11$). Eighty-five urine samples from MPS IV patients (1–66 years old) and 20 from other LSD patients (0–35 years old) were collected. Fifty-nine MPS IVA patients have been described previously (Tomatsu et al 2004). Four hundred and fifty control samples were also collected (0–64 years old). Overall, we collected 206 and 450 plasma samples from LSD patients and normal controls, respectively, and 232 and 450 urine samples from LSD patients and normal controls, respectively (Table 1).

Table 1A Age-dependent plasma KS (ng/ml) by ELISA method

	Mean KS (ng/ml)	*SD*	*p-value**	*Max.*	*Min.*	*N*	*Mean age* (y)
Control							
Newborn[a]	41	18.6		77	2	100	0
0 < age ≤ 5	119	65.2		279	18	100	1.53
5 < age ≤ 10	239	70.2		323	80	50	6.8
10 < age ≤ 15 y	197	61		328	130	50	12.3
15 < age ≤ 20 y	158	50.5		352	22	50	26.4
Over 20 y	151	48.2		250	15	100	42.6
MPS and ML patients except IVA							
Newborn[a]	104	27.3	<0.0001	145	87	4	0
0 < age ≤ 5 y[b]	315	186	<0.0001	916	71	38	1.9
5 < age ≤ 10 y	634	309	<0.0001	1167	191	20	6.4
10 < age ≤ 15 y	642	320	<0.0001	1034	227	6	11.6
15 < age ≤ 20 y	724	431	<0.0001	1363	213	6	17.7
Over 20 y	613	327	<0.0001	1102	147	11	31.6
Type of MPS and ML							
I	254	118		517	87	18	5.5
II	742	327		1363	206	28	12.9
III	411	175		871	173	20	5.9
VI	523	113		625	401	3	4.9
VII	219	128		427	89	5	12.1
ML	340	331		1034	71	11	6.6
MPS IV							
IVA	569	345		1525	101	59	14.2
IVB	226	123		362	123	3	11.3
Other LSDs	130	91		435	17	59	17.1

y, year; max., maximum; min., minimum
[a] Within 1 month after birth
[b] Over 1 month
*p value was calculated in comparison with the age-matched controls and patients

The urine and blood samples from the patients were collected at the following institutes: Department of Pediatrics, Shimane Medical University, Japan; Department of Pediatrics, Gifu University School of Medicine, Japan; Willink Biochemical Genetics Unit, Royal Manchester Children's Hospital, UK; Institute of Inborn Errors of Metabolism, Javeriana University, Colombia; Medical Genetics Service, Federal University of Rio Grande do Sul, Brazil; Department of Pediatrics, University of Mainz, Germany; Institute of Medical Chemistry, University of Vienna, Austria; Laboratory of Metabolic Diseases, Department of Pediatrics, University of Graz, Austria; Department of Pediatrics, University of Hamburg, Germany. The samples were sent to the Department of Pediatrics, Saint Louis University, for further analysis.

All the patients showed enzyme activity below 5% normal activity and one specimen of urine and/or blood was taken from each patient. Written informed consent was obtained from each patient at entry to the study at each institute. The study protocol was approved by the institutional review board at Saint Louis University.

Table 1B Age-dependent urine KS (mg/gCre) by ELISA method

	Mean KS (mg/gCre)	*SD*	*p*-value*	*Max.*	*Min.*	*N*	*Mean age*
Control							
0 < age ≤ 5 y	0.23	0.16		0.97	0	200	1.1
5 < age ≤ 10 y	0.21	0.13		0.49	0.07	50	6
10 < age ≤ 15 y	0.29	0.14		0.62	0.05	50	11.8
15 < age ≤ 20 y	0.14	0.14		0.2	0.07	50	16.2
Over 20 y	0.13	0.13		0.42	0.03	100	38.5
MPS and ML patients except IVA							
0 < age ≤ 5 y	0.51	0.39	<0.0001	2.2	0.055	69	2
5 < age ≤ 10 y	0.4	0.27	<0.0001	1.05	0.06	32	6.3
10 < age ≤ 15 y	0.6	0.55	<0.001	1.86	0.22	8	11.8
15 < age ≤ 20 y	0.32	0.24	<0.0001	0.78	0.075	6	18
Over 20 y	0.28	0.23	<0.0001	0.88	0.075	12	27.7
Type of MPS and ML							
I	0.48	0.4		1.9	0.06	34	4.1
II	0.49	0.33		1.86	0.17	34	10.1
III	0.42	0.28		1.05	0.06	32	5.1
VI	0.56	0.73		2.19	0.09	7	5
VII	0.3	0.26		0.93	0.08	9	14.2
ML	0.53	0.36		1.23	0.13	11	5.9
MPS IV							
IVA	9.1	9.75		46.3	0.11	78	12.7
IVB	2.6	2.55		7.02	0.5	7	16.9
Other LSDs	0.63	0.79		3.05	0.01	20	11

*p value was calculated in comparison with the age-matched controls and patients

Methods

Sandwich ELISA assay: The KS standards for ELISA calibration and the anti-KS monoclonal antibody (5-D-4) (Tomatsu et al 2004) were obtained from Seikagaku (Japan). The ELISA procedure was performed as previously described (Caterson et al 1983). The absorbance was measured at 450 nm with microplate spectrophotometer reference to 650 nm. The KS concentration was read by applying the absorbances of each sample to the calibration curve.

For urine samples, creatinine was measured by mixing 10 µl of a 10-fold diluted urine sample with 50 µl saturated picric acid (Sigma, St Louis, MO, USA) and 50 µl 0.2 mol/L NaOH. Absorbance at 490 nm was read after 20 min and compared to a standard. We evaluated the cross-reactivity (%) with other KS-related GAG compounds including HS, DS and CS obtained from Seikagaku in an acid mucopolysaccharide (AMPS) kit (#400610). The KS assays all showed cross-reactivity of <0.1% against HS, DS and CS compared with the same concentration of KS. Repeated freezing at −20°C and thawing of the plasma did not affect the level of KS detected.

Keratanase II digestion: To confirm that the anti-KS monoclonal antibody did not recognize any other GAG except KS, the keratanase II treatment, which digests

KS specifically, was performed for the urine and plasma samples. The enzyme solution was prepared by dissolving keratanase II (Seikagaku, #100812) in 50 mmol/L Tris-HCl buffer (pH 7.0) at a concentration of 500 mU/ml. Keratanase II digestion was initiated by adding keratanase II to the plasma and urine samples to give a final concentration of 5 mU/ml. The reaction mixture was then incubated for 3 h at room temperature, followed by the ELISA.

HPLC assay: To prove elevation of KS in specimens of MPS and ML, HPLC assay was also used for measurement of plasma and urine KS concentration. Each 0.5 ml aliquot of the urine sample was chromatographed on a Q Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden) and desalted with a PD-10 column (Pharmacia). This fraction was concentrated to 0.2 ml with a centrifugal evaporator centrifuge EC-57C (Sakuma Seisakusyo Ltd, Tokyo, Japan). Each 50 µl aliquot of the concentrated urine or plasma samples was pretreated with 1 mU of keratanase II in 200 µl of 0.02 mol/L sodium acetate buffer (pH 6.0) at 37°C for 3 h, which produced saturated monosulphated disaccharide of KS (di-mono-KS) and saturated disulphated disaccharide of KS (di-di-KS). The di-mono-KS and di-di-KS were ultrafiltered using an Ultrafree C3GC system (molecular size cut-off 10 000; Japan Millipore Ltd, Tokyo, Japan). HPLC analysis of di-mono-KS and di-di-KS was performed as previously described (Caterson et al 1983; Shinmei et al 1992). The area of each peak corresponding to di-mono-KS and di-di-KS was calculated by the integrator and converted to an amount of KS against the areas of standard di-mono-KS and di-di-KS from bovine cornea KS digested with keratanase II (Yoshida et al 1989).

Data analysis: The data obtained were analysed to determine whether the levels of KS varied significantly with respect to age and each type of MPS and ML. Student's *t*-test or Welch's *t*-test was used to compare KS levels in the patient samples with the control samples with age dependency. Correlation in blood (or urine) KS concentrations assayed by ELISA and HPLC was evaluated using regression plot (Pearson's correlation coefficient). The mean value for KS levels in each type of MPS or ML was compared with that of the controls by one-way ANOVA, followed by Dunnet's post-test analysis. The KS levels were also each compared across type of MPS or ML by one-way ANOVA, followed by Bonferroni's post-test analysis.

All data analyses were performed with Statview statistical software (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

Plasma KS concentrations: The age groups in the control and patient (MPS and ML except MPS IV) populations were divided into newborn, 0–5 years, 5–10 years, 10–15 years, 15–20 years, and over 20 years (Table 1A).

The level of plasma KS varied with age in both control and patient populations (Table 1A). In the control samples, the plasma KS values in each age group did not show the normal distribution pattern, the lowest KS level was observed in the newborn period (mean 41 ng/ml). The mean value of plasma KS level in the control

population peaked between 5 and 10 years (mean 239 ng/ml) and thereafter gradually became lower. In the patient samples, the lowest KS level was also observed in the newborn period (mean 104 ng/ml). Thereafter, mean plasma KS levels were over 600 ng/ml in the 5–10 years group and subsequently over 600 ng/ml in all age groups. The mean values of plasma KS in each age group showed significant differences between the control and patient groups, as shown in the fourth column of Table 1A.

There was a clear discrimination for an individual patient sample when it was compared to the age-matched control group. Plasma KS values in four newborn patients were above the mean + 2SD (78.2 ng/ml) of the age-matched controls. The four newborn patients were two diagnosed with MPS I, one with MPS VII and one with ML II (plasma KS 87, 145, 89, and 98 ng/ml, respectively).

The level of plasma KS was also compared between each type of MPS and ML and the age-matched controls (Table 1A and Figure 1). Plasma KS levels in MPS I showed that 16 out of 18 (88.8%) patients had above the mean + 2SD of the age-matched controls (Figure 1A). Thirteen of 15 (86.7%) patients with a severe form and all three patients with an attenuated form showed plasma KS levels above the mean + 2SD. MPS II patients had the highest mean KS in plasma among all types of MPS and ML patients (mean 742 ng/ml). All patients except one with a severe form (27 out of 28: 96.4%) had plasma KS values above the mean + 2SD of the age-matched controls (Figure 1B). Plasma KS values in 16 of 20 (80%) MPS III patients were above the mean + 2SD of the age-matched controls (Figure 1C). Twelve of 15 (86.7%) patients with a severe form and 2 of 3 patients with an attenuated form showed plasma KS levels above the mean + 2SD. All three MPS VI patients revealed plasma KS levels above the mean + 2SD (Figure 1E). None of three MPS VII patients with an attenuated phenotype had plasma KS levels above the mean + 2SD, while two patients less than 2 months old with hydrops fetalis and developmental delay showed plasma KS levels above the mean + 2SD (89.1 and 427 ng/ml). Plasma KS values in 9 of 11 (82%) ML patients, ranging between 71 and 1034 ng/ml, were above the mean + 2SD of the age-matched controls. Finally, 85.9% of individual values in other MPS and ML patients except MPS IV plotted above the mean + 2SD of the age-matched controls. Fifty of 62 (80.6%) MPS IV patients had plasma KS levels above the mean + 2SD of the age-matched controls (Figure 1D). These findings suggest that the magnitude of plasma KS elevation in these patients was comparable to that in MPS IV patients.

In the samples from the other 59 LSD patients, the mean KS value was 130 ng/ml (Figure 1F) and only 6 patients (10.2%) had plasma KS values above the mean + 2SD of the age-matched controls. Therefore, the current method does not seem to be suitable for screening of other LSD patients.

Urine KS concentrations: The age groups in the control and patient (MPS and ML except MPS IV) populations were divided into 0–5 years, 5–10 years, 10–15 years, 15–20 years, and over 20 years (Table 1B). The level of urine KS changed with age in control and patient populations (Table 1B). In the control samples, the urine KS values in each age group did not show the normal distribution pattern; the lowest urine KS level was observed in the over 20 years group (mean 0.13 mg/g creatinine),

while the highest value was in the 10–15 years group (mean 0.29 mg/g creatinine; $p < 0.001$). In the patient samples, the lowest urine KS level was also observed in the over 20 years group (mean 0.28 mg/g creatinine), while the highest was in the 10–15 years group (mean 0.6 mg/g creatinine). The mean KS values in both control and patient populations over 20 years of age were decreased at 0.13 and 0.28 mg/g creatinine, respectively. Throughout all the age groups, the patient group had

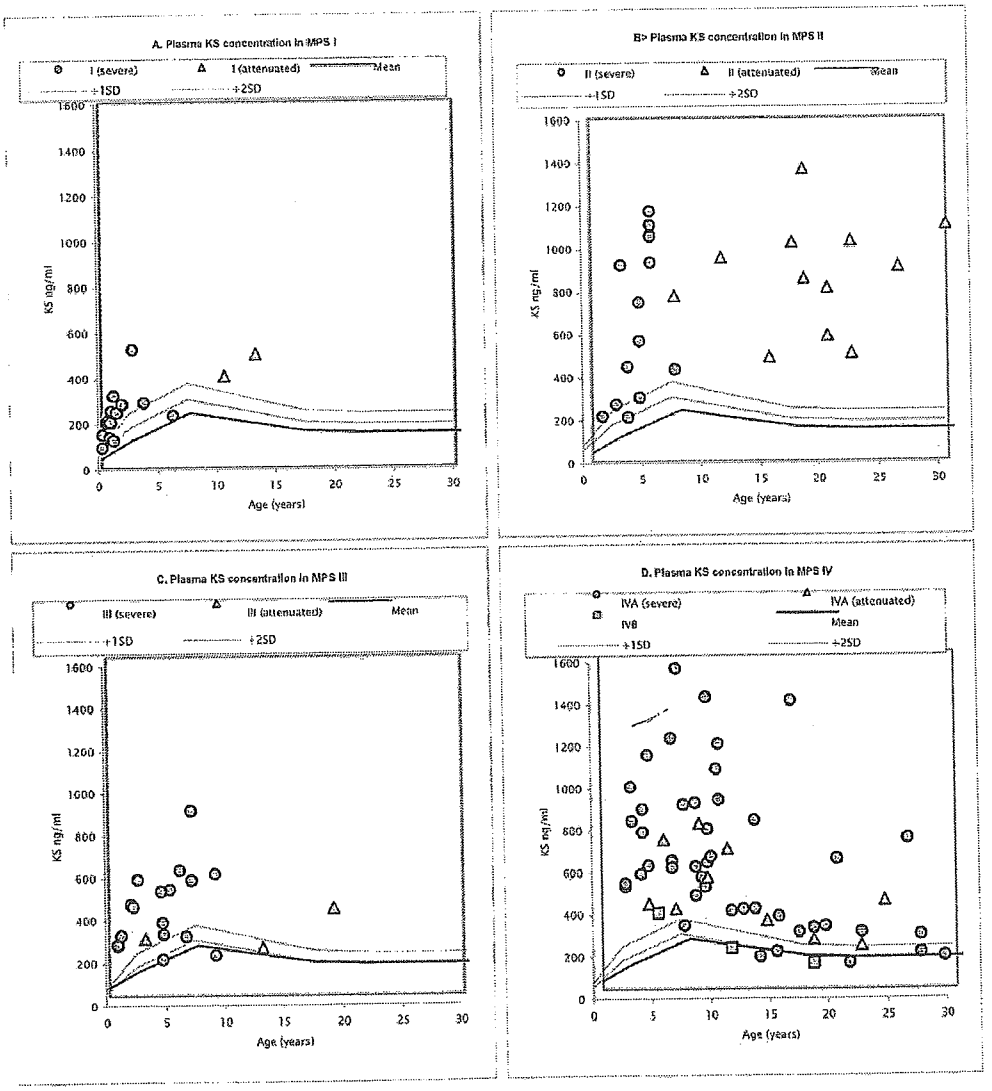

Figure 1 Concentrations of KS in plasma of patients with MPS, ML and other LSDs, and normal individuals. Results of all specimens from each individual were plotted with respect to age. The age was shown up to 30 years in each disease group

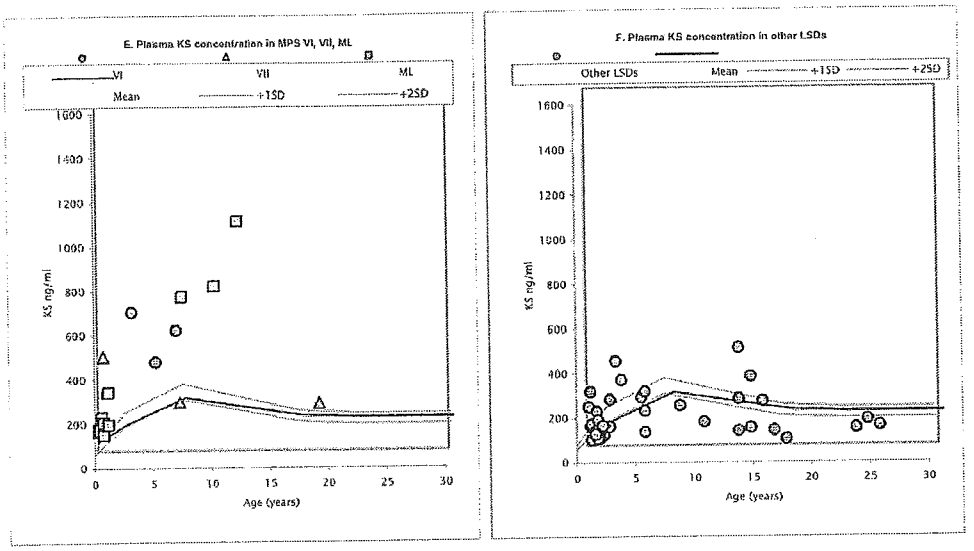

Figure 1 *Continued*

a significantly higher urine KS level compared with the age-matched control group, as shown in the fourth column of Table 1B.

When each type of MPS and ML and the age-matched controls (Table 1B and Figure 2) were compared, urine KS levels in MPS I showed that 6 out of 34 (17.6%) patients had above the mean + 2SD of the age-matched controls (Figure 2A). Twelve of 34 (35.3%) MPS II patients had urine KS levels above the mean + 2SD of the age-matched controls (Figure 2B). Urine KS values in 6 of 32 (18.8%) MPS III patients were above the mean + 2SD of the age-matched controls (Figure 2C). Only 1 of 7 MPS VI patients revealed urine KS level above the mean + 2SD (Figure 2E). One of 9 MPS VII patients showed urine KS levels above the mean + 2SD. Urine KS values in 5 of 11 (45.5%) ML patients were above the mean + 2SD of the age-matched controls. Overall, 24.4% of individual values in other MPS and ML patients plotted above the mean + 2SD of the age-matched controls, suggesting that urine KS concentration is not a suitable indicator as a screening test for these types.

In contrast, the mean values in MPS IVA and MPS IVB were 9.1 and 2.6 mg/g creatinine with 76 of 78 (97.4%) MPS IVA and 6 of 7 (85.7%) MPS IVB patients above the mean + 2SD of the age-matched controls (Figure 2D). Comparing plasma and urine KS levels in each type demonstrated that there is a difference in the blood KS to urine KS ratio between MPS IV patients and other MPS and ML patients.

Additionally, 6 of 20 (30%) other LSD patients had urine KS level above the mean + 2SD of the age-matched controls (Figure 1D).

To confirm that the elevation observed in MPS and ML patients is derived specifically from KS, the specificity of the immunoassay for KS was assessed by determining the cross-reactivity (%) to structurally related compounds. The KS assays all showed cross-reactivity of <0.1% against HS, DS and CS compared with the same