Dihydropyrimidine Dehydrogenase Deficiency

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Dihydropyrimidine dehydrogenase deficiency: homozygosity for an extremely rare variant in DPYD due to uniparental isodisomy of chromosome 1

--Manuscript Draft--

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Abstract:	Dihydropyrimidine dehydrogenase (DPD) deficiency is a rare autosomal recessive disorder of the pyrimidine degradation pathway and can lead to intellectual disability, motor retardation and seizures. Genetic variations in DPYD have also emerged as predictive risk factors for severe toxicity in cancer patients treated with fluoropyrimidines. We recently observed a child born to non-consanguineous parents, who demonstrated seizures, cognitive impairment, language delay and MRI abnormalities, and was found to have marked thymine-uraciluria. No residual DPD activity could be detected in peripheral blood mononuclear cells. Molecular analysis showed that the child was homozygous for the very rare c.257C>T (p.Pro86Leu) variant in DPYD. Functional analysis of the recombinantly-expressed DPD mutant showed that the DPD mutant carrying the p.Pro86Leu did not possess any residual DPD activity. Carrier testing in parents revealed that the father was heterozygous for the variant but unexpectedly the mother did not carry the variant. Microsatellite repeat testing with markers covering chromosome 1 showed that the DPD deficiency in the child is due to paternal uniparental isodisomy. Our report thus extends the genetic spectrum underlying DPYD deficiency.

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Dear prof. dr. Morava-Kozicz,

Please find enclosed our revised manuscript entitled: "**Dihydropyrimidine dehydrogenase deficiency: homozygosity for an extremely rare variant in *DPYD* due to uniparental isodisomy of chromosome 1**", by André BP van Kuilenburg, Judith Meijer, Rutger Meinsma, Belén Pérez-Dueñas, Marielle Alders, Zahurul A Bhuiyan, Rafael Artuch and Raoul CM Hennekam.

We are pleased with the favorable remarks and helpful suggestion of the Editor. We have revised our manuscript in accordance with the suggestion of the Editor.

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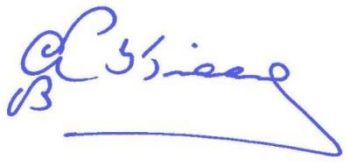

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We are very pleased with the favorable remarks and helpful suggestion of the Editor.

We should like to reply to the remarks of the Editor as follows:

COMMENTS FOR THE AUTHOR:

The authors have appropriately dealt with the recommendations for improvement of their manuscript.
However, the (newly introduced) control median value '0' for thymine in figure 1 looks strange. Wouldn't it be more appropriate to indicate that the value is below the quantitation (or detection) limit, which should be stated?

For the vast majority of the analyzed urine samples, we could not detect thymine as the concentration was below the limit of quantitation (LOQ). Although the LOQ is known for the absolute concentration of thymine (µM), there is no such LOQ value for the concentration of thymine expressed per mmol creatinine (µmol/mmol creatinine) since the latter will depend on the amount of creatinine in the urine of each individual.

For that reason, we have now stated in table 1 that the mean concentration and range of thymine in urine is < 1 µmol/mmol creatinine.

Dihydropyrimidine dehydrogenase deficiency: homozygosity for an extremely rare variant in *DPYD* due to uniparental isodisomy of chromosome 1

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) deficiency is a rare autosomal recessive disorder of the pyrimidine degradation pathway and can lead to intellectual disability, motor retardation and seizures. Genetic variations in *DPYD* have also emerged as predictive risk factors for severe toxicity in cancer patients treated with fluoropyrimidines. We recently observed a child born to non-consanguineous parents, who demonstrated seizures, cognitive impairment, language delay and MRI abnormalities, and was found to have marked thymine-uraciluria. No residual DPD activity could be detected in peripheral blood mononuclear cells. Molecular analysis showed that the child was homozygous for the very rare c.257C>T (p.Pro86Leu) variant in *DPYD*. Functional analysis of the recombinantly-expressed DPD mutant showed that the DPD mutant carrying the p.Pro86Leu did not possess any residual DPD activity. Carrier testing in parents revealed that the father was heterozygous for the variant but unexpectedly the mother did not carry the variant. Microsatellite repeat testing with markers covering chromosome 1 showed that the DPD deficiency in the child is due to paternal uniparental isodisomy. Our report thus extends the genetic spectrum underlying *DPYD* deficiency.

Synopsis: The c.257C>T (p.Pro86Leu) variant in *DPYD* results in a mutant DPD enzyme without residual activity and uniparental isodisomy should be considered in DPD deficient patients with only one parent being a carrier for a pathogenic variant in *DPYD*.

Compliance with Ethics Guidelines

Conflict of Interest

André van Kuilenburg, Judith Meijer, Rutger Meinsma, Belén Pérez-Dueñas, Marielle Alders, Zahurul A. Bhuiyan, Rafael Artuch and Raoul Hennekam declare that they have no conflict of interest.

Details of Ethical Approval

The study (W16_179 # 16.210) was approved by the Medical Ethics Committee of the Academic Medical Center.

Patient consent statement

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the parents of the child included in this study for publication.

Authors' Contribution

André van Kuilenburg and Raoul Hennekam: study design, data analysis and drafting of the article

Judith Meijer, Rutger Meinsma, Marielle Alders, Zahurul A. Bhuiyan: Experimental data acquisition and data analysis

Belén Pérez-Dueñas, Rafael Artuch: Patient care, drafting of the article

Guarantor and Corresponding Author

André B.P. van Kuilenburg accepts full responsibility for the work and conduct of the study, had access to the data, and controlled the decision to publish.

Details of Funding

None

Key-words: dihydropyrimidine dehydrogenase; *DPYD*; pyrimidines; uniparental isodisomy

Introduction

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme of the pyrimidine degradation pathway, catalyzing the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. In patients with a complete DPD deficiency (MIM 274270), a considerable variation in the clinical presentation has been observed ranging from severely (neurologically) affected to symptomless. Therefore, a DPD deficiency is probably a necessary, but not a sole prerequisite for the onset of a clinical phenotype (Fleger et al 2017; van Kuilenburg et al 1999). Delayed cognitive and motor development and convulsive disorders are relatively frequent manifestations whereas growth retardation, microcephaly, dysmorphia, autism, hypotonia and ocular abnormalities are less frequently observed (Chen et al 2014; Enns et al 2004; van Kuilenburg et al 2002a; van Kuilenburg et al 2009; van Kuilenburg et al 1999). In addition, patients with a DPD deficiency have a strongly reduced capacity to degrade the widely used chemotherapeutic drug 5-fluorouracil and therefore, an increased likelihood of suffering from severe and sometimes fatal multi-organ toxicity (Johnson and Diasio 2001; van Kuilenburg 2004).

DPYD is present as a single copy gene on chromosome 1p21.3 and consists of 23 exons (Wei et al 1998). A large number of variants have been described in *DPYD* including large genomic deletions and amplifications (van Kuilenburg et al 2009). The identification of novel disease-causing genomic aberrations is important to allow analysis of genotype-phenotype relationships in DPD-deficient patients and screening of cancer patients at risk. Our study identified a novel genetic mechanism underlying DPD deficiency and we present the first patient with a complete DPD deficiency due to paternal uniparental isodisomy of chromosome 1.

Materials and Methods

Sequence analysis of *DPYD*, including analysis of intragenic rearrangements, was carried out essentially as described before (van Kuilenburg et al 2017). Analysis of pyrimidine metabolites was performed using reversed-phase HPLC combined with electrospray tandem-mass spectrometry (van Lenthe et al 2000). Functional expression of a *DPYD* mutation in mammalian HEK293 Flp-In cells and subsequent analysis of recombinantly-expressed DPD protein levels and DPD activity was performed as described before (van Kuilenburg et al 2017).

Twenty six microsatellite repeat markers spreading over the full length of chromosome 1 were used for haplotype analysis. These included 21 markers from ABI-Prism Linkage Mapping Set MD panel 1 and 2 (PE Biosystems, Foster City, CA, USA) and 5 additional markers: D1S2775, D1S2719, D1S2793, D1S415 and D1S2753 (NCBI, UniSTS). After PCR, the amplified fragments were separated using the ABI Prism 377 automatic DNA sequencer (PE Biosystems, Foster City, CA, USA), and the length of the fragments was analyzed with Genemapper software (PE Biosystems, Foster City, CA, USA).

Results

Case report

The female patient was the first child of non-consanguineous Portuguese parents. Developmental delay was noticed during the second year of life: she walked unassisted at the age of 20 months and showed language delay. At the age of 3 years, she started to have seizures. Despite treatment with valproic acid and carbamazepine she continued to have seizures every few weeks to months. A neuropsychological study at 5 years 8 months using the McCarthy Scales of Children's Abilities (MSCA) showed significantly reduced scores [verbal: 22; perceptual-performance: 22; quantitative: 22; memory: 25; motor: 24 (Controls: mean ± standard deviation 50 ± 10), and general cognitive index: 50 (Controls: mean ± standard deviation 100 ± 15)]. Neurological examination at the age of 7 years revealed a non-dysmorphic child with normal growth and head circumference and poor fine and gross motor coordination. She was socially engaging and showed cognitive impairment and language delay. Magnetic resonance imaging (MRI) demonstrated symmetrically enlarged lateral ventricles and a thin corpus callosum. Cerebral white matter signal was normal. EEG showed generalized slow wave discharges with maximal amplitude in frontal lobes and poor organization of background activity. At 10 years, the Peabody Picture Vocabulary Test-IV (PPVT-IV) revealed markedly low verbal abilities (verbal age 4 years and 4 months). An attempt to withdraw valproic acid at 10 years increased epileptic activity. Currently, the patient is 12 years old and she has adapted to a mainstream school with the support of special education teachers and speech therapy. She suffers from occasional partial and secondarily generalized tonic-clonic seizures. Background activity on EEG recording has normalized and no paroxysms are registered.

Biochemical and genetic studies

As part of a screening for inborn errors of metabolism, purines and pyrimidines were analyzed in urine and plasma. Strongly elevated concentrations of uracil and thymine were observed in urine and plasma which suggested that the patient had a DPD deficiency (Table 1). Subsequent analysis showed no residual DPD activity in peripheral blood mononuclear cells. Sequence analysis of *DPYD* showed

that the patient was homozygous for the c.257C>T (p.Pro86Leu) variant (Table 1). Expression of the mutant *DPYD* construct containing the c.257C>T (p.Pro86Leu) variant in HEK293 Flp-In cells showed that the DPD mutant carrying the Pro86Leu variant possessed hardly any residual activity (0.7%) compared to the wild-type enzyme (Fig. 1). To exclude the possibility that the lack of DPD activity was the result of an inability to produce the mutant DPD protein in HEK293 Flp-In cells the DPD protein expression levels were analyzed by immunoblotting. Fig. 1 shows that the mutant DPD protein, carrying the Pro86Leu variant, was expressed in a comparable amount as the wild-type protein. Thus, the lack of DPD activity of the mutant DPD enzyme in HEK293 Flp-In cells is not due to rapid degradation of the mutant DPD protein in the HEK293 Flp-In lysates.

DNA sequence analysis in the father demonstrated that he was heterozygous for the c.257C>T variant in *DPYD* but in the mother the variant could not be detected. *DPYD* is prone to acquire genomic rearrangements due to the presence of an intragenic fragile site *FRA1E*, but MLPA analysis showed no intragenic deletions or amplifications of *DPYD* in the patient or parents. Haplotype analyses with 26 microsatellite repeats distributed over chromosome 1 to probe for homozygosity for the c.257C>T variant in *DPYD* by uniparental isodisomy for chromosome 1 demonstrated the patient to be homozygous for all 26 markers (Fig. 2). Fourteen markers were uninformative since they could have been inherited from either parent. For one marker only paternal uniparental disomy (UPD) could be proven. Paternal isodisomy was observed for 11 markers (Fig. 2).

Discussion

Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal recessive disease characterized by thymine-uraciluria in homozygous deficient patients. Here, we present the first case of DPD deficiency due to uniparental isodisomy. The phenotype in the patient, i.e. cognitive impairment, language delay and seizures, is similar to the phenotype typically observed in clinically-affected patients with DPD deficiency (van Kuilenburg et al 2002a; van Kuilenburg et al 2009; van Kuilenburg et al 1999). The MRI findings in the present patient are nonspecific and have been reported infrequently in DPD deficient patients (Chen et al 2014; Enns et al 2004). Chromosome 1 is not known to contain imprinted areas or imprinted genes, so UPD of chromosome 1 is not expected to cause a phenotype by a disturbed methylation.

The frequency of UPD in newborn is considered to be 1 in 3,500-5,000 (Liehr 2010). Chromosomes 7, 11, 14, 15 and 16 are most often involved in uniparental isodisomy formation and for chromosome 1 only a moderate frequency of uniparental isodisomy has been observed (Liehr 2010). The c.257C>T variant (rs568132506) is extremely rare in the general population (allele frequency 5.4 x 10^{-5} in gnomAD; http://gnomad.broadinstitute.org/variant/1-98206012-G-A) So far, this variant has been described in only one patient with a complete DPD deficiency (van Kuilenburg et al 2002a). Analysis of the crystal structure of DPD showed that Pro86 is in close proximity to one of the iron-sulfur clusters in the N-terminal domain (van Kuilenburg et al 2002a). The introduction of a leucine at this position would interfere with the binding of the iron-sulfur cluster, thereby inhibiting electron-transport and thus activity (van Kuilenburg et al 2002a).

The elucidation of genetic mechanisms underlying DPD deficiency is increasingly being appreciated since DPD deficiency has been recognized as an important determinant of fluoropyrimidine-associated toxicity in cancer patients (van Kuilenburg et al 2017; van Kuilenburg 2004). To date, many pathogenic variants have been described in *DPYD* and additional rare variants may collectively explain an appreciable fraction of patients with DPD deficiency. Therefore, the identification of novel genetic mechanisms underlying DPD deficiency will not only allow analysis of genotype-phenotype relationships in DPD-deficient patients but also screening of cancer patients at

risk. Our study showed that uniparental isodisomy should be considered in DPD deficient patients with only one parent being a carrier for a pathogenic variant in *DPYD*.

Titles and legends to figures

Fig. 1 DPD activity and immunoblot analysis of recombinantly-expressed wild-type and mutant DPD enzymes. The results represent the relative DPD activity (mean + SD, n=3) of the DPD mutant carrying the Pro86Leu variant compared to wild-type DPD enzyme. The insert shows the immunoblot analysis of the expressed wild-type and mutant DPD enzyme.

Fig. 2 Genotype analysis of chromosome 1 using 26 microsatellite repeats. The patient was homozygous for all 26 markers and showed paternal uniparental isodisomy for 11 markers, paternal UPD for one marker and 14 markers were uninformative. The presence of the c.257C>T variant (M) or wild-type sequence (WT) of *DPYD* is indicated for the patient and parents.

Table 1

Biochemical and genetic analysis of a DPD deficient patient

Subject	Urine (µmol/mmol creatinine)		Plasma (µM)		DPD activity [nmol/(mg total protein. h)]	DPYD[a]
	Uracil	Thymine	Uracil	Thymine		
Patient	236	131	13.4	15.2	< 0.025	c.257[C>T];[C>T]
Father	n.a.	n.a.	n.a.	n.a.	n.a.	c.257[C>T];[=]
Mother	n.a.	n.a.	n.a.	n.a.	n.a.	c.257C=
Controls					9.9 ± 2.8 (n=54)[b]	
Median	5	< 1	0.19	0.04		
Range	1-35 (n =112)	< 1 (n=112)	0.08-0.36 (n=100)	0.02-0.09 (n=100)		

n.a., not available; [a]nomenclature according to http://varnomen.hgvs.org/; [b]Data taken from (van Kuilenburg et al 2002b)

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Figure 1

Click here to download Figure Figure 1 (DPD activity and blot).tif

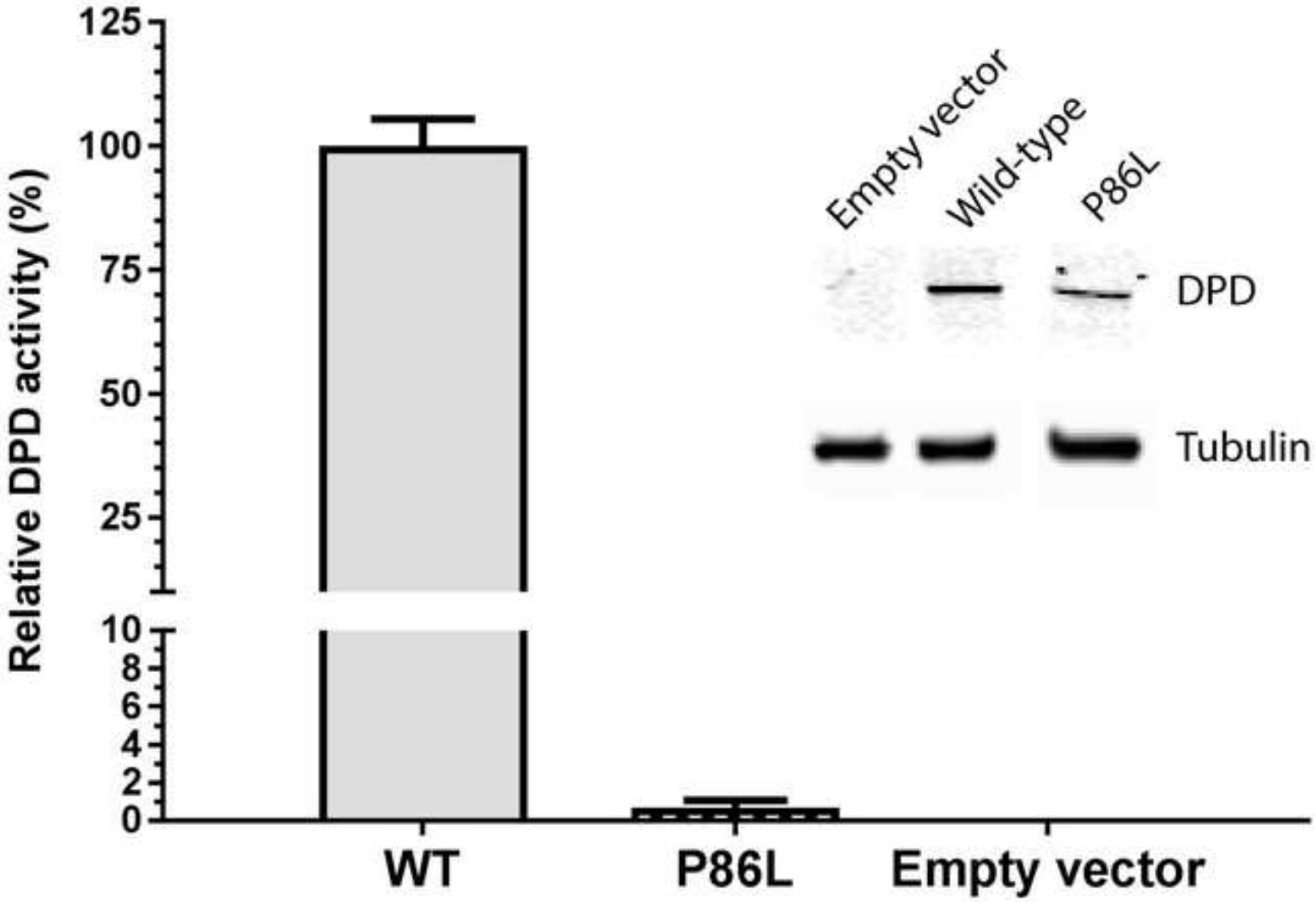
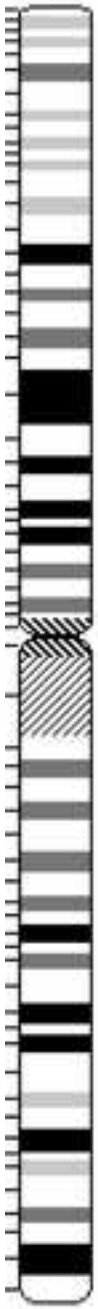


Figure 2



Locus	Patient	Father	Mother	Inheritance
D1S468	1,1	1,2	3,4	Paternal isodisomy
D1S214	1,1	1,2	1,3	Not informative
D1S450	1,1	1,2	1,3	Not informative
D1S2667	1,1	1,1	2,3	Paternal UPD
D1S199	1,1	1,2	3,4	Paternal isodisomy
D1S234	1,1	1,1	1,2	Not informative
D1S255	1,1	1,2	2,3	Paternal isodisomy
D1S2797	1,1	1,2	3,4	Paternal isodisomy
D1S230	1,1	1,2	2,3	Paternal isodisomy
D1S2841	1,1	1,1	1,2	Not informative
D1S207	1,1	1,2	3,4	Paternal isodisomy
D1S2868	1,1	1,2	1,2	Not informative
D1S2775	1,1	1,1	1,2	Not informative
D1S2719	1,1	1,2	3,3	Paternal isodisomy
D1S2793	1,1	1,2	1,3	Not informative
DPYD	M, M	M, WT	WT, WT	Paternal isodisomy
D1S415	1,1	1,1	1,2	Not informative
D1S2753	1,1	1,2	1,2	Not informative
D1S206	1,1	1,2	1,1	Not informative
D1S2726	1,1	1,2	1,2	Not informative
D1S252	1,1	1,2	3,4	Paternal isodisomy
D1S498	1,1	1,2	1,1	Not informative
D1S484	1,1	1,4	2,3	Paternal isodisomy
D1S2878	1,1	1,2	3,4	Paternal isodisomy
D1S218	1,1	1,1	1,2	Not informative
D1S413	1,1	1,2	1,1	Not informative
D1S2836	1,1	1,2	3.4	Paternal isodisomy

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