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A clinical, cytogenetic and molecular study in Prader-Willi patients

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Abstract. Twenty-three patients with a putative diagnosis of Prader-Willi syndrome (PWS) were reassessed clinically and then examined by cytogenetic and molecular techniques. Nineteen patients fulfilled the diagnostic criteria for PWS and the remaining four were judged to have atypical PWS. Definite molecular defects were detected in all clinically typical PWS patients but one. A deletion of part of chromosome 15q could be identified molecularly in 14 patients (74%) and maternal disomy for chromosome 15 in four (21%). In all, except one, PWS patients with molecularly detected deletions, the deletion was also identified by cytogenetic studies. Cytogenetic deletions were suspected in two of the atypical PWS patients. However, based on the results of scoring the diagnostic criteria for PWS and on the PW71B methylation test, we were able to rule out PWS in all of our atypical patients. Our study confirms observations that both clinical and cytogenetic investigations can provide misdiagnoses of PWS in some patients, and the first, simple and fast investigation, which can confirm the PWS in most, if not all PWS patients, is molecular analysis by the methylation test.

Key words: chromosome 15, deletions, Prader-Willi syndrome, uniparental disomy.

Introduction

Although Prader-Willi syndrome (PWS) is a well-known, complex, multisystem disorder, its clinical variability, which additionally changes dramatically with age, makes diagnosis difficult (GREENBERG et al. 1987, BUTLER 1990). Prompt diagnosis is important not only to allow initiation of early

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medical and dietary management, but also to provide appropriate genetic counselling to the family (most PWS cases are sporadic).

PWS is associated with a loss of the paternal contribution in 15q11q13, resulting from deletion of the proximal long arm of paternally-derived chromosome 15, maternal uniparental disomy (UPD), and rarely from an imprinting mutation (BUITING et al. 1995). This suggests that PWS genes are subject to parental imprinting, probably by DNA methylation. The finding of parent-of-origin specific DNA methylation at a few loci within 15q11q13 (gene ZNF127, marker PW71, gene SNRPN) supports this suggestion (DITTRICH et al. 1992, GLENN et al. 1993, LALANDE 1994, SUTCLIFFE et al. 1994, WEVRICK et al. 1994, BUITING et al. 1995, GILLESSEN-KAESBACH et al. 1995a). High resolution banding techniques (HRT) have shown that the majority of probands have a de novo deletion, and that other chromosomal abnormalities (mainly unbalanced and apparently balanced translocations) involving the proximal region of the long arm of chromosome 15 may account for about 5% of cases (BUTLER 1990). Recent molecular studies have indicated that almost all clinically typical PWS patients have a demonstrable deletion or maternal UPD (LAI et al. 1993, CHU et al. 1994). In order to evaluate the clinical and laboratory methods of diagnosing PWS, and to understand the nature of the genetic defect, we have assesed a series of 23 (all our patients) with a putative diagnosis of PWS by clinical, cytogenetic and molecular investigations.

Patients and methods

Patients

All of the probands (12 males and 11 females) who participated in this study were selected from among over 2200 patients registered between 1993-1995 at the Department of Medical Genetics of the Children's Memorial Health Institute, which is a reference hospital for the entire country. These patients have visited our centre at least twice during the last three years to undergo a standarized symptom review (HOLM et al. 1993), clinical examination by a geneticist, neurologist, endocrinologist, psychiatrist, dentist, photography, anthropometric measurements and specific investigations (bone age, psychological assessment, cytogenetic studies and DNA analysis). The diagnostic criteria used for PWS were modified by us from those described by HOLM et al. (1993). We excluded cytogenetic and/or molecular abnormalities from the scoring, but used the same total score as the diagnostic limit for PWS both in children below 3 years and above 3 years, as was recommended by HOLM

et al. (1993). Clinical evaluation showed that 19 of these patients fulfilled diagnostic criteria for PWS; four of them were classified as atypical PWS (three boys and one girl). The age of the typical PWS patients ranged from 2 to 15.5 years; the age of the atypical PWS patients was from 2 to 8 years.

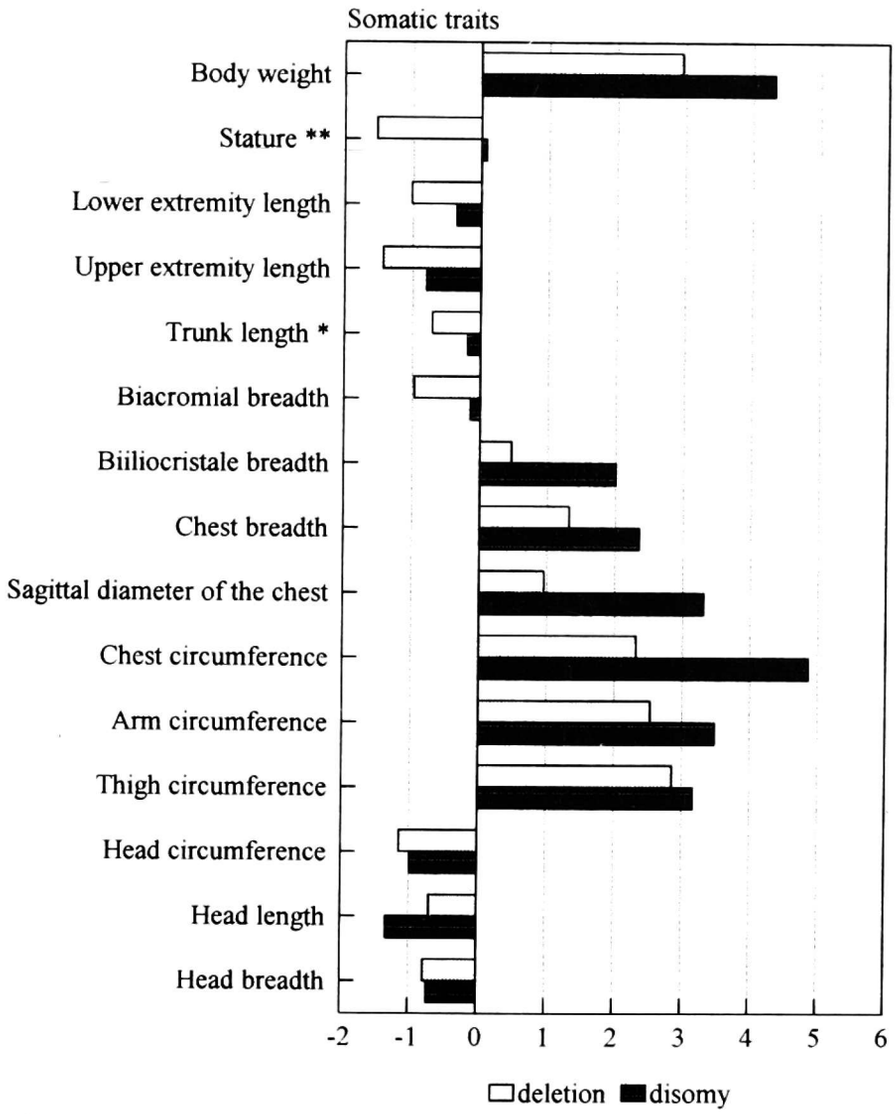

Fig. 1. Diagram of standard deviation score measurements of deletion (14) and disomy (4) patients with PWS (5-16 years) according to 1983 Warsaw Standards in respect to chronological age. * $P < 0.01$, ** $P < 0.05$

A detailed anthropometric study of 18 physical parameters, including 3 cephalic traits and the measurements of skin folds at 3 sites, was performed. The statistical significance of differences in somatic and cephalic traits between the PWS children with deletion and disomy in comparison with healthy children of the same chronological age were evaluated by the Student's t-test (Fig.1).

Cytogenetic studies

Cytogenetic examination was performed in all probands, and where possible, in their parents. We studied prometaphase (looking for 15q deletions)

and metaphase (analysing short arm variants for heteromorphism studies) chromosomes. Mainly, the GTG technique was used, but to confirm the parental origin of pair 15 chromosomes, QFQ and CBG techniques were applied as well.

Molecular genetic analysis

RFLP analyses

Genomic DNA was isolated from peripheral blood leucocytes. Aliquots of DNA were digested with restriction endonucleases: BglII, RsaI, ScaI, or TaqI, according to the manufacturer's instructions. Restriction fragments were electrophoresed on 0.8% agarose gels and then transferred to nylon membranes by Southern blotting. Membranes were hybridized to P-32 labelled DNA probes specific for chromosome 15q11-q13: IR-39 (D15S18), ML-34 (D15S9), IR4-3R (D15S11), 189-1 (D15S13) and IR-10-1 (D15S12), provided by American Type Culture Collection. After washing, the filtres were exposed to X-ray films at 70°C for 2-10 days. Deletions in patient's DNA were identified by the absence of the paternal alleles.

Methylation pattern analysis

DNA was digested with HindIII and then HpaII, separated on 1% agarose gels and analysed by hybridization with P-32 labelled probe PW71B (D15S63), provided by Dr. K. BUITING.

Microsatellite polymorphism analyses

The following $(CA)_n$ repeat markers were used:
– from the PWS/AS region: IR4-3R (D15S11), TD3-21 (D15S10), GABRB3*, D15S128*, D15S122*, D15S97*,
– from chromosome 15, outside the PWS/AS region: ACTC*, CYP19*, D15S108*, FES* (*primers provided by Research Genetics).
PCR was performed on 0.5 µg DNA with the following reaction mixture: $MgCl_2$ 1.5 mM, KCl 50 mM, Tris 10 mM pH 8.3, dNTP 0.2 mM, oligonucleotide primers 0.8 µM, Taq polymerase 1 u/50 µl (35 cycles: 94°C 40-60 s, 52-55°C 30-60 s and 72°C 30-120 s). 12 µl of PCR products with 8 µl denaturating loading buffer were heated to 94°C 5 min and then loaded onto 8% denaturating (6 M urea)polyacrylamide gel. The gel was run overnight at 6-8W or four hours at 60 W. The fragments on the gel were detected by silver staining.

Results and discussion

From among the 23 patients studied, 19 were found to have clinically typical PWS and 4 were defined as atypical PWS patients. Their results of clinical assessment, cytogenetic and molecular analysis are presented in Table 1. Clinical details of four atypical PWS patients are listed in Table 2. Cytogenetic deletions had been suspected in two of these patients, but all of them had a normal methylation pattern. Only one atypical patient had been diagnosed in infancy. He was suspected of PWS because of severe hypotonia, poor weight gain, characteristic facial features with dolichocephaly and genital hypoplasia (small penis). Neither difficult feeding nor cryptorchidism were observed. In addition, at the age of 2 years he was not obese and did not have hyperphagia, but slight global developmental delay was evident. The remaining three atypical PWS patients were seen by us for the first time at the age of 7-8 years. Their neonatal history could be assesed only on the basis of parental interviews and we doubt if that information is objective. All of these patients had global developmental delay in early childhood and multiple learning problems later. In addition, one of them was moderately mentally retarded, one had cryptorchidism, one had specific behaviour problems and one had short stature. In patient BG, diagnosis of Bardet-Biedl syndrome was also considered because of the obesity, postaxial polydactyly and genital hypoplasia. However, up to the age of 12 years, he had no sings of characteristic pigmentary retinopathy (LEPPERT et al. 1994). By using the PW71B methylation test and scoring the diagnostic criteria for PWS, we were able to rule out PWS in these four patients.

In 18 out of 19 patients who were found to have clinically typical PWS, definite molecular defects were detected on the basis of molecular analysis by RFLP, microsatellite markers and PW71B methylation test. Fourteen of them had a paternal deletion, and four were disomic. Almost all identified deletions were relatively large and covered many loci from the region (15q11q13) (Fig. 2). The most frequent deletions were defined by loci the D15S11, D15S13, D15S63, D15S10, GABRB3, D15S12 and D15S128. In six patients the deletions extended to the proximal loci D15S9 and D15S18 or to the distal loci D15S122 and D15S97 as well. Because of some uninformative polymorphic markers, the borders of the deletions were estimated approximately. Interestingly, in all except one PWS patient with molecularly detected deletions, the deletion was also identified by cytogenetic studies. Our heteromorphism studies showed that the origin of the deleted chromosomes could be assesed in three cases; nine families were uninformative and two families

Table 1. Individual results of clinical assessment, cytogenetic and molecular analysis

Patient No/Age,y/sex	Clinical assessment	Total clinical criteria score	Cytogenetics: karyotype/ variants analysis	RFLP and microsatellite polymorphism analysis	Loss of paternal methylation pattern at D15S63
KaK/2/F	Typical	6.5	Deletion/Paternal deletion	Paternal deletion	Yes
ZK/3/F	Typical	7	Deletion/N.I.	Paternal deletion	Yes
BS/3/M	Typical	7	Normal/Maternal heterodisomy	Partial maternal isodisomy	Yes
JM/4/F	Typical	11.5	Deletion/N.I.	Paternal deletion	Yes
PZ/4/F	Typical	11	Deletion/N.I.	Paternal deletion	Yes
BM/5/M	Typical	11	Normal/Maternal heterodisomy	Maternal heterodisomy	Yes
JuJ/5/F	Typical	11.5	Deletion/N.I.	Paternal deletion	Yes
RW/5/M	Typical	11	Deletion/N.I.	Paternal deletion	Yes
KM/7/M	Typical	10	Deletion/N.I.	N.I.	No
WN/7/F	Typical	11	Deletion/Paternal deletion	Paternal deletion	Yes
Słu/7.5/M	Typical	11	Normal/N.T.	Maternal heterodisomy	Yes
LM/8/M	Typical	11.5	Deletion/N.T	Paternal deletion	Yes
MJ/8/F	Typical	11	Normal/Maternal heterodisomy	Maternal heterodisomy	Yes
DG/8/M	Typical	10	Deletion/Paternal deletion	Paternal deletion	Yes
TA/8/F	Typical	11	Normal/N.I.	Paternal deletion	Yes
ZM/12/F	Typical	11.5	Deletion/N.I.	Paternal deletion	Yes
ZaM/12/M	Typical	11	Deletion/Paternal deletion	Paternal deletion	Yes
MP/13/M	Typical	12	Deletion/N.I.	Paternal deletion	Yes
SŁ/15.5/M	Typical	12.5	Deletion/N.I.	Paternal deletion	Yes
FP/2/M	Atypical	4	Normal/N.I.	N.T	No
BG/7/M	Atypical	6.5	Deletion/N.I.	N.T	No
SM/8/M	Atypical	7	Deletion/N.I.	N.I	No
CN/8/F	Atypical	7	Normal/N.T.	N.I	No

N.I. – not informative
N.T. – not tested

Table 2. Frequencies of common PWS features in atypical patients suspected of PWS

Clinical features	Atypical patients			
	BG/7y/M	SM/8y/M	FP/2y/M	CN/8y/F
Reduced fetal activity	+	+	+	+
Neonatal hypotonia	–	+	+	+
Poor sucking	+	+	–	+
Feeding problems	–	–	+	+
Psychomotoric retardation (IQ)	79	47	100	90
Hyperphagia	+	+	–	+
Personality problems	–	–	–	+
Obesity	+	+	–	+
Short stature (< -1SD)	–	–	+	+
Delayed bone age	?	+	+	+
Characteristic facies	+	+	+	+
Strabismus	+	–	+	–
Thick saliva	+	–	–	–
Caries	+	+	–	–
Enamel hypoplasia	–	–	–	–
Malocclusion	+	–	–	–
Cryptorchidism	+	–	–	
Hypogenitalism	+	+	+	–
Hypopigmentation	–	–	–	–

were not examined. In addition, in three patients without cytogenetic deletions it was possible to show the presence of two different maternal chromosomes 15 (maternal heterodisomy). On the other hand, molecular study revealed the existence of four patients with maternal disomy: three with heterodisomy and one with partial isodisomy. One clinically typical PWS patient, a girl aged 7 years (KM), showed neither molecular deletion nor disomy, although she had a cytogenetic deletion, and in addition, she had a normal methylation pattern at the locus D15S63. She fulfils the diagnostic criteria for PWS, but among the major clinical signs, feeding problems with poor weight gain in infancy were not observed.

Table 3 presents clinical features of 18 patients who were classified as having typical PWS; 14 with deletions and 4 with disomy. Although the number of PWS patients with deletions or disomy is too small to serve as a basis for

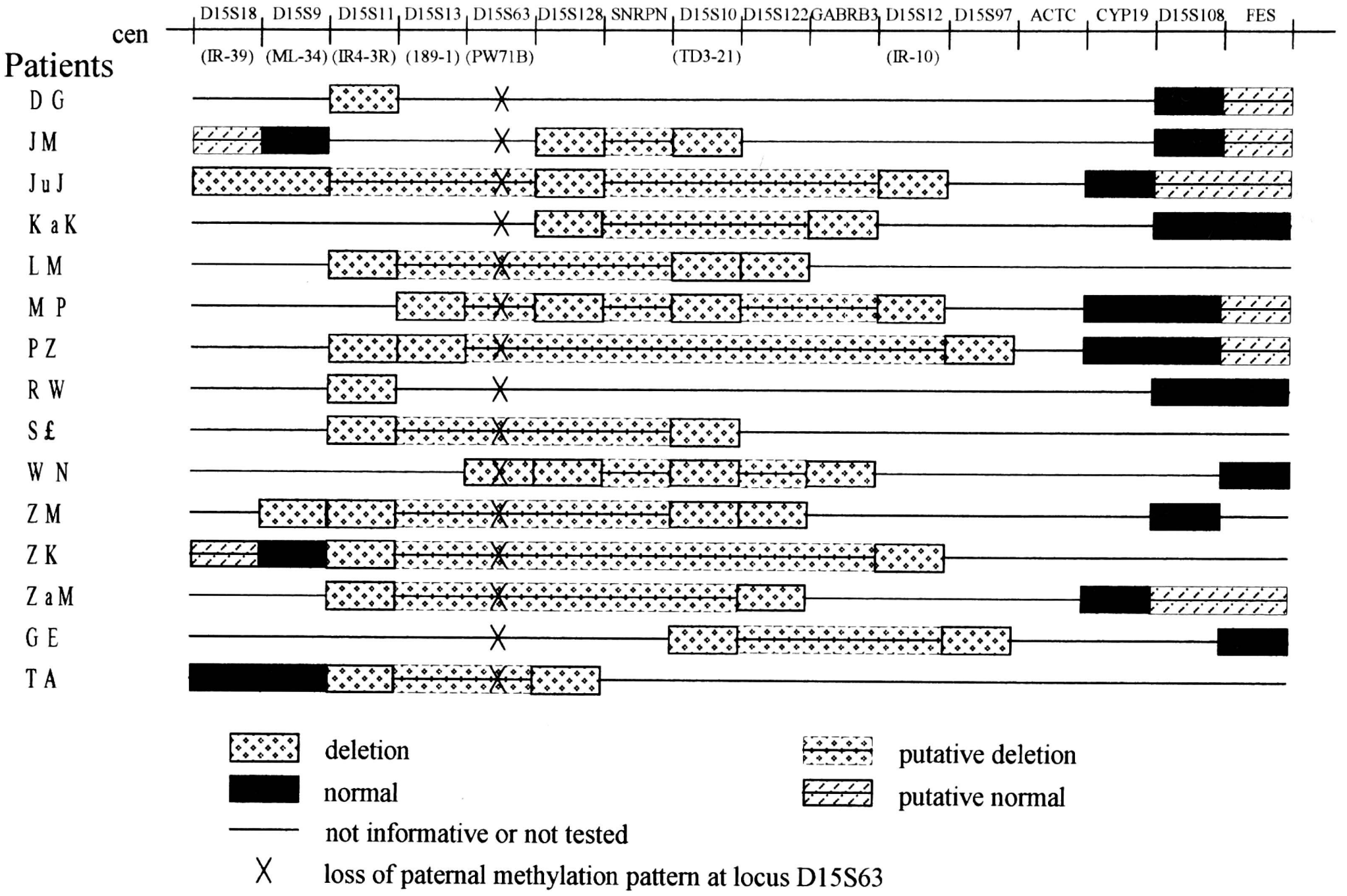

Fig. 2. Extent of molecular deletions based on RFLP and $(CA)_n$ repeat analyses of 15q11q26 markers

Table 3. Frequencies of common PWS features in patients with deletion and patients with disomy

Clinical features	No. with clinical features / total No.	
	Patients with deletion	Patients with disomy
Reduced fetal activity	12/14	3/4
Neonatal hypotonia	14/14	4/4
Poor sucking	14/14	3/4
Feeding problems	14/14	4/4
Psychomotoric retardation (IQ)	67	72
Hyperphagia	14/14	4/4
Personality problems	11/12	3/4
Obesity	14/14	4/4
Short stature (< -1SD)	8/14	1/4
Delayed bone age	9/12	0/2
Characteristic facies	14/14	4/4
Strabismus	9/14	3/4
Thick saliva	13/14	4/4
Caries	13/14	4/4
Enamel hypoplasia	10/14	2/3
Malocclusion	8/14	3/3
Cryptorchidism	6/6	3/3
Hypogenitalism	14/14	4/4
Hypopigmentation	10/14	0/3

statistical comparisons, patients with deletions at the time of evaluation seem to be shorter and to have a lower weight, length and head circumference at birth than patients with disomy. Like in the study of GILLESSEN-KAESBACH et al. (1995a), the birth weight and length of all our patients (both with deletion and disomy) were significantly lower compared with healthy newborns, whereas their head circumferences were normal. Neither of our disomy patients was hypopigmented. It has been suggested that hypopigmentation may be caused by hemizygosity at the P locus, which is located at the distal border of the PWS deletion region, and which is probably not imprinted (RINCHIK et al. 1993). It is also worth mentioning that the parental ages at birth of disomic patients were significantly higher than those of deletion patients; the mean maternal and paternal ages being 34.3 ($P < 0.05$) and 38 ($P < 0.01$), respectively (Table 4). Our data supports the assumption that nondisjunction, commonly associated

Table 4. Mean and standard deviation (SD) for age (years) of parents at birth of patient

Patient group	Mother			Father		
	mean	SD	n	mean	SD	n
Deletion	27.21	5.34	14	29.36	5.11	14
Disomy	34.25	4.50	4	38.00	1.41	4

with advanced maternal age, may be one of the mechanisms for development of maternal disomy (ROBINSON et al. 1991).

Our study confirms the observation that clinical diagnosis of PWS is complicated and misdiagnoses can still occur. Especially, the characteristic facies are difficult to score objectively as some features are subtle or nonspecific and change with age. From our observations and those of others, it appears that the most stable characteristic that does not change with age are "puffy" hands with delicate tapering fingers. Such hand abnormalities were present in all our typical PWS patients and maybe should be sought in all patients with this disorder.

In our study, similarly as in others, HRT misdiagnosed deletion in some cases (CHU et al. 1994). Recent application of FISH analysis with specific DNA probes proved to be a more sensitive approach than detection of deletions by HRT (BETTIO et al. 1995). However, standard cytogenetic analysis should be recommended in every PWS case to detect such chromosome abnormalities as translocations, inversion or ring chromosomes.

The presented study shows the advantage of the methylation test as the first, simple and fast molecular diagnosis in confirmation of a clinical diagnosis of PWS. The methylation test can identify PWS in most, if not all, PWS patients, both with deletion, disomy and imprinting mutation, but cannot distinguish between these aberrations (GILLESSEN-KAESBACH et al. 1995a, LERER et al. 1994, van den OUWELAND et al. 1995). To know the nature of the genetic defect, RFLP and/or microsatellite analyses, using polymorphic markers from the entire long arm of chromosome 15, should be done. Among 19 Polish patients with clinically typical PWS, deletions of part of chromosome 15q were identified in 74% cases and maternal disomy for chromosome 15 in 21% cases, which is similar to the data reported by others (KOKKONEN et al. 1995, ROBINSON et al. 1991, WEBB et al. 1995). In one patient suspected of having PWS, so far no molecular defect has been detected by us, so the type of mutation is still unknown or different etiology (phenocopy?) in patients such as this must be considered (CHU et al. 1994, LERER et al. 1994).

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