Paternal UPD15: Further Genetic and Clinical Studies in Four Angelman Syndrome Patients

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Among 25 patients diagnosed with Angelman syndrome, we detected 21 with deletion and 4 with paternal uniparental disomy (UPD), 2 isodisomies originating by postzygotic error, and 1 MII nondisjunction event. The diagnosis was obtained by molecular techniques, including methylation pattern analysis of exon 1 of SNRPN and microsatellite analysis of loci within and outside the 15q11-q13 region. Most manifestations present in deletion patients are those previously reported. Comparing the clinical data from our and published UPD patients with those with deletions we observed the following: the age of diagnosis is higher in UPD group (average 7 $^3/_{12}$ years), microcephaly is more frequent among deletion patients, UPD children start walking earlier (average age 2 $^9/_{12}$ years), whereas in deletion patients the average is 4 ½ years, epilepsy started later in UPD patients (average 5 $^{10}/_{12}$ years) than in deletion patients (average 1 $^{11}/_{12}$ years), weight above the 75th centile is reported mainly in UPD patients, complete absence of speech is more common in the deleted (88.9%) than in the UPD patients because half of the children are able to say few words. Thus, besides the abnormalities already described, the UPD patients have somewhat better verbal development, a weight above the 75th centile, and OFC in the upper normal range. Am. J. Med. Genet. 92:322–327, 2000. © 2000 Wiley-Liss, Inc.

KEY WORDS: Angelman syndrome; 15q deletion; uniparental disomy; genomic imprinting; meiosis II nondisjunction

INTRODUCTION

Angelman syndrome (AS) [Angelman, 1965] comprises hypotonia (90%), severe mental retardation (100%), absent speech (98%), seizures (80%), ataxia (100%), outbursts of laughter, micro and/or brachycephaly (90%), macrostomia (75%), and prognathism (95%). The gait is described as wide-based with arms held flexed and upheld at the elbows [Clayton-Smith and Pembrey, 1992; Fryburg et al., 1991; Robb et al., 1989].

The Prader-Willi (PWS) (hypotonia, obesity, and hyperphagia) and Angelman syndromes are clear examples of genomic imprinting in humans because the clinical manifestation of these syndromes depends on the sex of the parent-of-origin of mutations within the 15q11-q13 region. The genetic basis of AS is complex, and at present, it is unknown whether the syndrome is caused by the loss of function of a single gene or whether different genes are involved [Bürger et al., 1997]. About two thirds of the AS cases are due to maternal deletion within 15q11-q13 [Magenis et al., 1987; Knoll et al., 1989]; paternal uniparental disomy of chromosome 15 (UPD15) is detected in 2–3% [Nicholls, 1993]; approximately 2% of individuals show imprinting control center mutations identified by abnormal methylation pattern [Buiting et al., 1995, 1998; Glenn et al., 1997; Saitoh et al., 1996; Sutcliffe et al., 1994]; about 8% show mutations in the UBE3A gene [Kishino et al., 1997; Malzac et al., 1998; Matsuura et al., 1997], and there is a class of AS patients with undetected genetic mechanism. Recurrence risk in deletion and UPD cases is less than 1% and for familial imprinting mutation and UBE3A mutation may be 50% [Burger et al., 1997; Saitoh et al., 1997].

In general, AS is not suspected during the first year of life, but it is better recognized around 3–4 years of age. Some characteristics, such as seizures, outbursts of laughter, macrostomia, prognathism, and wide-based gait, become more evident after age 2 years; asymmetrical face and scoliosis can occur only at puberty [Buntinx et al., 1995]. An electroencephalogram (EEG) study can be useful for diagnosis if some of the AS characteristics are present; nevertheless, some of those manifestations are age-dependent and can disappear in older children [Clayton-Smith, 1992].

TABLE I. Manifestations Present in Our UPD and Deleted AS Patients*

	1[a] UPD (iso)	2[b] UPD (iso)	3 UPD	4 UPD (iso)	Deleted (19 cases)
Age at diagnosis (yr)	9	10 1/2	7	3 1/2	4 3/12
(range)					(1 5/12–11)
Maternal age (yr)	30	40	23	29	28 1/2
(range)					(18–39)
Paternal age (yr)	33	47	25	37	32 9/12
(range)					(26–52)
Birth weight (centile) (g)	3780	2400	3600	3150	2992.6 (p25)
Birth height (centile) (cm)	50	46	na	48	49.1 (p25)
Length (centile)					
<25					35.3% (6/18)
25–75			+		47.0% (8/18)
>75	+	+		+	17.6% (3/17)
Weight (centile)					
<25					33.3% (6/18)
25–75			+		44.4% (8/18)
>75	+	+		+	22.2% (4/18)
Developmental delay	+	+	+	+	100% (19/19)
Microcephaly	–	–	–	–	47.4% (9/19)
Absence of speech		+		+	88.9% (16/18)
Few words	+		+		11.1% (2/18)
Independent gait (yr)	2 8/12	na	3	–	3 1/2
(range)					(2 3/12–8)
Hyperactivity	+	na	+	–	93.3% (14/15)
Seizures	–	+	–	–	94.4% (17/18)
Onset of seizures (yr)		8			1 9/12
(range)					(6/12–4)

*+, presence; –, absence; na, data not available; iso, isodisomy.
[a]Fridman et al., 1998.
[b]Fridman et al., 2000.

Several authors suggested that AS patients with uniparental disomy (UPD) have a milder phenotype than patients with deletions [Bottani et al., 1994; Freeman et al., 1993; Gillessen-Kaesbach et al., 1995; Smith et al., 1997]. They pointed out that children with UPD have better physical growth, fewer or no seizures, less ataxia, and higher cognitive skills. Hypopigmentation is the only finding more common in deleted AS patients, because the gene responsible for pigmentation (P), located in the distal position of the 15q11-q13 region, is not imprinted.

In this study we describe the clinical and behavioral manifestations of four cases of paternal UPD15 in Brazilian AS children. We compared these cases and published UPD cases [Bottani et al., 1994; Freeman et al., 1993; Gillessen-Kaesbach et al., 1995; Malcolm et al., 1991; Nicholls et al., 1992; Prasad and Wagstaff, 1997; Smeets et al., 1992; Smith et al., 1997, 1998] to our deleted patients (n = 21) and show that better speech development, weight above the 75th centile and outer frontal circumference (OFC) in the upper normal range should be added to clinical variability present in patients with Angelman syndrome.

MATERIALS AND METHODS
Patients

Twenty-five patients (9 boys and 16 girls with ages ranging from 1 5/12 to 11 years) with Angelman syndrome were referred from neurologists of the Hospital das Clínicas, School of Medicine, University of São Paulo, Brazil.

Cytogenetic Studies

Chromosome of patients and parents were studied with the GTG-banding technique.

Fluorescence in situ hybridization (FISH) was performed by using a probe specific for the 15q11-q13 region. The probe *GABRB3* was obtained from Oncor, Inc. (Gaithersburg, MD), and the hybridization and immunochemical detection were performed according to the manufacturer's instructions. The probe also contains control chromosome 15 marker cosmids that detect specific sequences in 15q22; 20 cells were examined on each patient.

DNA Analysis
Methylation analysis. DNA was extracted from peripheral blood leukocytes by standard procedures. The methylation status of the PWS/AS region was assessed by Southern blotting [Southern, 1975]. Genomic DNA was digested with *Xba*I + *Not*I, separated by size on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized by using the probe that corresponds to a 0.6-kb *Eco*RI-*Not*I fragment that contains exon 1 of *SNRPN* [Glenn et al., 1996].

Dinucleotide repeat $(CA)_n$ polymorphisms within and outside the PWS/AS critical region. Microsatellite analyses were performed with three markers within the critical region 15q11-q13, 4-3RCA (*D15S11*), LS6-1CA (*D15S113*), and GABRB3CA (*GABRB3*), and two loci outside the PWS/AS region (D15S131 and D15S984) were studied to distinguish between deletion and UPD (data not shown). Deletion is disclosed if no maternal and only one of the two pa-

ternal alleles are present within the PWS/AS region and biparental inheritance is demonstrated outside this region. The presence of one paternal allele or two different paternal alleles within and outside PWS/AS region and no maternal alleles disclose isodisomy or heterodisomy, respectively. For UPD patients we also analyzed the loci D15S541 and D15S542, localized close to the centromere, which allow us to disclose the meiotic origin of the nondisjunction [Robinson et al., 1998] and additional loci outside the PWS/AS region (CYP19, D17S117, and D15S115). A postzygotic error was considered when all markers along the entire chromosome 15 showed an isodisomic state. The multiplex polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis of ^{32}P end-labeled amplification products followed the method described by Mutirangura et al. [1993].

Statistical Analysis

Statistical analysis was performed with Fisher's test, Student's t-test, and Mann-Whitney's test by using the level of confidence of $\alpha = 0.05$ as a statistically significant difference.

RESULTS

In our 25 AS patients, deletion was detected in 21 (14 girls and 7 boys) and UPD in 4 individuals (2 girls and 2 boys). Clinical characteristics of our UPD patients are presented in Table I and some patients are seen in

Fig. 1.   AS patients with (**a**) UPD and (**b–e**) deletion. a: 7 years; b: 5 4/12 years; c: 2 years; d: 3 10/12 years; e: 2 10/12 years.

TABLE II. Comparison of Clinical Findings Between Our Deleted Patients and
the UPD Cases Described[a] Plus Our Four Cases*

	UPD[a] (19 cases)	Deleted (21 cases)	P[b]
Age at diagnosis (yr)	**7 3/12**	**4 3/12**	(1) **0.0251**
(range)	(2 9/12–10 6/12)	(1 5/12–11)	
Maternal age (yr)	28 8/12	27 3/12	(1) 0.7943
(range)	(14–43)	(18–39)	
Paternal age (yr)	32 7/12	31 11/12	(1) 0.5443
(range)	(22–47)	(26–52)	
Birth weight (centile)	3232 g	2872 g	(2) 0.3337
Birth height (centile)	48 cm	48.8 cm	(2) 0.3712
Length (centile)			
<25	16.7% (3/18)	31.6% (6/19)	(3) 0.4470
25–75	38.9% (7/18)	52.6% (10/19)	(3) 0.5148
>75	44.5% (8/18)	15.8% (3/19)	(3) 0.0789
Weight (centile)			
<25	11.8% (2/17)	30.0% (6/20)	(3) 0.2455
25–75	17.6% (3/17)	50.0% (10/20)	(3) 0.0823
>75	**70.6% (12/17)**	**20.0% (4/20)**	(3) **0.0030**
Developmental delay	100% (19/19)	100% (21/21)	(3) 1.0000
Microcephaly	**15.8% (3/19)**	**57.9% (11/19)**	(3) **0.0170**
Absence of speech	**47.4% (9/19)**	**88.9% (16/18)**	(3) **0.0128**
Independent gait (yr)	**2 9/12**	**4 1/2**	(1) **0.0097**
(range)	(1 10/12–6)	(2 3/12–8)	
Hyperactivity	90.9% (10/11)	93.7% (15/16)	(3) 1.0000
Seizures	**42.1% (8/19)**	**81.7% (18/21)**	(3) **0.0072**
Onset of seizures (yr)	**5 10/12**	**1 11/12**	(1) **0.0010**
(range)	(1 1/2–12)	(6/12–4)	

*The bold data indicated those with statistical significance.
[a]Our 4 UPD cases plus the UPD patients already described [Bottani et al., 1994; Freeman et al., 1993; Gillessen–Kaesbach et al., 1995; Malcolm et al., 1991; Nicholls et al., 1992; Prasad and Wagstaff, 1997; Smeets et al., 1992; Smith et al., 1997, 1998].
[b]Statistical test using significance level of $\alpha = 0.005$. (1) Mann-Whitney's test; (2) Student's t-test; (3) Fisher's test.

Figure 1. A comparison between our deleted children and all published UPD patients is shown in Table II.

In the molecular investigation, all the patients showed only the 0.9-kb nonmethylated paternal band when SNRPN methylation pattern was tested, confirming the AS diagnosis (data not shown). Analysis of microsatellites within and outside the PWS/AS region was performed in all patients. The markers within PWS/AS region disclosed only one of the paternal bands, for at least 1 locus, in all cases except in patient 3 who had two paternal bands, indicating heterodisomy for this region; the biparental inheritance of the markers outside the PWS/AS region demonstrated deletion

in 21 cases. In four cases, with normal FISH results, UPD was confirmed when the loci outside the PWS/AS region were tested (Table III).

The study of loci D15S541 and D15S542 performed in the four UPD patients showed three isodisomies (patients 1, 2, and 4), and one noninformative case (patient 3). Patient 1 has the karyotype 45,XY, t(15;15) and was described previously [Fridman et al., 1998]. Besides being homozygous for the D15S542, D15S11, D15S113, and GABRB3 loci, patient 2 showed one crossover between the PWS/AS region (homozygous) and locus CYP19 (heterozygous) (Table III). So, we conclude that this patient results from a meiosis II non-

TABLE III. Microsatellites Analysis of the UPD AS Patients*

	D15S541	D15S542	*D15S11*	*D15S113*	*GABRB3*	CYP19	D15S117	D15S131	D15S984	D15S115
	0 cM	0 cM	15q11.2–q12, 3 cM	15q11.2–q12, 8 cM	15q11.2–q13, 9 cM	15q21.1 51 cM	15q15–q21, 59 cM	15q21–q22, 81 cM	15q24–q25, 90 cM	15q24–q25, 97 cM
Mother	**3,4**	**3,4**	**2,4**	1,1	1,2	**1,4**	**2,2**	3,3	2,3	**1,2**
1	**1,1**	**1,1**	**1,1**	1,1	3,3	**2,2**	**3,3**	3,3	2,2	**4,4**
Father	**1,2**	**1,2**	**1,3**	1,2	3,3	**2,3**	**1,3**	1,3	1,2	**3,4**
Mother	2,3	2,3	**1,2**	2,3	**3,3**	1,1	**2,3**	1,3	2,3	**2,2**
2	1,1	**4,4**	**1,1**	2,2	**1,1**	1,2	**1,4**	2,4	1,1	**1,3**
Father	1.1	**1,4**	**1,3**	1,2	**1,2**	1,2	**1,4**	2,4	1,1	**1,3**
Mother	1,1	1,1	—	**1,4**	2,3	1,3	1,2	1,2	1,3	1,2
3	2,2	2,2	—	**2,3**	1,2	2,2	1,1	1,1	2,2	2,2
Father	na	na	—	**na**	na	na	na	na	na	na
Mother	**1,3**	**1,3**	—	**2,2**	**1,1**	2,4	**1,2**	1,2	**1,3**	1,3
4	**2,2**	**2,2**	—	**1,1**	2,2	1,1	**4,4**	2,2	3,3	4,4
Father	**2,4**	**2,3**	—	**1,3**	2,3	1,3	**3,4**	2,3	2,3	2,4

*The markers are ordered from centromere to telomere, and the distances are based on Dib et al. [1996] and Robinson and Knoll [1997]. na, not available; italic, critical PWS/AS region; bold, the informative data.

disjunction because we documented an isodisomy with 1 crossover [Fridman et al., 2000].

Patient 3 had two different nonmaternal alleles in the PWS/AS critical region and only one allele (homozygous) at the centromeric loci D15S541 and D15S542; because the paternal DNA sample was not available, we cannot conclude the type of UPD in this case. The other two cases showed reduction (homozygous) for all tested markers, suggesting a postzygotic error. The molecular results of UPD patients are summarized in Table III.

All parents had normal karyotypes.

By comparing the two groups of AS patients, we observed statistically significant clinical differences besides those already described: delayed age-of-diagnosis and weight above the 75th centile in the UPD cases, prevalence of microcephaly in deleted patients, complete absence of speech more common in the deleted group and capacity to speak a few words in the UPD group, early walking in the UPD cases, and earlier onset of seizures in the deleted group.

DISCUSSION

This work is part of a research project on Brazilian AS children, and the diagnosis of AS was confirmed in all patients through the SNRPN methylation analysis. This test has been used as a diagnostic tool for AS and PWS because the methylation pattern is parent-specific in this region [Gillessen-Kaesbach et al., 1995; Glenn et al., 1996; Kubota et al., 1996; Saitoh et al., 1997] and detects 80% of the AS and 95% of the PWS patients.

Some findings in our patients are those commonly observed in AS children: brachycephaly (88%), macrostomia (100%) and wide-spaced teeth (100%). All of our children showed happy disposition with a constant smile or outbursts of laughter that sometimes looks inappropriate, and an easily excitable personality with hand flapping, ataxic gait with arms flexed and upheld at the elbows, sleep disturbance, love of water, and mirror reflection [Clayton-Smith, 1993; Clayton-Smith and Pembrey, 1992; Knoll et al., 1989; Williams et al., 1995; Zori et al., 1992]. AS children usually have a normal OFC at birth, but microcephaly can be detected around 1 year [Fryburg et al., 1991]. In our deleted patients microcephaly (OFC < 2nd centile) was present in 11 of 19 (57.9%) and in only 15.8% (3 of 19) of the UPD patients already described (Table II), but none of our four cases presented this trait (Table I).

Smith et al. [1996] described the clinical signs in 27 AS patients with deletion and concluded that the phenotypic variability observed in deletion patients can be observed in UPD patients and that there are no differences between these two groups, except for the lower incidence of seizures and gait improvement in UPD cases. By comparing the clinical data from 15 published UPD patients [Bottani et al., 1994; Freeman et al., 1993; Gillessen-Kaesbach et al., 1995; Malcolm et al., 1991; Nicholls et al., 1992; Prasad and Wagstaff, 1997; Smeets et al., 1992; Smith et al., 1997, 1998], including our four, with data from our 21 deletion patients, some significant differences were observed

(Table II): the age of diagnosis is higher in UPD children (average 7 $3/12$ years), mainly because the phenotypic and behavioral traits are more subtle; microcephaly is more frequent among deletion patients; UPD children start walking earlier (average age 2 $9/12$ years), whereas in our deleted patients it is on the average 4 ½ years; epilepsy started later in UPD patients (average 5 $10/12$ years) than in our deleted patients (average 1 $11/12$ years); weight above the 75th centile is reported mainly in UPD patients; although all patients showed speech impairment, complete absence of speech is more common in the deleted (88.9%) than in UPD patients where half of the children are able to say a few words (Table II).

The origin of a UPD individual depends on nondisjunction events that are associated with increased parental age [Robinson et al., 1993, 1996]. However, when parental ages of UPD and deletions patients were analyzed all together, no differences could be detected (Table II).

Despite clinical variability, UPD patients seem to have a milder phenotype with later onset of epilepsy and better motor development [Bottani et al., 1994; Gillessen-Kaesbach et al., 1995; Smith et al., 1998]. We suggest that the capacity to say some words and body weight and OFC in the upper normal range should be included in the clinical spectrum of AS. These findings corroborate the hypothesis that the severe phenotype of deleted patients is caused by haploinsufficiency of other genes in the deleted region [Ohta et al., 1999]. Because AS UPD patients could have a less typical phenotype, it is possible that they are underdiagnosed and the prevalence rate of 1:20,000 is underestimated [Kyllerman, 1995].

Recently it was suggested that microcephaly seems to be less frequent in AS subgroups without deletion [Saitoh et al., 1999] and that ataxia and seizures are absent in the UBE3A mutation patients [Moncla et al., 1999]. This information is important to decide which patient with normal methylation and microsatellites studies should be submitted to UBE3A mutation analysis.

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