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A combination of five short tandem repeats of chromosome 15 significantly improves the identification of Prader-Willi syndrome etiology in the Argentinean population

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ABSTRACT. Prader-Willi syndrome (PWS) is a multisystemic disorder caused by the loss of expression of paternally transcribed genes in the PWS critical region of chromosome 15. Various molecular mechanisms are known to lead to PWS: deletion 15q11-q13 (75% of cases), maternal uniparental disomy (matUPD15) (23%) and imprinting defects (2%). FISH and microsatellite analysis are required to establish the molecular etiology, which is essential for appropriate genetic counseling and care management. We characterized an Argentinean population, using five microsatellite markers (D15S1035, D15S11, D15S113, GABRB3, D15S211) chosen to develop an appropriate cost-effective method to establish the parental origin of chromosome 15 in nondeleted PWS patients. The range of heterozygosity for these five microsatellites was 0.59 to 0.94. The average heterozygosity obtained for joint loci was 0.81. The parental origin of chromosome 15 was established by microsatellite analysis in 19 of 21 non-deleted PWS children. We also examined the origin of the matUPD15; as expected, most of disomies were

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due to a maternal meiosis I error. The molecular characterization of this set of five microsatellites with high heterozygosity and polymorphism information content improves the diagnostic algorithm of Argentinean PWS children, contributing significantly to adequate genetic counseling of such families.

Key words: Microsatellite analysis, matUPD15, Prader-Willi syndrome, Microsatellites

INTRODUCTION

Prader-Willi syndrome (PWS; OMIN 176270) is a multisystemic disorder, mainly characterized by neonatal hypotonia, hypogonadism, hyperphagia leading to obesity, short stature, small hands and feet, behavioral problems, and mental retardation.

The incidence of this syndrome is approximately 1 in 15,000 (Cassidy, 1997). It is caused by the absence of the paternally derived PWS-Angelman syndrome (PWS/AS) critical region 15q11-13 of chromosome 15. Various molecular mechanisms have been recognized to cause PWS. The most common molecular defect, accounting for about 70% of the cases, is a large chromosomal deletion of the paternally derived 15q11-13 region, which includes a large cluster of imprinted and non-imprinted domain genes (Robinson et al., 1991). Approximately 25% of the cases are caused by maternal uniparental disomy (matUPD15), and 2% of the cases are caused by imprinting defects (Nicholls et al., 1989; Mascari et al., 1992).

Although most PW cases are sporadic, knowledge concerning molecular etiology is necessary for appropriate genetic counseling, since a significant recurrence risk (up to 50%) is associated with imprinting defects (Buiting et al., 1998). Also, several phenotype-genotype correlation studies have demonstrated varying degrees of severity in clinical, cognitive and behavioral manifestations according to the etiology (Cassidy et al., 1997; Webb et al., 2002; Bittel et al., 2003). Thus, the identification of the different genetic subtypes is also necessary for adequate care of patients with PWS.

A DNA-based methylation test is considered to be the first step in the PWS diagnosis algorithm, since it detects over 99% of affected individuals (Gillessen-Kaesbach et al., 1995; ASHG/ACMG Report, 1996; Monaghan et al., 2002). Once PWS is confirmed by methylation analysis, FISH and microsatellite studies are required to establish whether deletion, maternal uniparental disomy or imprinting defects are present (Monaghan et al., 1997). Deletions are detected by two-color FISH, using one or two probes within the typical deletion boundaries, along with a centromeric probe (Kuwano et al., 1992; Delach et al., 1994). On the other hand, a microsatellite analysis is required in order to establish whether there is matUPD15 or biparental inheritance. Many short tandem repeats that map to chromosome 15 have been identified. Initially, three dinucleotide repeat markers (D15S11, D15S113, GABRB3) in the PWS/AS critical region were identified and used as a diagnostic test for matUPD15 (Mutirangura et al., 1993). As these three markers were found not to be fully informative in all cases, various laboratories have included other microsatellites of chromosome 15 to improve the informativity

of microsatellite analysis, given the ethnic characteristics of each population and the local availability of technology (Christian et al., 1997; Fridman and Koiffman, 2000). Until now, there is no information available in Argentina about the frequency distribution of microsatellites mapping on chromosome 15. We examined several microsatellites of chromosome 15 in an Argentinean population in order to develop an appropriate cost-effective method to establish the parental origin of the 15q11q13 region in non-deleted PWS patients.

MATERIAL AND METHODS

Study population

General population

One hundred unrelated healthy Argentine blood donors (50 males, 50 females). The samples were collected randomly and anonymously, with the consent of the Ethics Committee of the Hospital de Pediatría "Prof. Dr. J.P. Garrahan" in the city of Buenos Aires, a national reference health center in pediatrics which receives patients and donors from all over the country.

PWS population

Twenty-five PWS patients who tested positive with the Southern blot methylation test (PW71b probe) (Gillessen-Kaesbach et al., 1995) and with no evidence of deletion after FISH analyses with the probes SNRPN, D15S10, D15S11, and GABRB3 (VYSIS) (Ozcelik et al., 1992). In our analysis of uniparental disomy, samples from both parents were available for 21 PWS patients; while for four of them, only maternal samples were available.

Molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes of all individuals using standard methods: phenol-chloroform extraction or the salting out procedure (Sambrook et al., 1989). The three markers within the critical region, D15S11, D15S113 and GABRB3, were amplified in a multiplex polymerase chain reaction (PCR), according to Mutirangura et al. (1993). The other two markers, D15S1035 (centromeric) (Dib et al., 1996) and D15S211 (telomeric) (Gyapay et al., 1994), were amplified individually. The oligonucleotide primer sequences were obtained from GenBank (accession number: Z24390 for D15S1035 and Z51470 for D15S211). One strand of each primer pair was labeled on the 5' end with $^{32}P\gamma$ ATP and T4 polynucleotide kinase. PCR reactions were performed in a total volume of 25 μL, using 150 ng of genomic DNA. The primers were added to each PCR mixture, with a final primer concentration of 0.40 μM for both microsatellites, D15S211 and D15S1035. The relation primer:labeled primer was 4 to 1. For PCR reactions, initial denaturation was at 94°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C (for D15S211) and 55°C (for D15S1035) for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 10 min. Two microliters of the PCR products was diluted in formamide loading buffer, loaded on a 6% polyacrylamide denaturating gel, and electrophoresed at 65 watts for 3 h. The dried gel was exposed to a Kodak

X-ray film. The number of repeats for each marker was estimated by comparing them with a fragment with a known sequence.

Statistical analysis

For each locus, the number of alleles, the allele frequencies, the observed and the expected heterozygosity (HET) were calculated using a Microsatellite Tools program. The heterozygosity for joint loci was calculated as ΣHET/r, where r is the number of loci considered.

The polymorphism information content (PIC) of each marker was calculated as:

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n} \sum_{j=i+1}^{n} p_i^2 p_j^2$$

where p_i is the frequency of the ith allele. The second term removes the homozygotes and the third term removes half the matings of similar heterozygotes (Strachan and Read, 1996).

RESULTS

Microsatellite characterization

Five microsatellites of chromosome 15 were characterized: D15S11, D15S113, GABRB3, D15S211 and D15S1035. The first three, which are within the 15q11q13 critical region, were originally described by Mutirangura et al. (1993). D15S211 and D15S1035 were selected, taking into account the distance to the centromere and the reported heterozygosity. The most centromeric one was D15S1035 (0.00 cM) (GenBank accession number: Z24390); it is described close to break point 1, involved in PWS deletion type I (Ungaro et al., 2001). The most telomeric one was D15S211 (75.85 cM); it is outside the PWS critical region (Gyapay et al., 1994; GenBank accession number: Z51470).

The distribution of allelic frequencies of these microsatellites was established based on the data from the 100 unrelated (healthy) individuals (200 alleles) that we sampled (Table 1). Five new alleles were described for both D15S211 and GABRB3. The expected heterozygosity ranged from 0.59 to 0.94, and the observed heterozygosity gave values similar to the expected values at all the loci. D15S211 was the most polymorphic locus. Another locus that showed high heterozygosity was D15S1035. The average heterozygosity for joint loci was 0.73, when the three microsatellites run in multiplex (D15S113, D15S11 and GABRB3) were considered. This value rose to 0.81, when the five microsatellites were considered altogether (Table 2).

Microsatellite analysis

Using the five microsatellites described above, the parental origin of chromosome 15 was analyzed by microsatellite analysis in the 21 non-deleted patients for which samples of both parents were available. As the American College of Medical Genetics strongly recommends, we considered at least two fully informative loci showing either matUPD15 or biparental inheritance for diagnostic reporting (Shaffer et al., 2001). Based on this criterion, we were able to recognize the parental origin of chromosome 15 in 19 of the 21 patients; in the remaining two

Table 1. Allele frequency distribution of the five short tandem repeat markers in an Argentinean population (200 alleles).

D15S1035			D15S11			D15S113			GABRB3			D15S211		
Size (bp)	No.	Frequency	Size (bp)	No.	Frequency	Size (bp)	No.	Frequency	Size (bp)	No.	Frequency	Size (bp)	No.	Frequency
166	1	0.005	242	123	0.615	123	25	0.125	182	70	0.35	198*	1	0.005
172	2	0.01	244	2	0.01	127	84	0.42	186	12	0.06	202	1	0.005
174	1	0.005	246	3	0.015	131	26	0.13	188	7	0.035	208	1	0.005
176	3	0.015	248	31	0.155	135	32	0.16	190	15	0.075	210	2	0.01
178	4	0.02	250	14	0.07	137	2	0.01	192	5	0.025	212*	3	0.015
180	2	0.01	252	5	0.025	139	23	0.115	194	6	0.03	214	2	0.01
182	2	0.01	254	4	0.02	141	8	0.04	196	3	0.015	218	1	0.005
184	1	0.005	256	2	0.01				198	4	0.02	222	1	0.005
188	1	0.005	258	6	0.03				202	2	0.01	224	4	0.02
230	1	0.005	260	6	0.03				204*	5	0.025	226	7	0.035
232	1	0.005	262	2	0.01				206	19	0.095	228	11	0.055
234	3	0.015	264	2	0.01				210*	17	0.085	230	17	0.085
236	1	0.005							214*	27	0.135	232	8	0.04
238	12	0.06							220*	5	0.025	234	13	0.065
240	6	0.03							228*	3	0.015	236	17	0.085
242	7	0.035										238	3	0.015
244	8	0.04										240	20	0.1
246	5	0.025										242	8	0.04
248	8	0.04										244	7	0.035
250	8	0.04										246	8	0.04
252	8	0.04										248	17	0.085
254	19	0.095										250	9	0.045
256	35	0.175										252	12	0.06
258	27	0.135										254	7	0.035
260	18	0.09										256	4	0.02
262	8	0.04										258	7	0.035
264	4	0.02										260	3	0.015
266	2	0.01										262*	1	0.005
268	1	0.005										264*	4	0.02
270	1	0.005										324*	1	0.005
Total	200	1	Total	200	1	Total	200	1	Total	200	1	Total	200	1

*New alleles found.

Table 2. Molecular characterization of the five microsatellites in 200 alleles.

Microsatellites	Size (bp)	Alleles	Observed heterozygosity	Expected heterozygosity	PIC
D15S1035	166-270	30	92%	92%	91%
D15S11	242-264	12	62%	59%	57%
D15S113	123-141	7	75%	75%	72%
GABRB3	182-228	15	83%	83%	83%
D15S211	198-324	30	94%	94%	94%

PIC = polymorphism information content.

families, only one microsatellite was fully informative. Seventeen patients presented matUPD15, and two were classified as having an imprinting defect, since they presented biparental inheritance. One of these latter two patients had an affected sibling.

In our study, the addition of the centromeric marker D15S1035 and the distal marker D15S211 to the three microsatellites already described by Mutirangura et al. (1993) proved beneficial, since the sensitivity of the microsatellite analysis increased significantly (from 24%, when only the three microsatellites were considered, to 91%); it was also possible to assess the origin of the matUPD15 in most cases. In these patients, 16 of 17 showed maternal uniparental heterodisomy, while two of them presented reduction of heterozygosity limited to centromeric markers. Only one child showed reduction of heterozygosity for all markers.

We also studied four families for which the samples from the fathers were unavailable. In these four cases, the patients showed an identical pattern to their mother for all loci, suggesting a matUPD15.

DISCUSSION

During the past 10 years, our understanding of the basic genetic defects underlying PWS has been greatly enhanced. Molecular advances have greatly contributed to the identification and characterization of PWS patients. In 1997, an interdisciplinary team in Hospital de Pediatría "Prof Dr. J.P. Garrahan" was organized for the diagnosis and follow-up of children with this pathology. So far, 99 PWS patients have been diagnosed with the DNA methylation test. This technique is particularly useful for primary diagnosis of PWS, since it detects all cases with deletion, matUPD15 or imprinting defects; however, it makes no distinction among the different etiologies. To achieve adequate genetic counseling, the identification of various genetic etiologies with different recurrence risk was necessary; therefore, additional studies were required. To detect deletions, FISH analysis (using probes SNRPN, D15S10, D15S11, and GABRB3) was included in the diagnostic algorithm. So far, 74 patients with deletion (75%) have been detected. A microsatellite analysis was needed for the non-deletion cases, to identify uniparental disomy versus biparental inheritance (a presumed imprinting defect). In order to develop a cost-effective method of microsatellite analysis using a small number of microsatellites, we selected five short tandem repeats (D15S11, D15S211, D15S113, D15S1035, and GABRB3) and we have now evaluated their allelic frequencies.

Argentina has a distinctive ethnic background conferred by the integration of different ethnic groups. It was originally a Spanish colony with a small native component. Since the late 19th century, it has received large immigration waves from Italy and Spain; smaller groups have come from other European countries and from the Middle East (i.e., Syria and Lebanon) (Maccio and Elizalde, 1996). In addition, during the last decades, a new Amerindian component coming from various other South American countries has also been contributing to the heterogeneity of this population.

In this first report about the frequency distribution of five microsatellites mapping in chromosome 15 in an Argentinean population, high levels of heterozygosity (0.81) and informativity (90.5%) were obtained. The values for both observed and expected heterozygosity of these markers were similar to those described for other Caucasian populations (Mutirangura et al., 1993; GenBanK accession number: Z24390-Z51470). The 10 new alleles found for D15S211 and GABRB3 did not significantly modify the heterozygosity values already reported for these markers (Mutirangura et al., 1993; GenBanK accession number: Z24390-Z51470). These results are similar to those from studies of other polymorphic markers made in an Argentinean population (Sala et al., 1997). However, the Argentinean population has shown a distinctive mutational pattern for various genetic disorders and susceptibility alleles (Dardis et al., 1997; Mangano et al., 2000; Visich et al., 2002).

The microsatellite analysis sensitivity allowed us to establish the parental origin of chromosome 15 in 19 of the 21 PWS patients. Similar sensitivity values are likely to be obtained when using this small number of microsatellites in other Caucasian PWS populations.

The frequency distribution of genotypic subtypes in this study was similar to that described in other populations (Nicholls et al., 1989; Robinson et al., 1991; Mascari et al., 1992; Mutirangura et al., 1993; Buiting et al., 2003). Taking into account that a certain number of PWS patients with imprinting defect are familiar (Buiting et al., 1998), the identification of 2% of cases with biparental inheritance is highly relevant for genetic counseling.

The use of these microsatellites also led to the assessment of the origin of the matUPD15. Taking into account the criteria described by Robinson et al. (1993), the origin of non-disjunction was established using the most-centromeric markers. The heterodisomic state of these markers (non-reduction) indicates a meiosis I error, and the isodisomic state (reduction) indicates non-disjunction at meiosis II or a post-zygotic event if all markers encompassing the entire chromosome 15 show reduction to homozygosity. In our study, 16 of the 17 UPD cases were caused by a meiotic error. We found only one child with reduction to homozygosity for all the markers, which is attributable to a post-zygotic event. Among the patients that presented a meiotic error, our results suggest that 14 of the cases are the result of non-disjunction at meiosis I and the remaining two are due to a meiosis II error. These results are consistent with previous findings that showed that maternal UPD is caused by a meiotic error in 90% of cases, of which 83% had arisen in the first meiotic segregation (Robinson et al., 1998, 2000; Robinson, 2000a).

Finally, the molecular characterization of these five microsatellites in our population allowed us to carry out a microsatellite analysis with a high level of informativity that could be added to the diagnostic algorithm of Argentinean PWS children. Knowledge of the molecular etiology is not only essential for appropriate genetic counseling, it is also useful to perform phenotype-genotype correlation studies with the aim of improving the clinical care offered to PWS patients.

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