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A MOLECULAR DELETION STUDY WITH SOUTHERN HYBRIDIZATION ON TYPICAL PRADER-WILLI SYNDROME (PWS) PATIENTS WITH VARIOUS CHROMOSOME ABNORMALITIES INVOLVING 15q11-12 AND ON AN ATYPICAL PWS PATIENT WITH APPARENTLY NORMAL KARYOTYPE

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Summary We previously proposed a phenotype-karyotype correlation in the Prader-Willi syndrome (PWS). In order to confirm this hypothesis, we analyzed the genomic DNA of 10 clinically typical PWS patients with various chromosome 15 abnormalities, consisting of four [del(15)(q11.1;q12)], one [del(15)(q11;q13)], one [−15,+der(15)(pter::?HSR::p11→q11.1::q12)], one [mos 45,X,del(15)(q11.1;q12)/46,X,del(15)(q11.1;q12), +mar], two [t(15;15)(p11.2;q12)] and one [del(15)(q11.1;q12),+inv dup(15)(q11.1;q11.1)], and that of one atypical PWS patient with an apparently normal karyotype. Densitometric analyses on autoradiographic bands of Southern hybridization using two DNA segments, pML34 and pTD3-21, as probes revealed that all 7 patients with an interstitial deletion of 15q11.2 or 15q11.2-12 band had only one copy for each of the probes. In the two patients with an unbalanced translocation 15q;15q, two copies of each sequence were retained in spite of a visible deletion of band 15q11. This suggests that both of the two sequences may localize in flank of a critical region for the PWS phenotype. The copy number for the two probes was two in the case

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of inv dup(15), indicating that the patient is not tetrasomic for the sequences corresponding to the two probes. Densitometric analysis of one clinically atypical patient revealed a copy number of two for the two probes. Our results may support both our previous hypothesis and a recently proposed conception that PWS is a contiguous gene syndrome. The results also show that the molecular-genetic analysis is useful for an early and unambiguous diagnosis of PWS. RFLP analyses performed on three patients with del(15q) supported the preferential paternal origin of *de novo* deleted chromosome 15 in PWS.

Key Words Prader-Willi syndrome, chromosome deletion, molecular deletion, contiguous gene syndrome

INTRODUCTION

Prader-Willi syndrome (PWS) is a multiple congenital anomalies/mental retardation (MCA/MR) syndrome characterized by severe hypotonia and a feeding difficulty in infancy, obesity beginning at early childhood, craniofacial anomalies, short stature, small hands and feet, hypopigmented skin, hypogonadism and mental retardation. At least 50% or more of the PWS patients have a chromosome-15 abnormality (Ledbetter *et al.*, 1982; Mattei *et al.*, 1983, 1984; Niikawa and Ishikiriyama, 1985; Labidi and Cassidy, 1986; Butler *et al.*, 1986; Takano *et al.*, 1986). The chromosome abnormalities so far known include: an interstitial deletion [del(15)(q11.1;q12)], a pericentric inversion [inv(15)(p13;q12)], a balanced or unbalanced translocation [t(15q;Dp or other)], an isodicentric or inverted duplication [idic(15)(q11) or inv dup(15)(q11)], and other rearrangements (reviewed by Mattei *et al.*, 1984). In these chromosome abnormalities, a breakpoint in the region 15q11-q12 was always involved, suggesting a localization of the PWS gene(s) within this region. On the other hand, one-third to a half of PWS patients have been reported to have a normal karyotype (Ledbetter *et al.*, 1982, 1987). Thus, there is confusion with regard to the cytogenetic findings on PWS: monosomy (deletion) *versus* disomy (normal karyotype) *versus* tetrasomy (inverted duplicated 15q). Mattei *et al.* (1983) showed that some of the patients with a normal karyotype do not fit the strict criteria for clinical diagnosis of PWS. Niikawa and Ishikiriyama (1985) further found that patients with clinically confirmed PWS always show an interstitial deletion of band 15q11.2 and proposed a hypothesis of phenotype-karyotype correlation of the disease. However, another confusion has occurred, since recent reports (Schwartz *et al.*, 1985; Reynolds *et al.*, 1987; Kaplan *et al.*, 1987; Magenis *et al.*, 1987) showed that patients with a chromosome 15q abnormality which was almost identical to that seen in PWS patients lacked some of the features of PWS or had some additional manifestations. Thus, alternative hypothesis was recently proposed by Schmickel (1986) that PWS is a contiguous gene syndrome in which a comprehensive loss of contiguous genes may be involved.

We present here the results of our molecular-deletion analyses on 10 clinically

typical PWS patients with various chromosome 15 abnormalities and one atypical patient with an apparently normal karyotype, with Southern blot hybridization using two cloned DNA segments specific for 15q11-q12 region as probes. The purposes of our study are to know whether all the cytogenetically visible deletions in PWS patients are confirmed or not at a molecular level, and to confirm the result of cytogenetic studies on the preferential paternal origin of *de novo* del(15q) by the RFLP analysis.

MATERIALS AND METHODS

Study subjects. The individuals studied included 10 patients whose phenotypes fit the strict clinical criteria of PWS, one patient who lacks some of the typical features for PWS, and the parents of some of those patients. Six (patient nos. 1–6) of the 10 PWS patients and the atypical patient (patient no. 11) were identical to those reported previously (Niikawa and Ishikiriyama, 1985) (Tables 1 and 2), while the remaining 4 (patient nos. 7–10) were new patients seen directly by us. The clinical manifestations of the 11 patients are shown in Table 1.

Cytogenetic analysis. Every new case was cytogenetically analyzed with high-resolution GTG-banding using either the ethidium bromide technique as described previously (Niikawa and Ishikiriyama, 1985) or the BrdU-Actinomycin D method (Rybak *et al.*, 1982).

Southern blot hybridization. Two probes, pTD3-21 and pML34 (kindly provided by Dr. S.A. Latt, Boston), are *Hin*dIII subclones of λ-phage libraries from a flow-sorted inverted duplication chromosome 15 [inv dup(15)(q13)] (Lalande *et al.*, 1985; Donlon *et al.*, 1986). Both pTD3-21 and pML34 (a 2.2 kb and a 6.4 kb inserts, respectively) were mapped to the region 15q11.2 (Donlon *et al.*, 1986). A 0.67 kb human prealbumin cDNA clone, pPA1 (Mita *et al.*, 1984) assigned to 18q11-q12.3 (Jinno *et al.*, 1986), was used as an internal standard for a comparative analysis of hybridization density. Genomic DNAs of the individuals were extracted by the standard method from their peripheral blood leukocytes or Epstein-Barr virus-transformed lymphoblastoid cells. Southern blots of each DNA sample on either nylon or nitrocellulose membranes were hybridized with the triple probes by the standard technique (Maniatis *et al.*, 1982).

Densitometric analysis. The gene doses of both pML34 and pTD3-21 in each individual were determined by taking the average of ratios of their hybridization densities to the PA gene-density obtained from densitometric analyses on several separate hybridizations.

RESULTS

Karyotypes

Of the 4 new PWS patients, one (patient no. 9) showed an interstitial deletion of band 15q11.2 [46,XY,del(15)(q11.1;q12)], one (patient no. 8) had a relatively large

Table 1. Clinical manifestations of the present patients.

Patient number	Previous number [a]	Age	Sex	Hypotonia in infancy	Feeding difficulty	Characteristic facies	Mental retardation	Small hands and feet	Hypoplastic genitalia	Hyperphagia	Obesity
1	2	18	female	+	+	+	+	+	−	+	+
2	1	22	male	+	+	+	+	+	+	+	+
3	11	7	male	+	+	+	+	+	+	+	+
4	10	8	male	+	+	+	+	+	+	+	+
5	4	15	female	+	+	+	+	+	+	+	+
6	16	4	male	+	+	+	+	+	+	+	+
7	—	9	female	?	?	+	+	+	+	+	+
8	—	3	female	+	+	+	+	+	+	−	−
9	—	7	male	+	+	+	+	+	+	+	+
10	—	13	female	+	+	+	+	+	+	+	+
11	18	4	male	−	+	−	+	−	+	−	−

[a] Niikawa and Ishikiriyama (1985).

Table 2. Gene dose of the genomic sequences corresponding to pML34 and pTD3-21 in the present 11 patients.

Patient	Karyotype	Density ratios [a] of p34 and p3-21 fragments to control fragment [b]		Corrected density ratios [c]		Copy number of fragment		Interpretation
		B/A	C/A	B/A	C/A	B	C	
1	del(15)(q11.1;q12)	0.594	1.335	0.7	1.0	1	1	DNA deletion
2	del(15)(q11.1;q12), 15p?HSR	0.816	1.549	1.0	1.2	1	1	DNA deletion
3	del(15)(q11.1;q12)	0.779	1.404	1.0	1 1	1	1	DNA deletion
4	del(15)(q11.1;q12)	0.965	1.559	1.2	1.2	1	1	DNA deletion
5	t(15;15)(p11;q13)	1.848	2.820	2.3	2.2	2	2	No DNA deletion
6	t(15;15)(p11;q13)	1.867	2.705	2.3	2.1	2	2	No DNA deletion
7	45,X,del(15)(q11.1;q12)/ 46,X,del(15)(q11.1;q12),+mar	0.958	1.261	1.2	1.0	1	1	DNA deletion
8	del(15)(q11;q13)	0.826	1.140	1.0	0.9	1	1	DNA deletion
9	del(15)(q11.1;q12)	0.933	1.959	1.2	1.5	1	1 or 2	DNA deletion
10	del(15)(q11.1;q12),+inv dup(15)(q11.1;q11.1)	1.505	1.828	1.9	1.4	2	1 or 2	DNA deletion and one copy gene from inv dup(15)
11	46,XY	1.629	3.199	2.0	2.5	2	2	No DNA deletion

[a] Density ratios were measured from several separate hybridizations. [b] A, 11 kb and/or 4.6 kb fragments for the prealbumin gene as an inner standard; B, 6.4 kb fragment for pML34; C, 2.2 kb fragment for pTD3-21. [c] Corrected ratios are calculated by dividing B/A or C/A ratio each by the average ratio among normal controls.

interstitial deletion of 15q [46,XX,del(15)(q11;q13)] (Fig. 1), one (patient no. 10) had a deletion of band 15q11.2 in one of the homologous members 15 and an additional inverted duplicated chromosome 15 [47,XX,del(15)(q11.1;q12),+inv dup(15)(q11.1;q11.1)], and one (patient no. 7) showed a mosaicism of [45,X,del(15)(q11.1;q12)/46,X,del(15)(q11.1;q12),+mar] (Fig. 1). No mosaicism was observed in any other patients examined. All parents examined had an apparently normal karyotype.

Gene dose studies

Probes pML34 and pTD3-21 hybridized to a 6.4 kb and a 2.2 kb *Hin*dIII-fragment, respectively, while the probe pPA1 showed 11 kb and 4.6 kb fragments (Jinno *et al.*, 1986). The results of the gene-dose studies on each individual were

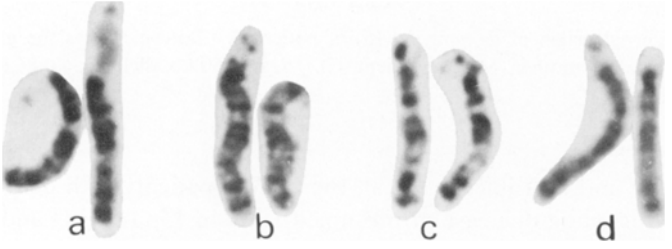

Fig. 1. Chromosome-15 pairs of patients 2(a), 7(b), 8(c), and 9(d). Right-sided chromosomes are those with 15q11.2 deletion. Note HSR-like short-arm of chromosome 15 of patient 2.

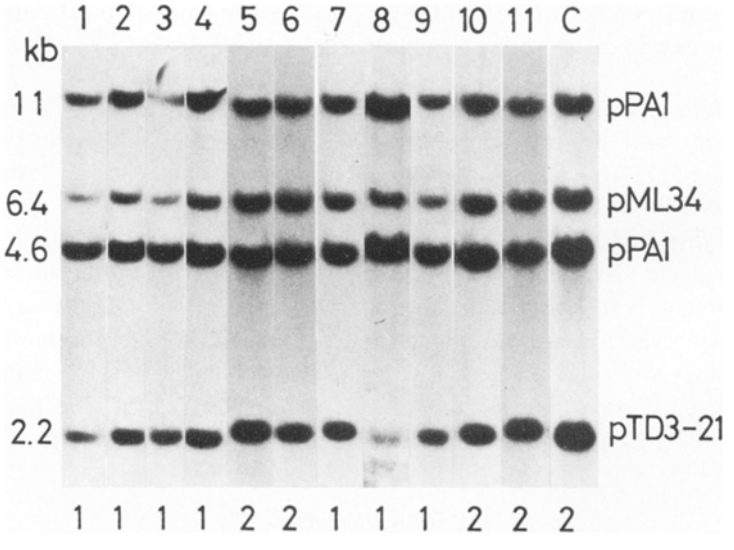

Fig. 2. Autoradiograms of patients with the use of pML34 and pTD3-21 as probes and pPA1 as inner control probe. Patient number is shown above the column and copy number of genes (genomic sequences corresponding to pML34 and pTD3-21) is shown at the bottom.

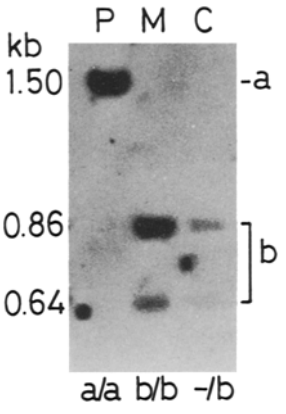

Fig. 3. Paternal origin of *de novo* del(15) of patient 4. Bottom shows the genotypes of father (P), mother (M) and patient (C). a, an 1.50 kb allele; b, a 0.86 kb+0.64 kb allele.

shown in Fig. 2 and in Table 2. In all the 7 PWS patients with del(15)(q11.1;q12) or (q11;q13), including one case of presumed HSR in 15p (Fig. 1) and another case of 45,X, the hybridization for both pML34 and pTD3-21 showed a one copy density compared with a density of the karyotypically normal control. A density indicating two copies was detected in the two patients with an unbalanced t(15q;15q). In one PWS patient with a 15q11.2 deletion and an inv dup(15q), the copy number was two for pML34 and one or two for pTD3-21. In the clinically atypical patient and in every parent examined, the copy number was also two.

Parental origin by RFLP analysis

A search for DNA polymorphism has been carried out for the two probes, pML34 and pTD3-21, using a total of 13 restriction endonucleases. An RFLP of 1.5 kb/0.86+0.64 kb was detected only with a combination of *Alu*I and pTD3-21. A study of this RFLP among ten normal individuals revealed that the frequencies for a major allele "A1" (a 0.86 kb+0.64 kb fragment) and a minor allele "A2" (an 1.5 kb fragment) is 0.80 and 0.20, respectively. Segregation analysis on this RFLP in one family revealed that the father is an A1/A1 homozygote, the mother was an A2/A2 homozygote, and the patient was an A2/− hemizygote, indicating that the *de novo* deleted chromosome 15 was of paternal origin (Fig. 3). In the other two patients, *Alu*I-RFLP was uninformative for the deleted chromosome 15.

DISCUSSION

Cytogenetic analyses on four new PWS patients in our series confirmed our previous conclusion of a phenotype-karyotype correlation in the syndrome (Niikawa and Ishikiriyama, 1985). The results of the present molecular study also confirmed

the previous findings by Donlon *et al.* (1986) and by Latt *et al.* (1987), except for the frequency of the molecular deletion. All our patients with an interstitial deletion of 15q11.2-q13 had a molecular deletion without any exceptions, while with the same probes, Donlon *et al.* (1986) found a DNA deletion in only one of their two PWS patients with the similar chromosome deletion as seen in our patients. A further study using four other chromosome-15 DNA clones (pIR4-3, pIR10-1, p189-1 and p190-2) as probes by the same group of investigators (Latt *et al.*, 1987) revealed a deletion at a molecular level in three of six PWS patients studied and probably in a PWS-like patient. The discrepancy of molecular deletion frequency between our and their studies is most likely due to a chance in collectioning patients in our study whose chromosome deletions would have been uniform.

Against all expectations, both of the patients with an unbalanced translocations 15q;15q were shown to retain the two DNA sequences in a double dose. This unexpected finding is hard to be explained by an assumption that the both of pML34 and pTD3-21 would be localized in flank to but apart from putative gene(s) responsible for the PWS phenotype, since the range of the deletion in each translocation is from 15pter to 15q12, which is wider than that in the interstitial deletion cases. Therefore, more complex rearrangements of DNA might have occurred in the genome of these translocation patients. Alternatively, although we could not have observed any abnormality other than the translocation in these patients (Niikawa and Ishikiriyama, 1985), the critical DNA segments might have inserted into another chromosome.

A two copy density of the sequence for each probe was detected in the genome of the patient with an interstitial deletion of band 15q11.2 accompanied with an additional inverted duplicated chromosome 15. This finding indicates that the patient is not tetrasomic at least for both the pML34 and pTD3-21 sequences, and is consistent with the result of the study on a cell line containing a small inv dup(15) by Donlon *et al.* (1986). One copy of each gene in our patient must have been derived from his one intact chromosome 15, while the other one copy may have come either from a deleted chromosome 15 or from an inverted duplicated chromosome.

Although we could not detect any cytogenetic and molecular deletions in an atypical patient, our finding may not universally be applicable to other atypical PWS patients, since the probes we used are the sequences probably flanking to the region responsible for the PWS phenotypes (Donlon *et al.*, 1986). More studies on such atypical patients are necessary using the same or the other probes as isolated by Latt *et al.* (1987).

The heterogeneity of the molecular changes observed in the present study on PWS patients may reflect instability around the 15q11 region, *i.e.*, a rearrangement hot-spot. This unstable region is physically closely linked to the critical region for the common phenotype of PWS. This "hot-spot" hypothesis first proposed by Riccardi (1981) and then by Mattei *et al.* (1984) may be compatible with the result of a study by Donlon *et al.* (1986) showing that among a total genomic library, two inserts, IR-4 and IR-10, localized at the 15q11.2 region contain an inverted repeat

DNA sequence which is belonging to the *Alu* family. Furthermore, chromosome rearrangements involving 15q11-q13 have been observed in patients with malformation syndromes or in those with well-known syndrome-like disorders other than PWS. These disorders include Cohen-like syndrome (Fuhrmann-Rieger *et al.*, 1984), Williams syndrome (Fryns *et al.*, 1982; Kaplan *et al.*, 1987), Angelman syndrome (Kaplan *et al.*, 1987), and many other disorders with dysmorphic manifestations (reviewed by Mattei *et al.*, 1984). An one copy density per genome for both pML34 and pTD3-21 was confirmed in one Angelman syndrome patient in our preliminary study (described elsewhere).

The chromosome abnormality of one of our PWS patients (patient no. 2) merits comment. The detailed clinical manifestations and family data were described previously (Kato *et al.*, 1965; Niikawa and Ishikiriyama, 1985). In short, he, his father and several other family members have an unusual chromosome 15 with an extremely long short-arm that appears a homogeneously staining region (HSR)-like structure by GTG- and QFQ-bandings (Fig. 1). A loss of genetic material in 15q11.2 region in this unusual chromosome 15 was detected only in the patient at both microscopic and molecular levels, and the HS-like region itself gives no phenotypic effects on any individuals who carry it. It is of great interest to know the molecular structure of this HS-like region. If we assume that this contains a highly repeated DNA sequence resulted from a pericentric inversion involving the 15q11 region and subsequent tandem repeats, it would reflect the instability of the region. Then, the loss of the sequences, pML34 and pTD3-21, in the patient could also be explained by the recombinant aneusomy mechanism as a result of a loop formation around the centromere. Thus, a further molecular study is necessary using a highly repetitive cloned DNA sequence as a probe, as isolated by Donlon *et al.* (1986).

The findings in our study together with the previous molecular, cytogenetic and clinical studies provide us the following conclusions: (1) The gene(s) located in 15q11.2 region play an important role in the cause of PWS; (2) there is not only genetic but also phenotypical heterogeneity in the rearrangements involving the 15q11-q12 region; (3) this heterogeneity may be compatible with a conception, "the contiguous gene syndrome," induced recently by Schmickel (1986). If we follow this conception, each DNA segment (gene) in the region 15q11.2 would be responsible for each component of the common phenotype of PWS. In addition, if we extend this conception into a syndrome scale, the gene(s) for PWS would flank or overlap to a segment for Angelman syndrome, and possibly those for Williams and Cohen syndromes. Among the heterogeneous classes of deletion, a comprehensive loss of a critical segment which may contain putative gene(s) would lead to a syndrome, while a loss of a larger or a smaller segment would result in variation of phenotypes.

Our results on the parental origin of the *de novo* deleted chromosomes 15 in PWS patients traced with molecular probes are consistent with the preferential paternal origin of a deleted chromosome in PWS patients that was ascertained in previous cytogenetic studies (Butler and Palmer, 1983; Hasegawa *et al.*, 1984; Niikawa and Ishikiriyama, 1985). This preferential paternal origin is also consistent

with the data in other *de novo* non-Robertsonian structural rearrangements (Chamberlin and Magenis, 1980).

A few PWS patients are familial (Hasegawa *et al.*, 1984; Lubinsky *et al.*, 1987; Cassidy, 1987). Prenatal diagnosis in a pregnancy following the birth of a PWS child have been performed cytogenetically (Emanuel *et al.*, 1983; Smith, 1986), but no recurrence has been found in the pregnancies studied (Schinzel, 1986). It is often difficult to obtain convincing prometaphase chromosomes from cultured amniotic cells. Thus, molecular-genetic analysis may be useful for prenatal diagnosis, especially in familial cases. Neonatal diagnosis of PWS is more important clinically to prevent patients early from obesity and its subsequent diabetes. However, the clinical diagnosis in early infancy is often difficult, because the time of onset of obesity is usually in around childhood. Although neonatal diagnosis of PWS by chromosome analysis was recently succeeded (Greenberg *et al.*, 1987), a molecular genetic analysis as described here is also applicable to the diagnosis of presumed newborn patients, especially of patients with a normal karyotype.

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