Original articles

Molecular and cytogenetic studies of the Prader-Willi syndrome

R J Trent, F Volpato, A Smith, R Lindeman, M-K Wong, G Warne, E Haan

Abstract

Twenty-seven subjects with the Prader-Willi syndrome (PWS) were studied. Sixteen (59%) had a cytogenetic deletion involving chromosome 15q11-13. Nine were non-deletional and two patients had structural rearrangements of chromosome 15: 47,XY,+del(15)(pter→q12), var(15)(p11) and 45,XX,t(14q15q). At the DNA level, a greater proportion of patients (74%) showed loss of one chromosome 15q11-13 allele using a combination of densitometry and RFLP analysis. Deletion sizes were variable with 13 of 20 detectable both cytogenetically and with probe pML34 (*D15S9*). The remaining seven had microdeletions at the pML34 locus. Heterogeneity was further seen in three subjects who had cytogenetic deletions but normal DNA studies. In one patient there was evidence of a duplication at the pML34 locus. A new molecular rearrangement was identified with probe p3-21 (*D15S10*) in two patients

and their mothers. Fifteen family studies were performed. In all 10 families where there was a molecular deletion, this was shown to have arisen *de novo*. DNA mapping confirmed that the paternal 15q allele was lost in three patients with PWS.

The Prader-Willi syndrome (PWS) is a rare multi-system disorder. Clinical features include hypotonia, hypogonadism, hypopigmentation, mental retardation, and obesity. The latter two are particularly significant in the natural history of this disorder. Mental retardation is usually not severe (mean IQ 60 with a range of 20 to 80), but obesity is a major clinical and psychosocial problem.¹ The newborn with PWS is floppy, sucks poorly, and fails to thrive. However, appetite increases rapidly at approximately 3 to 4 years of age. By the teens, excessive eating produces gross obesity. The diagnosis of PWS may be suggested in the neonatal period but cannot be made conclusively until early childhood. Thus, there is delay in initiating appropriate management.²

The aetiology of the PWS is unknown. Almost invariably only one member of the family is affected. However, some cases of familial recurrence have been described.³ Cytogenetic deletions involving the long arm of chromosome 15 were first described in 1981 and approximately half the patients with PWS have an interstitial deletion of 15q11-13.⁴ Five percent of patients have duplications or translocations while the remainder have normal karyotypes.^{5,6} DNA probes specific for the 15q11-13 region are now available.^{7,8} These have been used to characterise further the putative PWS locus called *PWCR* (Prader-Willi chromosome region). Recent results using these probes have provided further evidence that genomic imprinting secondary to loss of the paternal 15q locus is implicated in the pathogenesis of PWS.⁹ The present study describes DNA and

Department of Molecular Genetics, Royal Prince Alfred Hospital, Camperdown, NSW 2050, and Department of Medicine, University of Sydney, NSW 2006, Australia.

R J Trent, F Volpato, R Lindeman, M-K Wong

Cytogenetics Unit, Oliver Latham Laboratory, Department of Health, North Ryde, NSW, Australia.
A Smith

Endocrinology and Diabetes Department, Royal Children's Hospital, Melbourne, Victoria, Australia.
G Warne

Department of Medical Genetics, Adelaide Children's Hospital, Adelaide, SA, Australia.
E Haan

Correspondence to Professor Trent.

Received for publication 17 September 1990.

Revised version accepted for publication 19 February 1991.

cytogenetic findings in 27 patients with PWS, which is the largest cohort investigated in this way.

Patients and methods

PATIENTS

This group comprised 27 patients in whom clinical histories and phenotypes were entirely consistent with PWS. These included early stage I phase of neonatal hypotonia and failure to thrive, hypogenitalism in the male, followed by hyperphagia in early childhood, mental retardation, short stature, typical facies, small hands and feet, and a behavioural disorder. Twenty-four patients had been followed over a period of time.¹⁰ They were recently reviewed and three additional patients were added to this study.

DNA STUDIES

Detection of a new DNA restriction map

DNA was isolated from buffy coats obtained from 10 ml of blood. Aliquots of DNA (10 µg) were digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, and *Bgl*II. Additional restriction enzymes were used where indicated below. Digested DNA was electrophoresed on 0.8% agarose gels and then transferred to nylon membranes by Southern blotting. Membranes were hybridised to ³²P labelled DNA probes and washed using stringent conditions (0.1 × SSC at 65°C for 60 minutes).¹¹ *PWCR* specific DNA probes included pML34 (*D15S9*), p3-21 (*D15S10*) (donated by the late Professor S Latt, Boston), and 85(2.4) (*D15S53*) (donated by Professor F-T Kao, Denver). DNA polymorphisms identifiable with probes pML34 (*Sca*I, allele frequencies: 6.5 kb – 0.72; 6.3 kb – 0.28), p3-21 (*Taq*I, allele frequencies: 9.0 kb – 0.83; 8.2 kb – 0.17), and a multiallelic (CMW1) probe (*Eco*RI) were sought.^{12–14} The CMW1 probe was provided by Dr D Ledbetter (Houston). The 3'α HVR probe (a gift from Dr D R Higgs, Oxford) was used for paternity testing.¹⁵ For the latter, filters were hybridised at 55°C and washed in 5 × SSC.

Detection of loss of one *PWCR* specific allele

Membranes containing DNA digested with the restriction endonuclease *Hind*III were hybridised simultaneously to three DNA probes, pML34, OP1, and a β globin gene probe (*Pst* β) located on chromosome 11p15 (*Pst* β was donated by Professor T Maniatis, Boston). Probe OP1 is a 2.4 kb *Hinc*II insert in plasmid pBluescript. It was isolated from an EMBL3 phage library prepared from normal

DNA partially digested with *Mbo*I. The library was screened with probe p3-21 and so probe OP1 is located within 20 to 25 kb of p3-21 (Lindeman *et al*, in preparation). Band sizes obtained for the above *Hind*III digests were 6.0 kb (pML34), 3.0 kb (OP1), and 7.8 kb (β globin). Autoradiographs were scanned in an LKB 2202 Ultrosan Laser Densitometer. Normal DNA samples were included in all membranes to control for hybridisation and washing conditions. Autoradiograph band intensities and densitometric traces of bands identified by probes pML34 and OP1 were scored by three observers (RJT, RL, M-KW) as representing normal, reduced, or increased intensities compared to the control β globin.

Family studies

Both parents of 15 patients were studied.

KARYOTYPE ANALYSIS

Peripheral blood cultures were set up for high resolution banding after the method of Yunis¹⁶; 0.3 ml of blood were added to 4.5 ml of medium (either Ham's F10 or Iscoves, Flow Laboratory). After 48 hours, the cultures were blocked with 0.05 ml of a methotrexate solution (10⁻⁵ mol/l) and reincubated for 17 hours overnight at 37°C. The following morning the methotrexate was washed out and the block released with fresh medium to which 0.05 ml (10⁻³ mol/l) thymidine were added. The cultures were then incubated at 37°C for a further six hours. Harvesting was performed with colcemid (final concentration 1 µg/ml) for 15 minutes, hypotonic 0.075 mol/l KCl for 10 minutes, and fixed for one hour with 3:1 methanol:acetic acid. Slides were placed in a 37°C incubator for two to three days before GTG banding.¹⁷ A total of 15 to 20 cells was examined in each case. On scanning under the microscope, metaphase spreads were chosen for the criteria of a band stage of resolution greater than 750 bands plus readily distinguishable chromosomes 15 which were reasonably straight and not involved in a crossover. Chromosome 15 was scored as deleted when the q12 band was not seen and was not based on the absolute length of the q11–13 region (fig 1). Other banding techniques were used as indicated.

Results

PATIENTS

Sex distribution for the PWS patients was 21 males and six females. The predominance of males (21:6) may reflect bias in terms of recognising PWS more

Figure 1 Ideogram of chromosome 15 in the centre shows the bands at the 850 band stage. On the left are normal chromosome 15 from patient 11 and on the right is a deleted chromosome 15 (patient 9). The broken line joins the centromeres in each case and the solid line the q12 band.

easily in males. Ages ranged from 4 to 30 years (table).

DNA STUDIES

New DNA restriction map

Digestion of normal DNA with the restriction enzyme *Bam*HI and hybridisation to probe p3·21 gives a normal restriction fragment of 25 kb. Two unrelated males (patients 4 and 5) with PWS showed only an abnormal 20 kb restriction fragment. In the above two cases, maternal DNA had both the normal 25 kb and the new 20 kb restriction fragments. DNA samples from the respective fathers had the normal restriction fragment (fig 2, table). One of the above two patients (No 5) and his mother had an additional new band detectable with probe 85(2·4) and the restriction enzyme *Eco*RI. All other restriction enzyme digests (*Hind*III, *Bgl*II, *Bcl*I, *Taq*I, *Xmn*I, *Xba*I, *Eco*RV, *Hinc*II, *Pst*I) in patients 4 and 5 were normal with both p3·21 and 85(2·4) probes. The two new restriction fragments described above were found in separate DNA preparations but not seen in 167 (*Bam*HI) and 57 (*Eco*RI) normal DNA samples (total 334 and 114 chromosomes respectively). DNA from the remaining 25 PWS patients did not show abnormal bands when studied with

probes pML34, p3·21, 85(2·4), and restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Bgl*II.

Loss of one P_WCR specific allele

Normal DNA maps do not exclude extensive deletions of one allele. These were sought in the P_WCR locus by comparing intensities in autoradiograph bands. Intensity changes were detected with probe pML34. These showed that 20 patients (Nos 1 to 10 and 16 to 25) had loss of one allele, while six (Nos 11, 13 to 15, 26, and 27) were normal in this respect (fig 3, table). In the remaining case (No 12), there was increased intensity in the DNA band. Densitometry changes with probe OP1 were consistent with the pML34 results in all but six cases (patients 3, 10, 12, 14, 16, and 17). In these instances, intensities of hybridisation bands were considered equivocal (table).

DNA family studies

RFLPs (*Sca*I with probe pML34 and *Taq*I with probe p3·21) were informative in only two of the 15 families studied. The *Sca*I (pML34) RFLP showed loss of the paternal 15q allele in case 9. In the same family, the multiallelic CMW1 DNA probe detected the paternal allele at this locus (fig 4). Non-paternity was excluded by use of a second VNTR probe (3'αHVR) (data not shown). In a second family (No 14) the two *Sca*I alleles were present in the PWS patient thereby confirming the normal pML34 densitometry result at this locus (table). DNA samples from parents of 10 patients (Nos 1 to 10) with loss of one allele were also studied by densitometry. Both alleles in all parental DNA samples were present (table).

KARYOTYPE ANALYSES

Sixteen (Nos 3, 6, 8 to 10, 13 to 19, 21, 23 to 25) of the 27 PWS patients (59%) had a deletion of 15q detectable on cytogenetic studies and nine (Nos 1, 2, 4, 5, 7, 11, 20, 22, 27) patients (33%) were normal (fig 1). Of the remaining two (7%), one (No 26) had a familial Robertsonian translocation involving chromosomes 14 and 15.¹⁸ The second (No 12) had an additional 15 marker chromosome.¹⁹ A comparison of cytogenetic and DNA studies in the 27 patients is given in the table.

Discussion

In the present study cytogenetic deletions involving chromosome 15q11–13 were found in 59% of patients with PWS. A higher proportion (74%) showed a deletion at the 15q11–13 locus by DNA

Cytogenetic and DNA findings in 27 patients with PWS.

Case No	Sex/age (y)	Karyotype	Densitometry		Parental origin
			pML34	OP1	
1	M4	46,XY	Deletion*	Deletion	Indeterminate
2	M10	46,XY	Deletion	Deletion	Indeterminate
3	M12	46,XY, del(15q)	Deletion	Equivocal	Indeterminate
4	M14	47,XXY	Deletion	Deletion	Paternal deleted
5	M19	46,XY	Deletion	Deletion	Paternal deleted
6	M26	46,XY, del(15q)	Deletion	Deletion	Indeterminate
7	M19	46,XY	Deletion	Deletion	Indeterminate
8	M8	46,XY, del(15q)	Deletion	Deletion	Indeterminate
9	M21	46,XY, del(15q)	Deletion	Deletion	Paternal deleted
10	M9	46,XY, del(15q)	Deletion	Equivocal	Indeterminate
11	M14	46,XY	Normal	Normal	Indeterminate
12	M13	47,XY, + del(15) (pter→q12), var(15)(p11)	Duplication	Equivocal	Indeterminate
13	M12	46,XY, del(15q)	Normal	Normal	Indeterminate
14	F27	46,XX, del(15q)	Normal	Equivocal	Indeterminate
15	M6	46,XY, del(15q)	Normal	Normal	Indeterminate
16	M18	46,XY, del(15q)	Deletion	Equivocal	Not studied
17	M16	46,XY, del(15q)	Deletion	Equivocal	Not studied
18	M12	46,XY, del(15q)	Deletion	Deletion	Not studied
19	M17	46,XY, del(15q)	Deletion	Deletion	Not studied
20	F16	46,XX	Deletion	Deletion	Not studied
21	F18	46,XX, del(15q)	Deletion	Deletion	Not studied
22	M10	46,XY	Deletion	Deletion	Not studied
23	M5	46,XY, del(15q)	Deletion	Deletion	Not studied
24	F5	46,XX, del(15q)	Deletion	Deletion	Not studied
25	M8	46,XY, del(15q)	Deletion	Deletion	Not studied
26	F30	45,XX,t (14q15q)	Normal	Normal	Not studied
27	F19	46,XX	Normal	Normal	Not studied

*DNA deletions in patients 1 to 10 are not present in their parents. Microdeletions were found in patients 1, 2, 4, 5, 7, 20, and 22. DNA densitometry results at the pML34 locus in patients 9 and 14 were confirmed by RFLP patterns.

mapping. Using a similar combination of densitometry and RFLP analysis others have described variable changes at *PWCR*. In one report of 10 patients, six of nine had loss of the pML34 locus. More variable results were found at the p3-21 locus: normal (3/8), deleted (2/8), duplicated (3/8).¹² In the latter study four *PWCR* specific DNA probes (*D15S9*, *D15S10*, *D15S11*, *D15S12*) were used. Despite this, identification of deletions in four patients relied entirely on densitometric analysis. None of the deletions in the 10 patients was fully characterised on the basis of RFLP data alone. Knoll *et al*²⁰ using the same approach analysed DNA from PWS and Angelman's syndrome and categorised patients into three classes (I, II, III). Deletional heterogeneity observed in these two studies was also

found in the present report with 20 of 27 PWS patients showing loss of one allele involving at least the pML34 locus; one patient had a duplication at pML34 and two had a DNA rearrangement at the p3-21 locus. The 27 PWS patients can be grouped into the classes proposed by Knoll *et al*,²⁰ that is, class III is non-deletional, class II involves loss of the pML34/p3-21 locus, and class I is characterised by larger deletions which are also present cytogenetically. These data provide further evidence that *PWCR* plays a central role in the pathogenesis of PWS. However, the significance of molecular changes reported to date remains unclear and more extensive study of *PWCR* is required.

Interpretations based on intensities of autoradiographs can be subjective and may be influenced by

Figure 2 DNA mapping patterns for two PWS patients with new restriction fragments identified by the restriction enzyme *Bam*HI and probe 3-21. The normal restriction fragment (arrowhead) is present in the two paternal DNA samples. Maternal DNA shows both the normal 25 kb band and the new 20 kb fragment. Both patients with PWS (■) have inherited the new (maternal) band but not the normal (paternal) allele.

technical variables, including the quantity of DNA present, the uniformity of DNA transfer, hybridisation, and washing. In the present study the inherent variability was reduced by incorporating an internal control (β globin) within each filter track. Plasmid p3-21 did not give a *Hind*III restriction fragment of comparable size to pML34 or β globin and so was replaced by the closely related probe OP1. The latter

was more helpful but still proved difficult to interpret in some cases. In this context, it is interesting that Gregory *et al*¹² were unable to analyse this locus in two of 10 patients because of weak hybridisation signals with probe p3-21. Until more definitive mapping data based on pulsed field gel electrophoresis are available, the exact extent of the *PWCR* related deletions must remain speculative.

Three subjects (Nos 13 to 15) had cytogenetic deletions but normal DNA maps. These could represent DNA rearrangements which were not detected with the probes available or the DNA changes were outside the cytogenetically deleted locus. Donlon²¹ has reported one PWS patient (DON-5) with a similar finding when studied with multiple probes including pML34 and p3-21. One subject (No 26) had a Robertsonian translocation involving chromosomes 14 and 15. The same abnormality was present in the patient's mother and her brother and sister.¹⁸ Normal phenotypes in all the above family members would exclude this rearrangement as the sole factor in the pathogenesis of PWS. In this situation, a paternal abnormality of chromosome 15 is suggested (see below). A second interesting cytogenetic finding involved an extra segment of chromosome 15. This was a *de novo* mutation in the patient (No 12).¹⁹ DNA intensity studies were consistent with additional chromosome 15q material.

Two subjects (Nos 4 and 5) had normal karyotypes. DNA analysis showed loss of one *PWCR* specific allele as well as a new restriction enzyme map detectable with *Bam*HI and probe p3-21. In both cases maternal DNA had a similar abnormal band (fig 2). The molecular basis for this unique finding remains to be determined. One consideration is a DNA polymorphism since a new band was

Figure 3 Southern blots and densitometric traces in patient 7 (A), his father (B), and mother (C). The three DNA bands/densitometry patterns produced by restriction enzyme *Hind*III are identified by probes: (1) OP1, (2) pML34, and (3) β globin.

Figure 4 DNA polymorphism patterns in family 9. (A,B) 6.5, 6.3 kb RFLPs identified with restriction endonuclease *ScaI* and probe pML34. (C,D) Two alleles for the multiallelic CMW1 probe detected with restriction endonuclease *EcoRI*.

found with only one of nine restriction enzymes. Against this explanation is the failure to find the same band in normal control DNA samples and its presence in two of 27 patients with PWS. One of these same two patients also showed a new *EcoRI* restriction fragment with a second, unrelated probe (85(2.4)). To determine the significance of the 20 kb *BamHI* band will require mapping with additional DNA probes or cloning of this region or both. The above two cases, as well as one example of paternal allele loss shown by RFLP analysis (No 9, fig 4), provide further evidence for the proposal that PWS is the result of a mutation affecting the paternal allele.⁵⁹ DNA polymorphic data from family studies in patient 9 further define the paternal abnormality to include the PWS specific locus identified by probe p3-21 (15q11.2), but not the multiallelic CMW1 probe which maps distal to the deletion region in PWS.⁹

In 10 family studies, normal parental patterns confirmed that DNA deletions were de novo mutations. This had been noted previously on cytogenetic analysis and a small number of DNA studies and is consistent with the sporadic nature of PWS.^{5,13} Diagnosis of PWS can be difficult since there are no distinguishing laboratory findings and the clinical spectrum varies. However, a combination of both cytogenetic and molecular analysis will increase the number of subjects who can be shown to have chromosome 15q11-13 specific defects.

This work was supported by the National Health and Medical Research Council of Australia. We are grateful to the Prader-Willi Association of Australia for help and cooperation in conducting this study. We thank Dr Brian Learoyd (Macquarie Hospital) and Sister Sue White (Adelaide Children's Hospital) for collecting blood from many of the patients, and Miss C Yeung for typing the manuscript. DNA paternity studies were obtained by Ms A Kearney.

- 1 Bray GA, Dahms WT, Sverdloff RS, Fiser RH, Atkinson RL, Carrel RE. The Prader-Willi syndrome: a study of 40 patients and a review of the literature. *Medicine (Baltimore)* 1983;62:59-80.
- 2 Lurance BM. The Prader-Willi syndrome. *Matern Child Health* 1985;10:106-9.
- 3 Kousseff BG, Burke CM. Familial Prader-Willi syndrome: new developments. *Am J Med Genet* 1987;28:79.
- 4 Ledbetter DH, Riccardi VM, Airhart SD, Strobel RJ, Keenan BS, Crawford JD. Deletions of chromosome 15 as a cause of the Prader-Willi syndrome. *N Engl J Med* 1981;304:325-9.
- 5 Butler MG, Meaney FJ, Palmer CG. Clinical and cytogenetic survey of 39 individuals with Prader-Labhart-Willi syndrome. *Am J Med Genet* 1986;23:793-809.
- 6 Smith A. Chromosome findings in the Prader-Willi syndrome. *Aust NZ J Develop Disab* 1986;12:13-28.
- 7 Donlon TA, Lalande M, Wyman A, Bruns G, Latt SA. Isolation of molecular probes associated with the chromosome 15 instability in the Prader-Willi syndrome. *Proc Natl Acad Sci USA* 1986;83:4408-12.
- 8 Tasset DM, Hartz JA, Kao FT. Isolation and analysis of DNA markers specific to human chromosome 15. *Am J Hum Genet* 1988;42:854-66.
- 9 Nicholls RD, Knoll JHM, Butler MG, Karam S, Lalande M. Genetic imprinting suggested by maternal heterodisomy in non-deletion Prader-Willi syndrome. *Nature* 1989;342:281-5.
- 10 Smith A, Simpson E. Dermatoglyphic analyses of 24 individuals with the Prader-Willi syndrome. *J Ment Defic Res* 1982;26:91-9.
- 11 Old JM, Higgs DR. Gene analysis. In: Weatherall DJ, ed. *The thalassaemias. Methods in haematology* vol 6. Edinburgh: Churchill Livingstone, 1983:74-102.
- 12 Gregory CA, Kirkilionis AJ, Greenberg CR, Chudley AE, Hamerton JL. Detection of molecular rearrangements in Prader-Willi syndrome patients by using genomic probes recognizing four loci within the PWCR. *Am J Med Genet* 1990;35:536-45.
- 13 Nicholls RD, Knoll JH, Glatt K, et al. Restriction fragment length polymorphisms within proximal 15q and their use in molecular cytogenetics and the Prader-Willi syndrome. *Am J Med Genet* 1989;33:66-77.
- 14 Rich DC, Witkowski CM, Summers KM, van Tuinen P, Ledbetter DH. Highly polymorphic locus D15S24 (CMW-1) maps to 15pter-q13. *Nucleic Acids Res* 1988;16:8740.
- 15 Higgs DR, Wainscoat JS, Flint J, et al. Analysis of the human α -globin gene cluster reveals a highly informative genetic locus. *Proc Natl Acad Sci USA* 1986;83:5165-9.
- 16 Yunis JJ. High resolution of human chromosomes. *Science* 1976;191:1268-70.
- 17 Seabright M. A rapid banding technique for human chromosomes. *Lancet* 1971;ii:971-2.
- 18 Smith A, Noel M. A girl with the Prader-Willi syndrome and Robertsonian translocation 45,XX,t(14;15)(p11;q11) which was present in three normal family members. *Hum Genet* 1980;55:271-3.
- 19 Smith A, den Dulk G, Lipson A, Suter M. Classical Prader-Willi syndrome with trisomy 15(pter-q12) plus de novo variant 15p11. *Ann Genet (Paris)* 1989;32:39-42.
- 20 Knoll JHM, Nicholls RD, Magenis RE, et al. Angelman syndrome: three molecular classes identified with chromosome 15q11-q13-specific DNA markers. *Am J Hum Genet* 1990;47:149-54.
- 21 Donlon TA. Similar molecular deletions on chromosome 15q11.2 are encountered in both the Prader-Willi and Angelman syndromes. *Hum Genet* 1988;80:322-8.